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RESEARCH ARTICLE

NATURAL PRESERVATION OF POLLEN IN HONEY AND BEE BREAD: DIVERSITY, VIABILITY, AND *IN VITRO* GERMINATION IN THE INDIAN SUNDARBANS MANGROVE ECOSYSTEM

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Abstract: Pollen preservation is important for understanding plant reproductive biology, pollination ecology and biodiversity, but conventional storage methods often require controlled environmental conditions and specialised infrastructure. The present study investigated the diversity, viability and *in vitro* germination potential of pollen naturally recovered from bee bread (BB) and honey (HN) in the Sundarbans mangrove ecosystem of India. Bee bread and honey samples collected from five bee hives representing four regions of the Sundarbans were subjected to palynological analysis. Pollen viability was assessed using Aniline Blue, Acetocarmine and 2,3,5-triphenyl tetrazolium chloride (TTC), while germination was evaluated under *in vitro* conditions at different sucrose concentrations and incubation periods. A total of 20 pollen taxa representing 12 plant families were identified, comprising mangrove, mangrove-associated and terrestrial taxa and reflecting the diverse floral resources exploited by honey bees. Viability varied among pollen taxa, staining methods and storage matrices, with bee-bread pollen generally showing higher viability than pollen recovered from honey. Significant differences in viability between BB and HN were observed for all three staining methods, whereas the responses among staining methods varied according to the pollen matrix. *In vitro* germination was highest in 20% sucrose and reached a maximum at 72 h of incubation. Germination frequency was consistently higher in BB than in HN pollen, ranging from 70.46–87.64% in BB and 62.32–73.42% in HN. Linear regression indicated strong positive associations between incubation time and germination frequency in both BB (R^2 = 0.8941) and HN (R^2 = 0.9107). Thus, pollen recovered from bee bread showed greater retention of viability and germination potential than pollen recovered from honey, although responses were species-specific. The findings identify bee bread as a promising natural biological archive for investigating pollen biology, bee–plant interactions, pollination ecology and biodiversity, while providing a basis for future research on natural pollen preservation.

Keywords: Bee bread, Honey; Pollen preservation, pollen viability, Sundarbans, Mangrove ecosystem

INTRODUCTION

Honey bees (*Apis* spp.) are among the most important pollinators, contributing significantly to ecosystem stability, biodiversity conservation, and agricultural productivity. During foraging, worker bees collect nectar and pollen from flowering plants. Nectar undergoes enzymatic modification and dehydration within the hive to produce honey, whereas pollen is mixed with nectar, honey, and glandular secretions and packed into comb cells, where microbial fermentation, particularly involving lactic acid bacteria, leads to the formation of bee bread—the principal protein-rich food reserve of the colony (Bogdanov et al., 2008; Da Silva et al., 2016; Kieliszek et al., 2018). Within the hive, bee bread is generally stored adjacent to brood cells, facilitating rapid access by nurse bees, while honey serves as the colony's primary carbohydrate reserve and is commonly stored in the upper and peripheral regions of the comb (Seeley, 1995).

Honey is a complex natural product valued for its nutritional, medicinal, ecological, and commercial importance (Edo *et al*., 2023). It is composed mainly of carbohydrates together with water, proteins, amino acids, enzymes, vitamins, minerals, organic acids, phenolic compounds, flavonoids, and varying quantities of pollen grains (Bogdanov *et al*., 2008). The pollen spectrum of honey reflects the floral resources exploited by foraging bees and serves as a reliable indicator of its botanical and geographical origin (Ponnuchamy *et al*., 2014). The identification and analysis of pollen grains in honey, known as melissopalynology, is widely used for honey authentication, determination of floral sources, and reconstruction of regional vegetation (Louveaux *et al*., 1978; Bryant & Jones, 2001). Melissopalynological studies also provide valuable information on bee foraging behaviour, seasonal floral availability, habitat characteristics, and ecosystem health, making them indispensable in apicultural, ecological, and biodiversity research

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(Ahmad *et al*., 2024). Similarly, bee bread contains pollen collected from diverse flowering plants and has attracted increasing attention because of its nutritional value, ecological significance, and commercial potential (Baky *et al*., 2023). The fermentation process alters the biochemical characteristics of pollen and may contribute to its preservation within the bee bread matrix, although its influence on pollen physiological viability remains insufficiently understood (Kieliszek *et al*., 2018). Together, honey and bee bread represent naturally occurring storage environments in which pollen grains are retained under distinct physicochemical conditions, including low water activity, high sugar concentration, and fermentation-associated biochemical changes. Whether these natural storage conditions maintain pollen viability and germination potential, however, remains poorly understood.

The preservation of viable pollen is of considerable importance in plant sciences because stored pollen serves as a valuable resource for plant breeding, hybridization, germplasm conservation, *ex situ* genetic resource management, crop improvement, fertility assessment, and studies of plant reproductive biology (Dinato *et al*., 2020). Conventional methods for pollen preservation include desiccation, refrigeration, freezing, and cryopreservation, each requiring careful optimization of moisture content and storage conditions to maintain pollen viability (Dinato *et al*., 2021). However, these approaches often require specialised equipment, controlled environments, and continuous maintenance. Consequently, the search for naturally occurring and sustainable systems capable of retaining pollen structure and physiological competence has attracted increasing scientific interest.

Pollen viability is fundamental to successful sexual reproduction in flowering plants. Viable pollen grains retain the ability to germinate on compatible stigmas, produce pollen tubes, and achieve fertilization (Dafni, 2000). Their physiological competence depends on the integrity of cellular structures and the availability of stored carbohydrates, proteins, lipids, and other reserve materials that support pollen tube growth. Therefore, the assessment of pollen viability and germination has broad applications in reproductive biology, germplasm conservation, pollen storage, fertility evaluation, and studies of pollen–stigma interactions (Althiab-Almasaud, 2024). Cytochemical staining methods, including Acetocarmine, Aniline Blue, and 2,3,5-triphenyltetrazolium chloride (TTC), provide rapid estimates of pollen viability, whereas *in vitro* germination assays offer a functional measure of pollen performance by directly evaluating the ability of pollen grains to produce pollen tubes under controlled conditions (Ballantyne *et al*., 1974; Tushabe & Rosbakh, 2021).

The Sundarbans, a UNESCO World Heritage Site, constitute the world's largest mangrove ecosystem, extending across the Ganga–Brahmaputra delta of India and Bangladesh (Ghosh *et al*., 2015). The Indian Sundarbans are characterized by exceptional floral diversity and abundant melliferous plant species that support extensive foraging by wild honey bees and the production of multifloral honey (Chakraborti *et al*., 2019). The region comprises a rich assemblage of mangrove and associated plant species, including *Avicennia marina, Bruguiera gymnorhiza, Rhizophora mucronata, Ceriops decandra, Sonneratia apetala*, and *Excoecaria agallocha*, which provide abundant nectar and pollen resources for bees (Naskar & Mandal, 1999). Consequently, honey and bee bread produced in this ecosystem contain pollen derived from a diverse range of mangrove and associated flora, making them valuable materials for melissopalynological and ecological investigations.

Although several studies have examined the botanical origin, melissopalynological characteristics, and physicochemical properties of Sundarbans honey (Refat *et al*., 2026), much less is known about the physiological status of pollen naturally retained within honey and bee bread. In particular, whether pollen grains recovered from these bee products remain viable and retain the capacity for *in vitro* germination remains poorly understood. Comparative information on pollen diversity, viability, and germination potential in honey and bee bread from the Sundarbans mangrove ecosystem is especially limited. Addressing this knowledge gap may improve our understanding of pollen behaviour under natural storage conditions and provide new insights into the biological quality and potential applications of bee-derived products.

Therefore, the present study investigated the botanical diversity, pollen viability, and *in vitro* germination potential of pollen isolated from honey and bee bread collected from different locations in the Sundarbans. By integrating melissopalynological analysis with cytochemical viability assays and *in vitro* germination tests, this study provides new insights into the diversity of floral resources exploited by honey bees and evaluates the physiological integrity of pollen retained within bee products. The findings improve our understanding of pollen persistence under natural storage conditions in honey and bee bread and highlight the potential significance of these bee-derived products for plant reproductive biology, biodiversity assessment, melissopalynological research, and conservation studies. To the best of our knowledge, this is among the first studies to simultaneously investigate the botanical composition, viability, and *in vitro* germination potential of pollen recovered from both honey and bee bread collected from the Sundarbans mangrove ecosystem.

MATERIALS AND METHODS

Material

Bee bread (BB) and honey (HN) samples were collected during the summer season (May 2026) directly from wild honey bee colonies at four locations in the Indian Sundarbans (Fig. 1, 2). The sampling locations and corresponding sample codes were: Kumirmari (BB1/KUM, HN1/KUM, BB2/KUM, HN2/KUM), Parghumti (BB3/PGM, HN3/PGM), Shamsernagar (BB4/SMN, HN4/SMN), and Hemnagar (BB5/HMN, HN5/HMN). Samples were collected using sterile tools to minimize external contamination, immediately transferred into sterile containers, and stored at −20 °C until further analysis.

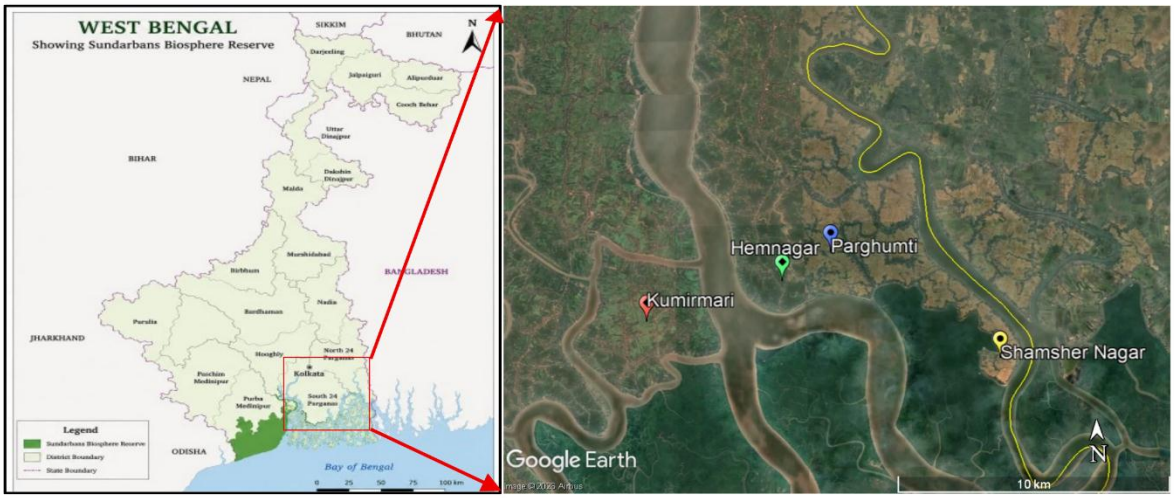


Fig. 1: Map of the sampling locations of bee bread and honey in the Indian Sundarbans.

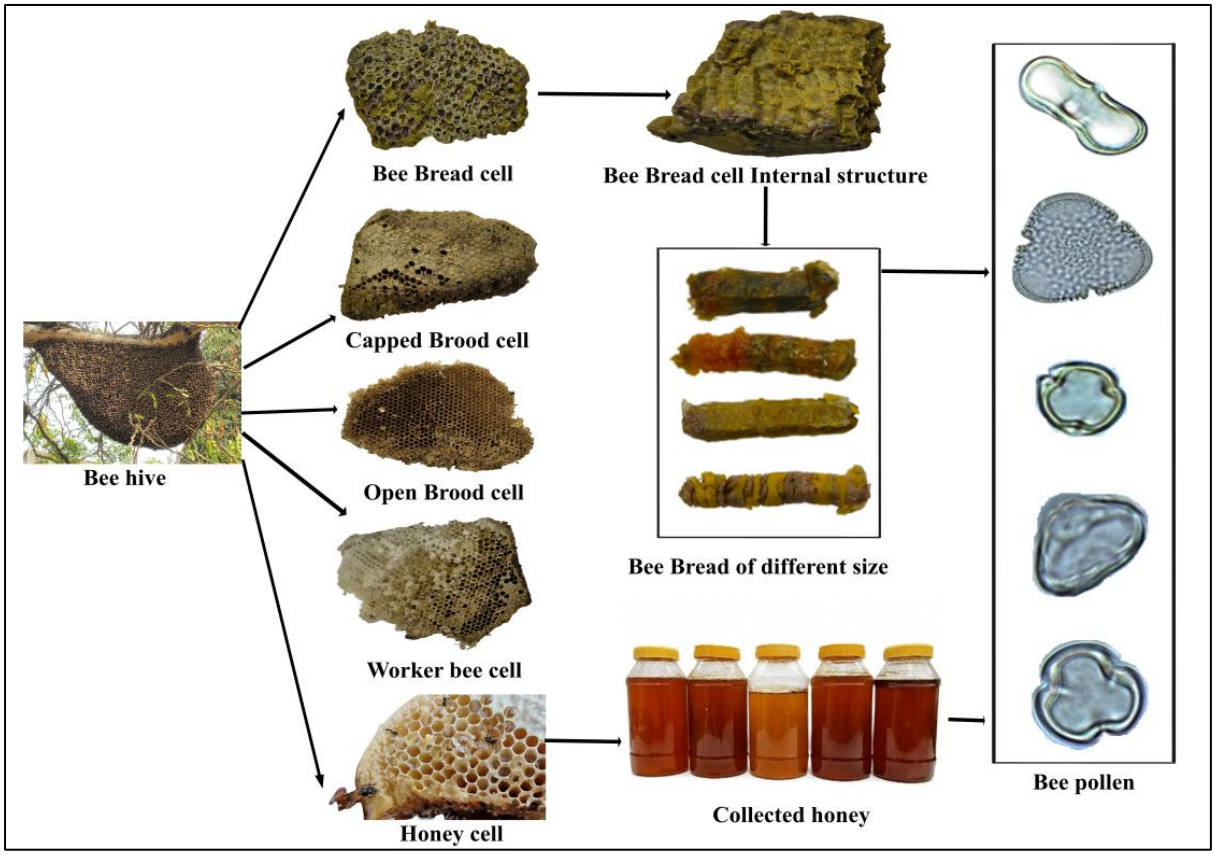


Fig. 2. Photographic workflow showing the collection and characterization of bee-hive materials, different cell types, bee-bread structures, honey, and representative pollen grains recovered from these materials.

METHODS

Sample Preparation

For each bee-bread sample, 20 bee-bread pellets were randomly collected from a single hive at each sampling location, pooled, and thoroughly homogenized. From the homogenized bee bread, 1 g was taken for pollen isolation. For honey, 10 g of sample was collected directly from the same hive and processed separately. Each sample was suspended in distilled water and thoroughly homogenized. The resulting suspensions were centrifuged at 2500 rpm for 10 min using a Remi RM-02 centrifuge to concentrate the pollen fraction. Following each centrifugation step, the supernatant was carefully decanted, and the sediment was resuspended in distilled water. The washing and centrifugation steps were repeated until a relatively clean pollen pellet was obtained. The recovered pollen fraction was subsequently used for palynological analysis, pollen viability assessment, and *in vitro* germination studies.

Pollen Viability Assessment

For viability assessment, the isolated pollen fraction obtained from each sample was divided into three sterile Eppendorf tubes, representing three technical replicates. Pollen viability was assessed using three cytochemical staining methods: 1% Aniline Blue, 2% Acetocarmine, and 5% 2,3,5-triphenyltetrazolium chloride (TTC) (El-Sofany *et al*., 2020). The pollen suspensions were incubated with the respective staining solutions for 5–6 h at room temperature. Following incubation, approximately 2000 pollen grains from each bee-bread sample and 1200 pollen grains from each honey sample were examined for each staining treatment under a Leitz Laborlux light microscope equipped with an E3 Scientific digital camera. Pollen grains showing intense blue, red, and red-to-yellow staining following treatment with Aniline Blue, acetocarmine, and TTC, respectively, were considered viable, whereas unstained or weakly stained pollen grains were considered non-viable. The percentage of pollen viability was calculated using the following equation (Bots & Mariani, 2005):

Pollen Viability (%) =

$$\left(\frac{\text{Number of viable pollen grains}}{\text{Total number of pollen grains observed}}\right) \times 100$$

In Vitro Pollen Germination Assay

The germination potential of pollen grains recovered from bee bread and honey was evaluated in vitro using a modified Brewbaker and Kwack (BK) germination medium (Brewbaker & Kwack, 1963). The germination medium was prepared in distilled water and consisted of 20% (w/v) sucrose, 100 mg L^{-1} boric acid, 100 mg L^{-1} calcium nitrate, 100 mg L^{-1} magnesium sulphate, and 100 mg L^{-1} potassium nitrate. Prior to the key germination experiment, the effect of sucrose concentration on pollen germination was evaluated using media containing 0, 10, 15, 20, and 25% (w/v) sucrose. The medium containing 20% sucrose showed the highest germination rate across the tested bee-bread and honey samples and was therefore selected as the standard germination medium for the subsequent experiments. The isolated pollen fraction from each sample was equally distributed into sterile Eppendorf tubes, and 1 mL of the germination medium was added to each tube. The pollen suspensions were incubated under laboratory conditions for 24, 48, 72, and 96 h. Three technical replicates were maintained for each sample at each incubation period. Following incubation, a drop of the pollen suspension was placed on a clean glass slide and examined under a Leitz Laborlux light microscope equipped with an E3 Scientific digital camera. Approximately 2,000 pollen grains from bee-bread samples and 1,200 pollen grains from honey samples were examined at each incubation period. A pollen grain was considered germinated when a pollen tube emerged through the germination aperture. The percentage of pollen germination was calculated using the following equation (Kakani et al., 2002):

Pollen Germination (%) =

$$\left(\frac{\text{Number of germinated pollen grains}}{\text{Total number of pollen grains observed}}\right) \times 100$$

Statistical Analysis

All experimental data are presented as mean ± standard deviation (SD). Pollen viability percentages obtained using Acetocarmine, Aniline Blue, and 2,3,5-triphenyltetrazolium chloride (TTC) staining were compared among the three staining methods. Similarly, *in vitro* pollen germination percentages were compared among the four incubation periods (24, 48, 72, and 96 h). One-way analysis of variance (ANOVA), followed by Tukey's Honestly Significant Difference (HSD) post hoc test, was used to determine significant differences among the pollen viability estimates obtained using the three staining methods and among pollen germination percentages recorded at different incubation periods. Linear regression analysis was performed to examine the relationship between incubation time and pollen germination percentage. Statistical analyses were performed using RStudio (version 2024.12.0+467), and differences were considered statistically significant at $p < 0.05$. Graphical representations, including heatmaps, box plots, and regression plots, were generated using RStudio.

RESULTS

Pollen Diversity and Distribution in Bee Bread and Honey

Five bee hives comprising bee bread (BB) and honey (HN) samples collected from four regions of the Indian Sundarbans were subjected to palynological analysis. A total of 20 pollen taxa belonging to 12 plant families were identified from the BB and HN samples. Of these, 12 taxa were common to both bee bread and honey, whereas four taxa were recorded

exclusively in bee bread and four taxa exclusively in honey (Table 1). Thus, 16 pollen taxa were identified in bee bread and 16 in honey, indicating substantial overlap in pollen composition between the two sample types along with differences in their representation among localities.

The identified pollen assemblage comprised both mangrove and associated terrestrial taxa. Rhizophoraceae was the most taxon-rich family, represented by *Bruguiera gymnorhiza*, *Ceriops decandra*, *Kandelia candel* and *Rhizophora apiculata*, followed by Malvaceae, represented by *Bombax ceiba*, *Heritiera fomes* and *Thespesia* sp. Among the identified taxa, *Ceriops decandra* was the most frequently represented, occurring in seven of the ten individual BB and HN samples. It was recorded in both BB and HN samples from Parghumti and Shamshernagar, in BB from Kumirmari and in both BB and HN from Shamshernagar, while it was absent from both samples at Hemnagar. *Avicennia* sp. was the second most frequently represented taxon, occurring in six of the ten samples and being recorded in both BB and HN from Kumirmari, Parghumti and Hemnagar, but absent from both samples at Shamshernagar. Twelve pollen taxa—*Acanthus ilicifolius*, *Avicennia* sp., *Bombax ceiba*, *Bruguiera gymnorhiza*, *Ceriops decandra*, *Coriandrum sativum*, *Desmodium* sp., *Excoecaria agallocha*, *Heritiera fomes*, *Lumnitzera littorea*, *Sonneratia apetala* and *Xylocarpus granatum*—were common to both bee bread and honey. Their occurrence in both sample types indicates substantial overlap in the floral resources represented in bee bread and honey. Four pollen taxa, namely *Aegiceras corniculatum*, *Laguncularia racemosa*, *Rhizophora apiculata* and *Thespesia* sp., were detected exclusively in bee bread. In contrast, *Kandelia candel*, *Nypa fruticans*, *Phoenix paludosa* and *Psidium guajava* were detected exclusively in honey. The occurrence of pollen from both mangrove and associated terrestrial plants demonstrates the diversity of floral resources represented in the samples. The differences in pollen composition among localities and between bee bread and honey suggest variation in the floral resources utilized by honey bees across the study area. The occurrence of several pollen taxa in both sample types further indicates overlap in the floral resources contributing to bee bread and honey, whereas sample-specific taxa may reflect differences in resource availability, collection site or the timing of foraging.

Table 1: Pollen taxa recovered from bee bread and honey samples collected from different localities of the Indian Sundarbans

IDENTIFIED POLLEN GRAINS	FAMILY	KUMIRMARI				PARGHUMTI		SHAMSHERNAGAR		HEMNAGAR	
		BB1/ KUM	HN1/ KUM	BB2/ KUM	HN2/ KUM	BB3/ PGM	HN3/ PGM	BB4/ SMN	HN4/ SMN	BB5/ HMN	HN5/ HMN
Acanthus ilicifolius L.	Acanthaceae	-	-	+	-	-	+	+	+	-	-
Aegiceras corniculatum (L.) Blanco	Primulaceae	-	-	-	-	-	-	+	-	+	-
Avicennia sp.	Acanthaceae	+	+	+	-	+	+	-	-	+	+
Bombax ceiba (L.)	Malvaceae	-	-	-	-	-	-	+	+	+	-
Bruguiera gymnorhiza (L.) Lam.	Rhizophoraceae	-	-	-	-	+	+	-	-	-	-
Ceriops decandra (Griff.) W.Theob.	Rhizophoraceae	+	-	+	+	+	+	+	+	-	-
Coriandrum sativum L.	Apiaceae	-	-	-	+	-	-	-	-	+	+
Desmodium sp.	Fabaceae	-	-	-	-	+	-	+	+	-	-
Excoecaria agallocha L.	Euphorbiaceae	-	-	-	+	-	-	-	-	+	+
Heritiera fomes Buch.	Malvaceae	-	+	+	+	-	-	+	-	-	-
Kandelia candel (L.) Druce	Rhizophoraceae	-	-	-	-	-	+	-	-	-	+
Laguncularia racemosa (L.) C.F.Gaertn.	Combretaceae	-	-	-	-	-	-	+	-	+	-
Lumnitzera littorea (Jack) Voigt	Combretaceae	-	-	-	+	-	-	-	-	+	-
Nypa fruticans Wurmb	Arecaceae	-	-	-	-	-	-	-	+	-	+
Phoenix paludosa Roxb.	Arecaceae	-	+	-	-	-	-	-	+	-	-
Psidium guajava L.	Myrtaceae	-	+	-	-	-	+	-	-	-	-
Rhizophora apiculata Blume	Rhizophoraceae	+	-	+	-	-	-	-	-	-	-
Sonneratia apetala Buch-Ham.	Lythraceae	+	+	-	+	+	-	-	-	-	-
Thespesia sp. L.	Malvaceae	+	-	+	-	-	-	-	-	-	-
Xylocarpus granatum J. Koenig	Meliaceae	+	-	-	-	+	-	-	+	+	+

Pollen Viability and Germination in Bee Bread and Honey

Pollen viability of the identified pollen taxa recovered from bee bread (BB) and honey (HN) samples collected from different localities of the Indian Sundarbans was assessed using three staining methods—Aniline Blue, Acetocarmine and 2,3,5-triphenyl tetrazolium chloride (TTC)—along with *in vitro* germination analysis. The viability and germination values presented in Table 2 represent the mean values obtained across the respective BB and HN samples from the study localities. Thus, the values provide an overall comparison of pollen viability and germination between the two mediums rather than locality-specific estimates.

Aniline Blue staining showed mean viability values ranging from 81.53% in *Acanthus ilicifolius* to 92.24% in *Heritiera fomes* for bee bread pollen. In honey pollen, the corresponding mean viability ranged from 82.33% in *Nypa fruticans* to 89.24% in *Excoecaria agallocha* and *Xylocarpus granatum*. Among the pollen taxa represented in both matrices, *Bruguiera gymnorhiza* showed identical mean viability (85.46%) in BB and HN, whereas *Acanthus ilicifolius* and *Xylocarpus granatum* showed higher mean viability in honey (86.34% and 89.24%, respectively) than in bee bread (81.53% and 83.33%, respectively).

Acetocarmine staining yielded mean viability values ranging from 80.67% in *Acanthus ilicifolius* to 93.32% in *Desmodium* sp. for bee bread pollen. In honey pollen, mean viability ranged from 82.34% in *Bruguiera gymnorhiza* to 90.75% in *Avicennia* sp. *Acanthus ilicifolius* and *Xylocarpus granatum* again showed higher mean viability in honey (89.47% and 85.47%, respectively) than in bee bread (80.67% and 81.19%, respectively).

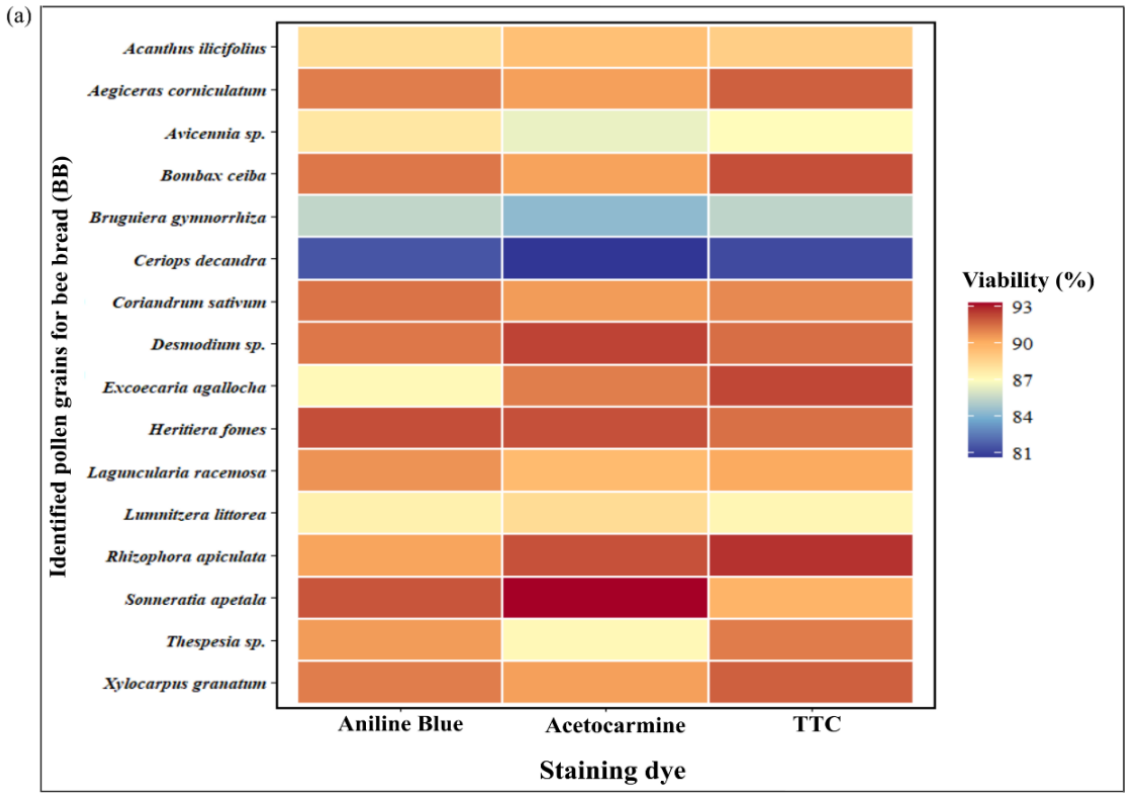
TTC staining recorded mean viability values ranging from 81.22% in *Acanthus ilicifolius* to 92.78% in *Laguncularia racemosa* in bee bread, whereas honey pollen showed mean values ranging from 81.01% in *Avicennia* sp. to 86.88% in *Kandelia candel*. *Acanthus ilicifolius* and *Xylocarpus granatum* again showed higher mean viability in honey (84.91% and 84.88%, respectively) than in bee bread (81.22% and 82.22%, respectively). Thus, among the taxa represented in both matrices, these two taxa consistently exhibited higher mean viability in honey across all three staining methods, whereas the other comparable taxa generally showed higher mean viability in bee bread.

Heatmaps (Fig. 3a, b) provide a comparative visualization of the mean pollen viability values obtained using Aniline Blue, Acetocarmine and TTC staining methods. Fig. 3a represents pollen recovered from bee bread, whereas Fig. 3b represents pollen recovered from honey. The colour intensity reflects the magnitude of pollen viability, with lighter (blue) gradients indicating relatively lower viability and darker (brown) gradients indicating relatively higher viability. Distinct gradient variations are evident not only between bee bread and honey pollen but also among the three staining methods within each matrix, indicating taxon- and stain-dependent differences in the estimated viability. In bee bread pollen (Fig. 3a), comparatively lighter gradients are observed for *Bruguiera gymnorhiza* and *Ceriops decandra*, particularly in comparison with taxa showing darker gradients, although the intensity varies among the three staining methods. In honey pollen (Fig. 3b), most taxa show relatively lighter gradients compared with the bee bread heatmap; however, some taxa exhibit distinct stain-specific patterns. For example, *Sonneratia apetala* shows noticeable differences in colour intensity among Aniline Blue, Acetocarmine and TTC, reflecting variation in its estimated viability according to the staining method. As such, the heatmaps demonstrate matrix-, taxon- and staining-method-dependent variation in pollen viability and provide a visual representation of the patterns presented quantitatively in Table 2.

The box plots in Fig. 4 illustrate the variation in pollen viability determined using Aniline Blue, Acetocarmine and TTC staining methods. Pairwise comparisons among the three staining methods showed no statistically significant differences in pollen viability for bee bread pollen (Fig. 4a). In contrast, significant differences were observed among the staining methods for honey pollen: viability obtained using Aniline Blue differed significantly from that obtained using Acetocarmine ($p < 0.05$) and TTC ($p < 0.01$), whereas the difference between Acetocarmine and TTC was not statistically significant. Pairwise comparisons between bee bread and honey pollen for each staining method revealed significantly higher viability in bee bread pollen (Fig. 4b). The difference between BB and HN was significant at $p < 0.05$ for both Aniline Blue and Acetocarmine, whereas a highly significant difference was observed for TTC staining ($p < 0.001$). These comparisons indicate that the choice of staining method did not significantly affect the estimated viability of bee bread pollen, whereas staining method influenced the viability estimates obtained for honey pollen, with bee bread pollen consistently exhibiting higher viability than honey pollen across all three staining methods.

Table 2: Mean pollen viability (%) ± standard deviation of 20 identified pollen taxa assessed using Aniline Blue, Acetocarmine, and TTC staining methods, together with the maximum germination frequency observed after 72 h of incubation.

Identified Pollen Taxa	Mean Pollen viability in different staining dyes						Germination (%)	
	Aniline Blue		Acetocarmine		TTC			
	Bee bread	Honey	Bee bread	Honey	Bee bread	Honey	Bee bread	Honey
Acanthus ilicifolius L.	81.53 ± 4.33	86.34 ± 2.13	80.67 ± 4.90	89.47 ± 1.78	81.22 ± 5.15	84.91 ± 4.66	70.46 ± 5.03	64.74 ± 3.22
Aegiceras corniculatum (L.) Blanco	91.24 ± 3.52	-	90.47 ± 4.16	-	91.88 ± 4.07	-	82.28 ± 4.45	-
Avicennia sp.	87.92 ± 3.35	87.52 ± 2.06	86.47 ± 3.83	90.75 ± 2.22	87.12 ± 3.72	81.01 ± 4.63	84.68 ± 4.42	71.68 ± 3.34
Bombax ceiba (L.)	91.37 ± 3.64	87.37 ± 2.33	90.39 ± 4.34	84.39 ± 1.15	92.23 ± 4.23	83.23 ± 4.56	78.56 ± 4.70	63.56 ± 2.98
Bruguiera gymnorhiza (L.) Lam.	85.46 ± 3.58	85.46 ± 2.21	84.34 ± 4.30	82.34 ± 1.42	85.38 ± 4.17	82.38 ± 4.47	76.34 ± 4.51	67.34 ± 2.83
Ceriops decandra (Griff.) W.Theob.	88.34 ± 3.33	84.53 ± 2.19	89.47 ± 3.88	85.67 ± 0.88	88.91 ± 3.78	86.22 ± 4.59	76.74 ± 4.32	67.46 ± 2.31
Coriandrum sativum L.	91.46 ± 3.46	87.46 ±2.22	90.56 ± 4.03	86.56 ± 1.37	90.98 ± 3.94	83.98 ± 4.61	83.42 ± 4.45	73.42 ± 3.29
Desmodium sp.	92.11 ± 3.29	88.37± 2.04	93.32 ± 5.08	84.47 ± 2.17	89.91 ± 4.74	84.58 ± 4.59	84.32 ± 5.22	63.58 ± 3.36
Excoecaria agallocha L.	90.56 ± 3.22	89.24 ± 2.45	87.26 ± 2.46	86.23 ± 1.18	91.26 ± 1.28	86.38 ± 4.68	82.18 ± 2.10	64.24 ± 3.00
Heritiera fomes Buch.	92.24 ± 3.27	87.24 ± 1.98	92.2 ± 3.77	85.20 ± 2.10	91.52 ± 3.55	86.52 ± 4.62	87.64 ± 4.69	71.64 ± 3.44
Kandelia candel (L.) Druce	-	88.24 ± 2.47	-	86.47 ± 1.02	-	86.88 ± 4.68	-	71.56 ± 2.61
Laguncularia racemosa (L.) C.F.Gaertn.	90.36 ± 3.28	-	92.18 ± 1.77	-	92.78 ± 1.80	-	78.54 ± 2.56	-
Lumnitzera littorea (Jack) Voigt	90.76 ± 3.42	83.56 ± 2.23	89.67 ± 2.42	84.38 ± 1.22	90.23 ± 2.54	84.35 ± 4.51	82.16 ± 2.48	64.68 ± 3.08
Nypa fruticans Wurmb	-	82.33 ± 2.13	-	86.19 ± 1.11	-	83.22 ± 4.51	-	68.68 ± 1.98
Phoenix paludosa Roxb.	-	86.36 ± 1.32	-	86.18 ± 1.34	-	86.78 ± 4.66	-	68.54 ±1.58
Psidium guajava L.	-	85.56 ± 0.57	-	84.26 ± 1.36	-	84.26 ± 4.58	-	65.18 ± 2.38
Rhizophora apiculata Blume	91.24 ± 3.33	-	90.47 ± 4.28	-	91.88 ± 4.27	-	80.56 ± 3.95	-
Sonneratia apetala Buch.Ham.	91.37 ± 3.64	88.11 ± 2.25	92.47 ± 3.81	83.32 ± 1.27	91.58 ± 3.63	85.91 ± 4.61	83.58 ± 4.40	62.32 ± 3.19
Thespesia sp. L.	87.56 ± 3.96	-	88.38 ± 4.71	-	87.35 ± 4.41	-	82.68 ± 5.03	-
Xylocarpus granatum J.Koenig	83.33 ± 3.62	89.24 ± 2.30	81.19 ± 4.71	85.47 ± 1.35	82.22 ± 4.72	84.88 ± 4.64	72.68 ± 4.48	68.28 ± 2.75



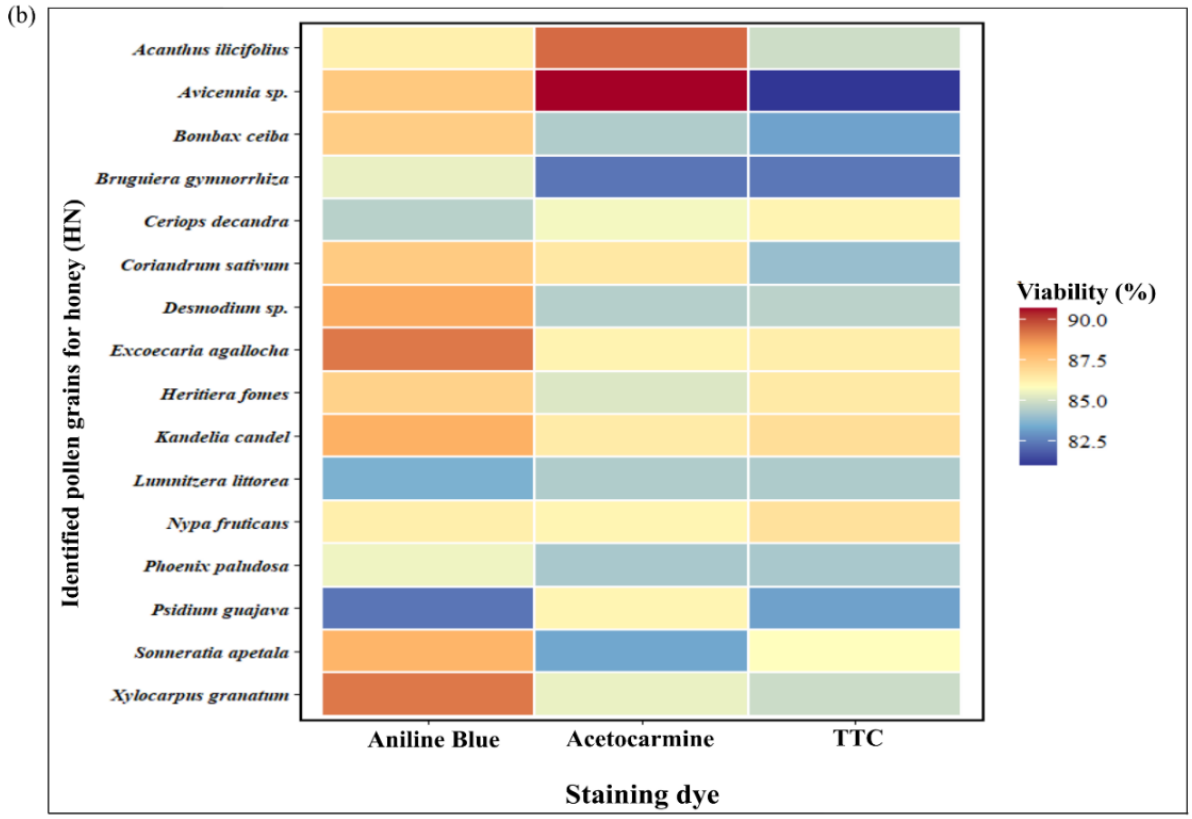
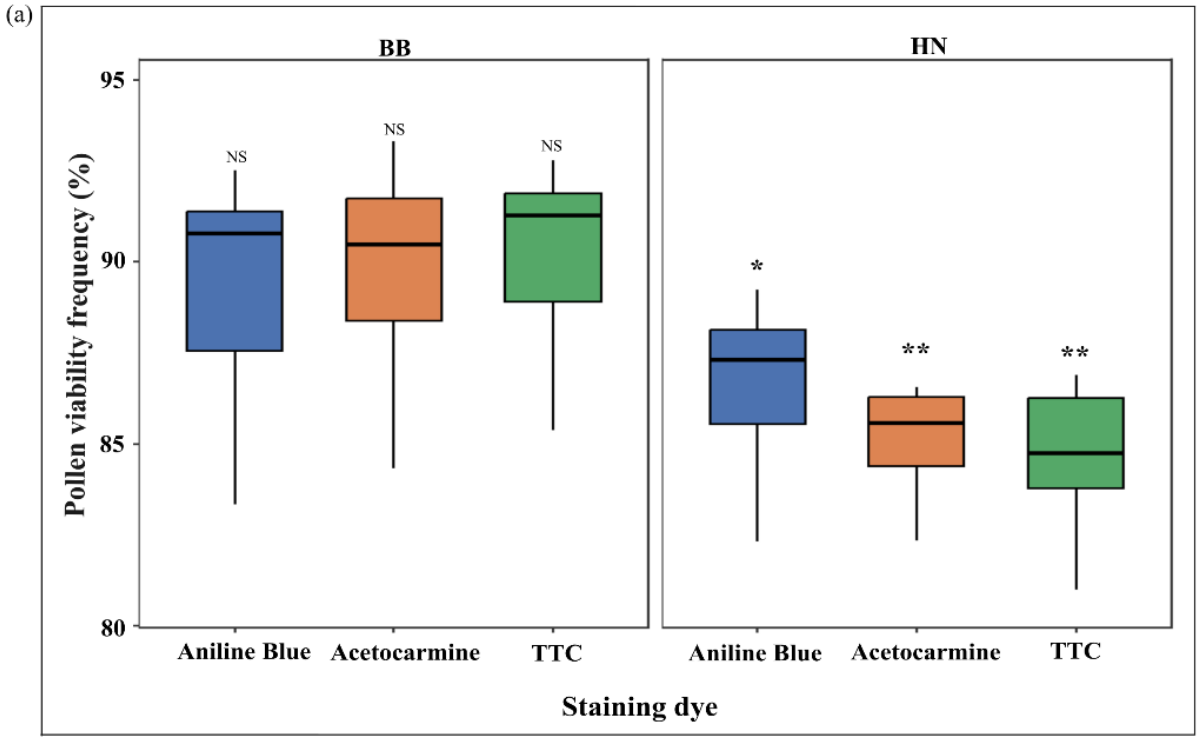


Fig. 3: Heatmap illustrating the pollen viability (%) of 16 identified pollen taxa recovered from **(a)** bee bread (BB) and **(b)** paired honey (HN), as assessed using three cytochemical staining methods: Aniline Blue, Acetocarmine, and TTC (2,3,5-triphenyltetrazolium chloride). The colour gradient from blue to dark brown represents lower to higher pollen viability (%), respectively.



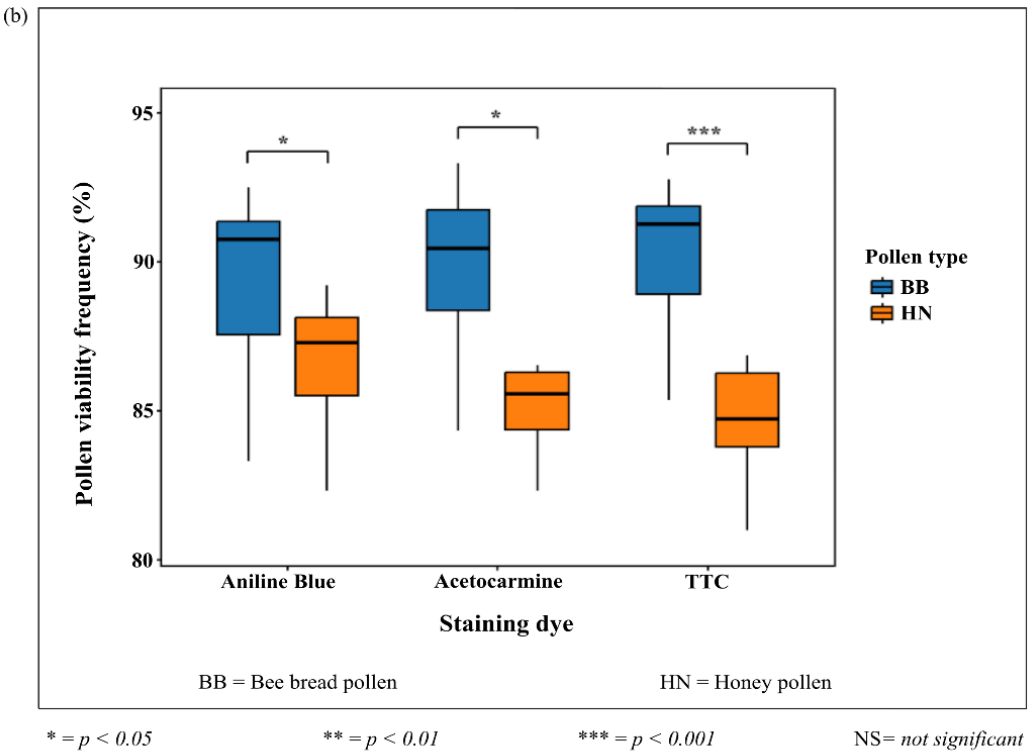


Fig. 4. Comparative assessment of pollen viability among staining methods and between bee bread and honey pollen. **(a)** Boxplots showing pollen viability (%) estimated using Aniline Blue, Acetocarmine, and TTC staining in bee bread (BB) and honey (HN) pollen. No significant differences were observed among the staining methods for BB pollen, whereas significant pairwise differences were observed between Aniline Blue and Acetocarmine and between Aniline Blue and TTC for HN pollen. **(b)** Boxplots comparing pollen viability between BB and HN pollen for each staining method. Significant differences were observed between BB and HN for Aniline Blue and Acetocarmine at $p < 0.05$ and for TTC at $p < 0.001$. Asterisks indicate statistical significance: $p < 0.05$, *$p <$ 0.01, and **$p < 0.001$.

The *in vitro* germination of pollen recovered from bee bread (BB) and honey (HN) samples (Fig. 5) was evaluated at 24, 48, 72 and 96 h of incubation using different sucrose concentrations (Fig. 6a, b). Germination frequency increased with increasing incubation time and reached its maximum at 72 h in both BB and HN pollen, followed by a slight decline at 96 h (Fig. 6b). The sucrose-concentration experiment showed that 20% sucrose produced the highest germination response under the experimental conditions, and this concentration was therefore selected as the working germination medium for subsequent experiments (Fig. 6a). Analysis of variance (ANOVA), followed by Tukey's Honest Significant Difference (HSD) test at $p \leq 0.05$, demonstrated significant differences in germination frequency among the incubation periods (Fig. 6b). Linear regression analysis demonstrated a strong positive association between incubation time and pollen germination frequency, with R^2 values of 0.8941 for bee bread pollen and 0.9107 for honey pollen (Fig. 6c, d). The corresponding regression equations were y = 1.0906x for BB pollen and y = 0.893x for HN pollen. Thus, the regression models explained 89.41% and 91.07% of the variation in germination frequency, respectively.

The mean germination frequency was consistently higher in pollen recovered from bee bread than in pollen recovered from honey. For BB pollen, germination frequency ranged from 70.46% in *Acanthus ilicifolius* to 87.64% in *Heritiera fomes*, whereas HN pollen showed values ranging from 62.32% in *Sonneratia apetala* to 73.42% in *Coriandrum sativum*. The relative germination frequencies of the identified pollen taxa recovered from BB and HN are presented in Fig. 7. For all pollen taxa represented in both matrices, the mean germination frequency was higher in BB than in HN. The difference was particularly pronounced for *Heritiera fomes*, *Desmodium* sp., *Excoecaria agallocha*, *Coriandrum sativum* and *Avicennia* sp., whereas the difference was comparatively smaller for *Xylocarpus granatum* and *Acanthus ilicifolius*. As such, the higher germination performance of pollen recovered from BB, together with its generally higher viability, indicates that the bee-bread matrix may provide more favourable conditions for maintaining pollen viability and germination potential than honey.

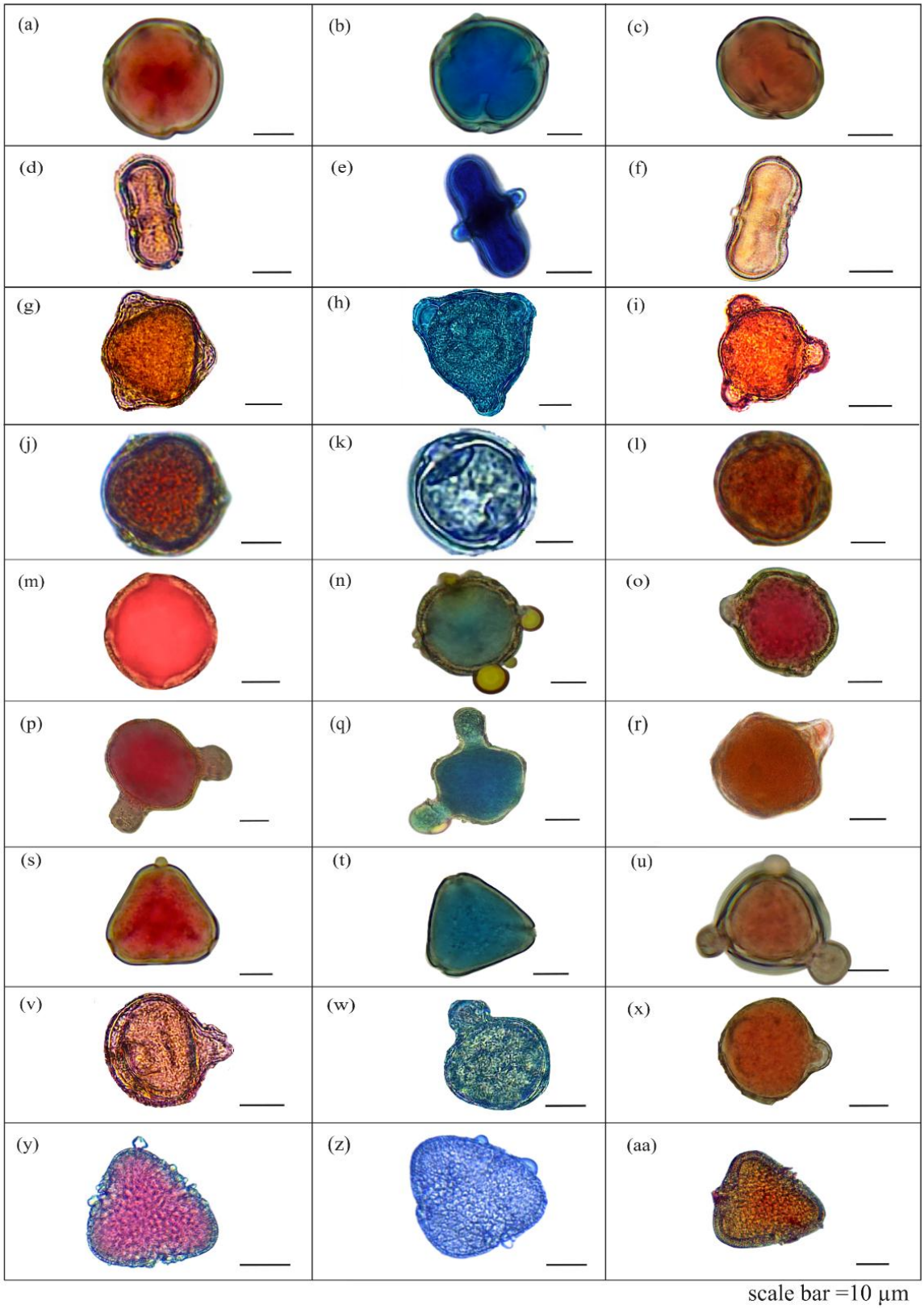


Fig. 5: Photomicrographs of pollen grains from 20 plant species stained with three cytochemical dyes for assessment of pollen viability, along with pollen germination and pollen tube formation. Each pollen type is represented under three staining protocols: **Acetocarmine** (left column), **Aniline Blue** (centre column), and **TTC [2,3,5-triphenyltetrazolium chloride]** (right column). **(a–c)** *Heritiera fomes*; **(d–f)** *Coriandrum sativum*; **(g–i)** *Sonneratia apetala*; **(j–l)** *Rhizophora apiculata*; **(m–o)** *Aegiceras corniculatum*; **(p–r)** *Ceriops decandra*; **(s–u)** *Bruguiera gymnorhiza*; **(v–x)** *Xylocarpus granatum*; **(y–aa)** *Bombax ceiba*.

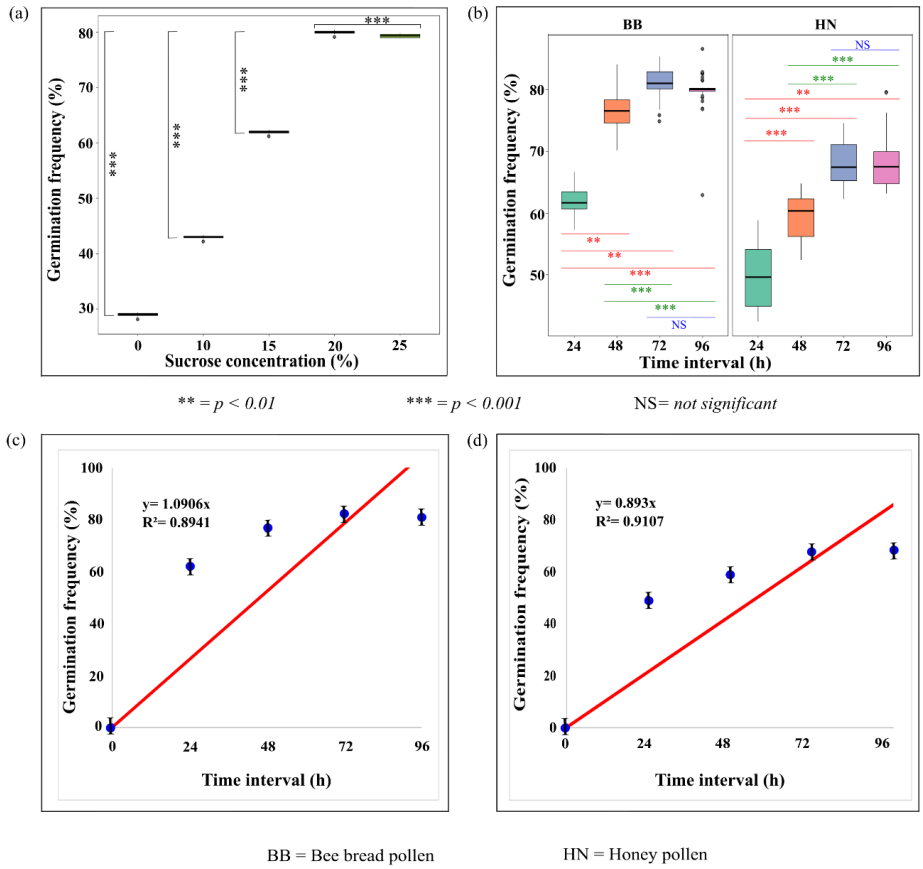


Fig 6: Effect of sucrose concentration and incubation time on pollen germination and comparative germination frequency of bee bread and honey pollen. **(a)** Germination frequency of pollen grains at different sucrose concentrations (0–25%). **(b)** Boxplots showing the temporal progression of germination frequency (%) at 24, 48, 72, and 96 h of incubation, with statistical significance brackets indicating pairwise comparisons among incubation periods. **(c–d)** Linear regression between incubation time and germination frequency for bee bread (BB) and honey (HN) pollen, respectively.

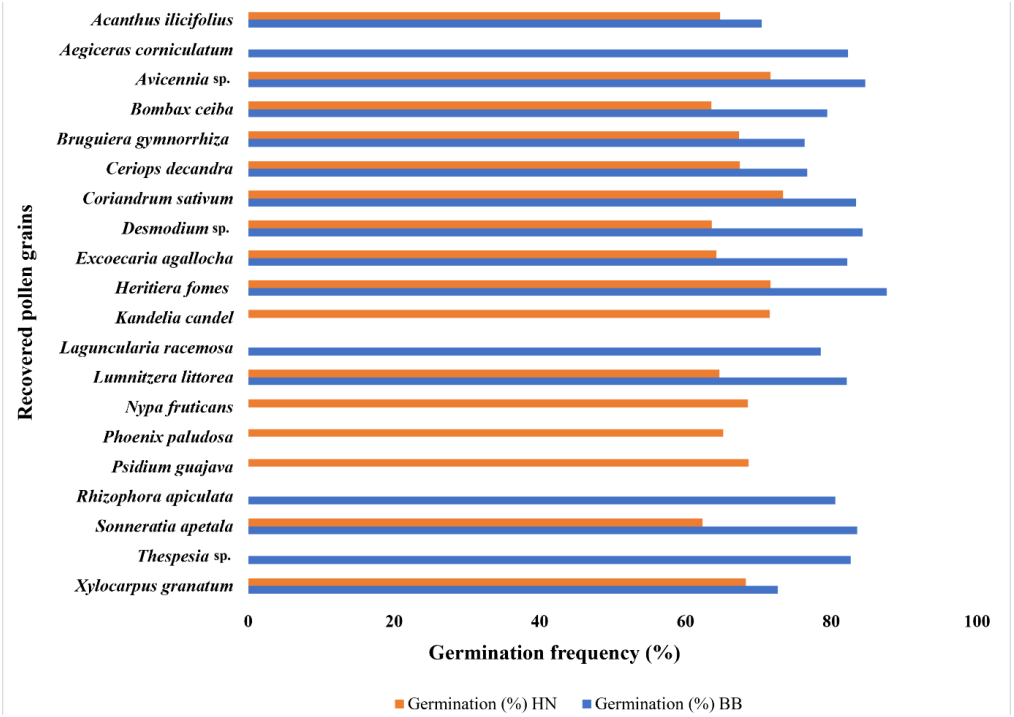


Fig. 7: Comparative germination frequency of identified pollen taxa recovered from bee bread and honey samples.

DISCUSSION

Conventional pollen preservation methods include chemical fixation, refrigeration, freezing, desiccation and cryopreservation, with the choice of method depending on whether the primary objective is preservation of morphological integrity, physiological viability or genetic material (Aguilera & Karel, 1997; Grizzle *et al*., 2008; Pegg, 2009; Huang & Yeung 2015; Parihar, 2023). Cryopreservation at −196 °C is widely regarded as an effective approach for long-term pollen storage when pollen moisture content is appropriately controlled. Excessive moisture may promote ice-crystal formation, whereas excessive dehydration can damage cellular membranes and reduce viability (Dinato *et al*., 2021). In addition, conventional preservation approaches may require specialised infrastructure, controlled environmental conditions and continuous maintenance. These limitations highlight the importance of exploring naturally occurring, low-cost systems that may contribute to the maintenance of pollen quality. In this context, bee-derived matrices such as bee bread and honey provide an interesting natural system for investigating the persistence of pollen viability and germination potential under hive-associated conditions.

The present analysis recovered 20 pollen taxa belonging to 12 families from bee bread and honey collected from different localities of the Indian Sundarbans. The assemblages included mangrove taxa such as *Acanthus ilicifolius, Aegiceras corniculatum, Avicennia* sp., *Bruguiera gymnorhiza, Ceriops decandra, Excoecaria agallocha, Heritiera fomes, Kandelia candel, Rhizophora apiculata, Sonneratia apetala* and *Xylocarpus granatum*, together with cultivated and associated taxa such as *Coriandrum sativum, Desmodium* sp., *Bombax ceiba* and *Psidium guajava*, reflecting the broad foraging landscape available to honey bees. The occurrence of taxa differed among localities and between bee bread and honey, indicating that the two hive products provide overlapping but not identical records of floral visitation. The presence of pollen taxa restricted to one matrix may reflect differences in the biological origin and accumulation of pollen in bee bread and honey. Bee bread represents pollen actively collected by worker bees and stored in comb cells, whereas pollen in honey may enter the product during nectar collection and subsequent hive processing. Consequently, the pollen spectra of the two matrices need not be identical. Differences in pollen production, nectar availability, flowering phenology and bee foraging behaviour may further contribute to the contrasting pollen assemblages.

The diversity of pollen types recorded in the present study is relevant to the interpretation of pollen preservation because the taxa differed considerably in their subsequent viability and germination responses. Mean viability values obtained using Aniline Blue, Acetocarmine and TTC were generally high, but the magnitude varied among pollen taxa and between the two matrices. For example, *Heritiera fomes, Desmodium* sp., *Coriandrum sativum* and several other taxa showed relatively high viability in bee bread, whereas *Acanthus ilicifolius* consistently showed comparatively lower viability in bee bread. Conversely, *Acanthus ilicifolius* and *Xylocarpus granatum* showed higher mean viability in honey than in bee bread across the three staining methods. These taxon-specific responses indicate that the effect of the storage matrix is not uniform across all pollen types and may depend on intrinsic pollen characteristics.

The three staining procedures used in the present study should be regarded as indirect indicators of pollen viability rather than direct measures of reproductive competence. Positive staining reflects particular aspects of cellular or physiological integrity, but staining alone does not establish the ability of pollen to germinate and successfully participate in fertilization (Stokes & Geitmann, 2025). Aniline Blue is particularly useful for detecting callose associated with pollen germination and pollen tube development (Currier, 1957; Heslop-Harrison, 1987), whereas Acetocarmine provides an indication of cytological integrity through staining of nuclear and cytoplasmic components (Stanley & Linskens, 1974; Shivanna & Rangaswamy, 1992). TTC staining provides a physiological indicator based on the reduction of tetrazolium salts by metabolically active cells and associated dehydrogenase activity (Lakon, 1949; Shivanna & Heslop-Harrison, 1981). Thus, the three methods provide complementary rather than interchangeable information about the condition of stored pollen.

Importantly, the present results do not support the conclusion that one staining method was universally superior to the others. In bee bread, no statistically significant difference was detected among the three staining methods, whereas significant differences were observed among staining methods in honey pollen, particularly between Aniline Blue and Acetocarmine and between Aniline Blue and TTC. The comparisons between the two matrices further showed significantly higher viability in bee bread than in honey for all three staining methods, with the difference being particularly strong for TTC. Therefore, the statistical results suggest that the storage matrix had a stronger and more consistent influence on pollen viability than the choice of staining method, although the magnitude of the staining response varied among taxa and matrices. This interpretation is more consistent with the heatmap and box-plot results than the earlier conclusion that Aniline Blue was universally the best staining method.

The taxon-specific patterns observed in the heatmaps further support this interpretation. The colour gradients varied not only between bee bread and honey but also among Aniline Blue, Acetocarmine and TTC within each matrix. In bee bread, *Bruguiera gymnorhiza* and *Ceriops decandra* showed comparatively lower viability profiles than several other taxa, whereas in honey *Sonneratia apetala* displayed noticeable variation among the three staining methods. Conversely, *Acanthus ilicifolius* and *Xylocarpus granatum* showed relatively higher viability in honey than in bee bread across the three staining approaches. These patterns indicate that pollen response to storage conditions is taxon-dependent and that no single staining method completely captures all aspects of pollen physiological status.

The consistently higher viability observed for many taxa in bee bread raises the possibility that the bee-bread matrix provides conditions favourable for maintaining pollen integrity. Bee bread is formed when worker bees collect pollen and incorporate nectar and glandular secretions before compacting it into comb cells. Subsequent microbial activity, particularly involving lactic acid bacteria, contributes to fermentation and changes in pH and other physicochemical properties (Herbert & Shimanuki, 1978). Such conditions may restrict the proliferation of undesirable microorganisms and contribute to the preservation of the stored pollen. However, the present study did not directly measure microbial communities, fermentation intensity, pH, water activity, antioxidant activity or biochemical degradation. Therefore, fermentation should be regarded as a plausible mechanism rather than a demonstrated explanation for the higher viability observed in bee bread.

Honey represents a substantially different storage environment. Pollen occurring in honey is not stored as a dedicated pollen reserve but may become incorporated during nectar collection and hive processing. The high sugar concentration and low water availability of honey can restrict microbial growth, while its acidic environment and other chemical properties may affect the physiological condition of entrapped pollen (Bogdanov *et al*., 2008). The present results suggest that pollen retained sufficient structural or cytochemical integrity to produce relatively high staining responses in honey, but its germination potential was generally lower than that of pollen recovered from bee bread. This distinction is important because it demonstrates that positive viability staining does not necessarily translate into equivalent germination competence.

The *in vitro* germination experiment provided a functional assessment that complemented the staining-based viability estimates. Germination increased with incubation time and reached its maximum at 72 h, followed by a slight decline at 96 h. The 20% sucrose medium produced the highest germination response under the conditions tested and was therefore selected for subsequent experiments. Linear regression showed strong positive associations between incubation time and germination frequency, with R^2 values of 0.8941 for bee bread pollen and 0.9107 for honey pollen. These values indicate that approximately 89.41% and 91.07%, respectively, of the variation in germination frequency was explained by the fitted regression models. The regression results therefore support a strong association between incubation duration and germination response over the experimental period, although the slight decline at 96 h indicates that the relationship should not be interpreted as indefinite or continuously increasing germination.

A clear difference between the two natural matrices was evident in the germination results. Mean germination frequency was higher for bee-bread pollen than for honey pollen across the taxa represented in both matrices. In bee bread, germination ranged from 70.46% in *Acanthus ilicifolius* to 87.64% in *Heritiera fomes*, whereas honey pollen ranged from 62.32% in *Sonneratia apetala* to 73.42% in *Coriandrum sativum*. The differences were particularly pronounced for *Heritiera fomes, Desmodium* sp., *Excoecaria agallocha* and *Bombax ceiba*, whereas the difference between the two matrices was comparatively smaller for *Xylocarpus granatum*. These taxon-specific differences again indicate that pollen response to natural storage conditions is not uniform.

The difference between staining-based viability and *in vitro* germination is particularly informative. In several taxa, viability estimates were substantially higher than germination frequencies. This is expected because staining methods detect selected aspects of cellular integrity or metabolic activity, whereas germination provides a more functional measure of the ability of pollen to initiate pollen-tube growth under the experimental conditions. Consequently, a pollen grain may retain sufficient structural or metabolic characteristics to stain positively while having reduced capacity to germinate. The present findings therefore support the use of both staining-based viability assessment and *in vitro* germination when evaluating the biological condition of naturally stored pollen.

The total pattern observed in this study suggests that bee bread has considerable potential as a natural matrix for maintaining pollen viability and germination competence. However, the results should not be interpreted as definitive evidence that bee bread acts as a long-term pollen preservation system. The study was based on naturally occurring pollen recovered from bee bread and honey and did not include freshly collected pollen as an initial control or a controlled time-course experiment in which the same pollen taxa were stored under defined bee-bread and honey conditions. Therefore, the observed

differences demonstrate an association between matrix and pollen condition, rather than directly establishing a causal preservation effect. Controlled experiments involving freshly collected pollen, defined storage periods, physicochemical characterization, microbial profiling and repeated viability and germination measurements would be necessary to determine the mechanisms and temporal stability of pollen preservation in bee bread.

Finally, the species-specific differences observed in both viability and germination indicate that bee bread should not be considered a uniformly effective natural preservative for all pollen taxa. Some pollen types, such as *Heritiera fomes* and *Coriandrum sativum*, maintained relatively high viability and germination responses, whereas others showed lower or contrasting responses between the two matrices. *Acanthus ilicifolius* and *Xylocarpus granatum*, in particular, exhibited comparatively unusual patterns in which honey-associated pollen showed higher viability than bee-bread pollen despite the overall trend favouring bee bread. These findings emphasize that the interaction between pollen characteristics and storage matrix should be considered on a taxa-specific basis. Thus, the Sundarbans bee-bread system provides a promising natural model for studying pollen preservation, while also highlighting the need for species-specific evaluation of pollen viability and germination.

CONCLUSION

The present study from the Sundarbans mangrove ecosystem provides baseline evidence of the diversity, viability and germination potential of pollen naturally preserved within bee bread and honey. Pollen recovered from bee bread generally exhibited higher viability and *in vitro* germination potential than pollen recovered from honey, suggesting that bee bread provides a more favourable natural environment for maintaining pollen physiological integrity and germination capacity. The study also revealed considerable pollen diversity associated with mangrove and surrounding vegetation, with taxon-specific differences in viability and germination between the two matrices. These findings highlight the potential of bee bread as a natural biological archive for investigating pollen biology, bee–plant interactions, pollination ecology, floral-resource availability and biodiversity, while also providing a promising basis for future research on natural pollen preservation and *ex situ* pollen conservation. Further controlled studies using freshly collected pollen and defined storage periods are needed to determine the long-term stability of pollen in bee bread and to elucidate the factors contributing to its preservation.

Credit Statement

Nirmallya Ghosh (NG): Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft. MD Ashfaque Molla (AM): Data curation, Methodology; Formal analysis; Investigation. Priyasi Ray (PR): Data Curation, Methodology; Formal analysis; Investigation. Radhakanta Nandi (RN): Data Curation; Methodology; Formal analysis; Investigation. Validation; Visualization; Writing – original draft. Sarupa Barman (SB): Data curation; Validation; Visualization; Writing – original draft. Anisha Satpati (AS): Data curation; Validation; Visualization; Writing – original draft. Sudha Gupta (SG): Conceptualization; Resources; Supervision; Validation; Visualization; Writing – review & editing.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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