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Field Conservation of Fresh Pollen for Rapid Ecological Studies of Pollination Biology

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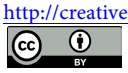

Abstract

Pollen is a resistance structure whose shape, size, and ornaments together make up a diagnostic method for species identification, comparable to a fingerprint. Traditional methods for cleaning this grain, such as acetolysis, not only seriously damage the pollen material of various botanical groups, but its use requires access to expensive reagents and equipment. Therefore, a low-cost proposal is presented for the fixation of fresh pollen in the field, whose preparations are suitable for depositing in biological reference collections, and can be reviewed with an optical microscope.

Subject Areas

Plant Science

Keywords

Biological Collections, Light Microscopy, Palynology, Pollinators

1. Introduction

The pollen consists of a resistance structure whose function is to protect the sperm nuclei of the plant; its external wall, called exine, is made of the natural polymer sporopollenin, which reacts to some environmental factors such as temperature and pH, allowing the generative cells to invade the stigma and fertilize the ovules of the flower [1].

This causes these same environmental factors to destroy the generative cells of the grains that do not reach the flower, but keeping the exine intact, which is why Erdtman [2] defines palynology as the study of this structure, considering its shape, ornaments, and thickness. As a fingerprint of each plant species and proposes acetolysis, a cleaning method that has been widely used by palynologists around

the world as a classic working method.

However, the grains of some plant species are seriously damaged by this and other traditional chemical processes used to remove the protein cover of the exine and eliminate the generative cells, such as some species of the Zingiberales group, within which the genus *Heliconia* (Heliconiaceae) has grains that are easily destroyed by the acetolysis method [3] [4].

Other families show slight damage to the exine, such as the species of Droseraceae [5], Araceae [6], Bromeliaceae, Cactaceae, and some whose grains have dispersion units of the tetrad and polyad type, such as Annonaceae [7], Fabaceae [8], Mimosaceae [9] and some genera of Rubiaceae [10] and Cucurbitacea [11].

This is a problem when carrying out pollination ecology studies, since valuable information is lost when processing samples, as well as it is important to have a laboratory and a series of reagents and equipment with high economic value that not all researchers or institutions have available provision.

This work aims to propose a fresh pollen fixation technique based on a glycerinated gelatin recipe that can be easily used in the field, and whose final preparations are suitable for long-term conservation in biological collections.

2. Methodology

2.1. Collection Site

Two collections were made in two different points of the Massif of Death, which is located in the Talamanca mountain range, between the Provinces of San José and Cartago, Costa Rica. The vegetation cover corresponds to the Mooreland ecosystem, with an altitude of 3200 - 3400 meters above sea level. The landscape presents a high richness of plant species, but a high dominance of *Chusquea subtessellata* (Poaceae). Considering that the paramo ecosystem of the Massif is constant, the species that were collected in Site 1 were not repeated during the collection in Site 2.

Site 1: Station of the Costa Rican Institute of Electricity (ICE), in the Province of San José, at 9˚33'13"N and 83˚45'31"W. December-2017. Rec. M. Sánchez-Ocampo.

Site 2: Sákira Hill, located on the border line between the Provinces of Cartago and San José, at 9˚35'32"N and 83˚45'27"W. March-2018. Rec. M. Sánchez-Ocampo.

2.2. Collection and Research Permits

The samples of tall plants were taken during a field trip of the project "Documentation and Biological Characterization of the Moorelands of Costa Rica", approved by resolutions 045-2017-ACC-PI and ACC-PI-100-2018 of the National System of Conservation Areas (SINAC) and the samples were collected using the digital scientific passports SINAC-ACC-PI-R-045-2017 and SINAC-ACC-255-2018.

2.3. Voucher Samples Deposit

The witness samples were deposited in the National Museum of Costa Rica, as part of the hymenoptera voucher collection and as a single specimen made up of samples from each site. The already prepared fixed slides were labeled using ad-

hesive craft paper labels and stored in cardboard envelopes and kept so that the coverslip was face down.

2.4. Preparation of Basic Fuchsin

- 0.5 g basic fuchsin powder ($C_{20}H_{20}N_3HCl$/mm = 337.86 g/mol).
- 20 ml of 96% ethanol (C_2H_5OH/mm = 46.07 g/mol).
- 3.5 ml of pure Xylol (C_8H_{10}/mm = 106.16 g/mol).
- 4.95 ml of distilled water.

This preparation does not require applying heat, but all the ingredients are mixed in the same container, preferably glass, beginning by dissolving the fuchsin powder in the alcohol, sifting it slowly (or sifting it slowly) and mixing with a glass stirrer, to avoid the formation of lumps. Then, the Xylol is added and stirred until homogeneous, and finally, the distilled water at the same point. It is stored in an amber glass bottle with a tight lid. It can be dispensed in smaller glass bottles, also amber in colour, and is fitted with a stopper or glass dropper for ease of use. Xylol can be replaced with Phenol or Thimol.

2.5. Preparation of Glycerinated Gelatin

- 1.40 ml of distilled water.
- 2.15 ml of glycerol ($C_3H_8O_3$/mm = 92.094 g/mol).
- 3.10 g unflavored gelatin powder.
- 4.3 ml of previously prepared basic fuchsin.
- 5.1 ml syringes with stopper (those used for insulin).

To prepare the gelatin, the water was first heated in a glass beaker in a water bath to avoid possible damage resulting from direct heat. Then, the gelatin powder was dissolved (it can also be in sheets), the glycerol was slowly added, stirring with a glass stirrer until incorporated. Finally, fuchsin was added, stirring at the same time as it was incorporated drop by drop (20 drops = 1 ml). Finally, while the preparation was still hot, the syringes were filled and immersed in ice water to accelerate the cooling of the gelatin.

2.6. Cleaning and Mounting of Pollen

The pollen was directly wiped onto the microscope slide by placing the pollen with the tip of a needle and adding a drop of white vinegar (5% acetic acid/CH_3COOH/mm = 60.052 g/mol) on it, then gently shaken with circular movements with the needle until all the lumps are dispersed. Next, with the help of a lighter, the slide was gently heated to evaporate the vinegar, and a small portion of the glycerinated gelatin was placed on the pollen, letting it melt with the heat, to finally place the upper slide. Once the jello cools, the edges should be sealed with clear nail polish to prevent air from entering.

2.7. Pollen Grain Photography

The review and photography of the pollen grains was carried out with an OMAX®

Microscope, an OMAX® 18 MP camera, and a slide micrometer. Grains were captured in polar and equatorial position at 40× magnification.

2.8. Fungal Growth Test

The sheets were placed from April 2018 to April 2021 on a shelf at an average ambient temperature of 25˚C - 26˚C with an average ambient humidity between 70% and 80%, and away from direct sunlight.

3. Results

In total, 23 successful samples were prepared in fixed slides (Table 1), 20

Table 1. Presence of pollen in slides.

Samples	Slides Final Register		
	Species	Site Number	Success
1A	*Vibrum costarricanum*	1	Viable
2A	*Cirsium subcoriaceum*	1	Viable
3A	*Diplostemum costarricense*	1	Viable
4A	*Gnaphalium roseum*	1	Viable
5A	*Senecio orstedianum*	1	Viable
6A	*Sigesbeckia agrestis*	1	Viable
7A	*Valeriana prinophylla*	1	Viable
8A	*Pernettya prostrata*	1	Viable
9A	*Trifolium repens*	1	Viable
10A	*Acaena cylindrisachya*	1	Viable
11A	*Solanum storkii*	1	Viable
12A	*Argeratina anisochroma*	1	Viable
Null	*Prunella vulgaris*	1	No Pollen
Null	*Hydrocotyle ribifolia*	1	No Pollen
Null	*Stereocaulon ramulosum*	1	No Pollen
1B	*Bomarea costarricensis*	2	Viable
2B	*Argeratina anisochroma*	2	Viable
3B	*Gaultheria gracilis*	2	Viable
4B	*Hyepricum costarricense*	2	Viable
5B	*Huperzia talamancana*	2	Viable
6B	*Lycopodium cotigum*	2	Viable
7B	*Lycopium thyoides*	2	Viable
8B	*Chaetolepis cufodontisii*	2	Viable
9B	*Chusquea subtessellata*	2	Viable
10B	*Arcytophyllum lavarum*	2	Viable
11B	*Castilleja irazuensis*	2	Viable
Null	*Diplostephium schultzii*	2	No Pollen

corresponding to pollen grains and 3 to Lycopodiaceae spores (Figure 1 and Figure 2). Four of the samples collected at the first site corresponding to the species *Argeratina anisochroma* (Asteraceae), *Prunella vulgaris* (Lamiaceae), *Hydrocotyle ribifolia* (Apiaceae) and *Stereocaulon ramulosum* (Stereocaulaceae) did not present pollen grains, and only the first could be collected again on Site 2 and get a workable mount to work with. Finally, the species *Diplostephium schultzii* (Asteraceae) was only found in Site 2, however, it was not possible to mount a viable sample, because the flowers found were already dry and did not contain pollen.

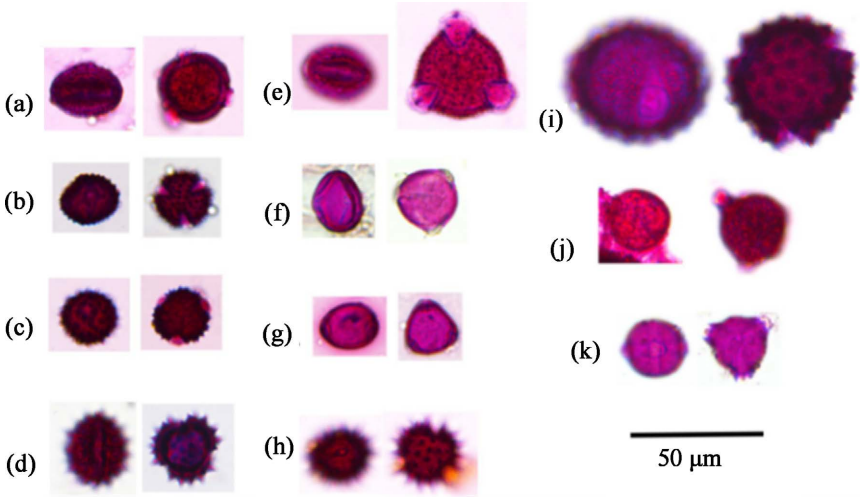

Figure 1. Species collected at Site 1. *Vibrum costarricanum* (a), *Diplostemum costarricense* (b), *Gnaphalium roseum* (c), *Senecio orstedianum* (d), *Valeriana prinophylla* (e), *Trifolium repens* (f), *Valeriana prinophylla* (g), *Sigesbeckia agrestis* (h), *Cirsium subcoriaceum* (i), *Acaena cylindrisachya* (j), *Solanum storkii* (k).

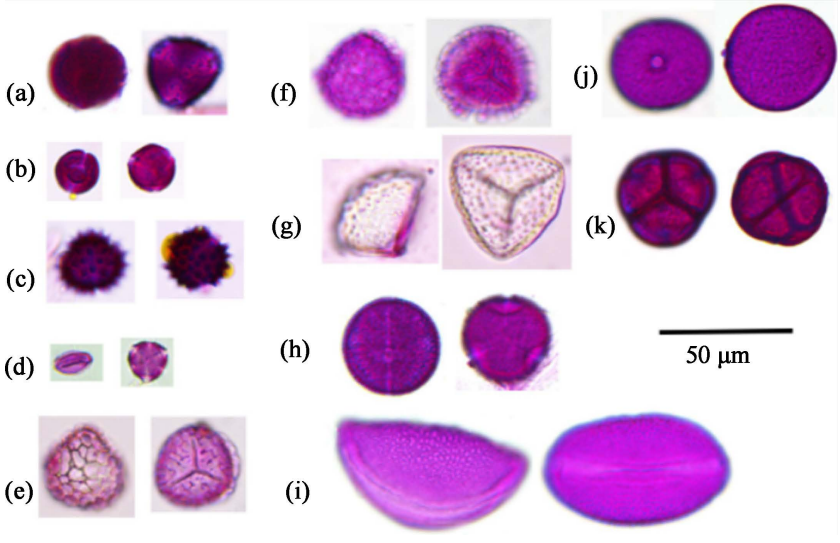

Figure 2. Species collected at Site 2. *Castilleja irazuensis* (a), *Hypericum costarricense* (b), *Argeratina anisochroma* (c), *Chaetolepis cufodontisii* (d), *Lycopium thyoides* (e), *Lycopodium cotigum* (f), *Huperzia talamancana* (g), *Arcytophyllum lavarum* (h), *Bomarea costarricensis* (i), *Chusquea subtessellata* (j), *Gaultheria gracilis* (k).

After the test time to verify the growth of fungi in the slides, no growth of the same was found in any of the samples, nor alteration in the structure of the pollen grains.

4. Discussion

The method for dispensing fuchsin in the field with syringes greatly facilitated the work, avoiding the loss of pollen that can sometimes occur when handling or storing some flowers in bags. On the other hand, the grains are perfectly clean and viable for photography, without the need to apply Erdtman Acetolysis [2], which saves many economic resources, mainly in terms of equipment and reagents, and avoids the need to mount a lab, or access one.

However, it is necessary to generate specific pollen collections by sites or regions, since many families have very similar morphologies that can create confusion, especially within the Asteraceae family.

On the other hand, although Xylol gave very good results in preventing pollen growth from 2018 to the present, in tests subsequent to the execution of this methodology, it was replaced with Herb.io® brand oregano essential oil because it contains Thimol, and in the same way as with Xylol, there was no fungal growth, so both reagents are suitable as a preservative for glycerinated gelatin.

5. Conclusions

We concluded that the field mounting method is completely feasible for rapid pollination ecology studies, providing the following advantages for researchers:

1) Samples with clear grains visible under an optical microscope.

2) Shorter sample processing time.

3) Conservation of samples in scientific collections without generating fungi even in natural environmental conditions.

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Conflicts of Interest

The authors declare no conflicts of interest.

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