OPTIMIZATION OF THE POLLEN EXTRACTION PROCESSFOR IMPROVEMENT OF IDENTIFICATION

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Abstract: Forensic palynology uses microscopic evidence that is resistant to external influences and displacement from the scene. Most suspects may overlook pollen as evidence because they do not know its importance. Pollen provides a myriad of opportunities in forensics: determining the time of year, locality, primary and secondary event venue, connecting participants in a single criminal event, etc. Pollen analysis consists of determining the species and estimating the percentage that each plant species represents in the evidence sample. All methods require preliminary preparation (pollen extraction). A couple of methods for pollen extraction are used, but they are usually either suitable for large samples or require expensive equipment. We tried to optimize some of the existing methods to improve yield. Best results have been achieved using water incubated samples combined with modified acetolysis. Further development and implementation of forensic palynology depend on the simplified procedure and involvement of forensic botanists.

Keywords: forensic palynology, identification, extraction optimization, microscopy

INTRODUCTION

It has been established that trace evidence can be very important in forensic investigations and pollen evidence in particular since it can provide valuable temporal information. Most of the time, the cases where pollen evidence was used represent cases of identification of a crime scene based on the pollen assemblages present on a suspect or victim or refuting an alibi. Palynology, which is the study of pollen and spores in an archaeological or geological context, has become a well-established research tool leading to many significant scientific developments. The term palynomorph includes pollen of spermatophytes, spores of fungi, ferns, and bryophytes, as well as other organic-walled microfossils,

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such as dinoflagellates and acritarchs (Babcock & Warny, 2014). Pollen is the male fertilizing agent of flowering plants, trees, grasses, and weeds. It is also a major allergen that causes symptoms of seasonal allergic rhinitis. While most people think of pollen only in terms of allergies, its story is much more interesting. Pollen carries the male sex cells from one plant to another of the same species. Pollination presents the precursor to fertilization and allows gene flow among plants.

Today, palynology is a tool that is necessary for various applied sciences such as systematics (Dransfield et al., 2008; Doyle and Endress, 2010), melissopalynology (Jones & Bryant 1996), and forensics (Mildenhall et al. 2006; Bryant 2014; Weber and Ulrich 2016), but should also be considered a basic, stand-alone scientific field. In general, compared to the sporophyte, the male gametophyte in seed plants is investigated rather poorly. Out of c. 260.000 to 422.000 plant species (Thorne, 2002; Govaerts, 2003; Scotland & Wortley, 2003) - The Plant List currently accepts 350.699 species - only about 10% have been studied for pollen grain morphology and an even smaller percentage regarding pollen ultrastructure. Therefore, we can conclude that it is of great importance to continue classical and more advanced palynological studies (Halbritter et al., 2018).

Pollen grains are utilized for forensic purposes because they are exceptionally impervious to chemical attacks. They can remain at a crime scene for a long time after the event under investigation has happened. Furthermore, they give one source of regularly moved material, which is frequently traded inside the setting of an exchange of mud, soil, and/or residue particles (Mildenhall et al., 2006). Moreover, pollens can also be transferred by direct contact with a part of a plant containing spores or pollen. Pollen grains are ideal forensic trace materials since they are small, highly variable and found on things that have been exposed to air, or interact with it. Pollen isolation from most things can be accomplished by submitting samples for forensic examination. Such samples may include soil, ropes and twines, clothing and fabrics, drugs, air filters, plant material, and animal and human material, such as fur, hair and stomach contents (Milne et al., 2005; Alotaibi et al., 2020)

RESEARCH

Scientific pollen studies have resulted in important breakthroughs not only in the world of forensic palynology but in the understanding of the history of Earth's vegetation as well. By using fossilized palynomorphs retrieved from sediment cores off the coast of Antarctica, geologists have been able to reconstruct the continent's climate from millions of years ago (Warny et al., 2009). Pollen's usefulness in forensic investigation, as well as the reconstruction of Earth's past, comes from its unique characteristics. Since it is small and produced in great numbers, it can be picked up by items associated with a crime (such as clothing, tools, or other objects) and transported without a perpetrator being aware of it, let alone being able to eliminate it (Babcock & Warny, 2014). Some types of pollen grains have characteristic hook-like structures on their exine wall that can attach themselves to the legs of pollinators, which lets them travel long distances even more easily. Just as every geographic location has its specific vegetation and ecosystem, it also possesses a unique pollen "fingerprint", allowing palynologists to ascertain where certain objects have been.

Pollen has had numerous applications in forensic crime investigation, starting in the 1930s in North America when samples of honey were studied in order to differentiate between the various types and their origins (Auer, 1930). Another one began in the 1950s in an Austrian murder mystery. A man went missing while travelling. The mud on the suspect's boots contained 20-million-year-old fossilized pollen grains that could have come only from a specific area of the Danube River. After seeing the

evidence, the suspect confessed to the crime and led the police to the body, which was exactly where the pollen suggested it would be found (Alotaibi et al., 2020)

Experimental pollen studies demonstrated certain inconsistencies in the persistence of tulip, lily, and daffodil pollens when exposed to high temperatures for periods between 0.5 min and 24 h. It was possible to identify all three pollen types after 30 min of exposure to 400°C. After shorter time frames the threshold for successful identification was 700°C after 0.5 min for all pollen types tested, and 500°C for lily and daffodil after a 5-minute heat exposure. For times longer than 18 h, all three pollen types were discovered to continue in a suitable structure for identification at 50°C (tulip), 200°C (daffodil), and 300°C (lily). These results proved the value and importance of seeking and collecting pollen as evidence even from extreme crime scenes such as vehicular fires (Morgan, Flynn, Sena & Bull, 2014)

METHOD AND MATERIALS

In this experiment, the process of acetolysis for pollen extraction was optimized to allow better extraction from a small sample. The species used in this experiment is *Delphinium Balcanicum*. This is one of the endemic species found in the Pčinja-Vražji Kamen area, in the southern part of Serbia.

Delphinium is a genus of about 300 species of perennial flowering plants in the family *Ranunculaceae*, native to the Northern Hemisphere, as well as the high mountains of tropical Africa.

All members of the genus *Delphinium* are toxic to humans and livestock.

The leaves are deeply lobed with three to seven toothed, pointed lobes in a palmate shape. The main flowering stem is erect, and varies greatly in size between the species, from 10 centimeters in some alpine species, up to 2 meters tall in the larger meadowland species.

Image 1. Flowers of Delphinium Balcanicum

In June and July (Northern Hemisphere), the plant is topped with a raceme of many flowers, varying in color from purple and blue, to red, yellow, or white. In most species, each flower consists of five petal-like sepals which grow together to form a hollow pocket with a spur at the end, which gives the plant, usually more or less dark blue, its name. Within the sepals are four true petals, small, inconspicuous, and commonly colored similarly to the sepals. The eponymous long spur of the upper sepal encloses the nectar-containing spurs of the two upper petals. (https://www.britannica.com/plant/Ranunculales, https://www.wildflowers-and-weeds.com/Plant_Families/Ranunculaceae.htm)

Class: Magnoliopsida

Ordo: Ranunculales

Familia: Ranunculaceae

Subfamilia: Ranunculoideae

Genus: Delphinium

Species: *Delphinium Balcanicum*

In this experiment we used 5 individual plants of *D. Balcanicum* and 5 flowers from each plant. We removed anthers from all the flowers using tweezers and distributed the pollen equally into 6 tubes (3 tubes as 3 samples for standard acetolysis and 3 tubes for optimized acetolysis). In the following section, we will take a brief look at standard acetolysis, as well as the optimized one.

The standard acetolysis procedure:

1. The anthers are preserved either in 70% aqueous solution of acetic acid or 70% ethyl alcohol.
2. The anthers are transported from the solution (aqueous solution of acetic acid or ethyl alcohol) to a centrifuge tube and washed with distilled water. The anthers are then transported to the watch glass and crushed with a glass rod. The crushed anthers are transported to the new tube with distilled water and the mixture is filtrated through cheesecloth. The mixture that passes through the cheesecloth contains washed pollen.
3. A freshly prepared acetolysis solution consisting of acetic anhydride and concentrated H_2SO_4 in the ratio of 9:1 (v/v) is poured in the centrifuge tube containing washed pollen. Before the solution is poured into the centrifuge tube with washed pollen, distilled water is discarded and glacial acetic acid is added. The acetolysis solution reacts violently with water. Glacial acetic acid wash is necessary to replace water. After washing the pollen with glacial acetic acid the acetolysis solution is added.
4. The centrifuge tube containing pollen in the suspension of the acetolysis solution (v/v) is placed in a water bath (Vims Electric, WKP-9) and heated at 70°C for 5-10 min, while the contents are stirred with a glass rod intermittently.
5. Acetolyzed pollen suspension is centrifuged and the supernatant liquid is decanted. (Bio-Rad Mini Centrifuge, #1660603,6000 rpm)
6. Pollen sediment in a centrifuge tube is immersed in glacial acetic acid for a few minutes and the superfluous liquid is decanted after 5 min of centrifugation (6000 rpm). This is washed with distilled water 2-3 times.
7. The pollen material is ready for mounting on the slides for microscopic examination. Alternatively, it can be stored in this condition in vials containing 50% glycerine.
8. Acetolyzed pollen material is mounted in glycerine jelly, polyvinyl alcohol, Canada balsam, etc.
9. Edges of the cover glass are sealed with paraffin wax.

Optimized procedure:

1. The anthers are preserved in 70% ethanol.
2. Anthers are transferred to a centrifuge tube with ethanol and centrifuged for 1 min. (6000 rpm)
3. After centrifugation, the top layer (supernatant) is decanted and pollen is emerged in water solution of acetic acid (70%; v/v) for a few min (2-5).

4. Pollen suspension with water solution of acetic acid is vortexed for 1 min. (Bio-Rad, BR-2000 Vortexer, #1660610)

5. The centrifuge tube that contains pollen and the water solution of acetic acid is pierced with a medical needle that was previously heated on a spirit lamp. Then the centrifuge tube with a pollen mixture is placed inside a new centrifuge tube and centrifuged for a few seconds (6000 rpm). This allows the acetic mixture with pollen to go through a hole into a new centrifuge tube.

6. The mixture is centrifuged for 3 min (6000 rpm) and the supernatant is decanted.

7. A freshly prepared acetolysis solution consisting of acetic anhydride and concentrated H_2SO_4 in the ratio of 9:1 (v/v) is poured in the centrifuge tube containing washed pollen.

8. The centrifuge tube containing pollen in the suspension of the acetolysis solution is placed in a water bath and heated at 75°C for 5-10 min, while the contents are stirred with a glass rod intermittently.

9. Acetolyzed pollen suspension is centrifuged and the supernatant liquid is decanted. (6000 rpm)

10. Pollen sediment in a centrifuge tube is immersed in glacial acetic acid for a few minutes and the superfluous liquid is decanted after 5 min centrifugation (6000 rpm).

11. After centrifugation, the supernatant is decanted and distilled water is added and centrifuged again for 2 min. (6000 rpm)

12. The supernatant is decanted again and the sample is dried at 65°C for 20 min in the incubator. (Vims Electronic, IT-33)

13. The pollen material is ready for mounting on the slides for microscopic examination.

14. Acetolyzed pollen material is mounted in glycerine jelly, polyvinyl alcohol, Canada balsam, etc.

15. Edges of the cover glass are sealed with paraffin wax.

RESULTS

As mentioned before, the optimized acetolysis process allows better extraction from a small sample. Better extraction is possible because the number of washings of the samples with acetic acid and distilled water is reduced to the minimum. This prevents excessive washing out of our samples, and it does not damage or have any bad effects on our pollen. Crushing anthers with a glass rod did not give expected results, since the anthers are too small, so very few of them are actually crushed because of this, and most of them tend to be difficult to handle due to their round shape, often causing them to end up outside the glass surface. Vortex and centrifugation break them faster and more easily. Filtration through cheesecloth gave a much smaller amount of pollen, since most of it stayed on the cheesecloth, so using a medical needle allowed us to filtrate more of our sample into the next tube. Bringing the drying step into optimized acetolysis allowed easier pollen collection from the tube with the glass rod or tweezers, since water residue was an obstacle for easier collection of our samples.

In the final results, the optimized process of acetolysis gave approximately 4 times more pollen per microscope field, than the standard acetolysis (Figure 1) (Figure 2).

Table1. Comparison of the standard and optimized acetolyses

<table>
<tr><th>Standard acetolysis</th><th>Optimized acetolysis</th></tr>
<tr><td>The anthers are preserved either in 70% aqueous glacial acetic acid or 70% ethyl alcohol.</td><td>The anthers are preserved in 70% ethanol</td></tr>
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	The supernatant is decanted again and the sample is being dried at 65°C for 20 min.
The pollen material is ready for mounting on the slides for microscopic examination. Alternatively, it can be stored in this condition in vials containing 50% glycerine. Acetolyzed pollen material is mounted in glycerine jelly, polyvinyl alcohol, Canada balsam, etc. Edges of the cover glass are sealed with paraffin wax.	The pollen material is ready for mounting on the slides for microscopic examination. Acetolyzed pollen material is mounted in glycerine jelly, polyvinyl alcohol, Canada balsam, etc. Edges of the cover glass are sealed with paraffin wax.

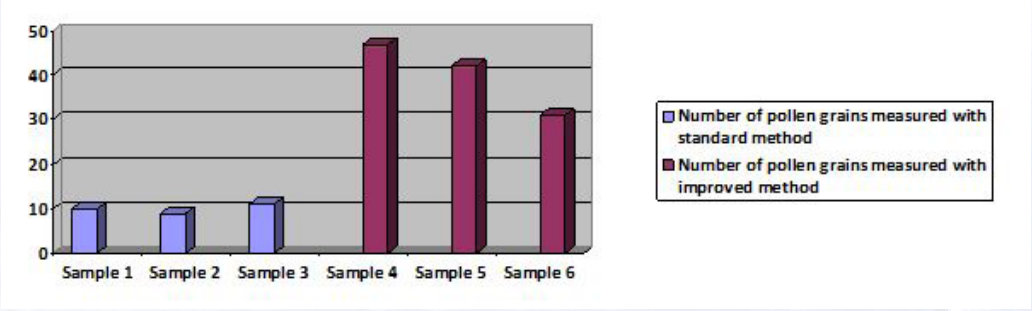

Figure 1. Comparison of number of pollen grains
Improved method in the legend refers to the optimized acetolysis method

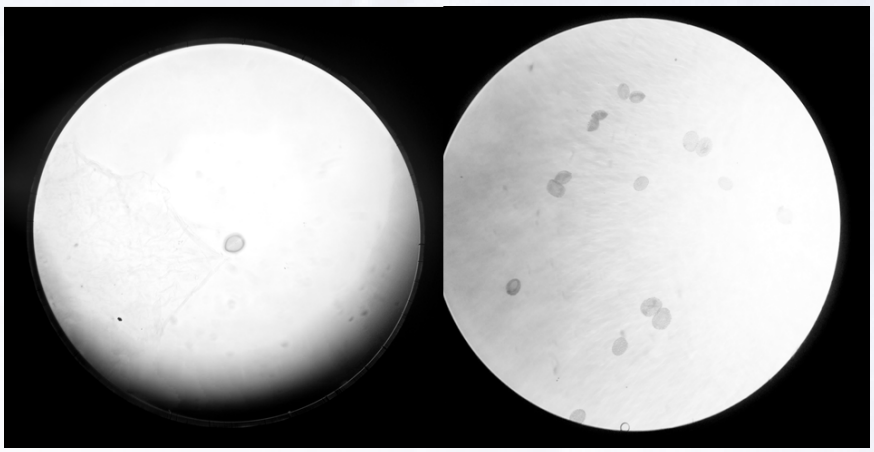

Figure 2. Pollen of Delphinium balcanicum under a microscope: left – standard acetolysis procedure; right – optimized acetolysis procedure

CONCLUSIONS

Pollen has been increasingly used in forensic investigations over the last couple of years and it is recognized as valid court evidence in countries such as New Zealand and the USA (Bryant 2014). Since sample quantities are usually limited, it is necessary to optimize the extraction process to ensure the highest possible yield. In this paper, we presented the optimized procedure of pollen extraction from one of the plant species that we treated in this experiment. In all of the samples, several times higher pollen yield was obtained. In the next phase, it is necessary to test the method on pre-prepared forensic samples, and finally on samples collected at the crime scene. We think there is a possibility of using this extraction method in forensic labs and since the ecosystems and vegetation have unique 'fingerprints', pollen investigation could have a significant place in common forensic and crime solving in the near future. However, for pollen identification to become a part standard forensic investigations we need to educate more crime scene investigators of its significance and to employ more botanists in forensic teams.

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