Solving adhesion questions. - The influence of different solvents on adhesive properties of *Ranunculus bulbosus'* pollen.

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Abstract

Although pollination has long been a topic of scientific research, the adhesion processes that regulate the transport of pollen from anther to stigma are poorly understood. A common assumption is that pollenkitt, a lipid-rich layer on the surface of pollen, acts as an adhesive to mediate the adhesion of pollen to insects and/or stigma. However, quantitative measurements on the influence of pollenkitt on pollen adhesion have been scarce. To provide more insight into the role of pollenkitt, the effects of different solvents on the adhesion of *Ranunculus bulbosus'* pollen were investigated in this thesis. For this purpose, pull-off forces of individual pollen from glass were measured by atomic force microscopy before and after washing the pollen in one of five different solvents. The results revealed that pollen adhesion is affected by solvents in complex and unexpected ways. Regardless of the solvent properties, there was a significant reduction in adhesion to a comparable level after only one minute of washing, with no apparent removal of pollenkitt. This questions the adhesion-related function and relevance of pollenkitt in this species and sheds light on other possible factors that could influence pollen adhesion.

Kurzzusammenfassung

Obwohl Bestäubung seit langem Gegenstand wissenschaftlicher Forschung ist, sind die Adhäsionsprozesse, die den Transport von Pollen von Anther zu Stigma regulieren, nur unzureichend verstanden. Eine gängige Annahme ist, dass Pollenkitt, eine lipidhaltige Schicht auf der Pollenoberfläche, als Klebstoff fungiert, um die Adhäsion von Pollen an Insekten und/oder das Stigma zu vermitteln. Quantitative Messungen zum Einfluss von Pollenkitt auf die Pollenadhäsion gibt es jedoch kaum. In dieser Arbeit wurden daher die Auswirkungen verschiedener Lösungsmittel auf die Adhäsion von Pollen des *Ranunculus bulbosus* untersucht. Zu diesem Zweck wurden die Abziehkräfte einzelner Pollen von Glas mittels Rasterkraftmikroskopie vor und nach dem Waschen der Pollen in einem von fünf verschiedenen Lösungsmitteln gemessen. Die Ergebnisse zeigten, dass die Adhäsion der Pollen auf komplexe und unerwartete Weise durch Lösungsmittel beeinflusst wird. Unabhängig von den Lösungsmitteleigenschaften kam es nach nur einer Minute waschen zu einer signifikanten Verringerung der Adhäsion auf ein vergleichbares Niveau, ohne dass der Pollenkitt erkennbar entfernt wurde. Dies stellt die adhäsionsbezogene Funktion und Bedeutung von Pollenkitt bei dieser Art in Frage und verlagert den Fokus auf weitere mögliche Faktoren, die die Pollenadhäsion beeinflussen könnten.

Contents

1. Introduction

Pollination is the mechanism of sperm cell transportation from male to female plant organs. The sperm cells are encapsulated into pollen grains and presented by the anther by most species. When a pollen arrives at the stigma of a matching individual, it fertilizes the plant by growing a pollen tube into the stigmatic tissue to the ovule where it releases the sperm cells (Kadereit et al., 2014). Despite a great diversity of species, the origin and destination of pollen are similar, but the strategies for transport are manifold. Known paths of pollen transportation are via wind (anemophily) and water flow (hydrophily) as well as by animals (zoophily), where insects (entomophily) are a major factor for angiosperm diversity (Kadereit et al., 2014). Even aquatic invertebrates have been reported to possess a role in pollination (van Tuessenbrok et al., 2016).

Pollination helps greatly with biological variety and, therefore, resilience against environmental changes by ensuring the exchange of genetic material through sexual recombination (Kadereit et al., 2014). It thereby provides the basis for plant reproduction which makes it an important process for our planet's ecosystem. Understanding pollination plays a vital role for our food chain as well, because as the world population grows steadily, the need for more efficient food production does as well. Some of the most important food producing plants like wheat and rice are anemophile (Cramer, 1967), but the vast majority of plants are pollinated by insects (Willmer, 2011). New findings in entomophily could help us to counter the effects of the global decrease in insect population (Kearns, 1998), which is crucial since many food plants like apple- and banana-trees rely on insect pollination for healthy growth. In addition to that, pollen are major allergens that pose a global threat to human health (Wüthrich, 1989; v. Mutius, 2004). They are therefore of high medical interest.

A wide range of biological and biochemical aspects of pollination is well researched and understood (Pacini, 2000; Willmer, 2011; Fattorini and Glover, 2020). But many questions regarding the physical processes are still unanswered. Only a few recent studies have quantified pollen adhesion, an essential part of pollination. They have shown that properties of the surfaces involved in the pollination process influence adhesive forces, ensuring proper pollen transfer (Amador et al., 2017; Ito and Gorb, 2019b). A key role to understand this phenomenon is played by the pollen coating substance pollenkitt.

Pollenkitt is the extracellular matrix covering the surface of most pollen grains. At the end of pollen grain production in the anther, a special tissue autolyzes. This tissue, which is called tapetum, is closely involved in the production of pollen. When the tapetum

decomposes, it covers the grains with pollenkitt (Pacini, 2000). Pollenkitt is thereby defined by the composition of the tapetal cells. This process generates a fatty acid shielding for pollen grains (Pacini, 2000). This material is a well-known but poorly understood component of pollination, particularly present in entomophilous species (Kadereit et al., 2014). What has been found is that the extent of pollenkitt on a pollen and its chemical and biophysical properties are critical factors for pollen quality, which is measured by the ability of successful fertilization. Pollenkitt is also proposed to have two antagonist functions: (1) to prevent drastic dehydration and (2) to promote water absorption from compatible stigmas (Chichiriccò et al., 2019). In addition to that, Pacini and Hesse postulated a variety of functions in 2005, e.g., to maintain sporophytic proteins, which are responsible for pollen–stigma recognition, to protect pollen from hydrolysis and extracellular enzymes and to provide a digestible reward for pollinators. It is also used by bees (*Osmia lignaria)* as a chemical cue (Guédot et al., 2006) and, beyond that, it was suggested by Piskorski et al. (2011) to be effective as a chemical cue for pollinators in general when foraging plants.

All these features are the consequence of pollenkitt being the outermost layer covering the pollen grains' outer cell wall (exine). Pollenkitt is in direct contact with the environment, mediating interactions with biotic and abiotic systems (Chichiriccò et al., 2019). Probably one of the most important of these interactions is to serve as an adhesive that facilitates adhesion to insects and stigmas during pollination (Pacini and Hesse, 2005). However, recent studies indicated that the influence of pollenkitt on pollen adhesion is more complex than assumed. For example, Ito and Gorb (2019a) showed that pollenkitt on wet surfaces like stigmas rather disturbed pollen adhesion and that adhesive properties of pollen depend on humidity, adding more evidence to findings of Lin et al. (2015). The role of humidity is especially interesting since pollenkitt influences pollen adhesion in different environments, like a dry insect leg and a wet stigma. To elaborate on this, as soon as the ripe pollen are presented to the environment by an open bursted (dehisced) anther, they need to withstand various stress factors, like direct UV-radiation and moisture i.e., humidity and rain in some cases. Especially when attached to an insect, the pollen grains are exposed to wind currents, high frequency vibrations and heat (Corbet et al., 2019). Pollenkitt helps to protect the pollen from these extreme microclimatic conditions and is assumed to serve as an adhesive liquid to mediate pollen transfer from anther to insect and from insect to stigma reliably. This poses complex demands to the pollenkitt's mechanical properties to be well tuned. The strategies used to accomplish this are not fully understood yet.

To gain more knowledge about those mechanisms, it is helpful to study the influence of pollenkitt on the adhesive properties of pollen grains. For this purpose, I utilize atomic force microscopy (AFM) to quantify and compare the adhesion of pollen with and without pollenkitt on glass. Pollen adhesion is quantified by determining the pull-off force necessary to detach a pollen grain from a glass slide. I compare the pull-off forces of pollen in native state (i.e., with pollenkitt) to the pull-off forces of the same pollen after removing pollenkitt via a defined washing procedure. This way, I expect a relative difference to show a trend in adhesion due to partial or complete removal of pollenkitt, thus quantifying the effectiveness of the washing process. To visualize the influence of a washing procedure on pollenkitt, pollen grains are imaged prior to and after the washing procedure employing a transmitted light microscope and a scanning electron microscope (SEM).

However, to do this comparison properly, it is necessary to understand how to remove pollenkitt and what exactly happens during a washing process. Previous studies used different organic solvents such as cyclohexane, chloroform and diethylether (Rejón et al., 2016) to wash off pollenkitt from pollen grains. Others used carbon disulfide (Chichiricco et al., 2019) or a combination of chloroform and methanol (Ito and Gorb, 2019a), but there are more solvents reported to be used, which raises the question of what the ideal way to remove pollenkitt really is. A good solvent should remove all coating molecules residue-free and at the same time not touch any properties of the pollen grain. Especially not the exine or intine (inner cell wall) which are the main structural layers beneath the pollenkitt.

This project aims to quantify the influence of different solvents on pollen adhesion and pollenkitt. I conduct experiments employing the pollen of *Ranunculus bulbosus,* commonly known as bulbous buttercup or St. Anthony's turnip, as this is one of the few species with characterized pollenkitt composition. This makes it easier to choose appropriate solvents for pollenkitt removal. Moreover, it was accessible at the time of my experiments since it is populated throughout Europe and produces pollen from May to July (FloraWeb, 2021).

Piskorski et al. provided information about the chemical composition of *R. bulbosus'* pollenkitt in 2011. They inspected the chemical composition of its coating material with gas chromatography followed by mass spectroscopy and detected aldehydes, fatty acid amides, saturated and unsaturated hydrocarbons, as well as secondary alcohols. Octadecenamide, Pentacosane, Heptacosane, Nonacosane and Nonacosan-4-ol (sorted according to their proportion) made up for around 80% of the compounds, which is a highly non-polar / lipophilic mixture. Based on these findings, the use of a hydrocarbon such as cyclohexane seems obvious since itself has highly non-polar properties which should be

able to dissolve aforementioned substances. Another promising solvent is a 1:1 molar mixture of diethyl ether, which is a weak H-bridge acceptor and chloroform, which is a weak H-bridge donor. Even though pollenkitt is broadly assumed to be of lipidic nature (Piskorski et al. 2011), pollenkitt also includes polar components that should not be neglected (Rejón et al., 2016). These polar parts consist of several proteins performing tasks like prolonged pollen adhesion, hydration, pollen–stigma recognition and communication, pollen germination and stigma invasion (Wu et al., 2014; Rejón et al., 2016). This shows the need to test the influence of a polar solvent like ethanol on pollenkitt as well. In addition to that, carbon disulfide (CS_2), with its good polarizability, seems to be another promising dissolver for pollenkitt. Water as the most common solvent in cell biology (Sargen, 2019) is tested as well, which is especially interesting because *R. bulbosus* does not shut its flowerhead during rain (own observation) contrary to many other species and thereby risking the contact of its pollen with water.

In this study, I quantify the effect of five different solvents, namely cyclohexane, carbon disulfide, a chloroform / diethyl ether mixture, ethanol, and water on the adhesion of *R. bulbosus'* pollen. I expect the pull-off force to decrease when comparing the native and washed states of a pollen grain since I assume that the adhesive pollenkitt is partially or completely removed by the washing procedure. Furthermore, different solvents should have different efficiencies in removing pollenkitt, which should be quantifiable based on the reduction in pollen adhesive forces.

2. Materials and Methods

Experiments were carried out employing a Nanowizard Atomic Force Microscope [JPK Instruments AG, Berlin, Germany] with an inverse light microscope [Axiovert 135, Carl Zeiss QEC GmbH, Germany] utilizing NanoWizard Control Software and JPK data Processing Software [both version 4.3.55, JPK Instruments AG, Berlin, Germany]. The microscope was equipped with a firewire camera [DFK 31AF03, The Imaging Source Europe GmbH, Bremen, Germany] on a 0.5x C-Mount adapter. I used tipless silicone cantilevers [Nanosensors TL-CONT-50, NanoWorld AG, Neuchatel, Switzerland] with spring constants ranging from 0.5259 to 0.5845 N/m. Pollen grains were prepared under a stereoscopic magnifier [SMZ 745T, Nikon Europe BV, Amsterdam, Netherlands] and glued employing [Uhu Plus Schnellfest, UHU GmbH & Co KG, Baden, Germany]. Scanning

Electron Microscopy (SEM) and cryogenic SEM images were recorded employing Hitachi S-4800 [Hitachi High-Technologies Corp., Tokyo, Japan]. The glass slides used in this study were Thermo Scientific Superfrost Ultra Plus [Gerhard Menzel GmbH, Braunschweig, Germany]. The following solvents were applied in washing procedures: min. 99.7% cyclohexane [BCD Chemie GmbH, Hamburg, Germany], min. 99% carbon disulfide [Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany], min. 99.5% diethyl ether [BCD Chemie GmbH, Hamburg, Germany], min. 99.9% chloroform [Carl Roth GmbH + Co. KG, Karlsruhe, Germany], min. 99.8% ethanol denatured [Carl Roth GmbH + Co. KG, Karlsruhe, Germany] and double distilled water. During long washing treatments, a flat reciprocal shaker with swivel motion [AS 250.3, IKA-Werke GmbH & CO. KG, Staufen, Germany] was utilized.

2.1 Specimen

Ranunculus bulbosus LINNÉ, 1753 (Ranunculaceae, Ranunculales) (Fig. 1A) is perennial, outlasting under the ground and storing energy in a bulb (Anderberg, 2004; FloraWeb, 2021). The corolla itself is brightly yellow and highly reflective on the inside, whereas the sepals are hairy on the outside. The flower consists of 5 petals, many (~ 50) anthers and a gynoecium that is composed of ~ 30 pistils (own observation). Its pollen (Fig. 1B) is mostly round with three apertures (Stebler, 2018). The porous exine structure forms spines and pollenkitt is evenly distributed on the surface.

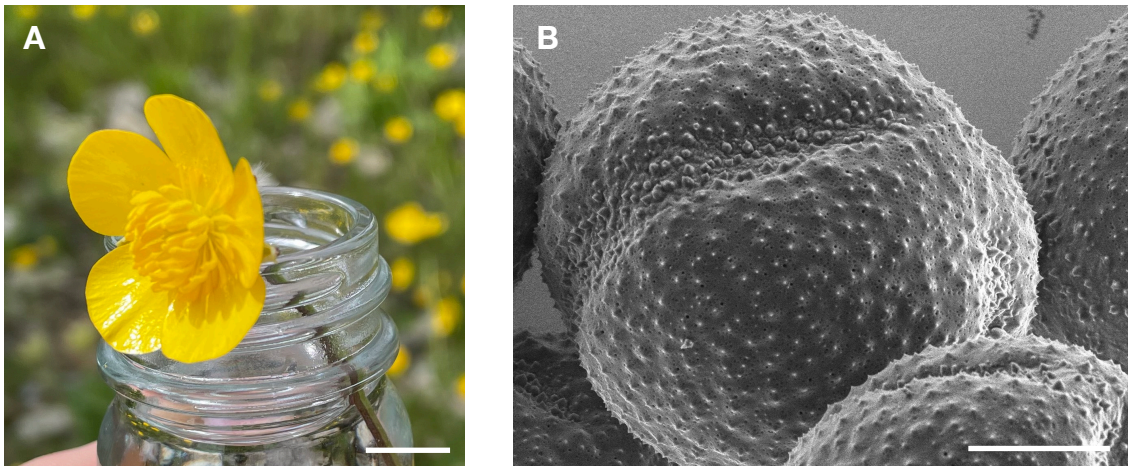


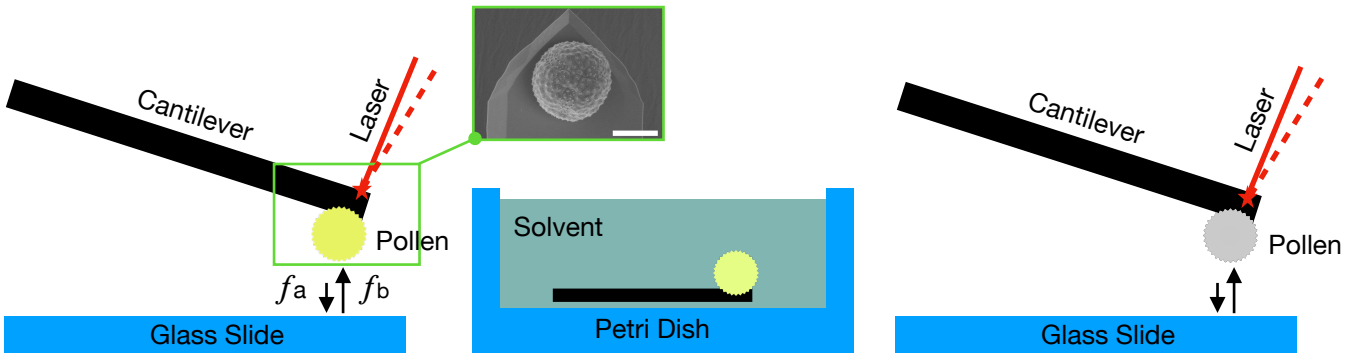
Figure 1: The pollen of *Ranunculus bulbosus* were employed in this study to quantify pollen adhesion. A) depicts a specimen of *R. bulbosus*. B) shows an SEM image of a pollen grain of *R. bulbosus.* The white scale bars represent A) 1 cm and B) 10 µm.

Samples were obtained from the Botanical Garden of the Christian-Albrechts-University of Kiel between May and July. Whenever fresh pollen were needed for an experiment,

anthers were picked from flowerheads, paying attention to visible pollen accumulation. Those anthers where then inspected under a stereoscopic magnifier while pollen grains were brushed onto a clean glass slide with an eye lash. Ideally, the pollen had to be fresh to avoid effects of dehydration or contamination. Therefore, only anthers with high amounts of pollen (which had not yet been visited by many insects), or anthers that had recently burst open (identified by incomplete dehiscence) were used. Subsequently, the glass slide carrying the pollen grains was installed into the atomic force microscope and an individual pollen was chosen employing inverse light microscopy. A suitable pollen grain was defined as mostly round with a diameter of ~ 35 µm, freely accessible and not clumped within a group.

2.2 Atomic Force Microscopy (AFM)

In order to quantify the influence of a solvent on pollen adhesivity, the adhesive properties of one and the same pollen grain were analyzed with an AFM before and after washing the grain with one solvent. Figure 2 depicts a schematic of the experimental design. The adhesive properties were quantified by measuring the pull-off force of a pollen grain from glass. The pollen grain was glued to a calibrated tipless cantilever with epoxy glue. After visual confirmation that the pollen was properly glued, the laser was switched off during the epoxy curing. This was done to prevent stress or damage on the pollenkitt. After a curing time of 20 minutes, measurements were carried out.



A: Measurement of fresh pollen **B**: Solvent treatment **C**: Measurement of washed pollen

Figure 2: A) The pollen was glued to a cantilever and approached a glass slide until a force of 1nN was detected. Subsequently, the cantilever was retracted. The cantilever was deflected due to the forces acting between pollen and glass. The deflection was read out with a laser beam reflected off the cantilever onto a position-sensitive photodiode. After measuring the adhesion of a native pollen to the glass slide, B) the cantilever carrying the pollen was washed in a solvent for 1 or 30 mins and subsequently, C) the adhesion onto glass was quantified again. Forces acting during the →

approach of the cantilever are called jump into contact forces (f_a), forces acting during the retract curve (see figure 3) are called pull-off forces (f_b). The white scale bar represents 20 µm.

The glued pollen was pressed onto the glass up to a certain setpoint force and was immediately retracted afterward. The bending of the cantilever provided information about the acting forces and was plotted in a force-distance curve, an example of which is presented in figure 3. The minimum of the retraction segment of a force-distance curve corresponds to the pull-off force (f_b), which is a measure for the sum of adhesion forces between pollen and glass. The difference between the lowest point of the approach curve and the baseline is the jump into contact force (f_a). This attraction force occurs during the approach in air in close proximity to the substrate. All force-distance curves were recorded with a pulling length of 1 µm and a cantilever speed of 10 µm/s. A setpoint force of 1 nN was employed. To achieve reliable datasets, the measurements were repeated five times at sixteen different positions in a 4x4, 100x100 µm grid. f_a and f_b were determined with the JPK data processing software by subtracting the baseline of the retraction or approach segment of each curve and determining the minimum force value.

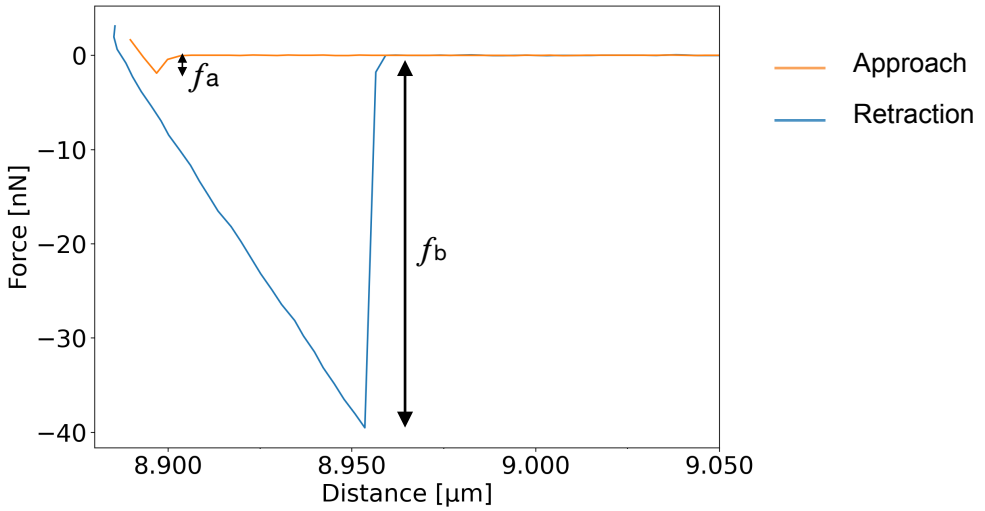


Figure 3: The deflection of the cantilever in combination with a piezo-controlled approach towards the glass slide resulted in a force-distance curve. The approach is colored orange and reads from right to left. The retraction is colored blue and reads from left to right. f_a represents forces responsible for a jump into contact while f_b resembles the force necessary to pull off the specimen (pollen grain) from the substrate (glass slide).

2.2.1 Cantilever calibration

Each cantilever was calibrated before a pollen was glued to it. The sensitivity was measured at sixteen different positions in a 4x4, 100x100 µm grid on a glass slide with a setpoint of 0.4 V and a cantilever speed of 10 µm/s. The mean value was employed. The spring constant was determined five times employing the well-established thermal noise method (Butt and Jaschke, 1995) and the mean was used for experiments. As the sensitivity depends on the position of the laser beam on the cantilever, it was recalibrated every time the cantilever was installed into the AFM (e.g., after the washing procedure).

2.3 Washing procedure

According to the manufacturer, the epoxy glue is solvent-resistant after a curation time of 60 minutes. Within this timeframe gluing, calibration and measurements of the native pollen state were completed. The whole cantilever carrying the pollen grain was then washed with one of the solvents for 1 min or 30 mins and the quantification of the pull-off force was repeated. For comparison, batches of pollen were washed for 24 h as well and examined by SEM.

Cyclohexane, carbon disulfide, a molar 1:1 mixture of chloroform and diethyl ether, ethanol and double distilled water were employed as solvents. Regardless of the solvent, the washing was done under a fume hood by placing the cantilever in a glass petri dish, pre-filled with one solvent, and removing the cantilever after 1 min. For longtime treatments, the petri dish was covered with a watch glass and put on a swivel motion shaker at 25 motions/minute to prevent evaporation and local buildup of dissolved substances.

2.4 Scanning Electron Microscopy (SEM)

To further understand the influence of a solvent on pollenkitt, the treated pollen were imaged employing SEM. The samples were air-dried for several days and sputter-coated with a ~ 10 nm thick gold-palladium layer to increase their conductivity. Images of fresh pollen were recorded as well. Their native state was preserved by rapid freezing with liquid nitrogen, the specimen was not sputtered and a conductive glass disk was used to achieve a high-contrast background. To image the aging process of pollen grains, SEM images of pollen grains were repeatedly recorded over a period of 3 days. These samples were not sputter-coated to allow them to change naturally and were analyzed without rapid freezing. All scans were done at 3 kV.

3. Results

3.1 Force measurements with AFM

Pollen adhesion was quantified by measuring the pull-off forces of pollen grains from glass. Figure 4 shows the pull-off forces of *R. bulbosus'* pollen measured before and after washing the pollen in one of five different solvents. Each box presents the results for two pollen grains. All solvents reduced pollen adhesion to glass significantly. It is striking to see that the distribution of adhesion forces of native pollen is much broader than the ones of the washed pollen. With carbon disulfide (CS_2) as an exception, the adhesion forces of washed pollen are similar for all solvents. The difference in the mean of pull-off forces between a native and treated pollen grain is significant for each treatment.

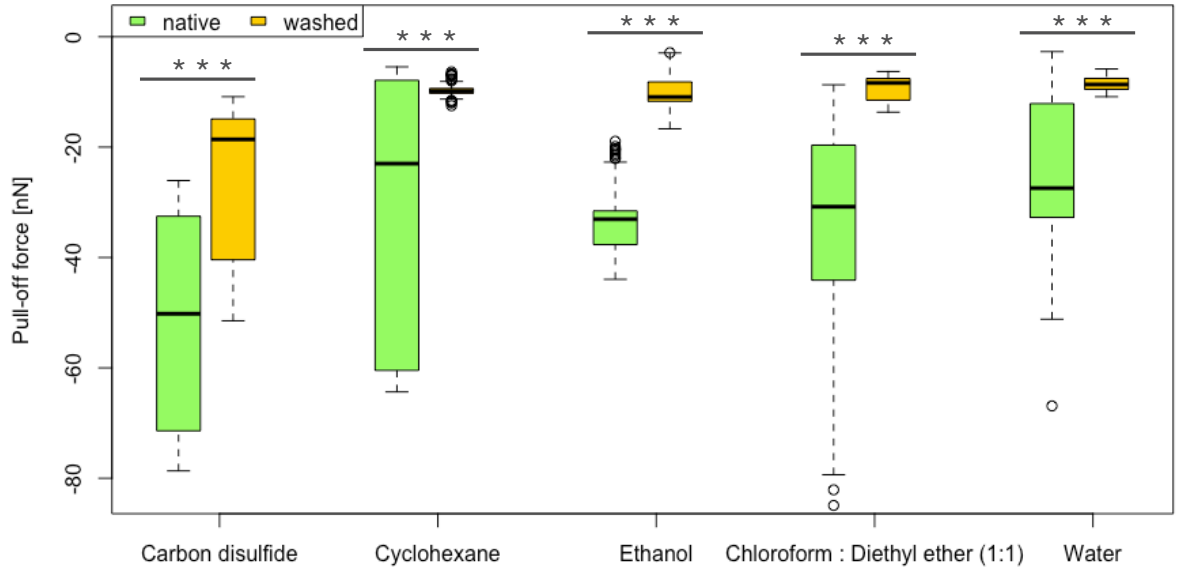


Figure 4: Pull-off forces of *R. bulbosus'* pollen from glass before (green) and after (orange) a one-minute solvent treatment (N=10, n=1600). The influence of different solvents on the pollen adhesion is quantified. Every pair of boxes represents the measurements for two individual pollen grains and within every group, the difference in mean values is significant (paired t-test).

In addition to two runs with a washing time of one minute, a 30-minute solvent application with the same conditions as the aforementioned was tested. Figure 5 shows a comparable range of pull-off forces for both the native state of the pollen grains and for some treated grains. Strikingly, in at least two groups (cyclohexane and water), the treated grain produces a greater pull-off force compared to the native state. This is not the case for any of the one-minute treatments. The values obtained for the ethanol- and 1:1 mixture-treatments are within a margin of error by the AFM and the medians in comparison are not substantially different.

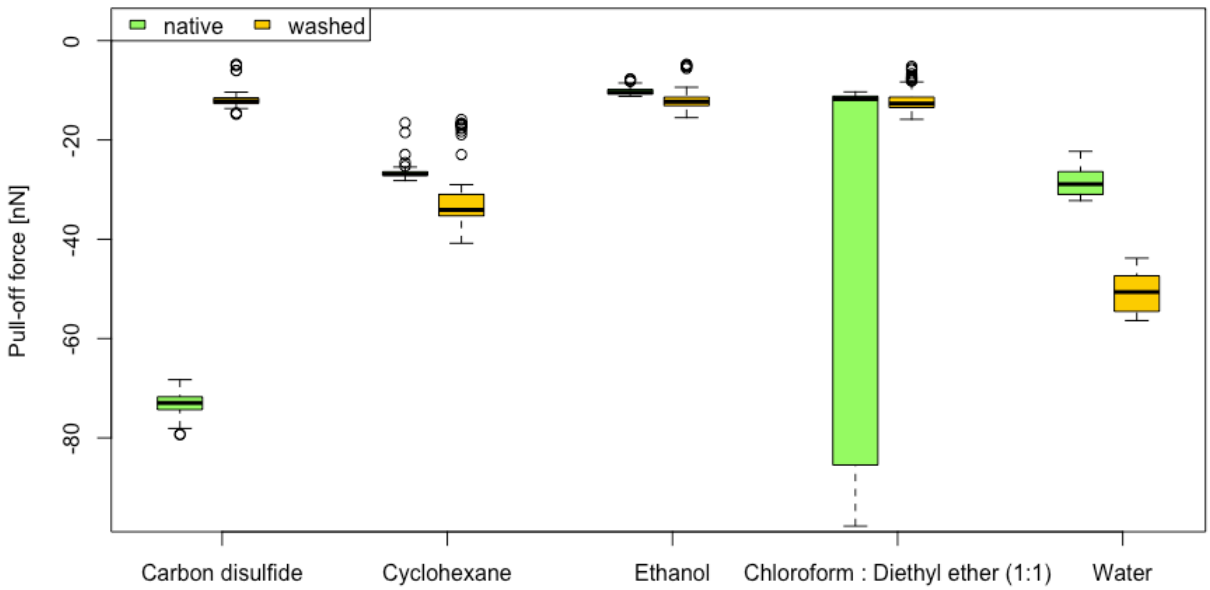


Figure 5: Pull-off forces of *R. bulbosus'* pollen from glass before (green) and after (orange) a 30-minute solvent treatment (N=5, n=800). The influence of different solvents on the pollen adhesion is quantified. Every pair of boxes (native and washed) represents the measurements for one individual pollen grain.

Moreover, possible changes in the adhesive properties of pollen over time were investigated after a washing procedure. This was tested for water, as it is the solvent with the highest volatility employed in this study. Figure 6 shows that the difference in treated conditions is within a margin of error by the AFM and, therefore, not substantial.

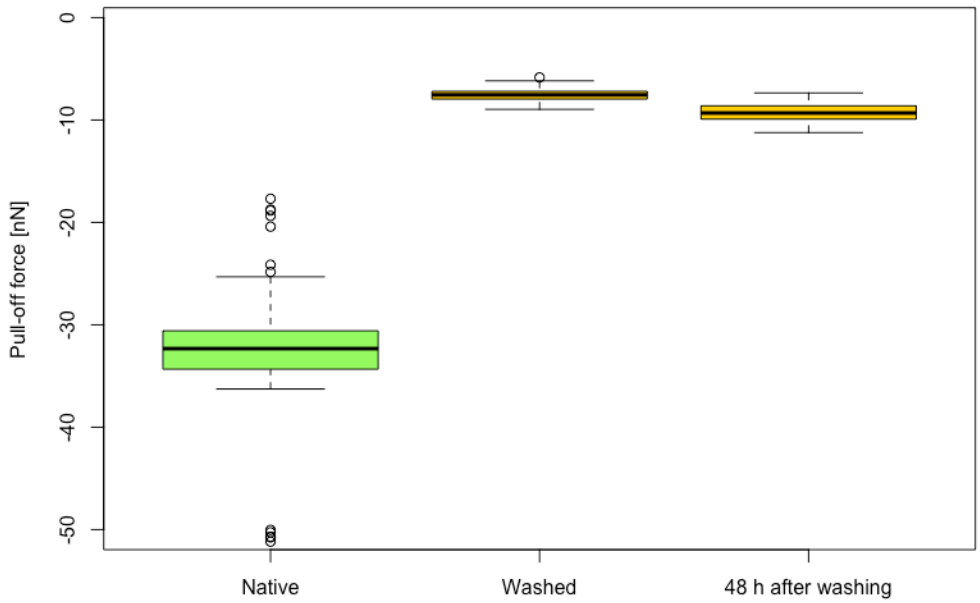


Figure 6: Pull-off forces of one and the same *R. bulbosus'* pollen from glass (native and washed), in comparison to its state 48 h after the water-treatment (N=1, n=240).

3.2 Visual inspections with SEM

Native and washed pollen grains were imaged after adhesion experiments in order to observe how the washing procedure affected the pollenkitt. Collected pollen from dozens of anthers had a wide variety of morphological appearances. They differed in overall size, expression of apertures and shape (Fig. 9A). Ranging from spherical to elongated and triangular or collapsed. Common fresh pollen grains were mostly round with three apertures and had a diameter of 30 to 35 µm. Their surface was equipped with spines (~ 30 spines / 10 µm^2) with an approximate height of 400 nm. Longer spines (~ 600 nm) were located in the aperture areas, where they were more dense as well (~ 50 spines / 10 µm^2). Most spines had 5 to 9 holes with a diameter of ~ 120 nm in close distance (300 nm) around them. Spines in the aperture areas had no visible holes around them.

With these common morphological characteristics in mind, figure 7 shows that there is no distinct difference in pollenkitt distribution or morphological anomalies when comparing the native state of *R. Bulbosus'* pollen to a one- or 30-minute treatment with SEM. Some pollen showed wrinkles on their outermost layer, some accumulated much pollenkitt in their apertures and some had more covered holes than others, but there was no obvious trend compared to native specimen (Figs. 1B and 8B).

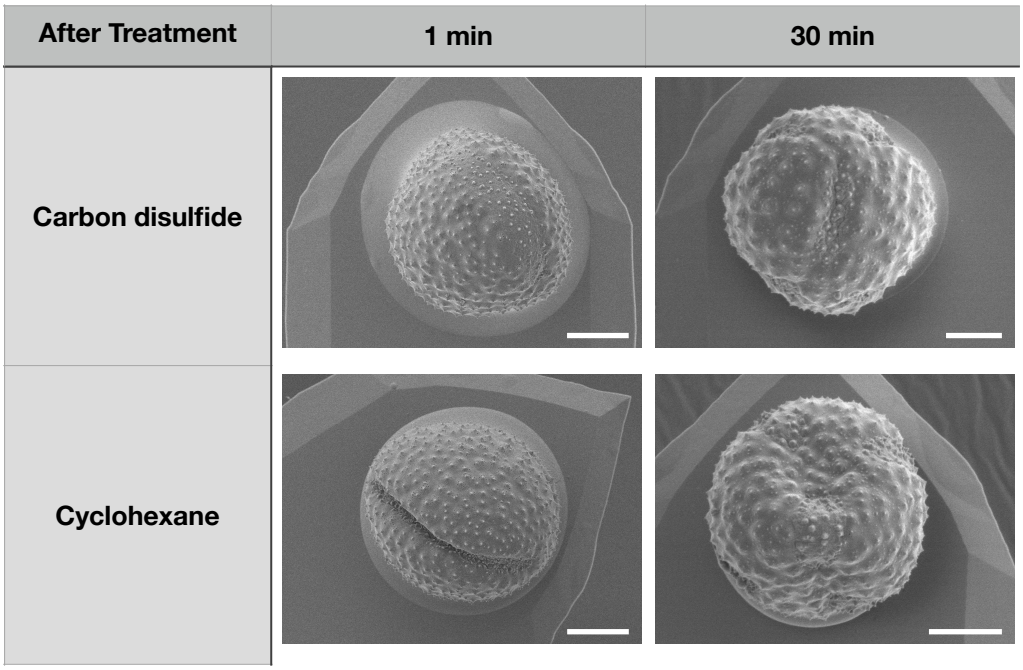


Figure 7: Continued on the following page.

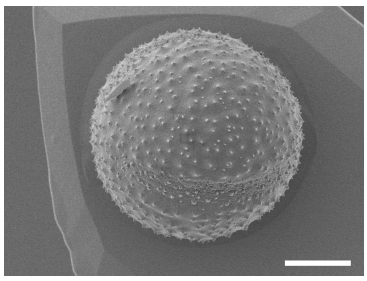
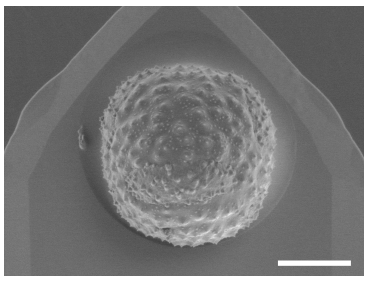
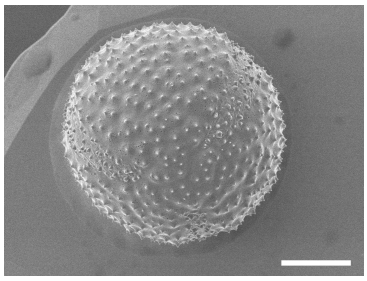
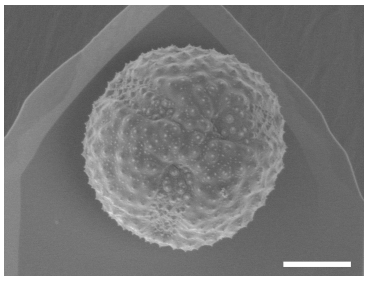
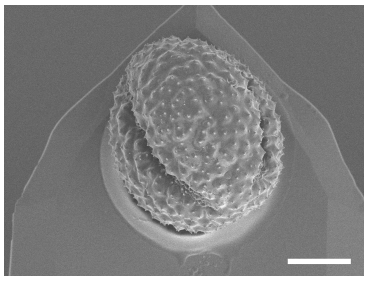
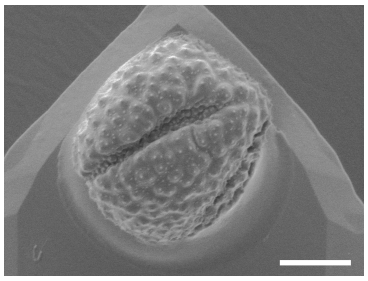
After Treatment	1 min	30 min
Ethanol		
Chloroform : Diethyl ether (molar 1:1)		
Water		

Figure 7: Each SEM image presents a different pollen of *R. bulbosus* glued onto a cantilever. This visualizes the influence of different solvents and washing times on the pollenkitt coating. The images were recorded after the pollen and cantilever had been washed with the respective solvent. Reference bars represent 10 µm each.

Clear changes in pollenkitt were only visible after a treatment with CS_2 for about 24 hours. Figure 8 shows a distinct redistribution and for the first time, a connection between neighboring grains can be seen. The apertures are filled with extracellular matrix as well.

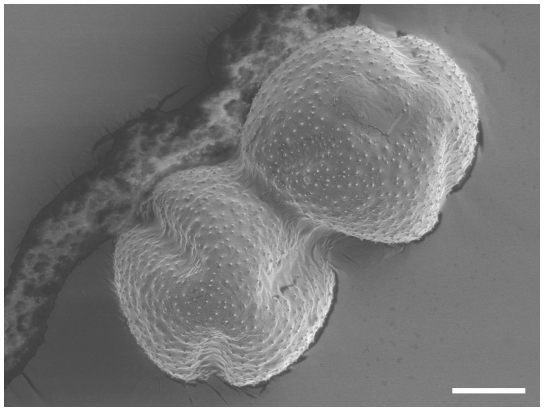


Figure 8: SEM image shows a liquid connection between two pollen grains after a 24 h solvent treatment with CS_2. The scale bar equals 10 µm.

Another aspect in context of morphology was discovered during the visual analysis of fresh pollen grains that were still in an anther and whose condition was preserved by liquid nitrogen. Figure 4A illustrates great diversity compared to conventional SEM (i.e., without cryogenic conservation) as shown in Fig. 9B, where the development states of apertures are much more equal and spherical grains occur extremely seldom. These changes in aperture depth only appeared under the conventional (warm) operation conditions. This change of aperture indentation on one and the same pollen grain was observed 5 minutes after the pollen encountered the vacuum needed to operate the SEM. The previously mostly round grain developed two defined apertures (Fig. 9C & D). The surface-spines sunk approximately 3 µm. Tracing the circled spine in figures 9C and 9D shows that the surface folds inwards instead of a substance filling the prebuilt apertures evaporating under vacuum. After revisiting the same pollen 24 days later, no further indentation has been observed (Fig. 9E).

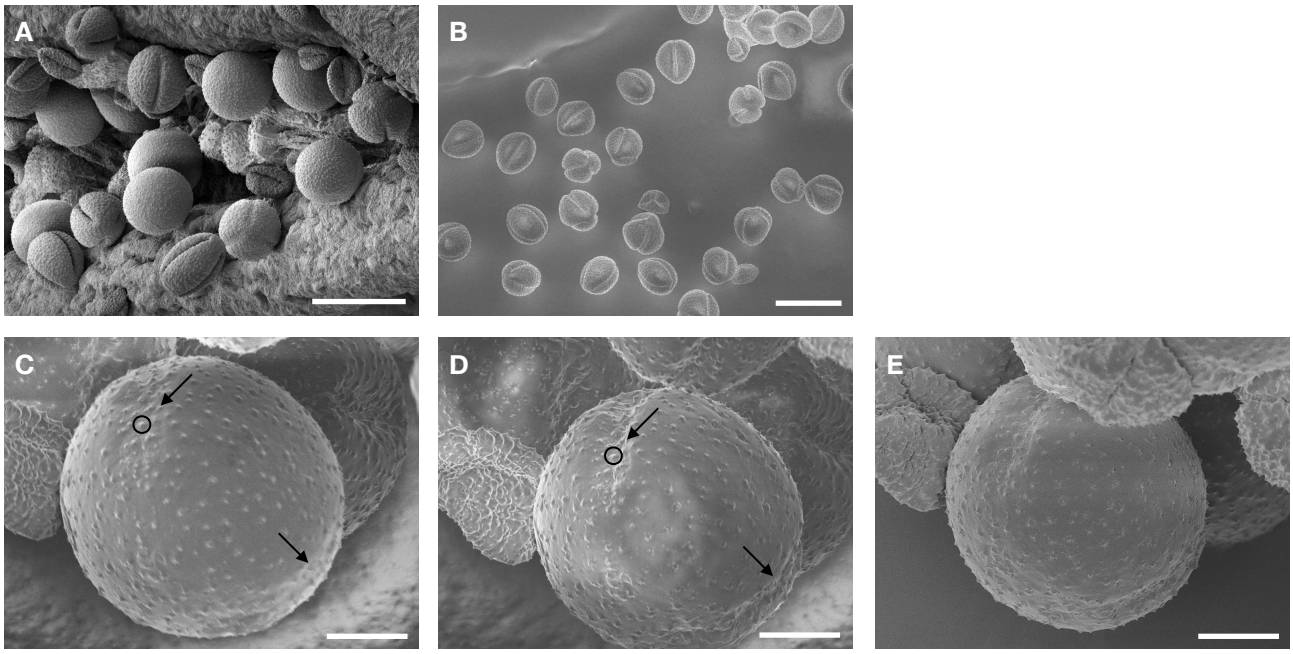


Figure 9: SEM images of pollen grains of *R. bulbosus.* A) shows a Cryo-SEM capture of the diversity of fresh pollen in an anther. B) depicts a representative diversity of a conventional (warm) SEM inspection. C-E) reveal the change in a pollen grain's apertures under vacuum, where C) was captured after 5 mins and D) after 10 mins under vacuum. E) was stored for 24 days under atmospheric pressure before re-examination. The arrows indicate the significant change in aperture depth and the circled spine can be tracked as it sacks down. The white scale bars represent the following lengths: A, B: 50 µm, C, D, E: 10 µm.

4. Discussion

Since *Ranunculus bulbosus*, as an entomophilous species, relies on the attachment of its pollen to insects, pollenkitt was expected to play a crucial role in adhesion. To test the adhesive properties of pollen grains themselves while limiting other influential factors from more complex surfaces, their adhesion to glass was quantified. The efficiency at which different solvents remove pollenkitt from pollen grains should be quantified by comparing pollen adhesion before and after a washing procedure.

Furthermore, the expected differences should only occur due to an altered pollenkitt, as it is in direct contact with the environment and the layers underneath should not be manipulated. This assumption is based on the fact that the cell wall (sporoderm) of a pollen consists of the particularly resistant sporopollenin (exine) (Heslop-Harrison, 1968). This material is inert to most solvents (Domínguez et al., 1999). However, the structure of the exine is porous, being composed of many nanoscale granules and the entire cell wall has many holes of unknown depth. Adding to this, the underlying intine of the sporoderm is composed of less resistant material, including cellulose (Heslop-Harrison, 1968). This was considered when applying CS_2, as it is used for industrial xanthogenation of cellulose because it has the ability to dissolve many components of plant cell walls and reorganize cellulose molecules (Bechtold and Schimper, 2010). Regardless, this solvent has been successfully used for the removal of pollenkitt from pollen grains before (Chichiriccò et al., 2019) and cannot chemically change the structural important sporopollenin. In addition, short treatment times should minimize the effect. Therefore it was employed as a promising solvent in this study as well. The other solvents used are not expected to be able to manipulate the intine.

As the chosen solvents have different chemical properties, they should vary in their ability to remove pollenkitt and thus affect pollen adhesion differently. However, the data obtained shows that the situation is complex and all solvents, regardless of their properties (e.g., polar or non-polar), achieve a similar adhesion-reducing effect. There are no distinct differences between the solvents, all of them reduce the pull-off force to similar values (Figs. 4 & 5), independent of the greatly varying pull-off forces measured in the native states. The question, therefore, arises of how the reduction in adhesion can be explained.

Assuming pollenkitt is predominantly involved in adhesion in this species, pollen freed from pollenkitt should exhibit different pull-off forces depending on the chemical properties of the solvent. Water, for example, should not be able to wash away many of the fatty and

other non-polar components of pollenkitt, especially since *R. bulbosus* does not particularly protect its pollen against rain (own observation). An irreversible reduction of adhesive properties of pollen by water exposure should have a significant impact on reproduction. Non-polar solvents, on the other hand, should be much more efficient in removing pollenkitt due to the non-polar character of pollenkitt. But SEM images do not clearly show that pollenkitt was removed in any group (Fig. 7). Instead of being removed, every solvent applied seems to have altered the pollenkitt such that its adhesion-interfering function, described for certain scenarios before (Ito and Gorb, 2019a), was increased. An indication of this is the considerably changed pollenkitt after 24 h of CS_2 application (Fig. 8). Although such drastic changes are not seen in the one- or 30-minute treatments (Fig. 7), it is thus clear that processes altering the pollenkitt are at work. However, it also shows that the pollenkitt is still present and not dissolved even after a long treatment period. Nevertheless, the solvents could have softened the pollenkitt or washed away functional components of it and "soaked" pollenkitt has already been demonstrated to reduce adhesion (Lin et al., 2015a).

If the pollenkitt's viscosity was changed by the solvent treatment, its changed viscoelasticity should be evident during pull-off. An increased viscoelasticity can be derived from the integral of the pull-off curve, the pull-off work. There are indications for a changed viscoelasticity in the comparison of the pull-off work between 1 and 30 minute applications (Fig. S3). Although no clear conclusions can be drawn in this respect since the shape of the curve has not clearly widened (Fig. S4), which would point to a changed release behavior. This may be due to the speed of measurement (10 µm/s). By reducing this speed, the measurement would have a higher resolution and corresponding (slower) effects could be made visible. In addition, this phenomenon likely changes after the solvent application because if pollenkitt is still moistened during the measurement, it should dry during storage. However, this is contradicted by a follow-up inspection of a one-minute water-treated pollen, which produced a comparable pull-off force even after two days of drying (Fig. 5). This shows that the drying period was adequate and artifacts caused by solvent residues can thus be ruled out for one-minute applications. Furthermore, since water has the slowest evaporation rate of all solvents used, this result allows a conclusion to be drawn regarding the faster-evaporating solvents. If the water-treated pollen already produces comparable pull-off values during the initial measurement, as it does after two days of storage under laboratory conditions (26±1.5 °C, 18±1 %rH), those with a different treatment probably will as well. This points to the conclusion that pollenkitt might be permanently altered after a solvent wash and that this manipulation

occurs within a short amount of time.

Another effect acting beyond this short time manipulation could be an altered wettability of the pollen's surface, which could have led to a reduced interaction with the ambient moisture and interfere with the formation of liquid bridges in the contact between pollen grain and glass surface, as described elsewhere (Harrison et al., 2015). Such liquid bridges can act when a meniscus is formed between the contact surface of pollen and glass, which in turn introduces capillary and viscous forces. How these act depends on surface texture, pollen grain size, humidity, and other factors (Jones et al., 2002; Harrison et al., 2015). However, it has been shown that a stable meniscus cannot form at humidity levels below 30% (Jones et al., 2002). Although a constant humidity of 18% prevailed in the laboratory when measurements were performed, these statements are only valid for the mean relative humidity in the room. It could be possible that a moistened pollenkitt changes the local humidity between the pollen and the glass slide in such a way that a meniscus and corresponding capillary and viscous forces have an effect on adhesion anyway. This might explain the dramatic increase in the pull-off forces of cyclohexane and water after 30 minutes of treatment. As these solvents represent the polar extremes of all solvents used, a comparable manifestation in pull-off forces is rather unexpected. But utilizing two different effects could have led to a comparable phenomenon that can be explained by the aforementioned stable meniscus. In this case, water might have caused a local increase in humidity, whereas cyclohexane might have liquefied the pollenkitt such that it could form a meniscus by itself. This phenomenon did not occur in the one-minute applications, which suggests that the interaction of the two solvents with the pollenkitt takes longer than one minute. In the future, the pull-off forces should also be retested for a 30-minute water or cyclohexane treatment after a drying period of several days.

However, it is also possible that the pollen of *R. bulbosus* is fundamentally not as reliant on the use of pollenkitt as other insect-pollinated species. Other properties like morphology, weight, ability to build up charges and probably more could be explanatory for the results obtained. This would explain why no clear changes in pollenkitt were seen in SEM, even though the chosen solvents except of water have been employed successfully in previous studies (Doughty et al., 1993; Bih et al., 1999; Piskorski et al., 2011; Lin et al., 2013, 2015; Wu et al., 2014; Rejón et al., 2016; Ito and Gorb, 2019a; Shin et al., 2019). Thus, the effect of altered or removed pollenkitt could be so small that it is overwhelmed by other factors. Such superimposed forces could act electrostatically and be diversely distributed in the

native state but neutralized by contact with liquids (Fig. S2B). This seems reasonable since Pacini (2000) described that pollen in insect-pollinated plants have the property to interact electrostatically. In nature, this effect comes into play when pollen get attracted by and „jump“ on pollinators, which generate electrostatic charges during their flight. Such an effect could also have occurred during the AFM measurements.

As shown in figure S2, the jump into contact forces correlate with the pull-off forces. This correlation suggests that forces like electrostatic charges and additional unknown forces acting during the approach also act during the retract phase of the cantilever (Fig. 3) and keep the pollen in contact with the glass. Although the forces mentioned are in different orders of magnitude, it is not necessarily contradicting because the forces measured depend on the speed of the approach. For example, as electrostatic forces act over a relatively large distance depending on the charge difference of the objects (Vaknin et al., 2000), it is decisive for the force measurement how long the pollen stays in the area where the force acts. Thus, if the approach speed is too fast, the measured force would not correspond to the forces effectively acting. It follows that too high a speed (displacement/time) can mask other attracting forces as it manipulates the x-axis (displacement). This consideration also applies to the cantilever itself. Because of the cantilever’s size and the fact that it was always washed together with the attached pollen grain, it could have been affected by long ranging forces even more. Those considerations should be tested in a future experiment by measuring only the tipless cantilever before and after a solvent treatment and reducing the approach speed.

Consequently, the demonstrated correlation between jump into contact and pull-off forces (Fig. S2) could indicate that attracting forces are crucial for pollen during pull-off and might be removed by the washing procedure, weakening adhesion.

In this context, the change in the approach curve can be assumed to indicate more than just an electrostatic force. Because the electrostatic force scales with $1/r^2$ according to Coulomb's law, the pollen, therefore, experiences a uniform increase in the effective force as it approaches the center of charge. This should result in a curve with a corresponding shape like shown in figure S1B but was rarely present during measurements with pollen. A spontaneous jump present in every plot captured (Figs. 3, S1A and S4) cannot be explained by this, which suggests the effect of other unknown forces. If considerations about such overlapping effects are true, a species with considerably more pollenkitt and an associated more dominant role of the latter should be investigated by the same method.

And if pollenkitt is not broadly distributed on the grain’s surface, the contact area between pollen and glass resulting from the morphology should also be taken into account as well.

Especially due to the high diversity in the native state (Fig. 9A) and the changes outside the anther (Figs. 8C and D), differences were to be expected. The pull-off values of washed pollen are nevertheless very similar despite different morphologies (Figs. 4, 5 and 7). It is therefore possible that the contact areas, especially due to the numerous spines (Fig. 1B), are comparable and play only a minor role. Additionally, it should be noted that the development of apertures probably occurs more slowly under normal atmospheric pressure but should not change dramatically between measurements.

Assuming that *R. bulbosus* might not utilize pollenkitt as intensively as related species demonstrates the relevance of ecological adaptation and challenges the simplification in categorization of mechanisms in nature. As every species caters to a certain ecological niche, the methods of transport vary in detail. Through this perspective it is clear that every pollenkitt is adapted in a way to serve to several unique demands, like structurally diverse stigmas (e.g. dry vs. wet and various micro- and nano-structures). This suggests that pollenkitt differs in its chemical composition, backed by several studies (Rejón et al., 2016; Chichiriccò et al., 2019), which necessitates a qualitative analysis of different species' pollenkitt in order to develop a better understanding and targeted methods. Moreover, the situation in nature is more complex than between pollen and glass because the adhesion properties of other surfaces and related interactions are added. This should be investigated in future research as well.

Apart from this, the determination of suitable solvents was based on an analysis of the washed out components of *R. bulbosus'* pollen. In the future, it would be even better to start one step prior because it has already been shown that across species, the components of the peritapetal membrane build the pollenkitt (Chichiriccò et al., 2019). Therefore, it would be interesting to focus more on these cells of the tapetal tissue and their apoptosis. This new focus could lead to a better understanding of pollenkitt effects, its potential properties and promising ways to reliably remove all components of the pollenkitt. Because this study shows, contrary to what was expected, pollenkitt-removal and its effects are non-trivial, hence more data should be generated to conclude statistically sound hypothesis on the function and properties of pollenkitt.

Importantly, since differences in pollenkitt composition could be minor within the same strategy of pollination (Piskorski et al., 2011), these findings are relevant for entomophilous pathways. However, it should be investigated which strategy *R. bulbosus* really utilizes in detail. Existing, as well as future data on adhesion of washed pollen, should take these unexpected effects into account when interpreting them.

5. Conclusion

Traditionally it was expected that pollen of an insect-pollinated species would rely primarily on pollenkitt to mediate adhesion and adhere to insects. Instead, it has been shown in this thesis that various solvents override another previously unknown factor of adhesion mediation. If this effect was dependent on pollenkitt, differences dependent on solvent properties should have been observed. However, the SEM observations showed no changes beyond the natural variability. Hence, it can be stated that pollenkitt, at least in *Ranunculus bulbosus*, might only have a minor role in the context of adhesion. This finding should lead to an increased emphasis on identifying forces that are independent of pollenkitt in further research on pollen adhesion mechanisms.

6. Acknowledgements

I would like to thank my Dad, without whose generous support my desired studies and this thesis would not have been possible. For this, I will always be grateful.

Additionally, I would like to address a special word of gratitude to Dr. Huth for his patience and enthusiasm when familiarizing me with scientific procedures or discussing unintuitive observations with me while always providing great input for me to think of new ways to understand previously unnoticed features of our environment.

I would also like to thank Prof. Dr. Gorb for the new challenge I was able to take on. I have learned an incredible number of things and experienced exciting insights into areas of functional morphology and biomechanics.

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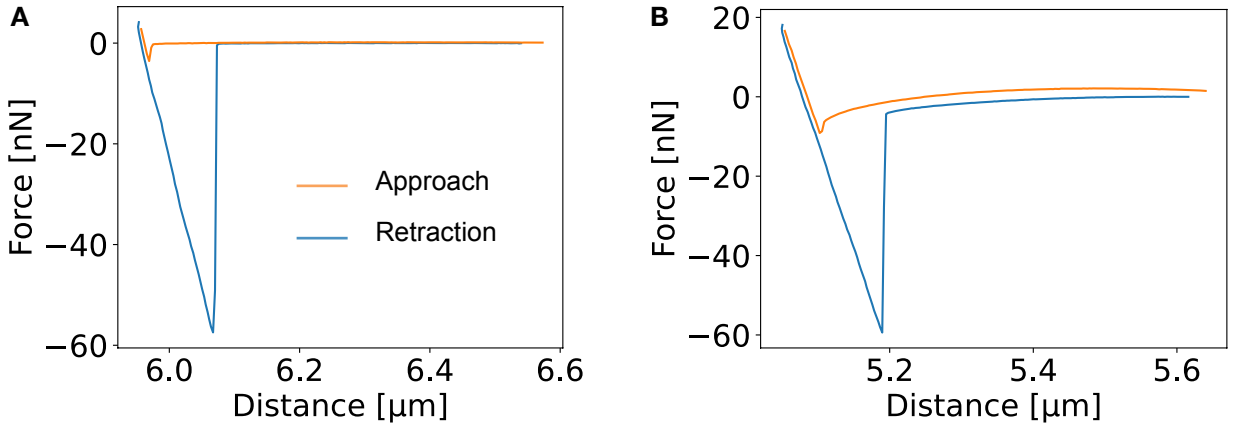
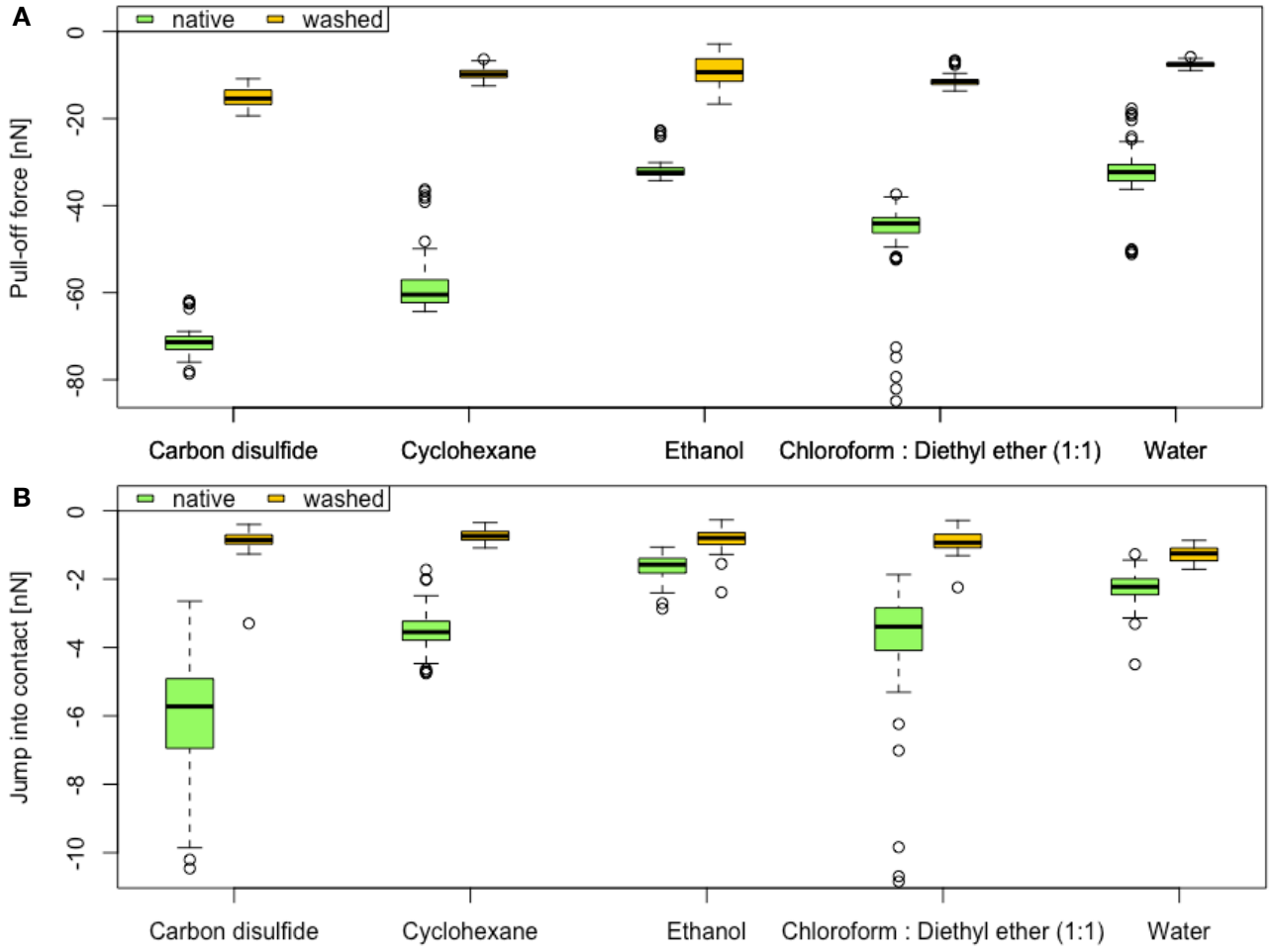
8. Appendix

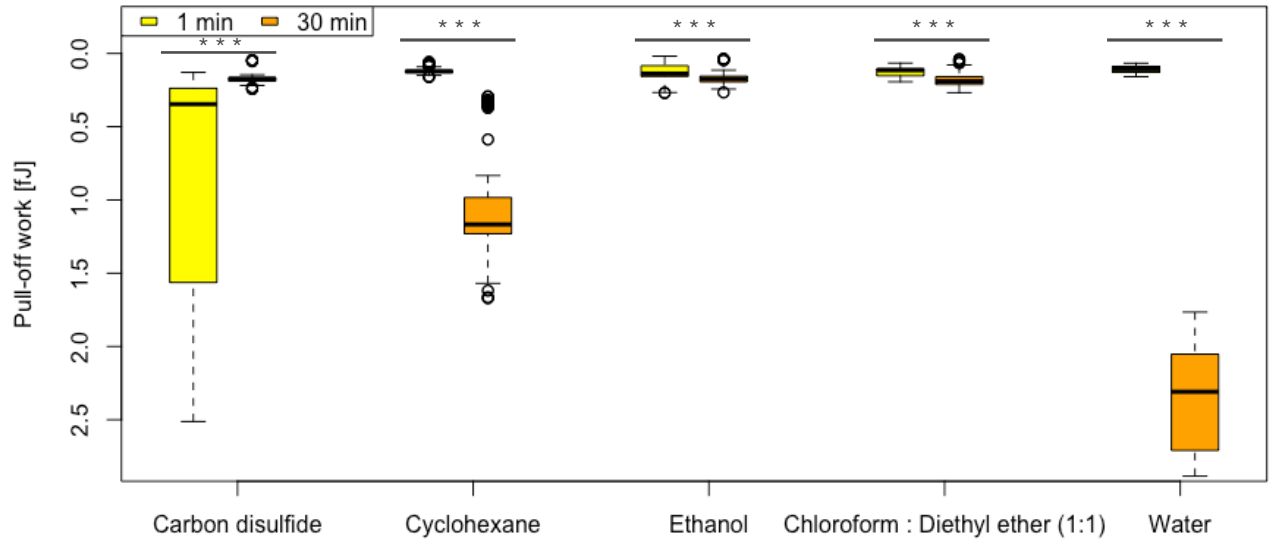


S1: These force-distance curves were manipulated with an ionizing blow-off gun. A) depicts a graph with no dominant effect of electrostatic charge visible. B) depicts a clearly visible impact of electrostatic charge in the approach curve, which describes a long-ranged force acting stronger the closer the distance between the cantilever and the glass substrate.

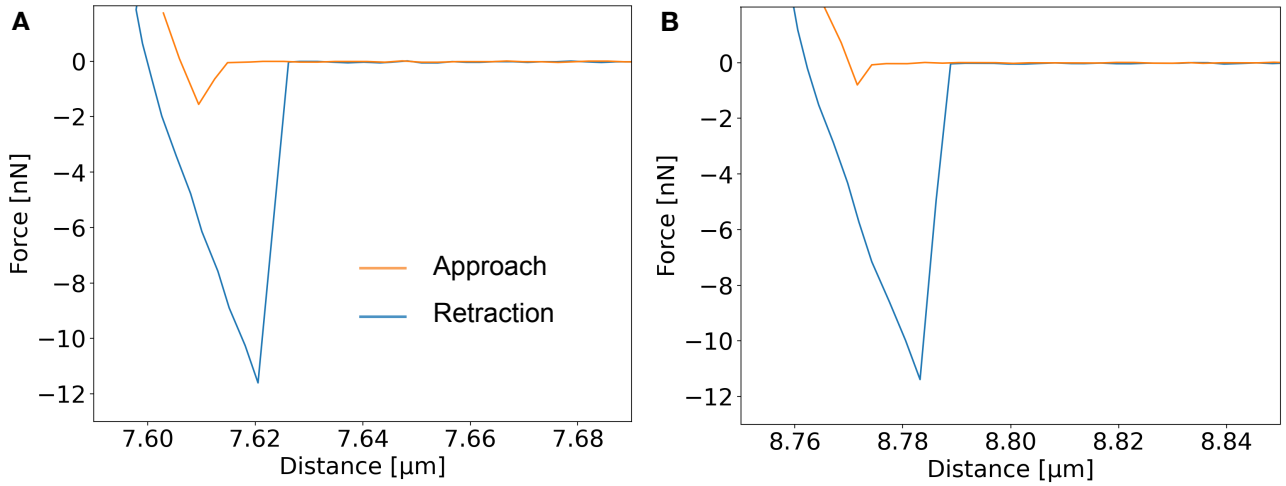


S2: Before any solvent treatment, the native pollen grains (green boxes) show A) a high pull-off force whenever B) the jump into contact action implies a high force as well in relative comparison. →

Although the magnitudes are different, the relative arrangement transfers from one to another. (N=5, n=1600)



S3: There are indications for a changed viscoelasticity in the comparison of the pull-off work after a one- or 30-minute applications as every group except carbon disulfide shows a significantly higher work for a 30 minute solvent application compared to a one minute treatment (unpaired t-test). (N=15, n=1200)



S4: The comparison of two representative force-distance curves does not show a change in viscoelasticity as the width of the curve A) after a one-minute ethanol treatment is the same as the width B) after a 30 minute ethanol treatment. As the distance (y-dimension) is to be compared, the force (x-dimension) needs to be at a similar level to observe a more viscose substance during retraction (pull-off).