


## Article

# Pollen Viability of *Fraxinus excelsior* in Storage Experiments and Investigations on the Potential Effect of Long-Range Transport

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**Abstract:** Fragmented ash populations due to ash dieback may lead to a limited gene flow and pollination success. Therefore, the viability of ash pollen plays a major role for the survival of the species. The extent to which the long-distance transport of pollen affects pollen viability was investigated with experiments in a climate chamber using ash pollen samples from a seed orchard in Emmendingen, Germany. Furthermore, experiments with a volumetric pollen trap were conducted. A suitable storage temperature for ash pollen was determined by using four viability tests; TTC test, pollen germination, Alexander's stain and Acetocarmine. An optimization of the germination medium was performed. We found a strong influence of prevailing temperatures on pollen viability, which decreased faster under warmer conditions. At moderate temperatures, viable pollen could still be observed after 28 days. Thus, a possible successful pollination can also be associated to long-range transported pollen. Storage experiments showed that pollen viability could be maintained longer at temperatures of  $-20\text{ }^{\circ}\text{C}$  and  $-80\text{ }^{\circ}\text{C}$  than at  $4\text{ }^{\circ}\text{C}$ . In particular, the TTC test has proven to be suitable for determining viability. Therefore, properly stored pollen can be used for breeding programs to support the survival of *Fraxinus excelsior*.

**Keywords:** Acetocarmine; Alexander's stain; ash dieback; pollen germination; TTC; volumetric pollen trap



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## 1. Introduction

The common ash (*Fraxinus excelsior* L.) was formerly thought of as a suitable tree species for Europe's forests in the course of climate change [1,2]. Nowadays, this tree species is severely threatened by ash dieback, caused by the fungus *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz and Hosoya [3]. Despite the current lack of effective conservation measures or opportunities to control ash dieback, there is hope for *Fraxinus excelsior* populations. According to the current literature [4–6], there is a natural variability in the susceptibility of ash trees to *H. fraxineus*. Different degrees of damage were attributed to a genetically determined resistance of ash tree individuals [7–9]. Less susceptible ash trees may have the potential to counter ash dieback through successful reproduction within breeding programs [10]. The proportion of ash trees that is (partially) resistant to ash dieback is estimated to be low, between 1% and 5% [11–14]. In addition, the natural regeneration of ash trees is believed to be of importance in fighting ash dieback since those seedlings, which are able to withstand competition, might constitute resistant phenotypes [15].

Within the next 30 years, an ash tree decline of 75% is expected in mixed stands in Europe [16]. A sharp decline in the ash populations is associated with stand fragmentation [13], probably resulting in the loss of genetic diversity and an increase in inbreeding

among ash individuals [17]. Studies of fragmented ash stands in Scotland have shown that gene flow between isolated ash trees can be maintained by pollen transport from distances of up to 2900 m [18,19]. However, it is known that pollen from anemophilous plants can be dispersed at mesoscales, i.e., 10–200 km from the source [20,21] and even synoptic scales [22,23]. These events are not frequent since they require specific environmental conditions including the characteristics of the landscape [19] and meteorological condition [24], however, they can be an important mean for geneflow providing that the pollen viability is maintained.

The success of atmospheric pollen transport depends crucially on pollen viability. At the same time, breeding programs with genetically more resistant ash trees can only be carried out successfully if viable pollen are available. The determination of pollen viability can be achieved using different methods. Besides the assessment of pollen germination, viability can also be studied via pollen staining tests [25,26]. The TTC test is a commonly used agent and proved successful determination of pollen viability for many species [27–31], although not all pollen species yielded successful staining [32,33]. For pollen of *Fraxinus excelsior*, this test was successfully applied by Castiñeiras et al. [34]. Furthermore, in our study viability was also tested using Acetocarmine, Alexander's stain and pollen germination. So far, no application of Alexander's stain has been documented for *Fraxinus excelsior*. For pollen germination, difficulties in achieving reliable results were reported [34]. In addition, no systematic optimization of the nutrient medium for pollen germination of ash pollen has been published as of yet.

We hypothesize that the viability of ash pollen decreases after their release from the flower and strongly depends on meteorological conditions during pollen flight. Thus, the aim of this study was to investigate pollen viability under different environmental conditions and to experimentally evaluate storage temperatures using different methods. In addition to climate chamber experiments that simulated the conditions pollen can face during atmospheric transport, the viability of pollen sampled from the atmosphere has been tested.

## 2. Materials and Methods

### 2.1. Study Material

For the experiments conducted in this study, ash pollen collected in an ash seed orchard were used. The seed orchard near Emmendingen (48°6'38.50" N, 7°52'20.49" E, 209 m NHN) is located in Baden-Württemberg, Germany. In 1995, the plantation was established on a previously agriculturally used area as a first afforestation on an area of about 2.7 ha. A total of 49 clones were planted, and in the summer of 2021 there were 84 ash trees on the plantation. The annual average temperature is 10.2 °C and the average precipitation sum is 882 mm (DWD station "Emmendingen-Mundingen", 1981–2010).

For the preparation of a pollen composite sample, shoots of male ash trees were cut shortly before flowering in March 2021. The shoots were then placed in vessels with water at room temperature in the laboratory. After approximately four days, the flowers of the shoots opened and pollen were released. These were then collected on paper underneath, mixed together and immediately used for the subsequent experiments.

### 2.2. Viability Tests

#### 2.2.1. TTC Test

The TTC test is an enzymatic test and detects the presence of enzyme activity in pollen, according to which the viability of the pollen is determined. For this purpose, a colorless, water-soluble tetrazolium salt was used, which is reduced to red formazan in the presence of dehydrogenase in the pollen. The red colored pollen thus signals the viability and is only produced when there is active enzyme activity in the pollen [26]. To perform the TTC test, a 1% TTC solution of 0.2 g 2,3,5-triphenyl tetrazolium chloride and 12 g sucrose in 20 mL distilled water was produced. A thin layer of the pollen from the mixed sample was dusted with a brush onto a microscope slide. Afterwards, two drops of the TTC

solution were added to the slide and covered with a coverslip. The slide was then kept in a petri dish with moist filter paper. To achieve the maximum intensity of coloring, an incubation period of 24 h was set. After 24 h, the stained pollen were counted under a light microscope (Olympus CX23, magnification  $\times 40$ ). Red stained pollen were categorized as “viable” and colorless or yellowish stained pollen were categorized as “non-viable”. We counted 400 pollen located on a random central row of the microscope slide. The evaluation of the TTC test was sometimes affected by a color gradient of the stained pollen, which made it difficult to distinguish clearly between viable and non-viable pollen. For this reason, we additionally included the classification “semi-viable” for pollen that were only colored slightly reddish. The coloration of pollen is often assessed very subjectively, and in particular, the boundaries according to which pollen are assigned to a certain category can vary depending on the processor [26]. Hence and in order to increase the accuracy, the microscope slides were only analyzed by one trained person in our study.

### 2.2.2. Alexander’s Stain

Malachite green, a component of Alexander’s stain is used to stain the cellulose of pollen walls green, while acid fuchsin and orange G are used to stain the cytoplasm pink. This test of pollen viability is based on the assumption that pollen containing cytoplasm can be regarded as viable [35]. Since Alexander’s stain contains toxic components, an adapted solution according to Peterson et al. [36] was used. The pollen were again finely spread on a microscope slide using a brush and then two drops of the solution were added. After covering the slide, it was heated on a hot plate for a few seconds until visible bubbles formed under the coverslip. Viable, semi-viable and non-viable pollen were counted after 10–15 min under the light microscope.

### 2.2.3. Acetocarmine

Acetocarmine stains existing chromosomes in the pollen [25]. Viable pollen are stained red, while non-viable pollen remain colorless. Again, when performing this test, pollen were first dusted onto a slide using a brush. Two drops of Acetocarmine were then applied to the slide and covered with a coverslip. The counting of pollen was conducted immediately after application using light microscopy.

### 2.2.4. Pollen Germination

Finally, the viability of the pollen was tested by pollen germination on a solid nutrient medium. For the storage experiments, a solution of 10% sucrose, 1% agar and 20 ppm boric acid in distilled water was used. After boiling the solution shortly, and allowing 14 mL to set in a 90 mm diameter petri dish, pollen were finely dusted onto the solid culture medium. Germination of the pollen then took place in the dark at room temperature for 24 h. After 24 h, pollen were counted under a light microscope and categorized as germinated or not germinated. Pollen were scored as germinated once the pollen tube was equal or larger than the diameter of the pollen grain [26]. As the germination values differed greatly from those of the other viability tests, an optimization of the culture medium was initiated.

For this purpose, a multi-stage experimental set-up was designed, according to which the ideal concentrations of the components of the culture medium for the pollen of *Fraxinus excelsior* were determined step by step. Different concentrations of sucrose, boric acid ( $H_3BO_3$ ), calcium nitrate ( $Ca(NO_3)_2$ ) and agar were investigated and three tests were conducted for each concentration. Five different culture media were prepared with different sucrose concentrations (0%, 5%, 10%, 15% and 20%). Since findings from our previous pollen germination experiments could be used, 20 ppm boric acid and 1% agar were also used for this experiment. The subsequent germination phase of 24 h took place in a regulated climate chamber (growth cabinet KBWF, Binder GmbH, Germany) at a constant temperature of 25 °C and 80% relative humidity in the dark. Then, the best sucrose concentration determined in the previous step was used and 20 ppm, 100 ppm, 200 ppm, 500 ppm and 1000 ppm boric acid were added to the prepared culture media with 1%

agar, respectively. The best concentration determined was further used in the third step of the optimization, which focused on calcium nitrate concentration. Calcium nitrate was investigated as an additional component of the nutrient medium, a substance that can often lead to a more successful pollen germination [37]. For this purpose, the concentrations 0 ppm, 100 ppm, 300 ppm, 500 ppm and 1000 ppm were tested. In the last step of the optimization, the concentration of agar was examined. Sucrose, boric acid and calcium nitrate concentrations, which were already determined, were used with 1% and 2% agar. Three repetitions were performed for each concentration for all components.

For the pollen viability derived from the different concentrations of sucrose, boric acid and calcium nitrate, a one-factor analysis of variance (ANOVA) and a subsequent post hoc analysis, the paired *t*-test, were performed in R (version 3.6.3). The suitability of the ANOVA was determined in advance by testing the variables for normal distribution (Shapiro–Wilk test) and variance homogeneity using Levene’s test. A two-sided *t*-test was used for the results of the two different agar concentrations.

Finally, a control with non-viable pollen was carried out according to Rodriguez-Riano and Dafni [38]. For this purpose, ash pollen were heated for approximately 15 minutes on a hot plate at 100 °C on a microscope slide. Afterwards, the non-viable pollen were tested for viability with the four viability tests, and thus, also for the validity of the tests used.

The TTC test, Alexander’s stain, Acetocarmine and pollen germination were used for the storage experiments, however, for experiments in the climate chamber and for the volumetric pollen trap, only the TTC test was used. For each experiment, we repeated each test three times.

### 2.3. Storage Experiments

Immediately after the preparation of the composite sample, the first viability tests were performed and the initial viability value was determined. Subsequently, the composite sample was stored at 4 °C in a fridge, at –20 °C in a common freezer and at –80 °C in a blast freezer (Fryka Cold box B 35-85). After one, two and three months, respectively, the viability of the samples was tested again. Please note, that results are not available for all investigated times due to delays in the delivery of individual chemicals and difficulties in carrying out pollen germination.

### 2.4. Experiments on Pollen Viability after Potential Atmospheric Transport

#### 2.4.1. Climate Chamber

For the investigation of pollen viability under simulated natural conditions, three experiments were executed with different settings in a climate chamber. Each trial covered a period of 28 days. The viability of ash pollen was tested with the TTC test initially, after 24 h, 3 days, 7 days, 14 days, 21 days and 28 days.

For experiment 1 (Table 1), we firstly determined the mean April temperature and relative humidity for Bavaria since the flowering of ash predominately occurs in this month. Hourly data of 17 weather stations from the German Weather Service (DWD) ([https://opendata.dwd.de/climate\\_environment/CDC/observations\\_germany/climate/hourly/](https://opendata.dwd.de/climate_environment/CDC/observations_germany/climate/hourly/) accessed on 15 November 2021) were averaged for the reference period 1961–1991. Data were divided into day (from 7 a.m. to 8 p.m.; 10 °C, 65% relative humidity) and night means (from 8 p.m. to 7 a.m.; 5 °C, 80% relative humidity).

**Table 1.** Climate chamber settings (temperature, relative humidity and UV radiation) for day and night conditions within experiments 1, 2 and 3 to test the influence of meteorology on the viability of ash pollen.

	Experiment 1		Experiment 2		Experiment 3	
	Day	Night	Day	Night	Day	Night
Temperature	10 °C	5 °C	12 °C	7 °C	20 °C	12 °C
Relative humidity	65%	80%	65%	80%	60%	80%
UV radiation	on	off	on	off	on	off

Experiment 2 represents a climate change scenario of +2 °C warming (Table 1), which can be expected according to the SSP2-4.5 scenario, by the middle of the 21st century for global means [39].

Experiment 3 investigated the influence of extreme temperatures on the viability of ash pollen with temperatures of 20 °C during the day and 12 °C during nighttime. These temperatures can obviously occur in April, although not constantly over a 28-day period. However, with these settings, the influence of higher temperatures can be studied in more detail.

The corresponding relative humidity for experiment 2 and 3 was estimated based on its relationship to the temperature using the hourly meteorological data of the DWD. UV radiation for all experiments was adjusted to the average potential sunshine duration prevailing in April (day 13 h) (Table 1).

#### 2.4.2. Volumetric Pollen Trap

To investigate the viability of pollen under partially natural occurring transport conditions, samples of pollen suspended in the atmosphere during four weeks of the common ash pollen season in 2021 were collected using a volumetric pollen trap of the Hirst type [40]. Pollen were caught within the trap for a duration of seven days and for four weeks in total. The viability resulting from these data was assessed on a daily basis. Therefore, the pollen that were adhered at the last day were exposed to ambient air and the air inside the sampler either for a very short time (hours or minutes in the case of a quick impaction after pollen release) or probably also even longer when prior transport took place. In turn, the pollen of the first sampling day of one week was exposed to the influence of surrounding air for seven days within the trap, and probably for an additional time in the ambient air.

The sampling was performed on the roof of a university building in Ingolstadt (48°76'58.06" N, 11°41'56.23" E), Bavaria, at 13 m a. g. l. There are numerous ash pollen sources surrounding the sampler, as there are parks and green areas close to the pollen trap.

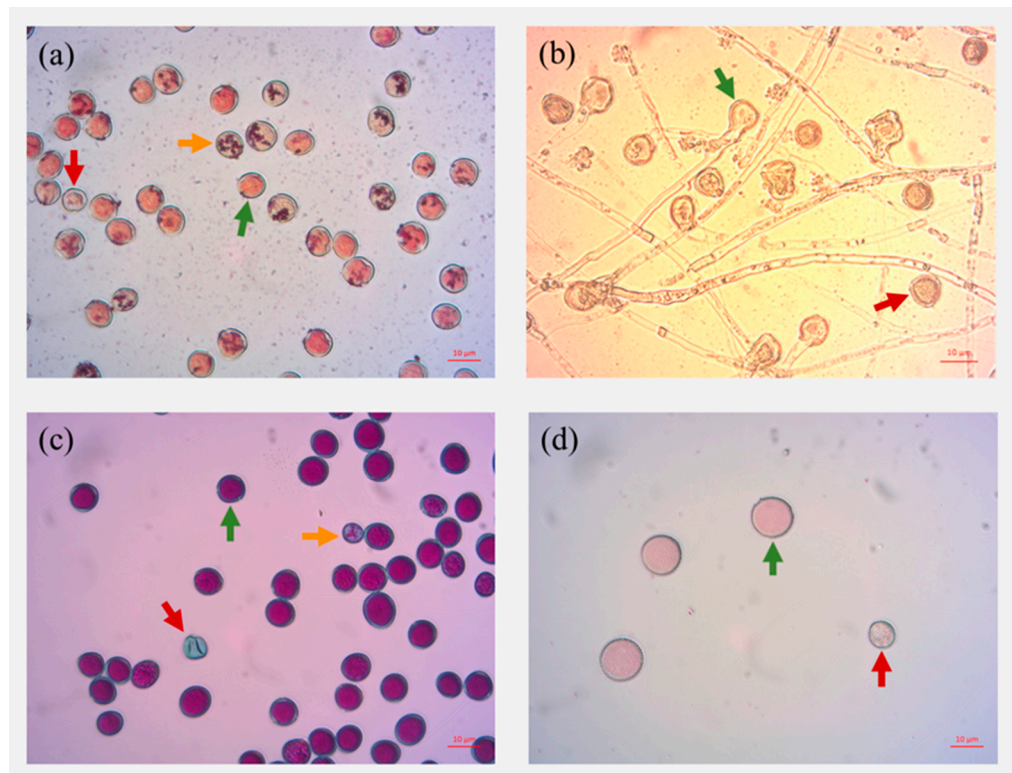
The drum of the pollen trap was changed weekly, starting on 15 April 2021. The silicone-coated foil strip attached to the drum was covered with a TTC solution shortly after each change and split into seven 48 mm long sections, each section corresponds to a 24 h sample, which was placed on the glass slide and examined under a light microscope at ×40 magnification. Ash pollen were checked for their viability along horizontal lines across the full range of the microscope slides and sorted into the categories of viable and non-viable until the number of 100 was reached. In total, on ten of 26 days the total count of all pollen on the slide was less than 100, due to a generally low pollen flight intensity on these days. The pollen concentration was calculated, after counting ash pollen of four random lines of the slide (representing approx. 9% of the impaction area), according to Galán et al. [41].

The air temperature was recorded throughout the study period, with a measuring device (Davis Vantage Pro 2) next to the pollen trap.

### 3. Results

#### 3.1. Viability Tests

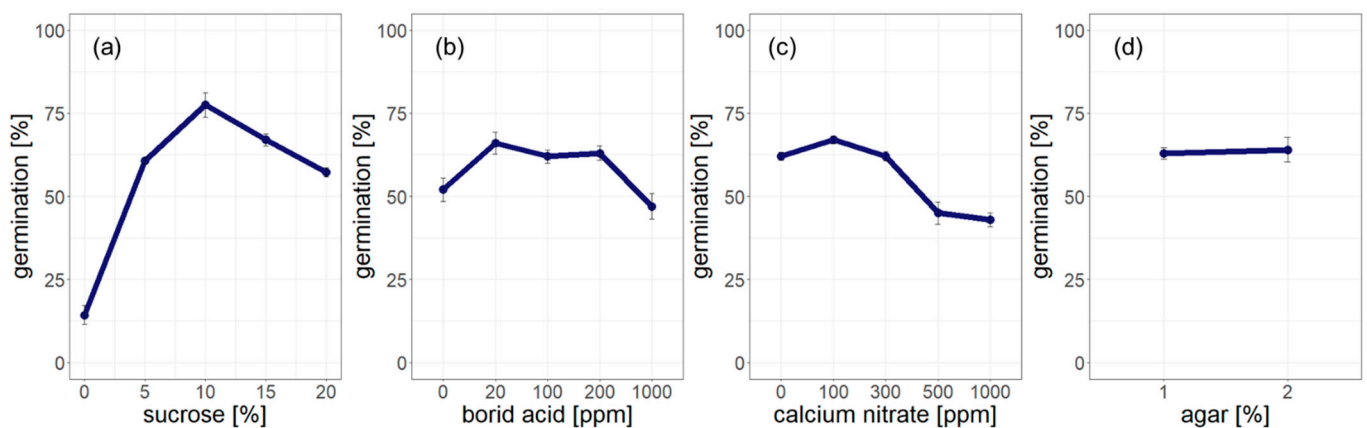
The applicability of the four viability tests for ash pollen was firstly evaluated on the basis of the quality related to distinction and recognition: The TTC test enabled a classification of the pollen into three categories (Figure 1a) based on the colors from dark red to pale yellow or colorless. Within the experiment of pollen germination, pollen tubes formed after 24 h, and could easily be recognized under the microscope (Figure 1b). Alexander's stain colored the pollen in clearly distinguishable pink and green colors (Figure 1c). In contrast to the TTC test and Alexander's stain, Acetocarmine led to a faint color, although viable and non-viable pollen could be differentiated based on pale reddish and colorless pollen. In addition to a coloration of the pollen, an increase in the size of the colored pollen was also observed after approx. 2 h when using Acetocarmine (Figure 1d), which decreased again after approx. 24 h.



**Figure 1.** Exemplary pictures of the conducted viability tests, (a) TTC test, (b) Pollen germination, (c) Alexander's stain, (d) Acetocarmine. The green arrow points to exemplarily viable pollen, the yellow arrow to semi-viable pollen and the red arrow to non-viable pollen.

### 3.2. Optimization of Pollen Germination

For optimizing the nutrient medium for pollen germination, the ANOVA analysis revealed a significant difference between the five sucrose concentrations ( $p = 0.020$ ). According to the post hoc analysis, there is no significant difference between 5% and 15%, and between 5% and 20%. The optimum sucrose concentration is 10%, which differs significantly to the other concentrations and was associated with the highest percentage of germinated pollen (Figure 2, Table 2).



**Figure 2.** Results of the optimization of the procedure for testing ash pollen germination for different concentrations of sucrose (a), boric acid (b), calcium nitrate (c) and agar (d). The standard deviation is visualized with error bars.

**Table 2.** Results (*p* values) of the ANOVA post hoc paired *t*-test for comparing different concentrations of sucrose, boric acid, and calcium nitrate concentrations in the optimization of ash pollen germination.

		0%	5%	10%	15%
Sucrose	5%	<0.001 *			
	10%	<0.001 *	<0.001 *		
	15%	<0.001 *	0.084	0.005 *	
	20%	<0.001 *	0.131	<0.001 *	0.008 *
		0 ppm	20 ppm	100 ppm	200 ppm
Boric acid	20 ppm	0.008 *			
	100 ppm	0.059	0.554		
	200 ppm	0.034 *	0.694	0.694	
	1000 ppm	0.524	0.001 *	0.007 *	0.004 *
		0 ppm	100 ppm	300 ppm	500 ppm
Calcium nitrate	100 ppm	0.190			
	300 ppm	0.880	0.190		
	500 ppm	<0.001 *	<0.001 *	<0.001 *	
	1000 ppm	<0.001 *	<0.001 *	<0.001 *	0.720

\* = significant difference with  $p < 0.05$ .

The optimal boric acid concentration was less clear. Even though the highest germination value was achieved at 20 ppm, the difference between 20 ppm, 100 ppm and 200 ppm was not statistically significant. Less suitable for pollen germination are 0 ppm and 1000 ppm. These groups were significantly different from the groups 20 ppm and 200 ppm and exhibited the lowest mean germination (Figure 2, Table 2). For the following tests, a concentration of 20 ppm was used.

For the calcium nitrate concentration, the highest germination value was observed at 100 ppm, however, this group was not significantly different from 0 ppm and 300 ppm. In particular, 500 ppm and 1000 ppm led to a lower germination of pollen (Figure 2, Table 2). For the following test, 100 ppm calcium nitrate were used.

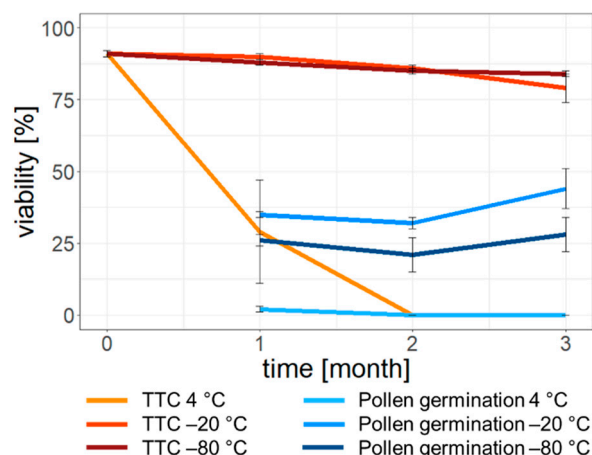
Performing the two-tailed *t*-test on the two agar concentrations studied, no significant difference between the germination of pollen at 1% and 2% agar ( $p = 0.913$ ) could be shown (Figure 2). For further tests 1% agar should be used.

Therefore, the optimum mixture yielding to the highest germination of ash pollen consists of 10% sucrose, 20 ppm boric acid, 100 ppm calcium nitrate and 1% agar, with calcium nitrate being an optional component.

### 3.3. Storage Experiments

The TTC tests showed that the viability of the pollen developed differently at the three investigated temperatures 4 °C, −20 °C and −80 °C after one, two and three months (Figure 3). In particular, the storage at 4 °C led to a more rapid decline in viability than at −20 °C and −80 °C and after two months, no viable pollen could be detected. The temperatures below freezing were associated with a decrease of 11.5% (−20 °C) and 6.7% (−80 °C) after three months.

Since the first test with pollen germination failed, the initial value is missing here. For pollen germination, pollen stored at −20 °C showed the highest viability values after one, two and three months. The viability of pollen stored at −80 °C was 11.9% lower. Pollen germination of pollen stored at 4 °C showed lower values in each month and no viable pollen from the second month onwards. In contrast to the expected decrease in pollen viability from the second to the third month, a slight increase ranging between 7% (−80 °C) and 12% (−20 °C) was recorded for all freezing temperatures (Figure 3). The documented increase can possibly be attributed to a higher room temperature and therefore a higher germination temperature during the incubation period (April vs. June).



**Figure 3.** Temporal development of ash pollen viability [%] after one, two, and three months at storage temperatures of 4 °C, -20 °C and -80 °C evaluated with TTC test and pollen germination. The standard deviation is visualized with error bars.

Although pollen viability derived from the TTC test and from pollen germination differed, both tests showed changes in viability over the three months studied. The Alexander's stain and Acetocarmine test, however, did not present any change in the viability of the pollen. Even though not all values were available, it still was evident, that there were neither remarkable differences between the different storage temperatures nor differences between the individual months. The proportion of viable pollen in both tests was between 95% and 97% and thus higher than in the TTC test and pollen germination. Within the control test using heat-killed pollen, the TTC test and the pollen germination showed no staining or germination and thus proved their reliability. The Alexander's stain and Acetocarmine test on the other hand, yielded values between 95% and 97% of viable pollen in the staining of the killed pollen. These values are comparable to those from the storage experiments.

### 3.4. Atmospheric Transport

#### 3.4.1. Simulations in Climate Chamber

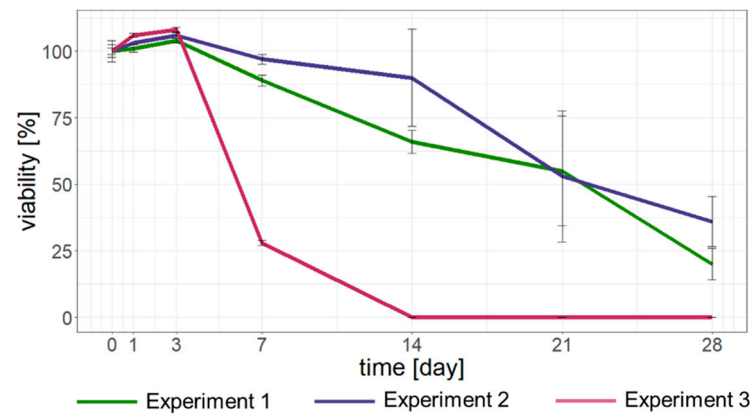
The viability of the pollen exposed to the settings of climate chamber experiment 1 (10/5 °C, 65/80% relative humidity) was increased by 4% after the third day; then there was a decrease in viable pollen (-15%) after seven days. After 21 days, about half of the pollen were still viable, compared to the initial value. Even after 28 days, viable pollen (8%) could still be detected.

Experiment 2 differed by only +2 °C from the settings of experiment 1 (12/7 °C 65/80% relative humidity). An increase in the viability of ash pollen could be observed until the third day; after seven days the viability of the pollen decreased by 9%. On day 14, the viability of the pollen was only 10% below the documented initial value, and on day 21, it was only less than half of the initial value. After 28 days, 15% viable pollen could be detected.

The results of experiment 3 (20/12 °C, 60/80%) also show an increase in the viability until day three. At day seven, a rapid drop in the viability of the pollen was documented (-80%). After 14 days, no more viable pollen were observed under the microscope.

The comparison of experiments shows, that experiment 3 in particular differs from the two previous experiments (Figure 4). While viable pollen were still documented after 28 days in both experiment 1 and 2, no viable pollen were present in experiment 3 after only two weeks.





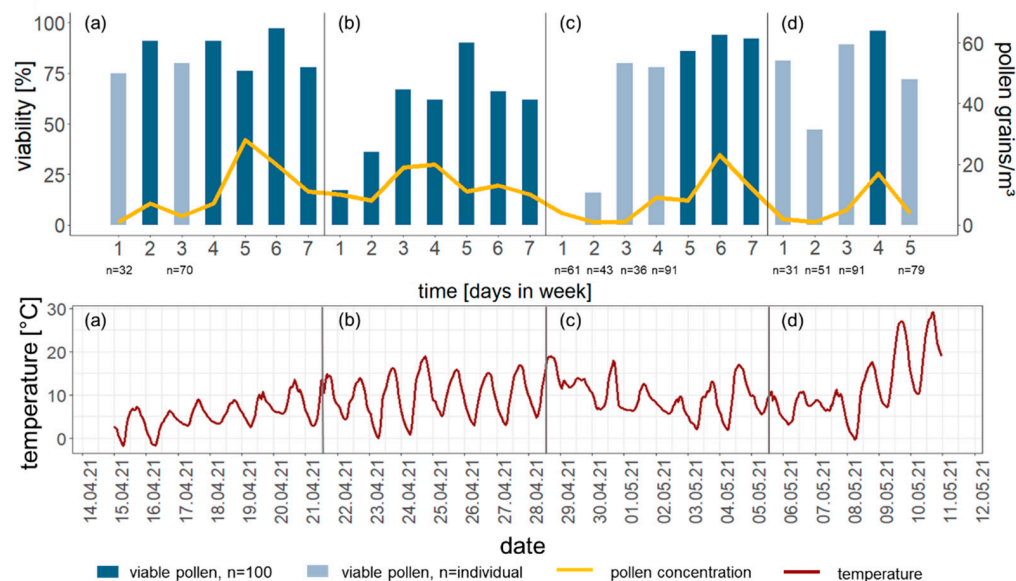
**Figure 4.** Temporal development of ash pollen viability [%] in the climate chamber for experiments 1 (10/5 °C, 65/80% relative humidity), 2 (12/7 °C 65/80% relative humidity) and 3 (20/12 °C, 60/80% relative humidity) within a total of 28 days. The standard deviation is visualized with error bars.

In all experiments, an increase in viability was observed up to day three. The initial viability value was determined using the TTC test, immediately after the pollen were removed from the blast freezer (−80 °C). These higher viability values can probably be explained by an increase in thawed pollen. This assumption is supported by the fact that in preliminary experiments, in which fresh pollen were used, no initial increase in viability was observed. However, it is also possible that the observed increases are due to counting inaccuracies, as the difference was only small (+4% to +8%).

#### 3.4.2. Samples from Atmosphere

Within the pollen trap experiments, different viability values could be observed for the four investigated weeks. In week one, high viability values between 75% and 97% were recorded on all seven days (Figure 5). Lower viability values (17%) were recorded on day one in week two. That means that pollen from this day were captured in the interior of the pollen trap for at least seven days. On day seven, 62% viable pollen were sampled. That means that sampled pollen on this day were one day old or older in the case of long-range transported pollen. The mean pollen concentration was the highest of all four investigated weeks in week two and the aspired sample size of 100 ash pollen was achieved on all seven days. Viability values ranged between 17% and 90%. Week three is characterized by a low pollen concentration in the first half of the week and high viability values from day three onwards. In week four, only on one day 100 ash pollen could be counted. Since the number of counted ash pollen was low in week four, the calculated viability values are limited in their reliability. Overall, the viability of the pollen varied greatly between 0% and 97% over the course of the four weeks investigated (Figure 5). However, it can be seen that especially in week two (and week three), viability values were lower in the first days of sampling, i.e., for pollen that have a minimum age of seven days.

Meteorological conditions show lower air temperatures, especially in week one, and higher temperatures at the end of week four (Figure 5).



**Figure 5.** Viability of ash pollen [%] captured with a volumetric pollen trap (blue bars) in Ingolstadt, Germany in 2021, the respective pollen concentration [pollen grains/m<sup>3</sup>] (yellow line) and the ambient air temperature [°C] (red line) for week one (15.04–21.04) (a), two (22.04–28.04) (b), three (29.04–05.05) (c) and four (06.05–11.05) (d). Light blue bars indicate that the total pollen catch was <100 pollen grains (total numbers are shown below these bars), dark blue bars indicate that 100 pollen grains were counted and assessed for their viability.

## 4. Discussion

### 4.1. Suitability of Viability Tests for Ash Pollen

Knowledge on pollen viability is important with respect to increasingly fragmented ash populations and therefore an adequate assessment is crucial. The first test, the TTC test resulted in a successful staining of the pollen with a recognizable color differentiation. In this study, the validation using a control group of killed pollen [38] showed that no staining appeared. For ash pollen, the TTC test was already evaluated as a suitable test for determining their viability [34].

The Alexander's stain resulted in a clear coloring of the ash pollen in pink and green. While we are not aware of any study related to the pollen of *Fraxinus excelsior*, the Alexander's stain is frequently used for other pollen species. In particular, the clearly separated coloring of pollen is considered an advantage [42]. Good results with the Alexander's stain have been obtained, among others, in the assessment of the viability of pollen of *Polygala paniculata* L. [43], *Crotalaria juncea* L. [44], *Centaureum Hill* and *Gentianella Moench* [45]. The easy application of the stain, and the rapid results in particular, were rated as positive [46], which can be confirmed by our study using ash pollen.

The test is based on the assumption that the presence of pink stained cytoplasm simultaneously signals the viability of the pollen. While pollen containing no cytoplasm is certainly not viable, conversely, it does not necessarily mean that all pollen containing cytoplasm are viable. Therefore, pollen viability can easily be overestimated [47,48]. The results obtained in this study, which was based on adapted staining agents [46,49], also call this issue into question: Regardless of the duration of pollen storage and the respective temperature, we found that the average viability ranged between 95% and 97% and was considerably higher than that determined by the TTC test. In the control test with killed pollen, these pollen were stained just like viable pollen, which further confirms the unsuitability of Alexander's stain for ash pollen. The application of Alexander's stain for the determination of the development of the viability of *Nothofagus Blume* pollen under defined storage conditions also proved to be not successful [50]. This was also observed in *Asparagus officinalis* L. pollen after storage at 4 °C, while other viability tests resulted in a

change in viability. Storage conditions probably modify structures and processes within pollen, which affect the viability of these, however, this cannot be made visible using Alexander's stain [51].

Alexander's stain is suitable for studying the absence or presence of cytoplasm, and thus, the integrity of pollen or the proportion of sterile pollen [52]. For the ash trees, whose pollen were examined in this study, the percentage of sterile pollen ranged between 3% and 5%.

The staining of ash pollen with Acetocarmine provided a less clear staining than the TTC test or the Alexander's stain. Nevertheless, a differentiation between slightly reddish colored pollen and colorless pollen was possible. Good results were also obtained for pollen of *Colocasia esculenta* L. [53], Liliium Oriental hybrids [33] and *Ficus carica* L. [29]. In the case of *Castanea mollissima* Blume and *Castanea henryi* (Skan) Rehder and E. H. Wilson, it was not possible to distinguish between viable and non-viable pollen [32]. For *Olea europaea* L. pollen, a reliable assessment of pollen viability could also not be achieved, as shown by a killed control [54]. In this study, the control test showed that killed pollen were colored like viable pollen. Thus, the determination of the development of viability of ash pollen with Acetocarmine is not suitable. These results are consistent with the findings of Castiñeiras et al. [34], who reported that the staining of *Fraxinus excelsior* pollen with Acetocarmine provided high viability values close to 100%, as obtained in our study.

The composition of the nutrient medium used for pollen germination and the method of pollen germination varies greatly depending on the type of pollen examined. In general, both a liquid, as well as a solid culture medium, can be suitable. The nutrient medium of Brewbaker and Kwack [37] is often used, e.g., for determining the viability of *Swainsona formosa* (G. Don) Joy Thomps pollen [55], for *Acacia podalyriifolia* A. Cunn. ex G. Don and *Acacia mearnsii* De Wild pollen [56] or for *Elaeis guineensis* Jacq. pollen [57], however, individual optimizations were also frequently applied [58–60].

For the storage experiments, we used a solid nutrient medium with sucrose, boric acid and agar, and pollen germination was carried out at room temperature in the dark. Germination at room temperature has already been successfully performed for numerous pollen species, including pollen of various legumes (Fabaceae) [61] or pollen of *Swainsona formosa* [55]. A successful germination at room temperature was also achieved for pollen of *Fraxinus lanuginosa* Koidz [62]. In our study, we found that germination at room temperature was unsuitable. Although germination of the pollen was achieved and a difference between the different storage temperatures was observed, a comparison between the viability of pollen for different storage durations was impossible.

For this reason, a uniform temperature of 25 °C was selected for the subsequent optimization of the culture medium. According to Kremer and Jemrić [63], the optimal temperature for germination of *Fraxinus pennsylvanica* Marshall pollen is between 20 °C and 25 °C, with an optimum closer to 25 °C. Castiñeiras et al. [34] also used a temperature of 25 °C for *Fraxinus excelsior* pollen germination.

According to our results, the optimum sucrose concentration for *Fraxinus excelsior* pollen is 10%. For *Fraxinus lanuginosa* pollen, satisfactory germination was also achieved at 5% and 10% [62]. The highest germination values in the investigation of boric acid were found at 20 ppm, those of calcium nitrate at 100 ppm. Due to high viability values related to other concentrations, we conclude that a range of 20 ppm to 200 ppm for boric acid and a range of 0 ppm to 300 ppm for calcium nitrate is suitable for ash pollen analyses. Boric acid in particular represents an important component of the nutrient medium [25] and promotes the germination of *Fraxinus excelsior* pollen. The enhancement of pollen germination by the addition of boric acid has already been shown in other pollen species including *Litchi chinensis* Sonn [64] or *Cunninghamia lanceolata* (Lamb.) Hook [65]. The concentration of agar, however, did not reveal any remarkable differences between 1% and 2%.

Problems related to the performance of ash pollen germination were reported by Castiñeiras et al. [34]. Therefore, the optimizing tests of a solid nutrient medium performed in our study can be very helpful for future research on ash pollen.

Nevertheless, the evaluation of germinated pollen is associated with difficulties, as a strong germination with long, tangled pollen tubes can hinder the counting and thus the evaluation of the experiment. In addition, pollen germination is highly dependent on the environmental conditions of the experiment.

#### 4.2. Effects of Storage Conditions on Ash Pollen

Breeding programs may benefit for the availability of viable ash pollen and therefore it is important to evaluate the optimal storage of pollen. The results of the Alexander's stain and the Acetocarmine test were not reliable in regards to the development of ash pollen viability under different storage conditions, the TTC test, however, provided good results. It was shown that a storage temperature of  $-20\text{ }^{\circ}\text{C}$  and  $-80\text{ }^{\circ}\text{C}$  is superior for preserving viability compared to  $4\text{ }^{\circ}\text{C}$ . Despite the limited evaluability of the results of pollen germination, pollen viability was also found to be least at  $4\text{ }^{\circ}\text{C}$ .

These results are consistent with studies on the viability of other pollen species under certain storage conditions. Pollen of strawberry have been shown to maintain their viability for 20 months at a storage temperature of  $-18\text{ }^{\circ}\text{C}$ , and for only eight months at  $4\text{ }^{\circ}\text{C}$  [66]. *Phoenix Dactylifera* L. pollen also showed higher viability values when stored at  $-20\text{ }^{\circ}\text{C}$  compared to  $4\text{ }^{\circ}\text{C}$  [67]. Pollen viability of date palm was maintained for up to one year at  $-20\text{ }^{\circ}\text{C}$  [68]. A higher viability was also observed in *Herbaceous peonies* pollen after storage at  $-76\text{ }^{\circ}\text{C}$  compared to  $4\text{ }^{\circ}\text{C}$  and  $-20\text{ }^{\circ}\text{C}$  [69]. In contrast, other studies did not detect remarkable differences in pollen viability between storage at  $-20\text{ }^{\circ}\text{C}$  and  $-80\text{ }^{\circ}\text{C}$  (e.g., for almond pollen [70] or for *Leonurus cardiaca* L. pollen [71]). In these cases, it can be concluded that a storage at  $-20\text{ }^{\circ}\text{C}$  is less expensive and therefore preferable. The observed slight decrease in germination of the frozen pollen in our study can probably be attributed to possible damage to the pollen due to the formation of intracellular ice during the freezing process, as also observed by Shekari et al. [71].

Our study covers only a storage duration of three months. Raquin et al. [72] found no decrease in viability of pollen from *Fraxinus excelsior* and *Fraxinus angustifolia* when stored at  $-70\text{ }^{\circ}\text{C}$  for up to eleven months. Therefore, storage of frozen and viable ash pollen for longer than three months seems to be possible. This offers the opportunity to preserve and use ash pollen a long time after sampling for breeding programs. In the light of ash dieback, pollen of potentially resistant trees can successfully be stored and bear the potential for future breeding of trees that are able to cope with the disease.

#### 4.3. Potential Effects of Long-Range Transport of Ash Pollen

We assume that long-range pollen dispersal affects pollen viability and we therefore evaluated the effects of meteorology on the quality of pollen in climate chamber experiments. We observed that pollen, which were distributed unevenly in the petri dish, probably led to shadow effects in the climate chamber experiments. That means that pollen that were located in the middle of a larger accumulation can be protected from the effects of UV radiation. In the first experiment, we were not aware of these shadow effects and from experiment two onwards, the pollen were then distributed in a very thin layer. However, due to the very small size of the individual pollen grains, shadow effects caused by surrounding pollen cannot be ruled out even in experiments two and three. Increased or prolonged UV radiation leading to a rapid reduction in pollen viability was demonstrated by studying maize [73,74], oak [75] and pine pollen [76]. To what extent, and in particular how quickly, the viability of the pollen examined is reduced differs greatly depending on the type of pollen. When interpreting our results, it has to be considered, that our experiments were designed to assume only cloudless days with a sunshine duration of 13 h. This is usually not the case for such a long period in the region (Emmendingen, Germany) from which we sampled the pollen. Under cloudy conditions, pollen viability of *Festuca arundinacea* Schreb. decreased more slowly [60]. For pollen of *Panicum virgatum* L., viability decreased five times faster under sunny compared to cloudy weather conditions [77].

The storage experiments have already shown that pollen viability can be maintained longer at lower temperatures. This was also confirmed by the climate chamber experiments. While the climate chamber experiments 1 and 2 with temperatures of 5/10 °C and 7/12 °C were associated with viable pollen that could still be observed after 28 days, in experiment 3 (12/20 °C) no viable pollen were present after only seven days. Experiments 1 and 2 differ from each other in the course of the development of viability, however, no clear conclusions can be drawn as to which of the two temperature settings ensures a longer maintenance of viability. Therefore, small changes in mean temperature were not associated with large effects on viability. However, long lasting heat extremes can have serious consequences for pollen viability.

The extent, and in particular, the time for which ash pollen maintains its viability after release thus depends on temperature (extremes) and the prevailing UV radiation.

The impact of natural environmental conditions on pollen viability was investigated in more detail by the volumetric pollen trap experiments. In particular, we determined whether a difference in viability could be detected between the days one to seven. Pollen sampled on day one may have been exposed to the prevailing temperatures and other meteorological conditions for six days longer. Since the foil band, on which the pollen were collected, was inside the pollen trap, the pollen already picked up were then protected from the influence of UV radiation. Meteorological conditions show lower temperatures at the beginning of the study period, however, a correlation with the viability values cannot be proven with the existing data.

In week two and three, lower viability values were documented for the first samplings days, which shows that pollen captured for a longer period may be negatively affected. With viable pollen ranging from 0% to 97%, viability of ash pollen differed greatly within the studied period. These data may present a rough proxy on the fraction of pollen, which was traveling a longer time before impaction.

A possible impact of the silicone adhered to the foil strip is unlikely, since we found a high viability of pollen on certain days.

## 5. Conclusions

We found that the duration of long-distance transport, and the environmental conditions prevailing at that time, play an important role in the successful pollination of female ash flowers by (long-range) transported pollen. In particular, high temperatures and sunny weather can have a negative effect on the pollination success since these meteorological factors reduce pollen viability.

Our methodological comparison showed that a successful pollen germination could be achieved using an optimized solid nutrient medium. Both Alexander's stain and Acetocarmine did not indicate the viability of the pollen correctly. However, the TTC test has proven to be suitable for determining viability, both in the storage experiments and in the long-distance transport investigations. The storage experiments have shown that storage of pollen at −20 °C or −80 °C is to be preferred to a storage temperature of 4 °C.

Overall, the viability of *Fraxinus excelsior* pollen in respect to ash dieback is shown to be of crucial importance. For future breeding programs using ash pollen, suitable methods to determine pollen viability are necessary, as well as an adequate storage preserving viability of the pollen. Natural reproduction of ash trees within and between prospectively fragmented ash populations is strongly dependent on pollen viability, and therefore, on the natural conditions during pollen dispersal. Future studies should focus on the influence of those natural conditions, especially UV radiation, however, they should also consider pollen production, which is potentially modified due to ash dieback.

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