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The Development of an Improved Medium for the *In Vitro* Germination of *Corylus avellana* L. Pollen

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Abstract: The European hazelnut (*Corylus avellana* L.) is a self-incompatible, wind-pollinated species of significant economic-productive interest, mainly cultivated between the Mediterranean basin and the Black Sea. Hazelnut breeding programs and high cropping in commercial orchards are necessarily linked to the availability of fertile pollen with wide germinability and high viability. The objective was to develop an improved method to determine the germinability of pollen, comparing the results with those found in the literature. Pollen germinability was firstly evaluated on a wild-type accession using boric acid (H_3BO_3), calcium nitrate ($Ca(NO_3)_2$), magnesium sulfate ($MgSO_4$), potassium nitrate (KNO_3), casein hydrolysate and different concentrations of sugars, including sucrose (10, 15 and 25%) and two monosaccharides, glucose (15%) and fructose (15%). The optimal composition included 15% sucrose in a semi-solid medium composed of 1% animal gelatin, containing collagen. This formulation was then tested on three cultivars of commercial interest, evaluating the effect of different concentrations of sucrose. The comparison between different *in vitro* germination methods showed that the hanging drop technique in a semi-solid medium provides a statistically higher germination index compared to the liquid and solid/agarized techniques. This study proposes an easy-to-use medium for the *in vitro* germination of *Corylus avellana* L. pollen.

Keywords: hazelnut pollen; pollen germination; pollen tube growth; carbohydrates; culture medium composition

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1. Introduction

Hazelnut (*Corylus avellana* L.) is one of the most important nut crops worldwide. According to the latest FAOSTAT reports [1], global hazelnut cultivation has increased intensively over the last 10 years. Furthermore, hazelnut farming is currently experiencing an expansion phase outside its native areas, particularly in the Southern hemisphere: Chile [2], South Africa [3] and Australia [4,5] are the new colonized frontiers. Although both wild hazelnut and cultivated varieties have faced a long history of domestication and selection, both are subject to a strong alternate bearing, characterized by biennial alternation in yield [6]. This phenomenon, mainly caused by reduced flower induction during the high-fruit-load year [7], is seasonally monitored by technicians and growers by detecting the atmospheric pollen concentration [8]. Reduced pollen emissions may negatively affect the seasonal fruit setting. In fact, fertilization and productivity are phenomena that strictly depend on the availability of viable and germinable pollen [9]. Where natural pollination is hindered by adverse weather conditions, artificial pollination can be applied to improve fruit set [3,10,11]. In hazelnut, the airborne pollen concentration can potentially be related

to yield. A positive correlation was recently observed between the annual sum of daily pollen concentrations emitted and the total hazelnut yield [12].

C. avellana L. is a monoecious, self-incompatible and wind-pollinated species [13] in which phenology and pollen viability during the pollen shedding period are largely influenced by climatic conditions [13–17]. *In vitro* pollen germination has so far been one of the most used methods for a rapid and reliable assessment of pollen viability [18]. However, hazelnut pollen, which is released at a relatively high moisture content (30%) compared to orthodox species (1–5% hydration) [19,20], is more susceptible to desiccation damage resulting in the rapid loss of viability [21]. For this reason, it is classified as recalcitrant or, more precisely, desiccation sensitive [22]. In the past, this characteristic has posed difficulties in the attempt to germinate hazelnut pollen. The first germination tests on hazelnut pollen date back to the first experimental studies on pollination and fertilization conducted at the beginning of the 20th century with the works of Rimoldi [23] and Schuster [24]. The substrates proposed did not involve complex formulations but an agar-based medium with the addition of variable concentrations of sucrose (8–12%) [25]. Between the 1930s and 1950s, Bodmer [26] and Visser [27] assumed the importance of the osmotic pressure of sucrose on the germination of hazelnut pollen. They showed how hazelnut pollen can also germinate on an 80% sugar medium, while the optimal conditions for the emission and growth of the pollen tube were between 35 and 50%. At the same time, Trotter [28] and Riera [29] began the first studies on the effect of temperature on pollen germination. They indicated how low temperatures (between −5 and −10 °C) can negatively influence pollen emission from catkins of late-flowering cultivars. They also observed how pollen could germinate between 1 and 4 °C, even though the optimal conditions found were between 20 and 22 °C. Cox [30] obtained the maximum hazelnut pollen germination at a 25% sucrose concentration after incubation at 25 °C. In the second half of the 20th century, conflicting responses on the effectiveness of specific sugar concentrations on pollen germination were reported [25,31–33]. Manuševr [33] achieved a better germination rate using 15% sucrose, while he observed a gradual increase in germination as the glucose concentration increased. On the other hand, Kim et al. [31] noted the abnormal growth of pollen tubes when germinated on glucose media, while germination on fructose media was poor or lacking at any fructose concentration or temperature combination. Only during the 1960s was the effectiveness of some micro- and macroelements that were selectively added to the germination medium evaluated. Brewbaker and Kwack [34] first formulated a single protocol for numerous species including boric acid (H_3BO_3), calcium nitrate ($Ca(NO_3)_2$), magnesium sulfate ($MgSO_4$) and potassium nitrate (KNO_3). Shortly after, Zielinski [21] observed that the addition of manganese and boron to the medium did not significantly improve hazelnut pollen germination. At the same time, he also observed that boron concentrations higher than 25 μg/mL significantly inhibited pollen tube emission, confuting what had been reported until that time. Subsequently, some studies were carried out on the use of boron and some macroelements in the hazelnut germination medium [35,36]. Only recently has the use of polyethylene glycol (PEG) been suggested in hazelnut pollen germination tests, but with conflicting results [3,37].

The aim of this study was to develop a suitable medium to improve *in vitro* pollen germination for *Corylus avellana* L. by comparing protocols and testing the effect of various methodologies and nutrient concentrations. Particular attention was paid to the use of different sucrose concentrations in a pool of cultivars of commercial interest and a wild-type accession. The aim of this study was to verify the possible existence of different sugar requirements at specific concentrations for different hazelnut cultivars.

The proposed methodology is intended to facilitate future studies on hazelnut plant breeding which often require a simple test to determine the quality of fresh pollen. This study emerges as part of the project on the European hazelnut reproductive biology and pollen viability analysis.

2. Materials and Methods

2.1. Plant Materials and Storage

Three early-flowering hazelnut cultivars, namely 'Tonda Rossa' (TR), 'Tonda Gentile' sin. 'Tonda Gentile delle Langhe' (TG) and 'San Giovanni' (SG), and a late-flowering wild-type (WT) accession were considered for this study. Pollen samples were collected from the *ex situ* collection of hazelnut varieties located at 'Le Cese' (Caprarola, Latium, Italy; latitude 42°20′00″ N; longitude 12°11′00″ E; altitude 570 m a.s.l.). The collection field was established by the Regional Agency for Agricultural Development and Innovation in Latium (ARSIAL) in the year 2000 in a typical hazelnut growing area. Plants were irrigated through a sub-irrigation system and managed with standard orchard management techniques. Namely, the soil was managed with a natural green cover crop, and annually, the orchard received applications of the following quantities of main fertilizers: 90 kg ha^{-1} nitrogen, 60 kg ha^{-1} phosphorus and 90 kg ha^{-1} potassium. The orchard management and the integrated pest and disease control were also applied yearly by ARSIAL according to the guidelines of the current regional Rural Development Plan [38].

Biennial alternation is a phenomenon mainly related to the age of the plant [39] and does not appear to influence pollen viability and germinability [40]; therefore, the samples were collected from 24-year-old plants and during the 2022/23 and 2023/24 flowering seasons. All the samples were harvested weekly by taking at least five bunches per plant from three different plants per cultivar. Only fully elongated catkins were selected from different parts of the canopy according to the different cardinal exposures and the extended BBCH reference scale [41]. Samples were transported to the laboratory where they were left overnight to dry at room temperature to facilitate pollen release, as reported by Ascari et al. [3,42]. The pollen was then classified based on the collection time and, after four hours of dehydration using a saturated aqueous solution of lithium chloride salts (15% RH) at room temperature (+20 °C) [43], it was stored at freezing temperature (−18 °C). Germination tests were carried out in the days following collection.

2.2. In Vitro Pollen Germination

Before performing pollen germination tests, the pollen viability analysis was carried out to establish samples with the highest viability. It has been extensively observed that wild hazelnut pollen has high levels of viability, higher than commercial cultivars [17,38,43]. A WT accession was chosen for preliminary tests because it was characterized by low levels of anomalous pollen [3,17,42]. These features ensured an easier interpretation of the results by limiting the classification of the pollen into germinated and non-germinated. For this reason, preliminary tests were conducted only on the WT accession during the 2022/23 flowering season, while during the following 2023/24 season, the methodology was validated on a pool of cultivars (TR, TG and SG). Pollen viability screening was performed using an impedance flow cytometer (Am-pha® Z32, Amphasys, Lucerne, Switzerland), according to the protocol described by Brandoli et al. [17] and Ascari et al. [42]. Pollen grains were sieved with a 50 µm filter to eliminate any debris before analysis. Samples were then suspended for 10 min in AF6 buffer (Amphasys, Lucerne, Switzerland), rich in mineral salts and sugars, specially designed to have a suitable osmolality for the analysis of pollen grains. Samples were sieved a second time to avoid clogging of the chip and pumped into a microchannel chip to which an electric field at intermediate frequencies (between 1 and 8 MHz) was applied. At these frequencies, each pollen grain modified the impedance signal according to its dielectric properties [44], providing information on its viability as the polarization of the pollen membrane decreased, and its capacitance and conductance were detected and measured [45].

Data were manually selected from an area on the scatterplot to classify pollen into viable, non-viable, and anomalous. The data were then normalized to 100% after filtering out debris or any air bubbles for which it was not possible to determine the category.

Once the best-performing germination medium had been assessed, the same methodology was then validated on the three selected cultivars: Tonda Gentile (TG), Tonda Rossa (TR) and San Giovanni (SG).

To evaluate the best germination medium among those already tested and available in the literature, some preliminary tests were performed. Three liquid media, commonly considered of reference for hazelnut pollen, were initially tested: those proposed by Brewbaker and Kwack [34], Thompson et al. [35] and Heslop-Harrison et al. [36]. Furthermore, the medium proposed by Novara et al. [37], consisting in the Heslop-Harrison medium with the addition of PEG, was evaluated. Since no indication regarding the concentration of PEG was reported, PEG at 1, 10, 15 and 20% (8000 u) was tested [46]. PEG is widely used in *in vitro* pollen germination tests, thanks to its effect of increasing the osmotic potential of media [47–53]. In fact, it can avoid a hyperosmotic shock to the pollen during the nutrient absorption phase, thus preventing pollen bursting during hydration [54]. In this context, the medium proposed by Ascari et al. [3] was also evaluated, as the authors incorporated PEG into a liquid medium. The medium proposed by Kim et al. [31] was also taken into consideration. These authors replaced the liquid medium used in Brewbaker and Kwack's protocol with 1% agar and increased the sucrose concentration up to 15%. In an attempt to improve the germination percentages, casein hydrolyzate (1 mg/mL) was added to the solid germination medium proposed by Kim et al. [31], as an additional source of organic nitrogen, as suggested by Mulcahy and Mulcahy [55] and Read et al. [50]. Lastly, the use of a commercial food-grade gelatin (1%) of animal origin containing collagen as an alternative gelling agent (pH 6.2–6.5) was tested. Different concentrations of sucrose (10, 15 and 25%) and two monosaccharides, glucose (15%) and fructose (15%), were tested on this last substrate. Finally, the effectiveness of the proposed semi-solid medium was compared with the solid agar one, using 1% plant agar (Duchefa Biochemie, Haarlem, the Netherlands). For the better readability of the applied methods and results, the formulations of media tested in the present study, along with the abbreviations used for the respective authors, are reported in Table 1.

Table 1. Abbreviations and formulations of the tested protocols: concentrations are reported as mentioned in the literature.

	Brewbaker and Kwack (1963) [34]	**Thompson et al. (1978) [35]**	**Heslop-Harrison et al. (1986) [36]**	**Kim et al. (1985) [31]**	**Novara et al. (2017) [37]**	**Ascari et al. (2018) [3]**	**Collagen-Based Gelatin**
Abbreviations	**(BK)**	**(Thompson)**	**(H-H)**	**(Kim)**	**(Novara)**	**(Ascari)**	**(Gelatin)**
	Liquid	**Liquid**	**Liquid**	**Solid**	**Liquid (Viscous)**	**Liquid (Viscous)**	**Semi-Solid**
H_3BO_3	100 mg/L	0.1%	10^{-3} M	100 mg/L	10^{-3} M	1.6 mM	-
$Ca(NO_3)_2$	300 mg/L	-	0.5×10^{-3} M	300 mg/L	0.5×10^{-3} M	-	-
$MgSO_4$	200 mg/L	-	-	200 mg/L	-	1.7 mM	-
KNO_3	100 mg/L	-	-	100 mg/L	-	-	-
$CaCl_2$	-	-	-	-	-	1.8 mM	-
KCl	-	-	-	-	-	1 mM	-
Sucrose	10%	0.7 M	10%	15%	10%	0.2 M	15%
Gelatin	-	-	-	-	-	-	1%
PEG	-	-	-	-	unreported	0.04 M	-
Agar	-	-	-	1%	-	-	-

A preliminary hydration phase of 150 min was carried out on stored pollen samples before each germination test, as suggested by Heslop-Harrison and Heslop-Harrison [36]. The effect of rehydration before the test has, in fact, a positive influence on the viability and germinability of the pollen, as also reported by Pacini et al. [56]. This procedure guarantees an almost complete recovery of the external membrane [57] and the reactivation

of metabolic activity [58]. Once rehydrated, pollen grains were plated onto semi-solid gel culture strips applying the hanging drop method [59–61] and incubated overnight at +20 °C, according to Zielinski [21] and Çetinbaş et al. [62]. For each sample, three slides were prepared and observed under a bright-field microscope (Nikon eclipse CV100ND, Nikon Instruments s.p.a., Florence, Italy) at one hundred magnifications (100×). At least 300 grains of pollen were counted for each picture. Only the grains with a pollen tube longer than pollen diameter were considered fully germinated (FG). The remaining pollen grains were then classified into two groups: pollen with partial germination (PG), showing a small pollen tube, and non-germinated pollen (NG), completely lacking a pollen tube. In the first group, turgid and partially germinated pollen (TPG) and dehydrated pollen which still showed a small pollen tube (DG) were distinguished. In the second group, three further categories were discriminated: turgid but non-germinated pollen (TNG), dehydrated/broken non-germinated pollen (DNG) and anomalous pollen (A), with the latter being characterized by morphological alterations in shape and size.

2.3. Analysis of the Microelements

The analyses on the chemical composition of the edible gelatin were carried out by an external laboratory (Water&Life Lab—https://www.waterlifelab.it/, accessed on 20 September 2023). The determination of total nitrogen was performed using the Kjeldahl method. The sample was heated to 400 °C and the organic substances present were oxidized with concentrated acids in the presence of Na_2SO_4 and K_2SO_4 salts. The reaction transformed the organic material into CO_2 and H_2O and the protein nitrogen (-NH_2) present into ammonium sulfate [$(NH_4)_2SO_4$]. The ammonium salt formed was then treated with alkali and the released ammonia was distilled and titrated. The macroelements were analyzed by inductively coupled plasma mass spectrometry (ICP-MS). The sample underwent a preparatory phase of acid mineralization (crumbling of the sample), followed, during ICP-MS spectrometry, by an atomization process through the use of a high-frequency current and heating at 5,000–10,000 °C. The process led to the ionization of the sample atoms and formation of plasma. The ions then flowed towards the analyzer driven by an electric field, and the individual elements and their isotopes were extracted and measured.

2.4. Statistical Analysis

Statistical differences among the accessions were evaluated by Student's T test, while the comparison of the data within each individual accession was performed using ANOVA tests and Tukey's tests (www.statskingdom.com/, accessed on 20 September 2023).

3. Results

3.1. Preliminary Results on the WT Accession

Germination protocols were compared based firstly on the three main media: liquid, solid and semi-solid [59]. Pollen grains were divided, as described in the Section 2, into three main categories: FG, PG and NG (Figure 1). In this preliminary evaluation on the WT accession, only results from the FG pollen count were compared.

The first tests were conducted starting from the liquid media. It was observed that there were non-significant differences among the three main liquid germination media, i.e., BK, Thompson and H-H (Figure 2).

The liquid media of Novara and Ascari (using PEG) were also compared separately. The use of PEG in the Novara protocol (i.e., HH with the addition of PEG) did not improve the germination percentage in our samples compared to the result obtained by applying the original H-H protocol at all concentrations tested (Figure 3A). Furthermore, an increase in pollen dehydration was observed, concomitant with the increase in the concentration of this polymer. In fact, from 15% PEG, germination dropped drastically until it stopped completely at a concentration of 20%. At the same time, it could be observed that there were no significant differences between the results obtained by applying 1% PEG to the

H-H medium with those obtained by applying the Ascari protocol, which made use of 1% PEG (Figure 3B).

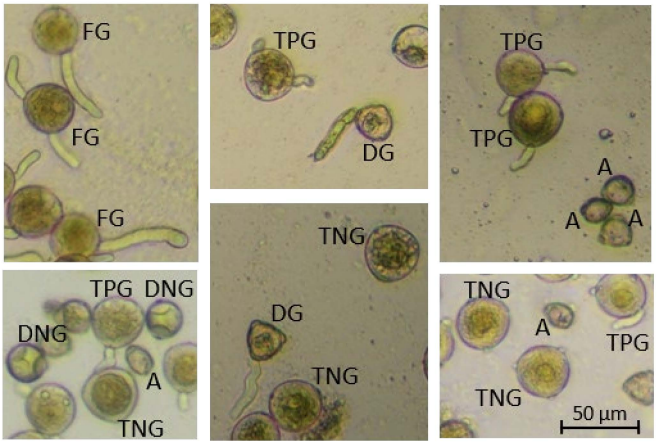

Figure 1. Representation of hazelnut pollen classified in the three categories of fully germinated pollen (FG); partially germinated pollen (PG), including turgid partially germinated pollen (TPG) and dehydrated pollen emitting a small pollen tube (DG); and non-germinated pollen (NG), including turgid non-germinated pollen (TNG), dehydrated/broken non-germinated pollen (DNG) and anomalous pollen (A). All the images have been taken from pollen samples cultured on gelatin substrate.

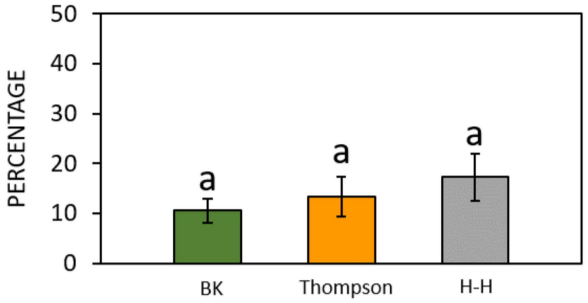

Figure 2. Comparison among substrates formulated for pollen germination and tested on a WT accession of *Corylus avellana* L. Data are shown as the average of at least three biological replicates. Error bars represent the standard error of the mean. Data from each substrate were analyzed using Tukey's test. Statistically significant differences ($p < 0.05$) are indicated with different letters.

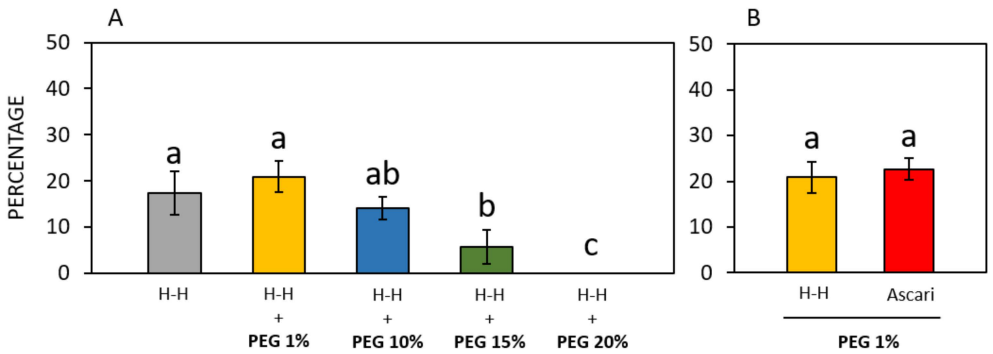

Figure 3. (**A**) Comparison of the use of different concentrations of PEG applied to the H-H protocol. (**B**) Comparison between the only two liquid media using PEG of Novara and Ascari. Data are shown as the average of at least three biological replicates. Error bars represent the standard error of the mean. Data were analyzed using Tukey's test. Statistically significant differences ($p < 0.05$) are indicated with different letters.

Solid media were also compared. Kim's protocol significantly improved the germination test result obtained by BK (+57.4%; p value 0.018). On the other hand, the addition of casein hydrolysate as a supplementary source of nitrogen in the Kim protocol did not substantially change the results (Figure 4).

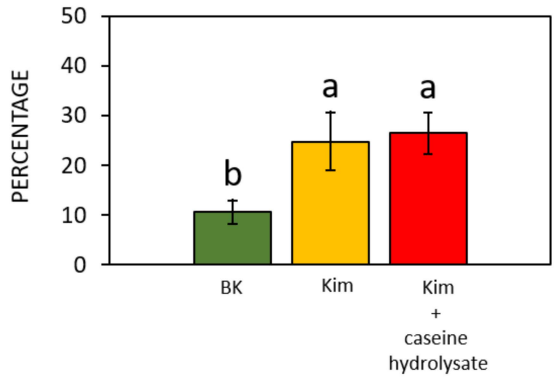

Figure 4. Comparison between some variants of the substrate proposed by Brewbaker and Kwak. Error bars represent the standard error of the mean. Data were analyzed using Tukey's test. Statistically significant differences ($p < 0.05$) are indicated with different letters.

The semi-solid gelatin medium was tested by applying the hanging-drop method and at different concentrations of sugars. It was observed in Student's T test that the 15% concentration showed a significant increase ($p < 0.001$) of +45 and +98% compared to 10 and 25% sucrose, respectively. Glucose and fructose, at a 15% concentration, were also tested. Both reported significantly lower values compared to 15% sucrose. In addition, 15% sucrose showed values substantially like those of 10% sucrose and 15% glucose with a significant reduction of 92% ($p < 0.001$) compared to 15% sucrose (Student's T test analysis), similar to what was observed with 25% sucrose (Figure 5). The semi-solid gelatin medium was finally compared with a solid-agarized one. The solid substrates at 10 and 15% sucrose gave values largely similar to each other and almost two-thirds lower than those obtained with 15% glucose in gelatin (Figure 5). Furthermore, comparing the concentrations of 10% and 15% sucrose applied on solid versus gelatin medium, both showed a significant reduction of −47% ($p = 0.005$) and −63% ($p = 0.0001$), respectively. On the contrary, the 25% medium on agar substrate showed a significant increase of +78% ($p = 0.001$) in germination compared to the corresponding gelatin medium, showing similar values to 15% glucose (Figure 5).

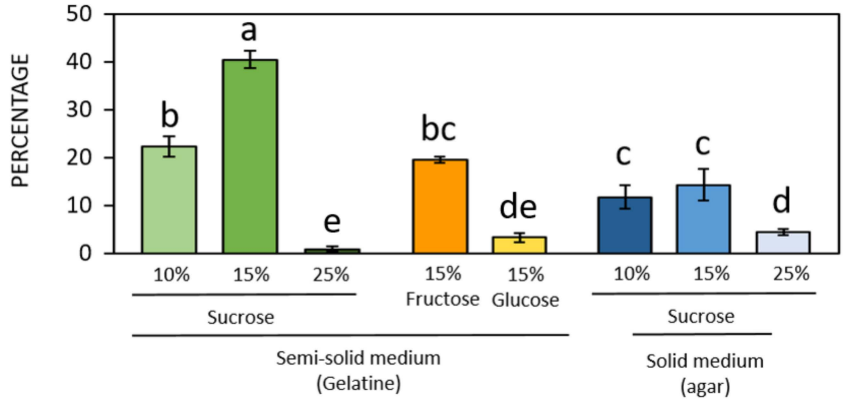

Figure 5. Comparison of the use of different concentrations of sucrose (10%, 15%, 25%) applied to both gelatin and agar substrate, as well as 15% fructose and 15% glucose applied to gelatin medium. Error bars represent the standard error of the mean. Data were analyzed using Tukey's test. Statistically significant differences ($p < 0.05$) are indicated with different letters.

All tested protocols were finally compared with each other. Overall, the experimental medium, gelatin with 15% sucrose, reported significative higher values compared to all the above-mentioned media (Figure 6). In particular, the liquid media, BK, Thompson and H-H, have been shown to poorly promote pollen germination. Similarly, the solid media (Kim and Kim plus casein hydrolysate) and viscous liquid media (Novara and Ascari), as well as the H-H liquid medium, gave similar results to each other (Figure 6).

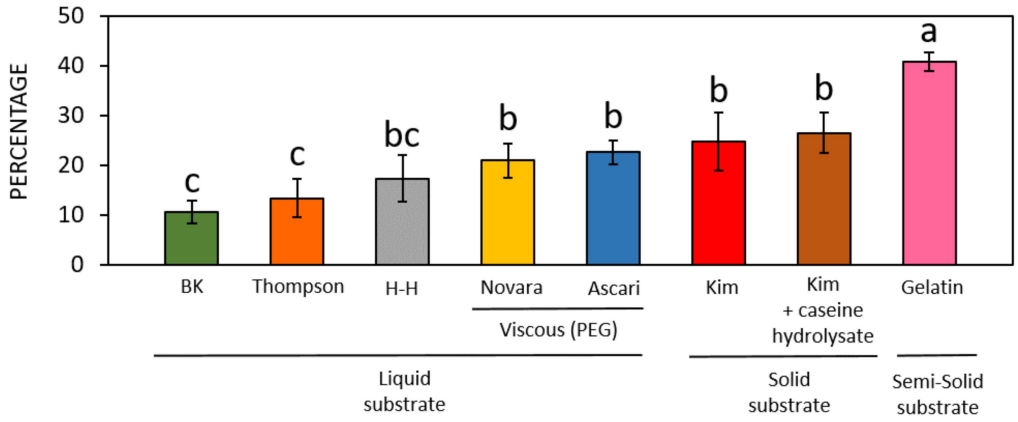

Figure 6. Comparison among substrates formulated for hazelnut pollen germination and tested on a WT accession. Data are shown as the average of at least three biological replicates. Error bars represent the standard error of the mean. Data from each protocol were analyzed using Tukey's test. Statistically significant differences ($p < 0.05$) are indicated with different letters.

When comparing protocols in terms of macro- and microelement composition, it was found that all substrates exhibited relatively low nitrogen values, whereas the gelatin medium was characterized by high nitrogen values (Table 2). Boron was the only microelement found in all formulations, mainly at concentrations between 10 and 17 µg/mL, with the exception of Thompson's medium, where it was found at 175 µg/mL, and in gelatin, where its presence was 0.006 µg/mL. Calcium, detected in all media except Thompson's, was found in concentrations varying between 20 and 89 µg/mL, while magnesium varied between 40 and 60 µg/mL in the BK and Ascari media. On the other hand, gelatin substrate was characterized by low levels of all macroelements (boron, calcium, magnesium and potassium) (Table 2).

Table 2. Comparison of the content of microelement (boron) and macroelements for pollen germination. Data are reported as µg/mL, except for *, which are reported in mg/mL.

		B^{3+}	Ca^{2+}	Mg^{2+}	K^{+}	Nitrogen
Brewbaker and Kwack (1963)	[34]	17.48	73.26	40.38	3.06	65.03
Thompson et al. (1978)	[35]	174.8	-	-	-	13.99
Heslop-Harrison et al. (1986)	[36]	10.82	20.04	-	-	14
Ascari et al. (2018)	[3]	17.31	88.55	59.96	21.74	-
Collagen-based gelatin		0.006	0.564	<0.25	0.777	160 *

3.2. Germination of Pollen of Different Hazelnut Cultivars

Similar results to those observed in the WT accession were observed on the TG, TR and SG cultivars using the hanging-drop technique. The three cultivars were analyzed using the gelatin media at different sucrose concentrations (10, 15 and 25%). Only the medium containing 15% sucrose showed a positive effect on germinability, while the medium with 25% sucrose exhibited the lowest germination levels (Figure 7).

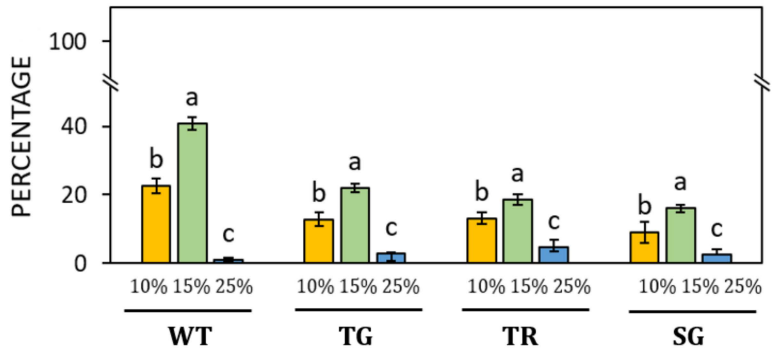

Figure 7. Effect of different sucrose concentrations (10%, 15%, 25%) applied to gelatin medium on pollen germination in the hazelnut cultivars, Tonda Gentile, Tonda Rossa, San Giovanni and wild-type accession. Data are shown as the average of at least three biological replicates. Error bars represent the standard error of the mean. Data among different sucrose concentrations within each cultivar were analyzed using Tukey's test. Statistically significant differences ($p < 0.05$) are indicated with different letters.

Regarding PG pollen, it was found in all samples. At the 15% sucrose concentration, its percentage was significantly lower than in FG pollen. On the other hand, in the 10% and 25% sucrose formulations, the percentages of FG and PG pollen were largely similar to each other. In the WT accession, 25% sucrose had higher percentage values of PG pollen than FG pollen (Figure 8).

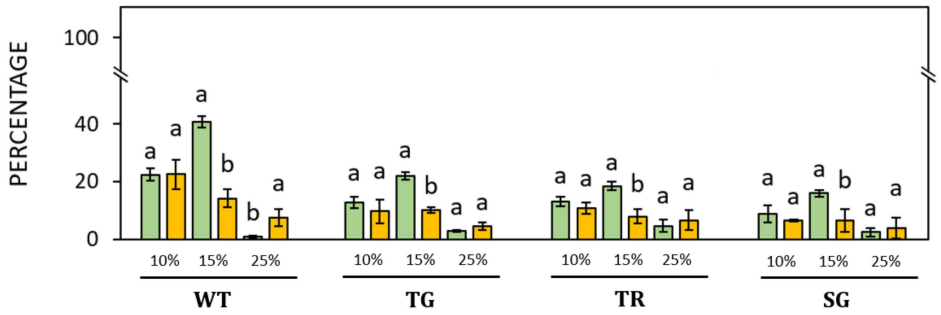

Figure 8. Comparison between the percentages of fully germinated pollens (green bars) and partially germinated pollens (yellow bars) at different sucrose concentrations (10%, 15%, 25%) applied to gelatin medium in the Tonda Gentile, Tonda Rossa and San Giovanni cultivars and in the WT accession. Data are shown as the average of at least three biological replicates. Error bars represent the standard error of the mean. Data among different sucrose concentrations within each cultivar were analyzed using Tukey's test. Statistically significant differences ($p < 0.05$) are indicated with different letters.

The percentage of total non-germinated pollen, represented by the sum of TNG, DNG and A pollen, was always significantly higher in media with 25% sucrose. Similarly, the lowest percentage of total non-germinated pollen was always found in the substrates containing 15% sucrose. In TR and SG, both the 10% and 15% sucrose media showed similar results (Figure 9). A similar trend was also reported by DNG pollen, which was always significantly higher on media with 25% sucrose and significantly lower on media with 15% sucrose (Figure 10). On the other hand, TNG pollen showed a significant reduction in values in 25% sucrose media in all samples. The anomalous pollen did not show significant variations at any concentration, with values between 18 and 31% in the cultivars. On the contrary, the WT accession was characterized by values lower than 3% (Figure 10).

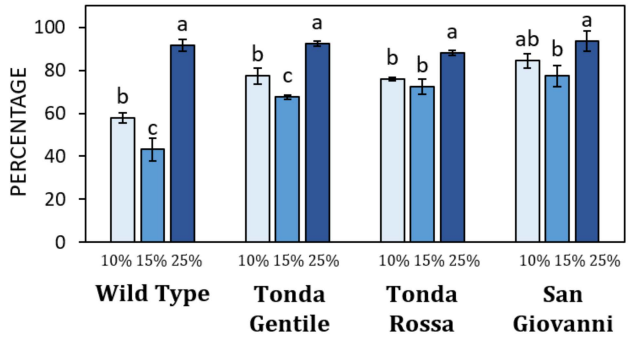

Figure 9. Comparison of total non-germinated pollen percentages obtained at different sucrose concentrations (10%, 15%, 25%) applied to gelatin medium in Tonda Gentile, Tonda Rossa, San Giovanni and WT accession. Data are shown as the average of at least three biological replicates. Error bars represent the standard error of the mean. Data among different sucrose concentrations within each cultivar were analyzed using Tukey's test. Statistically significant differences ($p < 0.05$) are indicated with different letters.

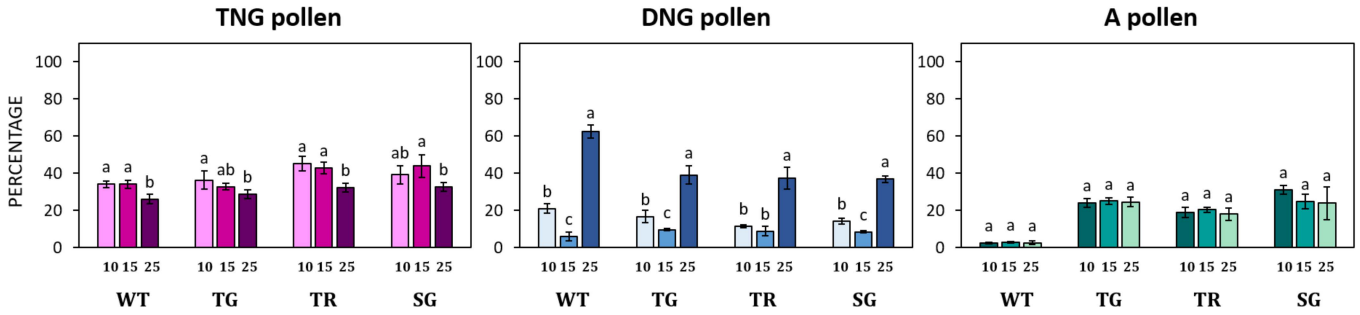

Figure 10. Comparison of the percentages of turgid non-germinated pollen (TNG), dehydrated/broken non-germinated pollen (DNG) and anomalous pollen (A), obtained at different sucrose concentrations (10%, 15%, 25%) applied to gelatin medium in Tonda Gentile (TG), Tonda Rossa (TR), San Giovanni (SG) and a wild-type accession (WT). Data are shown as the average of at least three biological replicates. Error bars represent the standard error of the mean. Data among different sucrose concentrations within each cultivar were analyzed using Tukey's test. Statistically significant differences ($p < 0.05$) are indicated with different letters.

4. Discussion

In this work, a series of protocols formulated for the germination of hazelnut pollen were tested and compared. Among the liquid substrates, BK, Thompson and H-H are those that showed values largely resembling each other (Figures 2 and 6). Both the viscous-liquid media with the addition of PEG, proposed by Novara and Ascari, showed similar values to each other (Figure 3), but they were significantly higher than those obtained with BK and Thompson (Figure 6). Similarly, the Kim solid medium achieved higher germination levels than the liquid media, but the levels were largely like those obtained with the H-H, Novara and Ascari media. Finally, the proposed semi-solid substrate gelatin outperformed all the previous media by exhibiting a significant increase in germination percentage (Figure 6). Our results would indicate, on the one hand, a greater effectiveness of high-viscosity substrates compared to liquid ones. In particular, solid and semi-solid media seem guarantee a uniform distribution of pollen tubes on the medium, allowing a better absorption of moisture and microelements. Kim's solid substrate significantly improved the BK liquid medium, providing adequate support for pollen germination, avoiding possible negative effects of embedding and agglomeration [63]. At the same time, the excessive rigidity of the agarized medium could act as an obstacle to the proper exchange of water and micronutrients between the pollen and the germination medium, causing pollen dehydration, as reported by Connor and Towill [64]. The positive results

obtained with semi-solid media could be related, on the other hand, to their ability to let the pollen adhere to the surface, allowing an adequate rate of hydration and exchange of micronutrients. This type of substrate is particularly suitable for species with pollen considered recalcitrant to germination, such as poplar, walnut, helm and hazelnut [22]. Hazelnut pollen indeed does not undergo drying during maturation and is released with a relative humidity content, about 30% [19]. As a consequence, it could be susceptible to desiccation and could be particularly demanding in terms of moisture requirements at the time of germination [36].

The comparison among the germination media also highlighted the role of a particular composition in micro- and macroelements, especially concerning boron, calcium, and the total organic nitrogen. In particular, boron and calcium are some of the nutrients with the greatest impact on cell growth, especially in the structure of the cell wall as well as in the structure and functions of the plasma membrane [65–68]. Boron plays a key role in the development of pollen tubes, regulating the concentration of cytosolic [Ca^{2+}], fundamental for polar growth [69,70] as well as influencing the accumulation and distribution of callose, pectins and arabinogalactan proteins [67]. In fact, the pectic substances of the pollen tube wall must cross-link with each other for the normal growth of the tube, a process mainly regulated by boron and calcium [71]. Zielinski [21] reported that concentrations greater than 25 µg/mL of boron in germination solution significantly inhibited pollen tube emission. Similarly, concentrations of just 10 µg/mL of borate reduced germination up to almost seven times when compared to the control. Likewise, Fang et al. [72] demonstrated that in pollen of *Malus domestica* Borkh, boron concentrations higher than 0.01% had a toxic effect on pollen germination. Finally, Kim et al. [31] reported, specifically regarding hazelnut pollen, that boron and other elements would have no real beneficial effect on pollen when the germination medium reaches pH values around 6.5. Considering all these data together, it can therefore be hypothesized that boron has a certain inhibitory effect on hazelnut pollen germination, especially in the liquid and solid media analyzed where the concentration found was between 10 and 174 µg/mL (Table 2). On the other hand, the very low levels of micro-nutrients found in the experimental medium gelatin (Table 2), in association with pH values between 6.2 and 6.5, would confirm what was previously reported by Kim et al. [31]. These elements could have contributed to improving the performance of gelatin medium but may not be essential for germination. In fact, as reported in the first works at the beginning of the 20th century on hazelnut pollination and fertilization by Rimoldi [23] and Schuster [24], the substrates used included only agar and variable concentrations of sucrose (8–12%). In addition, all the liquid and solid media taken into consideration in this study were almost uniformly characterized by low nitrogen content. On the contrary, the commercial food-grade gelatin presented high nitrogen content, which was the main macroelement (Table 2). Nitrogen is the main component of macromolecules such as proteins and nucleic acids, considered a limiting factor for plant growth and reproduction. It is known that nitrogen availability can strongly influence the number and size of pollen grains produced [73]. In fact, in addition to carbon, anthers and pollen accumulate nitrogen during development. During the initial phase, anthers mainly require amino acids to support the growth of the microspores; mature pollen grains require osmo-protective molecules such as prolines to cope with desiccation during dispersal [74–77]. For instance, Fang et al. [78] described the presence of abnormal cells in the tapetal layer of the anthers in sterile lines of pepper plants (*Capsicum annuum* L.). The tapetal tissue was characterized by low levels of expression of enzymes related to the synthesis of amino acids, especially prolines, showing wrinkled pollen. It is therefore possible that a deficiency of nitrogen during pollen germination can adversely affect the performance of the pollen in liquid and solid media. It has actually been reported by Mattioli et al. [79] that pollen development and fertility are closely linked to the *in situ* synthesis of prolines, i.e., within the developing microspores and in mature pollen grains. Our results would therefore indicate that in the germination processes of hazelnut pollen, the addition of organic nitrogen would

play an essential role, outweighing the addition of microelements such as boron, calcium, magnesium and potassium.

Our results suggest that the use of PEG (8000 u) in the medium for hazelnut pollen germination, even at low concentrations of 1%, does not improve the germination outcome. Unlike what was reported by Novara, no improvement was noted with the addition of PEG in H-H substrate. Accordingly, a notable limitation of pollen germinability was found, concomitant with an increase in PEG concentrations, as reported also in *Dioscorea* spp. by Mondo et al. [80]. Many authors agree on the beneficial effect of PEG addition to pollen germination media because of its key role in osmoregulation [48,50,81,82]. Thanks to its effect of increasing the osmotic potential of the medium, it could avoid a hyperosmotic shock to pollen grains during the nutrient absorption phase, thus preventing pollen bursting during hydration [54]. However, in the tests conducted, it was observed that the use of PEG caused a clearly dehydrating effect. This effect increased as its concentration in the germinative substrate increased precisely due to its effect of increasing the osmotic potential of the medium. Typically, variable concentrations of PEG, with molecular weights from 4,000 u to 8,000 u and percentages between 15 and 25% are added to the germination medium to lower the water potential, generally associated with sucrose levels no higher than 15% [46,49,83,84]. It is well known that pollen germination is supported by sucrose [85–87], used as one of the main sources of carbon and energy. Sucrose also plays a fundamental role in osmotic regulation by maintaining pollen turgor pressure and ensuring pollen survival [22,86,88]. Preliminary tests were performed on both semi-solid and solid substrates, using a WT accession with a sucrose concentration range of 10, 15 and 25%. The results obtained on the gelatin medium showed that 15% was the best condition for the germination of WT hazelnut pollen, while at 10% and 25%, germination was reduced or did not occur, respectively (Figure 5). The solid media seemed to confirm to what was previously hypothesized: their rigidity limited the exchange of water and micronutrients between pollen and the germination medium, thus reducing germination. At the same time, the results obtained with different sucrose concentrations confirmed what was observed in the gelatin substrate. These results are in agreement with those previously obtained by Manuševv [33], who tested the germination performance of 22 hazelnut cultivars at 12, 15 and 20% sucrose, identifying 15% as the best concentration. It should be noted, however, that Cox [30] reported maximum hazelnut pollen germination with a 25% sucrose concentration. Similarly, Kim et al. [31] concluded that the best condition was between 15 and 20%, indicating that some of the cultivars tested showed maximum germination at 20% and only low levels at 4% and at 15% sucrose. On the contrary, a consistent reduction in germination was observed at 25% sucrose. Our data seem to confirm the negative effect of higher sucrose concentrations than 15% on hazelnut pollen germination, as also previously reported in caprifig [89] and some stone fruits [90]. The different results obtained could be attributed to the different levels of osmotic pressure exerted on pollen during the rehydration phase. During dispersal, cytoplasmic sugars, especially sucrose, can be interconverted to regulate osmotic pressure and prevent water loss [88]. Pollen turgor pressure can be described as being proportionally linked to the cytoplasmic sucrose content and inversely related to the relative humidity of the environment. Regarding the use of other sugars, 15% fructose and 15% glucose, the results reported in the literature are somewhat conflicting. Okusaka and Hiratsuka [91] reported similar levels of germination in Japanese pear (*Pyrus pyrifolia* Nakai) pollen when plated on 10% sucrose or 10% glucose agar medium. On the contrary, the use of fructose has been shown to have an inhibitory effect on germination, showing specific dose-dependent characteristics. Similarly, Hirsche et al. [92] reported an inhibitory effect of both fructose and glucose in the pollen germination of *Arabidopsis thaliana* (L.) Heynh. Portnoi and Horovitz [93] highlighted high germination rates in pollen of *Yucca aloifolia* L. when placed on media containing either glucose or sucrose, while fructose produced high *in vitro* pollen germination only when borate and calcium were added to the medium. Our results indicated that 15% fructose does not improve germination, maintaining values around 20% (Figure 5), largely comparable to what was obtained by

applying the liquid-viscous media of Novara (20.9% ± 3.4) and Ascari (22.7% ± 2.4) or the solid agar of Kim (24.8% ± 5.8). Manuševv [33] observed a gradual increase in hazelnut pollen germination starting from a glucose concentration of 10% up to 25%. On the other hand, Kim et al. [31] reported opposite results: as glucose concentration increased, the germination index decreased until it stopped at 25%. According to these last results, our data showed that 15% glucose significantly increased the percentage of dehydrated pollen. In these conditions, none of the samples exceeded the value of 4.8% FG pollen, suggesting an inhibitory effect of 15% glucose on germination (Figure 5). The great variability found regarding the use and concentrations of different mono- and disaccharides for pollen germination tests reflects the diversity of plant species. Different types of responses are expected in different plant species.

Once the effectiveness of the gelatin germination medium with the addition of 15% sucrose on the WT accession had been verified, the methodology was validated on a small group of cultivars of agronomic interest. The availability of a common, rapid and reliable method to evaluate pollen quality is certainly of fundamental importance for all those processes aimed at genetic improvement and hybridization. For this purpose, the three cultivars, TG, TR and SG, were examined. These cultivars were chosen due to their high genetic compatibility (S allele) with the majority of commercial varieties used in high-production orchards [94–96], as well as for their ability to shed pollen early during the first part of seasonal flowering [14], acting as pollinizers. The results obtained confirmed that the optimal formulation for the WT was also applicable to the cultivars (Figure 7). In fact, they showed a drastic decrease in germination with 25% sucrose due to pollen dehydration. The 10% and 25% sucrose media gave similar percentages of FG and PG pollen in the cultivars but not in the WT (Figure 8), suggesting an inadequate sugar content to support the germination process. It has been shown that sub- and supra-optimal conditions of sucrose concentrations in the germinating substrate induce both a decrease in the percentage of germination and a decrease in the length of the pollen tube [97]. Since germinability was evaluated after overnight incubation, the presence in the WT of a higher percentage of PG pollen compared to the FG one could therefore be due to a slowdown in the growth of the pollen tube. Furthermore, it was possible to observe a completely identical effect between the cultivars and the WT accession also with regard to non-germinated pollen. At high sucrose concentrations (25%), a decrease in TNG pollen and a concomitant increase in DNG pollen was observed due to the increasing effect of the osmotic potential of sucrose on the medium [22,86,88]. Similarly, it was possible to observe a reduced presence of DNG pollen at the 15% concentration, thanks to an adequate rate of hydration and exchange of micronutrients between the 15% gelatin substrate and the pollen. It was also observed that the anomalous pollen within each cultivar maintained substantially similar values despite the different sucrose concentrations (Figure 10). This result would therefore support the genetic theory on the formation of anomalous pollen, characterized by morphological and functional alterations [98–100], excluding the possible involvement of external factors in its formation.

Overall, the obtained results seem to indicate that the applied technique supports hazelnut pollen germination requirements and offers an easy and quick-to-use method for germination assessment.

5. Conclusions

The results of this study indicate that semi-solid media more optimally support the *in vitro* pollen germination of *C. avellana* cultivars than liquid and solid-agar ones. The gelatinous constitution of the proposed gelatin substrate seems to guarantee a better absorption of humidity and microelements, as well as avoiding those inclusion and agglomeration phenomena that can inhibit pollen germination. Furthermore, findings suggested that sugars, in addition to their roles as carbon and energy sources, may act differently in pollen germination depending on their structural properties. Sucrose optimally supports germination, particularly at a concentration of 15%, maintaining pollen turgor pressure

and ensuring pollen survival. Contrariwise, both monosaccharides, 15% glucose and 15% fructose, did not promote pollen germination. In particular, 15% glucose had an inhibitory effect on germination, causing strong dehydration in pollen, similar to sucrose at high concentrations. The nature of the monosaccharide constituents could therefore influence the germination capacity of hazelnut pollen through selective molecular recognition. The results obtained in the three cultivars, TG, TR and SG, reflect what was previously observed on the WT accession, validating the new proposed methodology.

In light of these findings, this method can be considered applicable to the *C. avellana* varieties and new selections, allowing easy execution, high repeatability and results that are more consistent with the real pollen viability levels.

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