horticulturae

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Standardization of an In Vitro Seed Germination Protocol Compared to Acid Scarification and Cold Stratification Methods for Different Raspberry Genotypes

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Abstract: In raspberry (*Rubus idaeus* L.) breeding programs, seed double dormancy causes delayed and low germination rates. In this study, an in vitro germination protocol was developed to increase raspberry seed germination rate and decrease germination time. This protocol was optimized on the cultivars "Polka" and "Tulamagic", specifically, their open-pollinated seeds, by applying two different cutting types (transverse, or longitudinal), followed by two different treatments (direct in vitro culture at 24 °C, or 2 weeks at 4 °C followed by in vitro culture at 24 °C). The best results for both cultivars were obtained when seeds were cut transversally and directly cultured in vitro at 24 °C (germination rate 73–78%, starting germination time 4 days after treatment). The optimized in vitro protocol was applied to 14 different combination crosses, observing a germination frequency around 87%, and speed in line with "Polka" and "Tulamagic". These results were compared to those from the 14 cross combinations having undergone acid scarification (95% H_2SO_4 for 30 min) and cold stratification (3 months), which, in comparison, only yielded a 25% average germination rate. The optimized in vitro protocol favors high germination efficiency for all raspberry genotypes tested, and presents advantages for the standardization of germination time and reduction in the parental genotypes' impact on germination rate.

Keywords: *Rubus idaeus* L.; plant tissue culture; seed dormancy; germination rate

Citation: Pergolotti, V.; Marcellini, M.; Contreras, E.; Mezzetti, B.; Gambardella, M.; Capocasa, F.; Sabbadini, S. Standardization of an In Vitro Seed Germination Protocol Compared to Acid Scarification and Cold Stratification Methods for Different Raspberry Genotypes. *Horticulturae* **2023**, *9*, 153. https://doi.org/10.3390/horticulturae9020153

Academic Editors: Ju-Sung Cho and Sergio Ruffo Roberto

Received: 6 December 2022
Revised: 16 January 2023
Accepted: 20 January 2023
Published: 25 January 2023

1. Introduction

The genus *Rubus* is extensively spread across the world's temperate zones [1]. In fact, *Rubus* includes several subgenera [2] with different ploidy levels. More specifically, regarding the raspberry crops, the most domesticated plants are *Rubus idaeus* L. subsp. *Idaeus* in Europe, and *R. idaeus* subsp. *Strigosus* Michx in North America [3]. Worldwide raspberry production increased from 522.004 tons in 2010 up to 895.771 tons in 2020, with the major contribution by Europe, which represents 68.2% of the total production [4]. This increase in production in the last decade is directly connected to a heightened interest found among consumers, which has pushed farmers and consequently breeders to invest in the production of raspberry. The increase in the consumption of small fruits is influenced by the presence of healthy compounds that enrich the fruit, giving it added value [5]. Indeed, recent studies have highlighted the beneficial bioactive compounds of red raspberries [6]. This growing interest in raspberries has encouraged new breeding programs focused on the development of new cultivars.

Raspberry breeding programs are focused on the improvement of specific plant parameters such as high yield, improved pathogen resistance, and adaptation to the local environment, and fruit parameters such as high quality, long shelf life, brightness, easy

picking, and suitability for shipping [7]. Fortunately, the reproductive cycle of raspberry is relatively short, especially in primocane varieties, which has allowed new breeding programs to achieve good results in a short time, including the release of a wide range of new varieties. These genetic improvement processes are based on the availability of genetic resources to be used in cross combinations. After guided pollination, seeds are germinated to produce new seedlings, which will be evaluated to obtain a final selection. The creation of large genetic diversity depends on the number of crosses performed, the number of seeds produced, and on the efficiency of seed germination protocols [8]. However, one of the limits in *Rubus* species' breeding is determined by the long germination time and low germination rate of seeds, even with the application of pre-treatment protocols [9].

Rubus seed germination is a complex process due to double deep dormancy, one external and one internal, influenced by both genetical and environmental factors [10,11]. The first dormancy is represented by a protective physical coating of the seed, defined as hard endocarp, which limits the entry of air and water, preventing the embryo from swelling and the seed from germinating. The permeability of the hard endocarp in *Rubus ideaus* L. is dependent on several factors, including the presence of lipids, phenolic compounds, and flavonoids, located mainly in the testa and endocarp of the seed. Together, these metabolites contribute towards hardening the coat, and reducing water uptake and oxygen availability to the embryo, as well as in inhibiting seed germination and plant growth [12]. In addition, the presence of compounds such as hydrogen cyanide (HCN), accumulated between the endosperm and the cotyledons of some *Rosaceae* species, including *Rubus*, was also demonstrated to be responsible for low germination rate [12,13].

The second dormancy (the internal form) in *Rubus* spp. is switched off by biochemical and physiological reactions that occur in the seed after a chilling period of about 3–4 months at 0–5 °C, usually called the "stratification phase" [11,14]. Raspberry seed dormancy usually combines two main phases; one consists in the use of a pre-treatment aimed at breaking or damaging the seed coating, such as chemical scarification through concentrated sulfuric acid solutions [8,9,11,15–17], by physical damage with sandpaper [16,18], or by nicking or cutting the seed [19]. These pre-treatments are normally followed by a second phase of cold stratification; however, poor germination rates are obtained [9,11,20]. This low efficiency can be improved by applying other treatments after the scarification phase, such as the imbibition of seeds in a solution of gibberellic acid (GA3), a well-known phytohormone able to interrupt seed dormancy and stimulate seed germination [11,13,21,22]. However, this approach was not effective in reducing the dormancy of some genotypes of black raspberry seeds, compared to GA-untreated seeds [23]. On the contrary, when seeds of different *Rubus* species were treated with a solution combining GA3 and potassium nitrate (KNO3), after the acid scarification phase, a more effective germination efficiency, ranging between 14% up to 97%, was observed, compared to other treatments [11]. Additionally, in other studies, a range of nitrogen-containing compounds, including KNO3, have been shown to help break seed dormancy in different plant species, such as *Arabidopsis* [24]. Another treatment based on the use of smoke-derived compounds from cellulose fires, a well-known seed germination stimulant in several species [25], was effective at increasing germination efficiency of the treated seeds up to 69% [11].

Despite assessing the most effective treatments, the germination efficiency remains heavily dependent on genotype, and germination results may vary due to the interaction between parental genotypes used in breeding programs [11,16]. Thus, there is no standard procedure to achieve a high seed germination rate from different crossing combinations following these strategies [20].

The embryo rescue technique has also been used to accelerate the maturation of embryos in many crops, although it is a time-consuming and laborious technique, often due to the size of the embryo itself [26,27]. To overcome this laborious task, a different protocol has been developed as an alternative to embryo rescue, based on cutting the seed in half [19,28]. This mechanical treatment could be comparable to acid scarification, as they both involve the breakage of the external seed dormancy. In vitro techniques could

be used in conjunction with this mechanical treatment as a combined strategy, as they are well known to greatly enhance germination and growth rates [29]. Therefore, in vitro culture germination might be advantageous to shorten breeding cycles and accelerate the breeding process.

The objective of this study was to develop a new efficient protocol for raspberry seed germination based on the use of the seed cutting technique combined to in vitro culture. The optimized in vitro seed germination protocol resulted in much higher germination rates in a shorter time period of seed germination, in comparison with a standard chemical scarification and stratification protocol. The efficiency of this protocol was first demonstrated on seeds produced by open pollination of two raspberry genotypes, "Polka", a primocane fruiting cultivar, and "Tulamagic", a floricane fruiting cultivar. Both these genotypes have been included in several breeding programs mainly aimed at improving the quality of raspberry fruit [30,31]. These two cultivars can be considered representative of several raspberry genotypes, due to their different fruiting cycles and their importance in the breeding of this crop. For these reasons, they were chosen as the two starting genotypes to carry out the experimental trials described in this study.

In a second phase, by applying the optimized protocol on seeds from 14 different cross combinations, it was demonstrated that the in vitro method remains the more efficient when applied to seeds originating from different cross combinations. Therefore, we propose a new seed germination technique that can maximize seed germination and speed up breeding programs in *Rubus spp.* and other crops with similar problems of low seed germination.

2. Materials and Methods

2.1. Plant Material and Seed Source

Plants of two raspberry (*Rubus ideaus* L.) cultivars, "Tulamagic" and "Polka", were cultivated in pots (28 × 28 cm) containing growing medium (FR-LA 21V, Vigorplant Italia Srl) in the greenhouse at the Department of Agriculture, Food and Environmental Sciences, Ancona, Italy. Six pots for each cultivar were used for the test. Ripe fruits obtained through open pollination of these two cultivars were harvested during spring season in 2020. In addition, during the same period, ripe fruits from 14 cross combinations (D3A-UNIVPM raspberry breeding program) were collected from commercial raspberry varieties cultivated in the same protected conditions. Seeds were extracted from each drupelet of the collected ripe fruits using a hand blender, and washed under running tap water. The lightest/empty seeds (floating) were discarded, while the remaining seeds, representing those with a hard endocarp and with a mature embryo, were dried at room temperature for 24 h in a paper towel and used for the germination tests.

2.2. Seed Germination through Scarification and Stratification

2.2.1. Seed Scarification

Seeds obtained from the 14 cross combinations were subjected to pre-sowing treatment in glass flasks with a concentrated sulfuric acid solution (H_2SO_4—95%), using enough volume to cover the entire surface of seeds, with continuous shaking at 100 rpm for 30 min. During this step, the flasks were placed in an ice bath to avoid excessive exchange of heat released in exothermic reactions. Seeds were successively washed under running water for 1 min and subsequently treated with a calcium hydroxide ($Ca(OH)_2$) solution (5 g L^{-1}) for 5 min with continuous agitation. The seeds were then washed under running water for 1 min and soaked in distilled water for 10 min, changing the water twice during this step. Finally, the seeds were soaked in a 10% sodium hypochlorite solution for 20 min, then rinsed under running water, as described for the previous step. To conclude the scarification treatment, the seeds were placed in a falcon tube with distilled water and stored at +2 °C for 1 week, replacing with clean water at least three times a week.

2.2.2. Seed Cold Stratification and Sowing

The cold stratification treatment immediately followed the scarification phase. Seeds were transferred to a hermetic plastic bag with wet and sterilized sand, and stored in this condition for 3 months at +2 °C. After the seed stratification period, the seeds were rinsed with running water to remove excess sand and sowed in a substrate consisting of peat and perlite in 1:1 portion. Sowing was conducted in rectangular, perforated polystyrene containers (cm 50.5 × 32.5 × 11.5, Poliart s.r.l) filled with about 2 cm of substrate. The seeds were distributed on the soil with the help of tweezers and then covered with sifted peat (0.5 cm thickness). All containers were placed on benchtops in the greenhouse. Finally, the substrate was moistened with a sprayer. In total, 100 seeds for each combination of crosses were sown, with three replications each, for a total of 300 seeds for each crossing. All containers were covered with plastic film to maintain a high level of humidity (95–98%). After 2 weeks from sowing, the plastic film was partially opened to reduce the humidity level. Germinated seeds were then transplanted to 360-cell paperpots (HP D360/2.6-HerkuPlast-Kubern, GmbH, Daninmark) containing 6 cc of substrate (Substrat2, Klasmann-Deilmann, GmbH, Germany). Two months after sowing, germinated seedlings were transferred to 60-cell paperpots (HP D60/4P-40 HerkuPlast-Kubern, GmbH, Daninmark) containing 76 cc of the same substrate used previously, and were then transferred to the greenhouse with a 16 h photoperiod, at 25 °C. Data on the percentage of germination, expressed as the (number of germinated seeds/total scarified seeds) ×100, were collected 4 months after sowing.

2.3. In Vitro Seed Germination

Open-pollinated seeds from the two *Rubus ideaus* L. cultivars, "Tulamagic" and "Polka", were collected as described above, then sterilized in a solution of 1% (*V*/*V*) sodium hypochlorite and incubated at room temperature on a shaker (100 rpm) for 24 h. Then, the seeds were washed three times with sterile distilled water for 1, 5, and 10 min, respectively. The sterilized seeds were then blotted on sterile filter paper and treated with two types of cuttings (Figure 1b,c) for comparison with the uncut seeds used as the control (Figure 1a). In detail, for the in vitro germination test, seeds were cut in half by making a transverse section perpendicular to the embryonic axis (Figure 1b) [19], or they were incised longitudinally, without halving the seed (Figure 1c). The mechanically treated seeds plus the control were cultured on germination medium (GM) comprising MS salt and vitamins medium [32] supplemented with 30 g L^{-1} sucrose and 7 g L^{-1} of plant agar (Duchefa Biochemie, Haarlem, The Netherlands). The pH of the medium was adjusted to 5.7–5.8 with KOH. GM was autoclaved at 121 °C for 15 min, then poured into 9 cm diameter Petri plates. For each type of mechanically treated seed and the control seeds, ten Petri plates were prepared, each containing 25 seeds, making up a total of 250 seeds for each condition. Half of the seeds on the Petri plate from each group (125 seeds in total) (aT1, bT1, cT1; see Figure 1) were placed at 4 °C in the dark for 14 days, to evaluate the effect of cold temperature on germination efficiency. Meanwhile, the remaining half (aT2, bT2, cT2; see Figure 1) was directly placed into a growth chamber at 24 °C ± 1 under a photoperiod of 16 h light (70 µmol/m^2/s) provided by white fluorescent tubes. Successively, the first group of treated seeds (aT1, bT1, cT1) was also transferred to the growth chamber under the same conditions as described above. Three independent experiments were carried out for each condition: a(T1-T2), b(T1-T2), c(T1-T2).

Data on seed germination percentage, and the time of seed germination for each type of method (type of mechanical cutting, temperature condition, and the control), expressed as (the number of germinated seeds/total seeds treated) ×100, were recorded for a period of 60 days.

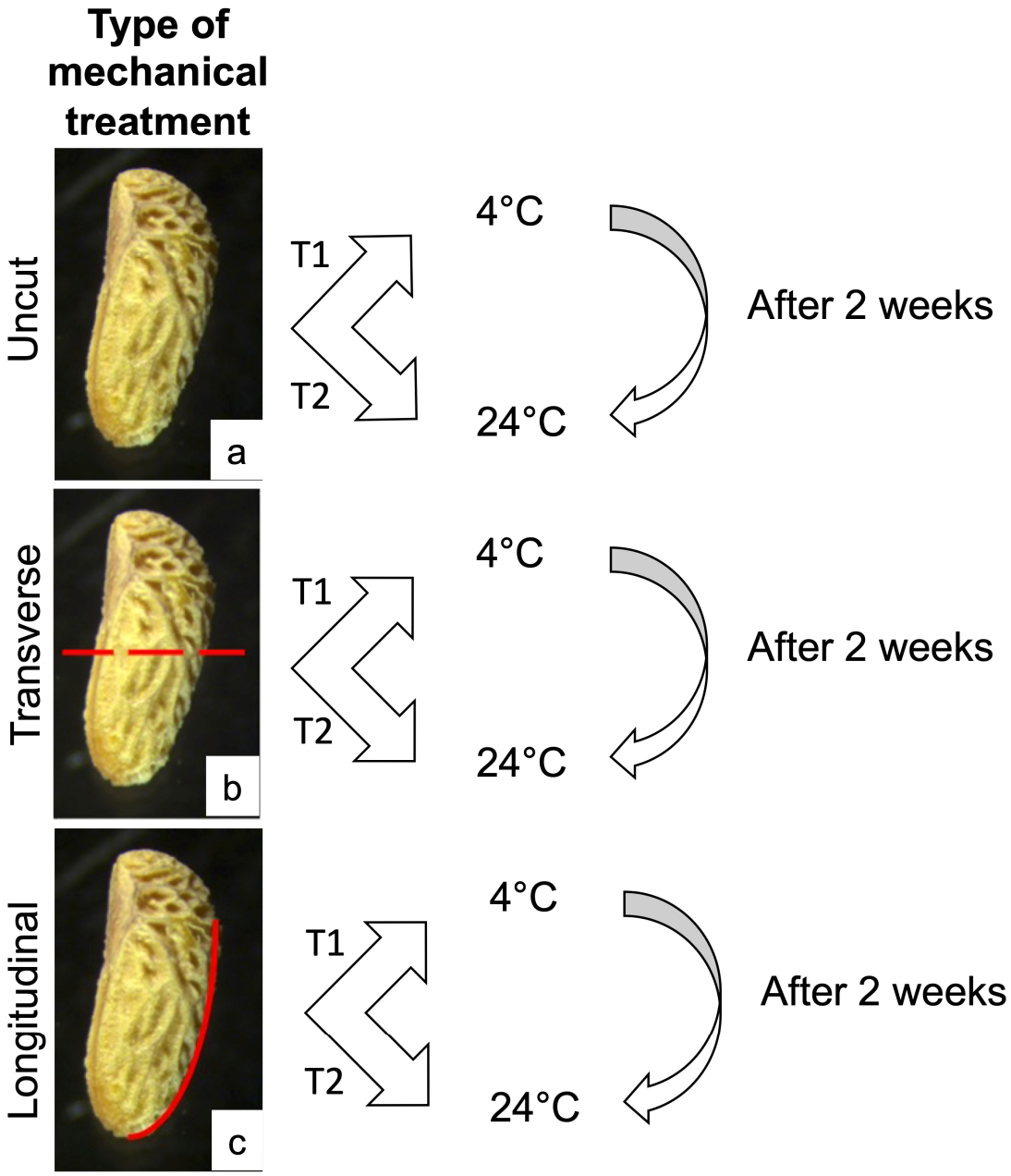

Figure 1. Type of mechanical and temperature treatments applied on seeds before in vitro culture: (**a**) uncut seed, (**b**) transverse cutting (halved seed), (**c**) longitudinal incision, stored at 4 °C for 14 days and then in vitro cultured at 24 °C (aT1, bT1, cT1), or placed directly in a growth chamber at 24 °C (aT2, bT2, cT2).

2.4. Seedlings In Vitro Rooting and Acclimatization

All germinated seeds were transferred to glass pots containing elongation/rooting medium, which consists of MS salt and vitamins medium [32] supplemented with 30 g L^{-1} sucrose and 7 g L^{-1} of plant agar (Duchefa Biochemie, Haarlem, The Netherlands). The pH of the medium was adjusted to 5.7–5.8 with KOH. Seedlings remained 3–4 weeks in the elongation phase, and were finally transferred to 60-cell paperpots (GmbH, Daninmark) containing 76 cc of substrate (GmbH, Germany), covered by a plastic tunnel with high humidity level for their acclimatization, for 2 weeks at 25 °C ± 1 under a photoperiod of 16 h light. Gradually, the plastic tunnel was opened and then the seedlings were transferred to the greenhouse.

2.5. Application of the Optimized In Vitro Seed Germination Protocol to Combination Crosses

Seeds obtained from the 14 programmed cross combinations were sterilized following the same protocol described above. Then they were cut in half (transverse cut), placed directly on GM medium, and placed in a growth chamber as described previously (bT2; see Figure 1). For each genotype, 25 seeds were cultured in each Petri plate, making a total of 10 Petri plates. Data on seed germination percentage for each type of cross, expressed as (the number of germinated seeds/total seeds treated) ×100, were acquired after 30 days of culture.

2.6. Statistical Analysis

All the data acquired from each trial were analyzed by one-way ANOVA using Statistica 7 software (Statsoft Tulsa, CA, USA), and means were separated using the Duncan test ($p < 0.05$).

3. Results

3.1. Seed Germination after Scarification and Stratification

The scarification and stratification procedures were characterized by three main phases with different timings: 1 week for the scarification phase, 3 months for the stratification step, and from 1 up to 3 months for the germination of seeds after sowing. A seed was defined as germinated when the first two cotyledons had formed. All the combinations of crosses were tested for their germination efficiency in 3 replications, each with 100 seeds (300 seeds in total). In general, seed germination efficiency was 24.6% for all the different combinations of crosses (Figure 2). In particular, the highest germination efficiency was observed for crosses AB20,22; AB20,09; and AB20,17, which reached 52.7%, 52%, and 48% of seed germination, respectively. Meanwhile, the lowest germination efficiency was obtained for AB20,13 and AB20,21 crosses, which generated the lowest number of germinated seeds, with 5% and 5.7% of germination efficiency, respectively.

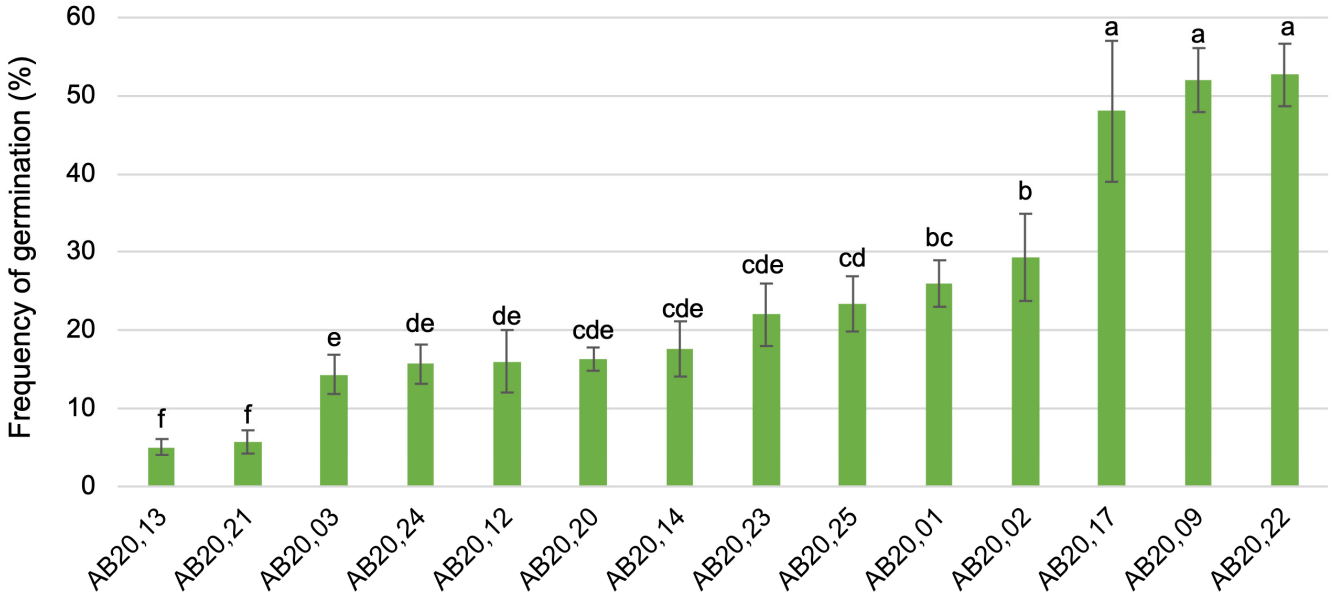

Figure 2. Data on seed germination frequency for different crosses of raspberry following the scarification and stratification method. Results are expressed as the (number of germinated seeds/total scarified seeds) ×100 at 3 months after sowing. One-way ANOVA was used to analyze the data. Different letters show significant differences at $p < 0.05$ by Duncan's test (n = 100). Each value represents the mean ± standard deviation of three independent experiments.

3.2. In Vitro Seed Germination Experiments

Raspberry seeds were germinated in vitro by applying different mechanical treatments (transverse cutting or longitudinal incision) combined with two temperature treatments at in vitro cultivation conditions (Figure 1). The best in vitro seed mechanical treatment, in terms of germination efficiency, proved to be transverse cutting (Figure 1b), regardless of the applied temperature, in comparison with the uncut control. Through this treatment, only one of the two halves germinated and gave rise to a seedling (Figure 3a,b). This treatment did not negatively affect the development of the germinated seedlings, which were elongated and rooted in vitro, and after were easily acclimatized to in vivo conditions in the greenhouse (Figure 3c–f).

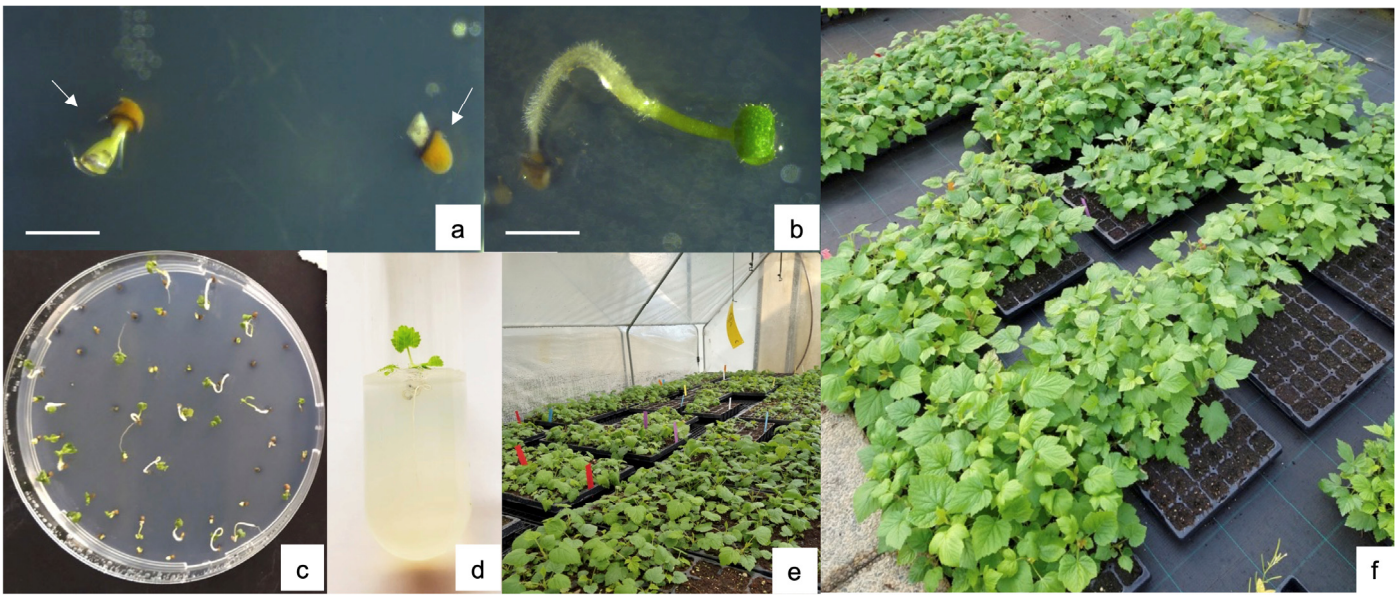

Figure 3. In vitro germination, rooting, and acclimatization of Polka seeds cut in half and placed directly under 24 °C (bT2) treatment. (**a**) The two halves of the seed at 4 days after treatment are indicated by white arrows; (**b**) a well-developed seedling from germinated seed after 7 days of in vitro culture (scale bar = 2 mm); (**c**) in vitro germination of seedlings after 10 days of in vitro culture; (**d**) in vitro raspberry shoot elongation and rooting; (**e**) gradual acclimatization of raspberry seedlings under plastic tunnel; (**f**) well-established in vivo plants in the greenhouse ready to be transferred to field conditions.

Figure 4 shows that there were no statistical differences between the seeds cut in half and stored at 4 °C in dark conditions for 14 days (bT1) and those placed directly under 24 °C (bT2) treatment. Specifically, seeds originating from open pollination of both cultivars, "Polka" and "Tulamagic", treated under bT2 conditions (i.e., seed cut in half and directly in vitro cultivated at 24 °C) generated the highest germination rates, 78% and 72.6%, respectively, similar to that observed for seeds treated under bT1 conditions (i.e., seed cut in half and stored 2 weeks at 4 °C), which induced a germination rate of 78.7% and 71% for "Polka" and "Tulamagic", respectively. These results demonstrate that at these conditions, the cold period of 2 weeks after cutting is not needed to improve germination efficiency. In addition, both bT1 and bT2 conditions induced a high germination efficiency for seeds originating from all the different cross combinations, thus demonstrating that this protocol is not influenced by the genetic differences of the seed population.

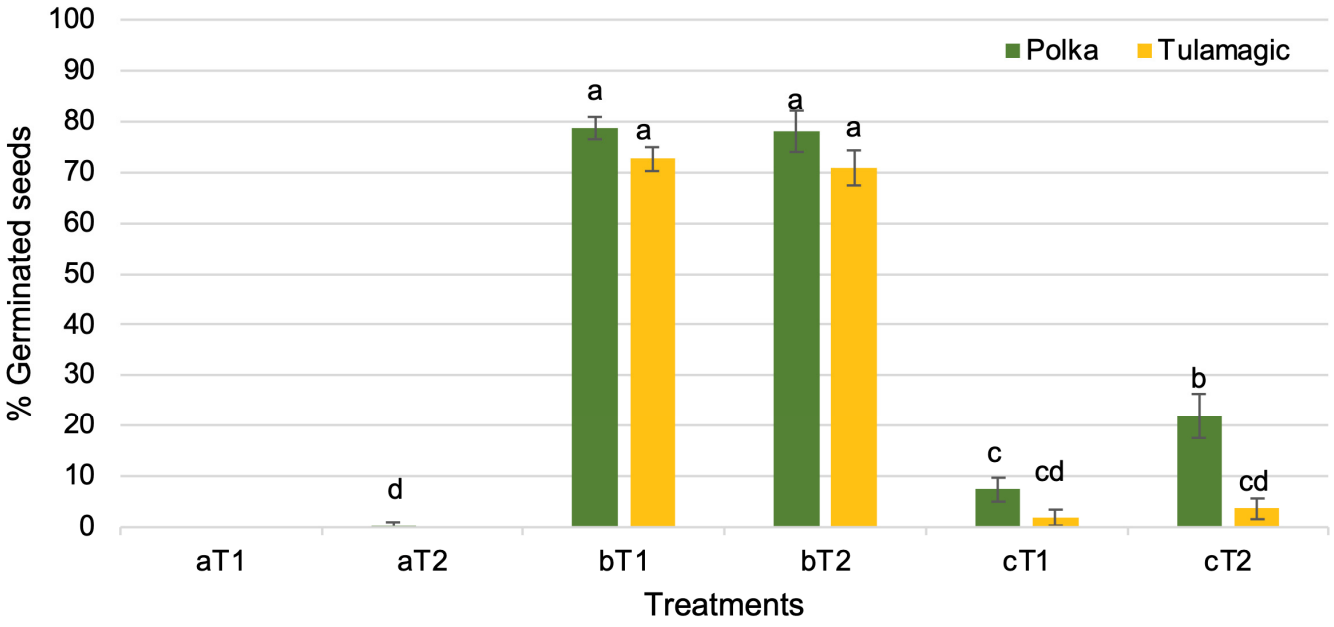

Figure 4. Data on in vitro germination frequency of seeds from "Polka" and "Tulamagic" open pollination exposed to different types of mechanical and temperature treatments: uncut seed (aT1), transverse cutting (bT1), and seed with longitudinal incision (cT1) stored at 4 °C for 14 days and then placed in a growth chamber at 24 °C; uncut seed (aT2), transverse cutting (bT2), and seed with longitudinal incision (cT2) placed directly at 24 °C. Results are expressed as the (number of germinated seeds/total treated seeds) ×100 at 60 days after treatment. One-way ANOVA was used to analyze the data. Different letters show significant differences at $p < 0.05$ by Duncan's test (n = 125). Each value represents the mean ± standard deviation of three independent experiments.

The second mechanical treatment (cT1, cT2) (i.e., longitudinal incision of the seed, Figure 1c), showed a lower germination rate compared to bT1 and bT2. Seeds obtained from "Polka" open pollination, treated with the cT2 condition, reached a maximum of 22% of germination rate, followed by seeds of "Tulamagic" open pollination, with a maximum of 3.7% germination efficiency, which demonstrates that with this type of incision, the germination rate seems to be influenced by the genotype used. In addition, when the same type of mechanical treatment was combined with low temperature (cT1), seed germination rate of Polka was reduced to almost half (7.4% of regeneration) compared to the cT2 condition.

The lowest percentages of germination were obtained from untreated seeds (control) of both cultivars, regardless the temperature; only one seed of "Polka" germinated in total (Figure 4). This result confirms that without any mechanical treatment, the *Rubus* seed is almost unable to germinate in vitro.

Figure 5 shows the time for seed germination, expressed as the days necessary for seeds to germinate after the different treatments applied to seeds belonging to both "Polka" and "Tulamagic" open pollination groups. The data show that bT1 and bT2 were the more efficient treatments in terms of time required for the germination of both cultivars. Seeds treated with bT2 conditions germinated after 4 days from cutting and in vitro culturing, and they reached the plateau level in 30 days.

Seeds in bT1 germinated with more than 14 days of delay, which corresponds to the time that seeds spent under the low temperature conditions of bT1. Indeed, in this condition, seeds started to germinate after 20 days from the beginning of the experiment and reached the plateau level after 40–42 days of in vitro culture. This is clearly visible in Figure 5a,b, in which seeds from both cultivars show similar germination timing.

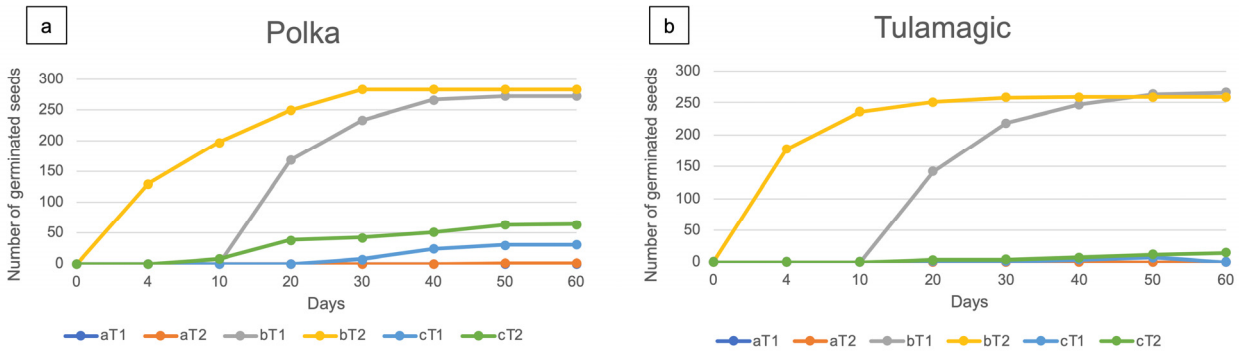

Figure 5. In vitro germination trend of "Polka" seeds (**a**) and of "Tulamagic" seeds (**b**) derived from open pollination, reported as the days required for seeds to germinate after the different treatments. Uncut seed (aT1), transverse cutting (bT1), and seed with longitudinal incision (cT1) stored at 4 °C for 14 days and then placed in growth chamber at 24 °C; uncut seed (aT2), transverse cutting (bT2), and seed with longitudinal incision (cT2) placed directly at 24 °C. The graphs represent the total number of germinated seeds, starting from 125 seeds, of three independent experiments (total number seeds for each condition = 375).

3.3. Application of the Optimized In Vitro Seed Germination Protocol to Combination Crosses

The most efficient protocol for in vitro germination of seeds obtained from open pollination of both "Polka" and "Tulamagic" varieties was also applied for the in vitro germination of seeds obtained through the programmed cross combinations. In particular, the bT2 conditions were found to be the best in terms of both seed germination efficiency and the time to germination; therefore, it was applied to all the combination crosses.

In Figure 6, all the cross combinations are reported with regards to their frequency of in vitro germination obtained by applying bT2 conditions. By applying this optimized in vitro germination protocol, the germination frequency obtained was homogeneous for all the 14 different crossings tested. The average frequency of germination was around 87%, with the highest germination rates recorded of 95.6% and 95.2% for AB20, 24 and AB20,03, respectively, and the lowest rate of 76.8% obtained for AB20,20.

Figure 6. Data on in vitro germination frequency of seeds obtained from the programmed crosses after bT2 treatment. Results are expressed as the (number of germinated seeds/total treated seeds) ×100 at 30 days after treatment. One-way ANOVA was used to analyze the data. Different letters show significant differences at $p < 0.05$ by Duncan's test ± standard deviation (n = 250).

4. Discussion

Seeds of the genus *Rubus* are characterized by their poor germination ability due to their deep dormancy and hard endocarp. Therefore, chemical or physical pre-treatments

are required to injure the hard seed coat that surrounds the embryo, allowing air and water to enter [2,33,34]. The most widely used pre-treatment is based on sulfuric-acid-mediated scarification, followed by cold treatment and seed stratification [9,20]. However, seeds subjected to scarification and stratification are observed to germinate very slowly, or often fail to germinate [8,11]. Usually, to achieve the number of seedlings requested, one needs to prepare and place under germination conditions at least 2–3 times more seeds than requested (Figure 7). In this study, 3 months after sowing, on average, less than 25% of all seed crosses sown had germinated. The low germination efficiency observed, coupled with the long time period required for stratification (i.e., 3 months), make this procedure lengthy and inefficient. Therefore, finding a more efficient protocol is paramount to the overall raspberry breeding activity.

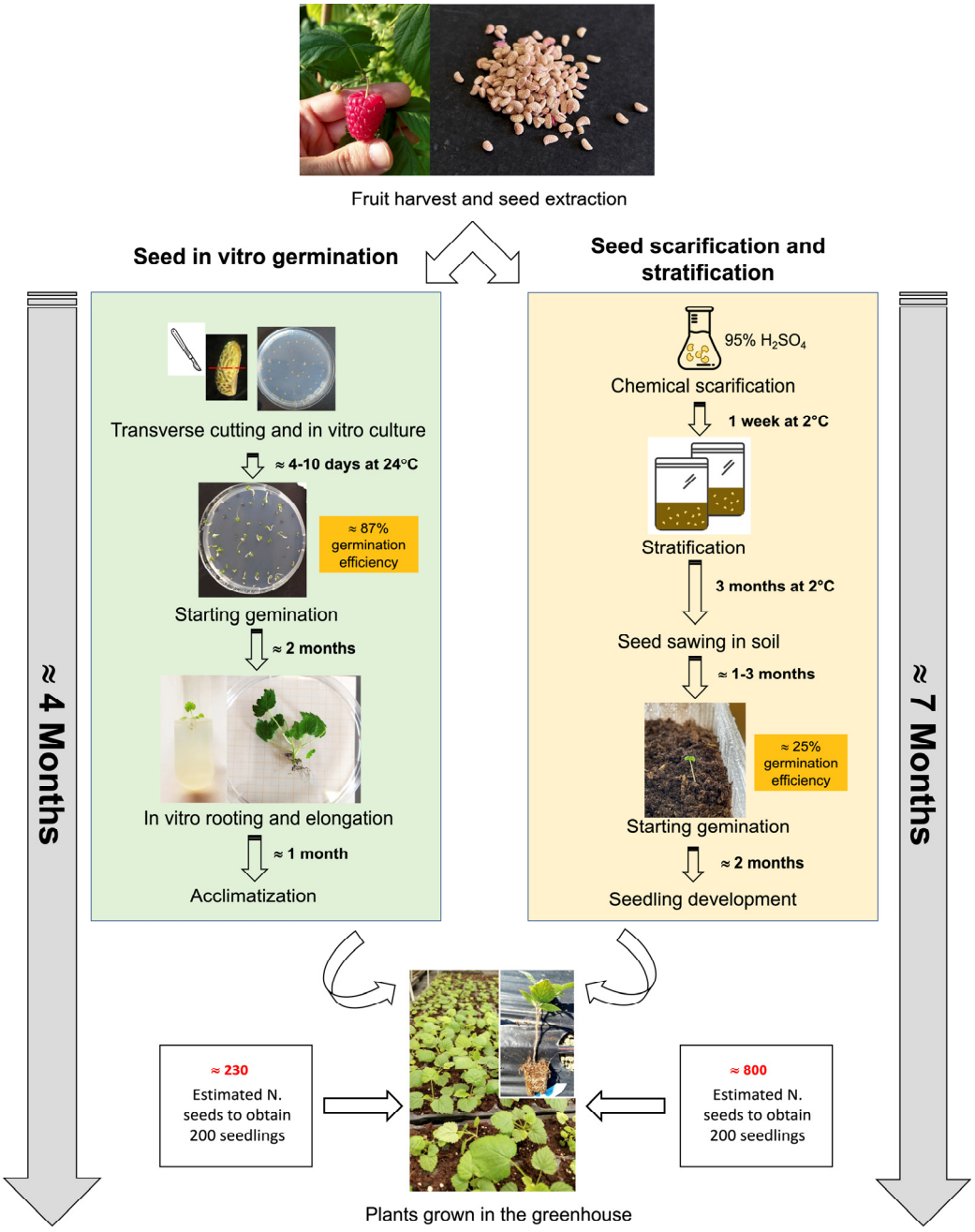

Figure 7. Schematical drawing of the steps required to obtain well-established seedlings of raspberry by following the optimized in vitro germination protocol in comparison with the scarification and stratification method used in this study.

In the present research, the application of an optimized in vitro protocol offered the guarantee of producing a large number of seedlings with a reduced number of seeds available, thus saving time during pollination, not needing to have a large number of fruits for each cross combination, and seeds in treatments and germination (Figure 7).

This study evaluates and compares the raspberry scarification pre-treatment described above with the in vitro germination method, based on a previously optimized protocol [19]. From the experiment with seeds derived from "Polka" and "Tulamagic" open pollination, it was possible to identify the best mechanical treatment, i.e., cutting the seeds in two halves (treatment "b", Figure 1). On the other hand, the longitudinal incision of the seed (treatment "c") was less effective than the cutting in half of the seeds in terms of germination efficiency. This result could be related to the ineffective lesion of the raspberry seed endocarp, which, on the contrary, is completely broken with treatment "b" (cutting the seed in two halves), leading to the highest observed seed germination rate (Figure 4). Similar results were revealed in another study, where raspberry seeds with nicked endocarps failed to germinate until the testa and endosperm were injured and exposed to the external environment [16].

In our study, the optimal temperature to culture the seeds in vitro was confirmed to be 24 °C under a photoperiod of 16 h light (70 µmol/m^2/s). These conditions (temperature and cutting protocol) favored the germination frequency and speed of seed germination, most of which germinated 4 days after treatment. It was also observed that low temperatures only favored a lag in germination, and no statistical differences were detected compared to the 24 °C conditions, in contrast with what has been observed in other studies applying pre-treatments for seed germination [11,33].

The in vitro germination protocol described in this study represents a useful guideline for the application of the same strategy to improve the seed germination efficiency in other plant species that are also characterized by seed dormancy and low germination rates. In fact, a similar result was already described in strawberry (*Fragaria* × *ananassa*), where an in vitro protocol was optimized to increase the efficiency of seed germination in a shorter germination time, in which achenes were cut across the embryo axis and were placed in contact with the culture medium [35]. In another study aimed at increasing seed germination of *Ilex dumosa* R., transversal cutting of sterilized pyrenes and their subsequent in vitro culture induced a maximum germination rate of around 70% after 2 months of in vitro culture compared to the whole pyrenes, which reached a maximum germinability of 37% at 8 months after sowing [28].

Therefore, the in vitro seed germination protocol described in this work can contribute towards speeding up breeding programs of *Rubus* spp. and in all other plant species having seeds with a low germination rate. Clearly, the use of the in vitro germination protocol requires laboratory facilities, more specialized personnel, and a well-established protocol for in vivo acclimatization, which represents one of the most critical phases of in vitro culture techniques. However, this procedure offers the advantage of obtaining sufficient seedlings for subsequent evaluation, even for cross combinations with low pollination and seed germination efficiencies. Of no less importance is the possibility of accelerating the germination times, and therefore, the production times of the seedlings to be taken to the evaluation environments.

Although the in vitro protocol has been confirmed to be the best protocol for efficiency and timeliness, it is important to note that there are statistical differences among the various combination crosses, both germinated in vitro and in soil.

As described in the previous literature [2,11,36,37], the parental genotype influences germination success rates. Through scarification and stratification methods, the range of germination rates was 5% to 52%, which is substantially wider than that observed in the in vitro protocol (from 76.8% up to 95.6%). Thus, the optimal and standardized growing conditions in the in vitro protocol have favored the high germination rates, which represents an incredible advantage to face challenges typical of breeding programs related to pollen vitality, compatibility between parental genotypes, environmental factors, and standardization of germination time. In addition, the data presented in this study point

toward the possibility that the in vitro protocol may reduce the impact of parental genotypes on germination rates.

Seedling populations obtained from the different cross combinations with the two germination protocols were easily developed in the greenhouse, showing similar levels of development, and then were planted in the field (Supplementary Figure S1). At this stage, it was not possible to assess the potential effect of the seed germination method on the genetic or phenotypic variability of the seedling populations. This is because the germinated seeds obtained by applying the two protocols described (in vitro germination and chemical scarification/stratification) had different timings of germination, and were thus transferred to the field at different times.

The variability generated in the different populations of seedlings obtained with the different germination methods will be the objective of subsequent work that, in order to have scientific value, must be carried out in the field, and will require the pheno-morphological assessment of all seedlings for at least two cultivation cycles.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/horticulturae9020153/s1. Figure S1: Raspberry plants belonging from in vitro germinated seeds by following "bt2" treatment (seed cut in half placed directly at 24°C), after two months of cultivation in the open field in Cesena (FC) Italy, located at 44°09′39.3″N latitude 12°14′29.8″E longitude.

Author Contributions: Author Contributions: Conceptualization, S.S., F.C. and B.M.; methodology, V.P., E.C., M.G., F.C. and S.S.; validation, V.P., M.M. and E.C.; formal analysis, V.P. and F.C.; investigation, V.P., E.C. and M.M.; data curation, S.S., V.P, M.M. and F.C.; writing—original draft preparation, V.P.; writing—review and editing, S.S., B.M., M.M, E.C., M.G., F.C. and V.P.; supervision, S.S., B.M. and F.C.; project administration, B.M., S.S. and F.C.; funding acquisition, B.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the BreedingValue project that has received funding from the European Union's Horizon 2020 research and innovation program under grant agreement No. 101000747.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data sharing not applicable.

Acknowledgments: We gratefully acknowledge financial support by Battistini Piante Vivai, Italy.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

1. Hall, D.; Shepherd, T.; Fountain, M.; Vétek, G.; Birch, N.; Jorna, C.; Farman, D.; Cross, J. Investigation of attraction of raspberry cane midge, Resseliella theobaldi, to volatiles from wounded raspberry primocanes. *WPRS Bull.* **2011**, *70*, 1–9.
2. Jennings, D.L. *Raspberries and Blackberries: Their Breeding, Diseases and Growth*; Academic Press: London, UK, 1988; p. 230.
3. Graham, J.; Smith, K.; MacKenzie, K.; Jorgenson, L.; Hackett, C.; Powell, W. The construction of a genetic linkage map of red raspberry (*Rubus idaeus* subsp. *idaeus*) based on AFLPs, genomic-SSR and EST-SSR markers. *Theor. Appl. Genet.* **2004**, *109*, 740–749. [PubMed]
4. Faostat. Available online: http:/FAOSTAT.FAO.ORG (accessed on 5 December 2022).
5. Sabbadini, S.; Capocasa, F.; Battino, M.; Mazzoni, L.; Mezzetti, B. Improved nutritional quality in fruit tree species through traditional and biotechnological approaches. *Trends Food Sci. Technol.* **2021**, *117*, 125–138. [CrossRef]
6. Palonen, P.; Weber, C. Fruit Color Stability, Anthocyanin Content, and Shelf Life Were Not Correlated with Ethylene Production Rate in Five Primocane Raspberry Genotypes. *Sci. Hortic.* **2019**, *247*, 9–16. [CrossRef]
7. Graham, J.; Jennings, N. Raspberry Breeding. In *Breeding Plantation Tree Crops: Temperate Species*; Springer: New York, NY, USA, 2009; pp. 233–248.
8. Contreras, E.; Grez, J.; Gambardella, M. Scarification and Stratification Protocols for Raspberry (*Rubus idaeus* L.) Seed Germination. *Acta Hortic.* **2016**, *1133*, 153–157. [CrossRef]

9. Zurawicz, E.; Masny, A.; Kubik, J.; Lewandowski, M. Germination of Red Raspberry Seeds as Affected by Origin and Chemical Scarification. *Hortic. Sci.* **2017**, *44*, 133–140. [CrossRef]
10. Tiwari, A.K.; Tiwari, T.N.; Prasad, S.R. Seed dormancy in ornamental plants: A review. *Indian J. Agric. Sci.* **2016**, *86*, 580–592.
11. Wada, S.; Reed, B.M. Standardizing Germination Protocols for Diverse Raspberry and Blackberry Species. *Sci. Hortic.* **2011**, *132*, 42–49. [CrossRef]
12. Wada, S.; Kennedy, J.A.; Reed, B.M. Seed-coat anatomy and proanthocyanidins contribute to the dormancy of Rubus seed. *Sci. Hortic.* **2011**, *130*, 762–768. [CrossRef]
13. Warr, H.J.; Savory, D.R.; Bal, A.K. Germination studies of bakeapple (cloud-berry) seeds. *Can. J. Plant Sci.* **1979**, *59*, 69–74. [CrossRef]
14. Taylor, K. Biological Flora of the British Isles: Rubus Vestitus Weihe. *J. Ecol.* **2005**, *93*, 1249–1262. [CrossRef]
15. Moore, J.N.; Brown, G.R.; Lundergan, C. Effect of Duration of Acid Scarification on Endocarp Thickness and Seedling Emergence of Blackberries1. *HortScience* **1974**, *9*, 204–205. [CrossRef]
16. Nesme, X. Respective effects of endocarp, testa and endosperm, and embryo on the germination of raspberry *(Rubus idaeus* L.) seeds. *Can. J. Plant Sci.* **1985**, *65*, 125–130. [CrossRef]
17. Peacock, D.N.; Hummer, K.E. Pregermination Studies with Liquid Nitrogen and Sulfuric Acid on Several *Rubus* Species. *HortScience* **1996**, *31*, 238–239. [CrossRef]
18. Grzesik, M.; Romanowska-Duda, Z. Quality of Seeds as a Key to Commodity Cultivation of *Lavatera Thuringiaca* L.—Plants with High Potential for Multi Directional Use. *Acta Innov.* **2015**, *15*, 5–12.
19. Ke, S.; Skirvin, R.M.; McPheeters, K.D.; Otterbacher, A.G.; Galletta, G. In vitro germination and growth of Rubus seeds and embryos. *HortScience* **1985**, *20*, 1047–1049. [CrossRef]
20. Masny, A.; Kubik, J.; Żurawicz, E. Seed Germination of Raspberry (*Rubus Idaeus* L.) Depending on the Age of Seeds and Hybridization Partners. *J. Hortic. Res.* **2022**, *3*, 61–66. [CrossRef]
21. Koornneef, M.; Bentsink, L.; Hilhorst, H. Seed dormancy and germination. *Curr. Opin. Plant Biol.* **2002**, *5*, 33–36. [CrossRef]
22. Finkelstein, R.; Reeves, W.; Ariizumi, T.; Steber, C. Molecular aspects of seed dormancy. *Annu. Rev. Plant Biol.* **2008**, *59*, 387–415. [CrossRef]
23. Rehman, S.; Cho, H.R.; Jamil, M.; Yun, S.J. Effect of GA and ABA on germination behavior of Black Raspberry (Rubus coreanus Miquel) Seeds. *Pak. J. Bot.* **2011**, *43*, 2811–2816.
24. Batak, I.; Dević, M.; Gibal, Z.; Grubišić, D.; Poff, K.L.; Konjević, R. The effects of potassium nitrate and NO-donors on phytochrome A-and phytochrome B-specific induced germination of Arabidopsis thaliana seeds. *Seed Sci. Res.* **2002**, *12*, 253–259. [CrossRef]
25. Nelson, D.C.; Riseborough, J.A.; Flematti, G.R.; Stevens, J.; Ghisalberti, E.L.; Dixon, K.W.; Smith, S.M. Karrikins discovered in smoke trigger Arabidopsis seed germination by a mechanism requiring gibberellic acid synthesis and light. *Plant Physiol.* **2009**, *149*, 863–873. [CrossRef]
26. Sharma, D.R.; Kaur, R.; Kumar, K. Embryo rescue in plants—A review. *Euphytica* **1996**, *89*, 325–337. [CrossRef]
27. Mroginski, L.; Dolce, N.; Sansberro, P.; Luna, C.; Gonzalez, A.; Rey, H. Cryopreservation of Ilex Immature Zygotic Embryos. In *Plant Embryo Culture*; Thorpe, T., Yeung, E., Eds.; Humana Press: Totowa, NJ, USA, 2011; pp. 215–225.
28. Dolce, N.R.; Mroginski, L.A.; Rey, H.Y. Enhanced Seed Germination of Ilex Dumosa R. (Aquifoliaceae) through in Vitro Culture of Cut Pyrenes. *HortScience* **2011**, *46*, 278–281. [CrossRef]
29. Fay, M.F. Conservation of rare and endangered plants using in vitro methods. *Vitr. Cell. Dev. Biol. Plant* **1992**, *28*, 1–4. [CrossRef]
30. Andrianjaka-Camps, Z.N.; Baumgartner, D.; Camps, C.; Guyer, E.; Arrigoni, E.; Carlen, C. Prediction of raspberries puree quality traits by Fourier transform infrared spectroscopy. *LWT Food Sci. Technol.* **2015**, *6*, 1056–1062. [CrossRef]
31. Maksimović, J.J.D.; Milivojević, J.M.; Poledica, M.M.; Nikolić, M.D.; Maksimović, V.M. Profiling antioxidant activity of two primocane fruiting red raspberry cultivars (Autumn bliss and Polka). *J. Food Compos. Anal.* **2013**, *31*, 173–179. [CrossRef]
32. Murashige, T.; Skoog, F. A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiol. Plant.* **1962**, *15*, 473–497. [CrossRef]
33. Jennings, D.L.; Tulloch, B.M.M. Studies on factors which promote germination of raspberry seeds. *J. Exp. Bot.* **1965**, *16*, 329–340. [CrossRef]
34. Dale, A.; Jarvis, B.C. Studies on germination in raspberry (*Rubus idaeus* L.). *Crop Res.* **1983**, *23*, 73–81.
35. Miller, A.R.; Scheereus, J.C.; Erb, P.S.; Chandler, C.K. Enhanced strawberry seed germination through in vitro culture of cut achenes. *J. Am. Soc. Hortic. Sci.* **1992**, *117*, 313–316. [CrossRef]
36. Jennings, D.L. Some genetic factors affecting fruit development in raspberries. *New Phytol.* **1971**, *70*, 361–370. [CrossRef]
37. Żurawicz, E.; Studnicki, M.; Kubik, J.; Pruski, K. A Careful Choice of Compatible Pollinizers Significantly Improves the Size of Fruits in Red Raspberry (*Rubus Idaeus* L.). *Sci. Hortic.* **2018**, *235*, 253–257. [CrossRef]