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Standardization of an Effective Scarification and Germination Protocol for Strawberry Seeds That Is Useful for Gamic Propagation

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Abstract: In strawberry (*Fragaria* × *ananassa* Duch.) breeding programs, seed dormancy adversely affects germination, resulting in delayed seedling emergence and low germination rates. This study investigated the best solution to enhance strawberry seed germination both in terms of efficiency and timing by evaluating the effect of three key factors: genotype, pre-sowing treatment, and germination medium. Chemical scarification treatment with the sulfuric acid of seeds from three different genotypes was optimized; treated seeds were placed to germinate on three germination media (Murashige and Skoog medium, peat, and filter paper) in a growth chamber. Seedlings obtained were acclimatized for evaluating post-acclimatization survival rate and possible phenotypic differences regarding seedling development. Chemical scarification treatment produced the best results, with germination rates of around 100% and the highest speed of germination compared to the not-treated controls. Indeed, more than 90% of the seeds germinated 14 days after sowing, regardless of the genotype or germination medium tested. Seedlings germinated on filter paper gave the poorest performances in terms of post-acclimatization survival rate and showed lower average plant height. In conclusion, it was demonstrated that excellent germination rates can be achieved through proper seed scarification, which is not genotype dependent; furthermore, when this method is combined with the correct germination medium, excellent seedling quality can be achieved.

Keywords: *Fragaria* × *ananassa*; plant tissue culture; gamic propagation; chemical scarification; seed dormancy

Citation: Giovanetti, G.; Marcellini, M.; Pergolotti, V.; Mecozzi, F.; Mezzetti, B.; Capocasa, F.; Sabbadini, S. Standardization of an Effective Scarification and Germination Protocol for Strawberry Seeds That Is Useful for Gamic Propagation. *Horticulturae* **2024**, *10*, 1345. https://doi.org/10.3390/horticulturae10121345

Academic Editor: Júlia Halász

Received: 8 November 2024
Revised: 11 December 2024
Accepted: 12 December 2024
Published: 14 December 2024

1. Introduction

Strawberry fruits are currently consumed all over the world due to their highly appreciated taste and aromatic profiles; moreover, they belong to the broader category of small fruits that are recognized by the scientific community as an important source of vitamins, minerals, and antioxidants which have beneficial effects on consumers' health [1]. Strawberry consumption has increased significantly over the last decade [2] together with consumer demand for a qualitatively superior product, both from an organoleptic and nutraceutical point of view [1,3]. In this evolving context, farmers rely on scientific research to provide them with technical and scientific means to cope with the current socio-economic and climate change impacts on the entire sector. The answer to these challenges can be achieved through the development of new, low-impact cultivation techniques that give better results in terms of quality and yield of production, adapted to the different cultivation systems [4], and through the development of new strawberry cultivars that are genetically advanced, resilient, and able to adapt to the environmental and cultural changes in the new millennium by using traditional and new breeding techniques [5]. Genetic studies on seedling populations are now essential for identifying genes that control traits that are important for strawberry breeding [5–7] but also open the possibility of obtaining F1 hybrid lines that can be propagated by seeds [8].

In recent years, in addition to public strawberry breeding programs affiliated with research institutions and/or universities, a diversification of breeding lines on this species has been observed due to the implementation of several private breeding programs carried out by companies of reference for the strawberry nursery and/or production sector [9]. This change has increased the number of cultivars licensed annually and has expanded the varietal offer, differentiating varieties by market sectors and increasingly distinguishing them through registered trademarks [10]. Thus, it is necessary to improve all the critical steps in a breeding program to make the whole process efficient, from the production of new genotypes, to their evaluation, selection and their long-term conservation for future progress in breeding programs [11,12]. One of the crucial initial steps for strawberry breeding is the process of controlled hybridization. Problems in this phase often arise from the interaction of several factors, including the viability of the pollen to be used as a male parent [13–15], gametic incompatibility between the pollen and the gynoecium of a different genotype or a different genus (interspecific hybridization) [16], as well as the growing environment of the parent plants and their agronomic quality.

The second criticality is encountered in the next step, namely the germination of seeds obtained from controlled crossbreeding; strawberry achenes indeed present an accentuated tegumentary dormancy that greatly affects the germination phase. The germination rate is usually around 30%, with high-scalar seedlings sprouting [17–19]. At present, all stakeholders, especially private institutions, are struggling to reach a high percentage of seed germination and, in order to obtain the requested number of seedlings for a representative breeding population, are obliged to pollinate a large number of flowers in order to obtain a large number of seeds to be used in the in vivo germination standard procedure. Therefore, the low germination rate contributes to increasing costs for the germination program and also to restricting the characterization to only the low-rate germinated seedlings. This leads to a loss of genetic variability because each seedling presents a different, potentially superior, genotype. Scalar seedling emergence, on the other hand, results in the evaluation of seedlings with different vegetative development [18–20].

Several research groups have already evaluated and validated different methods, which are more or less efficient, to overcome seed dormancy that were mainly based on either chemical or mechanical scarification processes. This method consists of removing or weakening the outer tegument of seeds, allowing them to be imbibed with water and promoting germination [21].

Concerning physical/mechanical methods of scarification, seeds have been subjected to cold treatments [22,23], exposure to light [18], and cutting of achenes [24], while the best results obtained through chemical scarification were produced by the use of sodium hypochlorite (NaClO) [25,26], hydrochloric acid (HCl) [24,27], and sulphuric acid (H_2SO_4) [17,28–30].

Tegument dormancy affects the germination of strawberry seeds due to the outer hard shelter, which can be removed by several techniques to facilitate the germination. El Hamdouni and collaborators [31] used chemical treatment in the achenes of two commercial cultivars which were subjected to different treatments, such as scarification with H_2SO_4 (36N), hydrogen peroxide for different timings (from 5 to 60 min), tegument cutting, and light scarification with H_2SO_4 (1N). Finally, all treated embryos were isolated and placed to germinate on a Murashige and Skoog (MS) medium free of hormones. The highest germination rate was obtained by scarifying seeds with H_2SO_4 (36N), demonstrating, along with the treatments involving lesioning or complete removal of the outer integument, the effect of the scarification protocols on strawberry achene dormancy. From these treatments, in addition to maximizing the germination rate, germination time was significantly reduced by up to 50%.

Another important aspect to be considered is the growing medium to be used in germination trials; there are many different substrates that are useful for germination and they are referred to under the generic term of "Growing media". A distinction is made between paper, sand, and other materials, such as organic mixtures of peat, sand, perlite, etc.

It is important to use the culture medium that best reflects the parameters of water retention, pH, and conductivity specific to the species, and it must also meet the prerequisites of cleanliness and harmlessness [32,33].

The use of in vitro growing media for strawberry seed germination has also been evaluated by some researchers as a valuable option for more standard substrates. Miller and collaborators [24] increased the germination rate up to 100% and decreased the germination delay by longitudinal cutting of achenes on Murashige and Skoog (MS) medium free of plant growth regulators (PGRs). This technique was particularly efficient from the point of view of germination rate but much too laborious for the cutting of the achenes.

Hongxiang and collaborators [32] adopted the same technique of removing the outer integument of the achenes by cutting and germinating them in vitro or on moist filter paper. Again, the best results were obtained through in vitro germination on MS media. In this study, different concentrations of PGRs were used and an almost 100% germination rate was obtained. The different germination rates observed were imputed to be genotype dependent.

The current private and small breeding programs for the genus *Fragaria* require standardized and effective seed scarification and germination protocols that enable a high germination percentage to be achieved with short germination times without the need for particularly expensive facilities and equipment. This study investigated the best solution, also on a technical–practical level, to achieve these results using repeatable methodologies and readily available materials. In particular, we investigated the efficiency and practicality of using some of the commonly used culture media for strawberry seed germination, comparing them with an in vitro substrate. The combination of chemical scarification combined with in vitro germination seems to positively influence the results in terms of germination rate and speed of germination on the strawberries genotypes tested.

2. Materials and Methods

2.1. Mother Plant Material and Seed Source

Seeds derived from the open pollination (OP) of three advanced breeding selections, called 68, 71 and 97, of strawberry (*Fragaria* × *ananassa* Duch.) were used for the experimental trials. These plants were obtained through the D3A breeding program of Marche Polytechnic University (UNIVPM), Italy, and grown in single plots (eight plants per plot) at the field of the Didactic–Experimental Farm "Pasquale Rosati", UNIVPM, located in Agugliano (Ancona, Italy), about 20 km away from the sea and at an altitude of 46 m above sea level. Genotypes 68 and 71 are both June-bearing advanced selections from the D3A strawberry breeding program. These two selections are late-ripening genotypes originated from crosses, including commercial cultivars, combined with advanced selections of the same breeding program. Genotype 97 is an advanced everbearing selection produced by the same breeding program.

Mature fruits derived from open-pollinated flowers of these three genotypes were harvested during the spring season of 2022 and subsequently grinded using a blender to extract seeds from the pulp. Seeds were collected by decanting them in a 500 mL beaker with water to allow for the efficient separation of the achenes from the receptacle and to assess their function and viability through water. This is possible because the viable achenes will be deposited on the bottom of the beaker as a result of decanting while the not-vital (lighter) ones will remain in suspension and thus can be easily removed [33,34]. The seed extraction protocol was repeated for each crossing combination. The achenes were allowed to dry on adsorbent paper for 24 h, thus preventing the possible development of fungal diseases, then placed in 5 mL Cryo.s$^{\mathrm{TM}}$ Freezing Tubes and refrigerated at 4 °C for 11 months before use in germination trials.

2.2. Seed Scarification and Sterilization Protocol

Seeds from the three genotypes were dehydrated in a methacrylate desiccator with quartz salts for 2 days at +4 °C before the pre-sowing treatments. Once the humidity of the seeds was stabilized (3–4% R.U.), they were placed in cold storage at 4 °C in closed Cryo.s^{TM} Freezing Tubes for 1 week. A portion of the extracted seeds were treated with a pre-sowing scarification using glass Erlenmeyer flasks containing a concentrated sulfuric acid solution (H_2SO_4 from 95% to 97%), with enough volume to cover the whole surface of the seeds, and placed in continuous agitation at 100 rpm for 35 min.

In this stage, the Erlenmeyer flasks were positioned in an ice bath to prevent undue heat transfer resulting from the exothermic reaction. Subsequently, the seeds underwent a 1 min rinsing under flowing water followed by treatment with a calcium hydroxide ($Ca(OH)_2$) solution (5 g L^{-1}) to stabilize their pH. This treatment occurred in a beaker with continuous stirring for 5 min. Afterward, the seeds were subjected to a 1 min wash under running water and immersed in distilled water for 20 min.

Finally, all seeds, including the not-scarified seeds (control), were sterilized by soaking in 15 mL falcons with 10% sodium hypochlorite (NaClO) solution for 20 min and then rinsed for four washes with sterile milli-Q water for one, five, and ten minutes, respectively. This was followed by a fourth 5 min wash; then, seeds were sown on the different germination media (GM) described in the next paragraph.

2.3. Seed Germination Experimental Design

After the sterilization protocol, scarified and not-scarified OP-derived seeds of the selections 68, 71 and 97 were placed on three different GM (A, B, C), following International Seed Testing Association (ISTA) guidelines [31], to evaluate their germination efficiency. GM-A consists of an in vitro substrate based on MS salt and vitamins [35]; GM-B is a filter paper (Whatman® quantitative filter paper, ashless, Grade 41, Merk Life Science S.r.l., Milan, Italy) that is often used for germination tests of different species; GM-C is made of a fine mixture (0–5 mm) of black peat and blond peat (Traysubstrat-Klasmann, Klasmann-Deilmann GmbH, Emsland, Germany) that is used in in vivo germination tests (Figure 1).

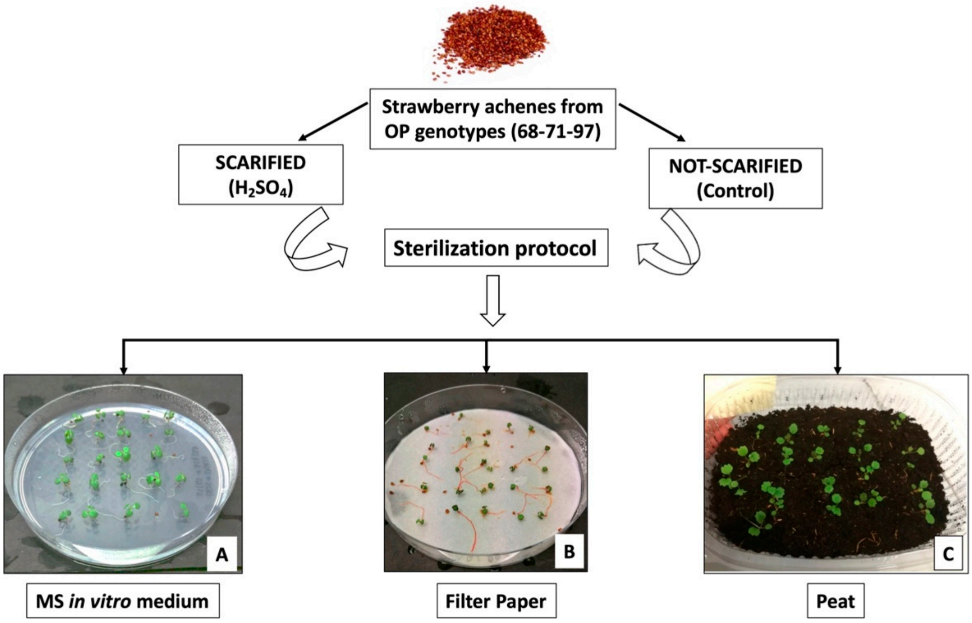

Figure 1. Experimental design of the germination trial. All genotypes, scarified and not-scarified (control), were subjected to the sterilization protocol before sowing in the different GM (A–C).

Scarified and not-scarified seeds were placed to germinate directly in a growth chamber equipped with white, fluorescent tubes (70 $\mu mol/m^2/s$) at a temperature of 24 ± 1 °C, which is optimal for the germination stage [28], and with a 16 h photoperiod of light. Three independent experiments were conducted for each condition. Data on seed germination efficiency and speed of germination were acquired weekly for each condition for a period of 42 days. Seed germination efficiency was expressed as the (number of germinated seeds/total sowed seeds) × 100. The time of seed germination was also acquired starting from T1 (7 days after sowing). A seed was considered to be germinated when its radicle reached a length greater than 1 mm.

2.4. In Vitro Seed Germination

Scarified and not-scarified OP seeds for each of the three seedling populations, 68, 71, and 97, were placed to germinate on GM-A composed of MS salts and vitamins [35] supplemented with 30 g L^{-1} sucrose and 7 g L^{-1} plant agar (Duchefa Biochemie, Haarlem, The Netherlands). The pH of the medium was adjusted to 5.7–5.8 with potassium hydroxide (KOH). GM-A was autoclaved at 121 °C for 15 min and then poured into 9 cm-diameter Petri dishes. Scarified and control seeds for each genotype were distributed in six Petri dishes (25 seeds per plate) to germinate for a total of 150 seeds per genotype and condition.

2.5. Filter Paper Seed Germination

Scarified and not-scarified OP seeds of the three genotypes were sowed onto singular sterilized 90 mm-diameter Whatman® filter paper disks (GM-B). The disks were placed in 9 cm-diameter Petri dishes and imbibed with 2 mL of sterile milli-Q water each. The same number of seeds described for GM-A were distributed on Petri dishes after the sterilization protocol. GM-B moisture was re-established weekly by supplementing 400 µL of milli-Q water per Petri plate, avoiding the dehydration of paper disks.

2.6. Seed Germination on Peat

Scarified and not-scarified OP-derived seeds of the three genotypes were placed to germinate in clear food-grade plastic containers filled with peat substrate (GM-C) (traysubstrat 70 lt seeding substrate- Klasmann-Deilmann GmbH, Germany) that were previously autoclaved at 121 °C for 15 min.

Each container was filled with 200 g of GM-C and sieved through a 4 mm mesh sieve, taking care to make the seeding surface as uniform as possible; then, 25 seeds per container were arranged in lines of five seeds and sprayed with 25 g of sterile milli-Q water. All containers of the different treatments were placed to germinate in the growth chamber under the same conditions described above.

2.7. Acclimatization of Seedlings and Phenotyping

Two weeks after the last data collection, and 56 days after sowing, 20 plants for each population and condition (scarified or not) were acclimatized in 60-hole honeycomb trays to assess their post-acclimatization survival rate and the height of seedlings. We selected only 20 plants to have the same amount of seedlings for each condition, GM, and genotype. The acclimatization process was carried out in a heated tunnel with a constant temperature of between 23 and 25 °C and relative humidity at saturation for the first week. After acclimatization, a fungicide treatment with Previter was carried out, respecting the doses reported on the label regarding treatments on seedlings. Data on plant height were collected for 10 seedlings per each condition and population; the height of the plants was measured starting from the crown at the ground level to the end of the peduncle of the most developed leaf. Phenotypic data were collected at three different intervals, with the first one being 25 days after acclimatization (T1), followed by 40 days (T2), and 55 days (T3) after acclimatization. The survival rate was calculated as the number of plants that survived at T3.

Data collection after acclimatization was performed considering a single plant as a replicate, and the phenotype parameters were analyzed to create an average of all genotypes for each condition at the three different time intervals (T1, T2, T3).

2.8. Statistical Analysis

All data acquired from each trial were analyzed to observe the differences and relations between genotype, scarification treatment, and GM compared to not-scarified seeds by using one-way ANOVA through the software Statistics 7 (Statsoft, Tulsa, OK, USA), and the averages were separated by a Duncan's Test ($p < 0.05$).

3. Results

3.1. Effect of Scarification Protocol and Germination Medium on the Germination Rate of Different Breeding Populations

The scarification protocol was carried out on seeds of three different populations of *Fragaria* × *ananassa* advanced breeding selections (68, 71, 97), which were then sterilized before being placed to germinate on the different germination media (GM): (A) MS in vitro medium; (B) Whatman® filter paper; (C) peat. Results in terms of germination efficiency and speed of the germination of scarified seeds were compared with those of not-scarified (but only sterilized) seeds (controls) sown on the different GM. For each genotype and condition, germination efficiency was tested by six replicates of 25 seeds each (150 seeds in total). In general, a significant difference was observed between the scarified (condition n. 1) and not-scarified seeds (condition n. 2) for all genotypes, regardless of the GM used (Figure 2). A germination rate of above 95% was observed for all scarified seeds regardless of the breeding population and GM used. Meanwhile, concerning the not-scarified seeds, statistically significant differences were observed. In particular, the breeding population 68 (blue histograms in Figure 2) showed the best germination efficiency in all GM, with the highest rate being obtained when seeds were sowed on GM-C2 (97%) (similar to the germination efficiency observed for scarified seeds), followed by GM-B2 (64%) and GM-A2 (13%). The breeding population 97 showed the lowest germination rate in all three GM when seeds were not scarified. In general, the GM that induced the lowest germination rate for not-scarified seeds in all three genotypes was A2 (MS in vitro medium), which led to 13% germinated seeds for the genotype 68 and 1% for the other two genotypes (71 and 97).

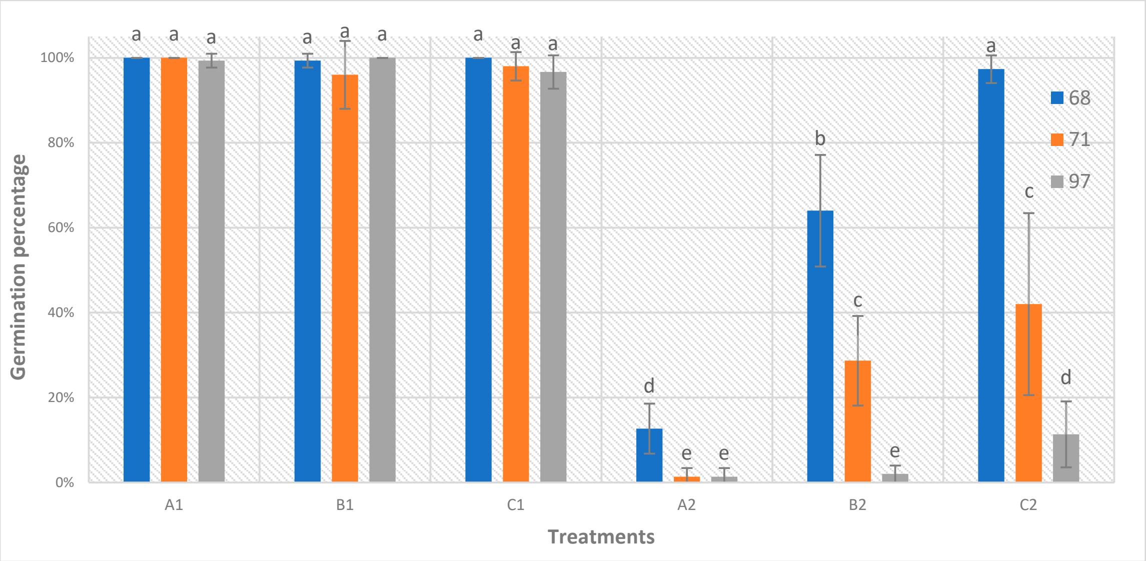

Figure 2. Data on seed germination efficiency for scarified and not-scarified seeds belonging to the three different breeding populations (68, 71, 97) sowed on the different GM. Germination percentage of scarified seeds on (A1) MS in vitro medium; (B1) Whatman® filter paper; (C1) peat. Germination percentage of not-scarified seeds on (A2) MS in vitro medium; (B2) Whatman® filter paper; (C2) peat. Results are expressed as the (number of germinated seeds/total sowed seeds) × 100 at 42 days after sowing for each different genotype. One-way ANOVA was used to analyze the data. Different letters show significant differences at $p < 0.05$ by Ducan's test ($n = 150$). Each value represents the mean ± standard deviation of six independent replicates for the three different genotypes.

The scarified seeds sown on the three GM (A1, B1, C1) already started to germinate 7 days after sowing (T1), with a higher germination efficiency and speed of germination being seen compared to not-scarified ones, with more than 90% germinated seeds at 14 days after sowing (T2). The not-scarified seeds started to germinate at T2 when sowed on GM-C2 and GM-B2. At 21 days after sowing (T3), peat medium (GM-C2) led to the higher germination efficiency (34%) and speed of germination of not-scarified seeds compared to the other substrates. GM-B2 reached similar germination efficiency (32%) at 42 days after sowing (T6) (Figure 3).

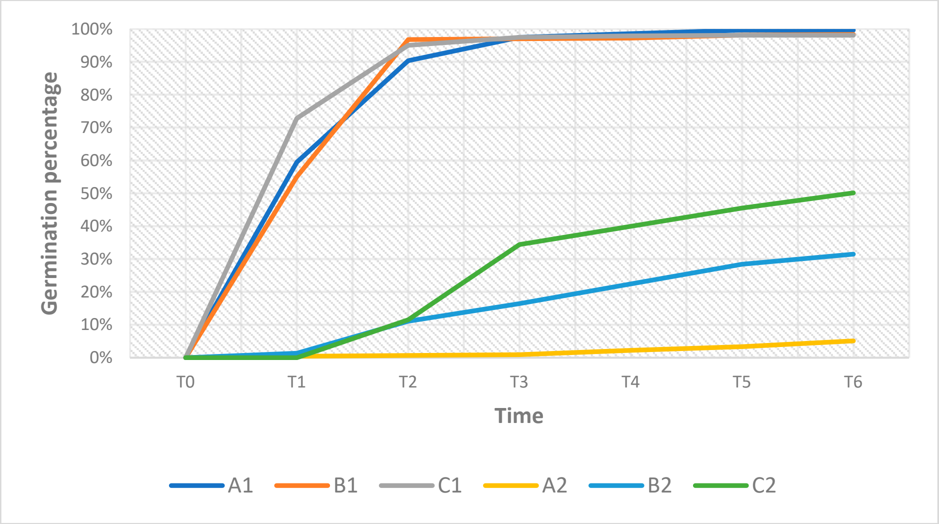

Figure 3. Germination efficiency and the speed of germination of scarified and not-scarified seeds sowed on different GM. Germination percentage of scarified seeds on (A1) MS in vitro medium; (B1) Whatman® filter paper; (C1) peat. Germination percentage of not-scarified seeds on (A2) MS in vitro medium; (B2) Whatman® filter paper; (C2) peat. Results are expressed as the (number of germinated seeds/total sowed seeds) × 100 from the sowing (T0) to the last data collection, 42 days after sowing (T6). The graph represents the average number of germinated seeds of the three genotypes (n = 450).

3.2. Post-Acclimatization Survival Rate

Following germination, where possible, 20 seedlings for each genotype and condition were acclimatized. For population 97, a lower number of seedlings were acclimatized from not-scarified seeds for all three GM (A2, B2, C2) because only a small number of seedlings were germinated from the 150 total sowed seeds. For population 97, the following seedlings were acclimatized: two seedlings from GM-A2, three seedlings from GM-B2, and 17 seedlings from GM-C2. For the genotype 71, only a low number of seedlings (three seedlings) were germinated on GM-A2; for all other conditions, a standard number of 20 seedlings was acclimatized.

Due to this low number of seedlings obtained for some genotypes in specific experimental conditions, an average analysis of the three genotypes was carried out, being only used to evaluate the effect of scarification and GM on the survival rate of seedlings post-acclimatization. As shown in Figure 4, with seedlings from the A1, C1 and C2 conditions, an average survival rate of 100% was obtained. Meanwhile, an average survival rate of 92% was obtained for A2, which was not a significantly different rate from the previous three. The lowest survival rate was obtained for seedlings germinated on GM-B, which corresponds to Whatman® paper filters, being precisely 82% for seedlings derived from scarified seeds and 72% for those derived from the not-scarified ones.

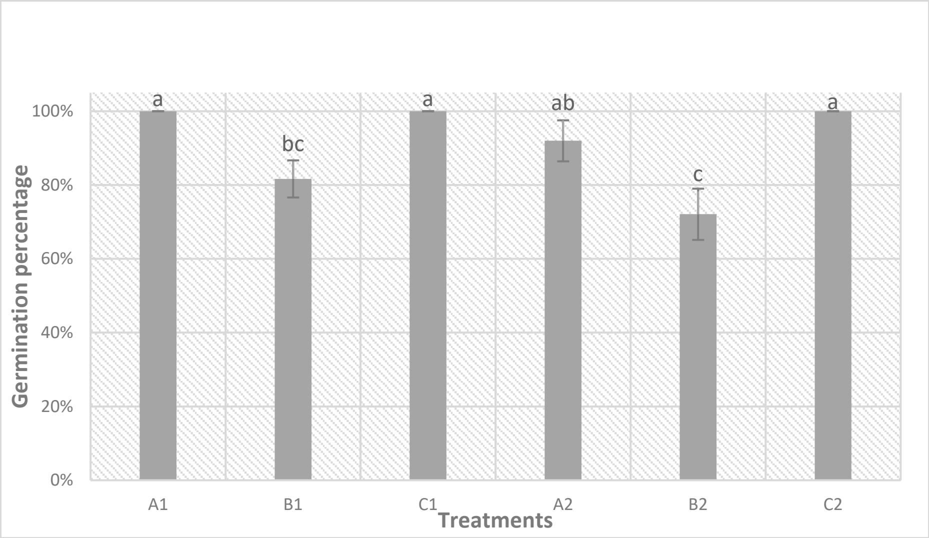

Figure 4. Survival rate post-acclimatization of seedlings from three genotypes. The graph represents the average of three populations in all conditions. Seedlings from scarified seeds germinated on (A1) MS in vitro medium; (B1) Whatman® filter paper; (C1) peat. Seedlings from not-scarified seeds germinated on (A2) MS in vitro medium; (B2) Whatman® filter paper; (C2) peat. Results are expressed as the (number of survival seedlings/total acclimatized seedlings) × 100 at 55 days after acclimatization. One-way ANOVA was used to analyze the data. Different letters show significant differences at $p < 0.05$ by Ducan's test ($n = 60$). Each value represents the mean ± standard error of 20 independent plantlets.

3.3. Phenotypic Characterization of Acclimatized Seedlings

In addition to the post-acclimatization survival rate, data were also collected on the phenotype of seedlings at 25 days (T1), 40 days (T2), and 55 days (T3) post-acclimatization specifically on the height of the most developed leaf for ten seedlings for each condition. Data were collected on the total number of seedlings obtained from the acclimatization stage. Notably, for population 97, no seedlings derived from not-scarified seeds sowed on GM A and B survived to the acclimatization phase; thus, we could not evaluate the differences between genotypes, and only the average height of the three genotypes together was evaluated. In Figure 5, the total average height of the three genotypes is shown. At T1 (25 days after acclimatization), the highest height was noted for seedlings germinated on C1 (4.9 cm), significantly different from the other conditions and GM. C1 was followed closely by A1, C2, and A2, with an average height of 3.8 cm for the first two conditions and 3.1 cm for the third one. A lower average height was obtained from seedlings germinated on GM B, from both scarified and not-scarified seeds, resulting in the GM with the worst performance (Supplementary Figure S1). Looking at the averages of heights collected at T2 and T3 (orange and gray histograms), it can be seen that seedling height increased in all GM and for both scarification treatment and control. At T3, the highest average height was recorded for seeds from A1 and C1, both leading to an average seedling height of 11.9 cm with no significant differences. For not-scarified seeds, seedlings from A2 and C2 showed the highest mean height values of 9.7 cm and 10.8 cm, respectively, with significant differences being seen between the two conditions.

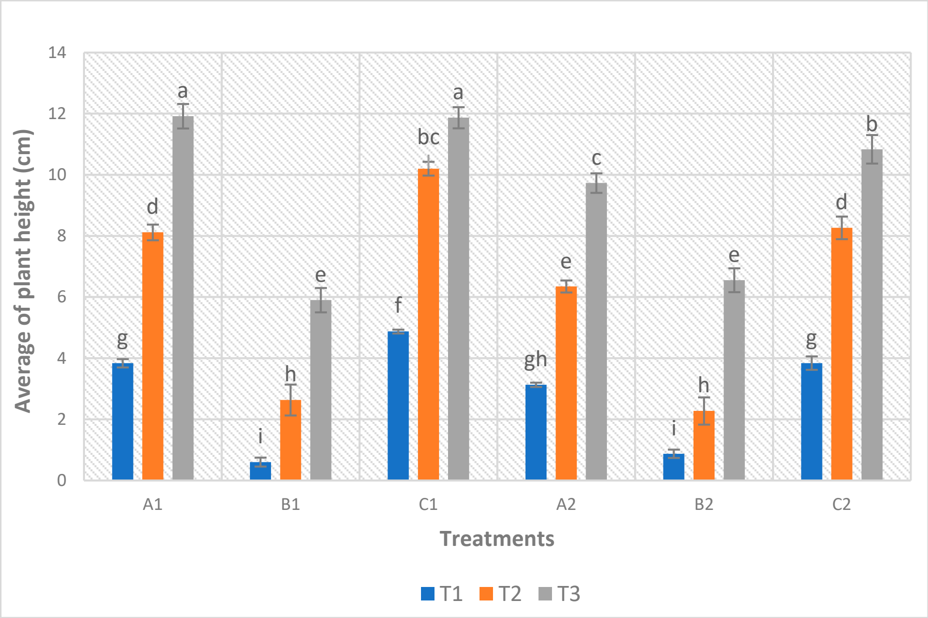

Figure 5. Average of plant height post-acclimatization. Seedlings from scarified seeds germinated on (A1) MS in vitro medium; (B1) Whatman® filter paper; (C1) peat. Seedlings from not-scarified seeds germinated on (A2) MS in vitro medium; (B2) Whatman® filter paper; (C2) peat. Results are expressed as the average among three genotypes at 25 days (T1), 40 days (T2), and 55 days (T3) post-acclimatization. One-way ANOVA was used to analyze the data. Different letters show significant differences at $p < 0.05$ by a Ducan's test ($n = 60$). Each value represents the mean ± standard error of 20 independent plantlets.

4. Discussion

Seeds of the species *Fragaria* × *ananassa* and those of related ones are characterized by a poor germination capacity caused by tegumentary dormancy due to the presence of the esocarp [21,25,33,36,37]. This issue is keenly felt in breeding programs where genetic variability resulting from controlled hybridizations needs to be explored. To do so, it is necessary to achieve a high number of seedlings from each cross combination to allow us to visualize all the genetic variabilities expressed [38,39]. To achieve a high germination rate while trying to concentrate it in a short time scale, various chemical or physical seed scarification treatments are used to remove the outer tegument facilitating imbibition [15,19,22–24,28]. These seed pretreatments have a positive effect on germinability, with the most widely used and effective being chemical scarification with H_2SO_4 sulfuric acid [25].

Seeds, once scarified, must be placed in the best environmental conditions to promote rapid germination and further development. In current breeding programs, the aim is to obtain a high number of seeds in each cross combination to mitigate the expected low germinability [38,40]. Therefore, research and standardization of an efficient and easy-to-implement scarification and germination protocol is essential, even in farm settings that lack special equipment, such as a micropropagation laboratory. In the present experiment, the application of a standardized scarification protocol and subsequent germination on different culture media, including MS medium without phytoregulators (MS0), which is considered the best GM in terms of germination speed and rate [29,32], allowed for clear and expendable results in private breeding programs, ensuring a high germination rate (close to 100%) and uniform seedling emergence. This provides a high number of seedlings derived from controlled crosses using a small seed lot, thus offering the possibility to explore all the genetic variability produced in the different crossing combinations.

Furthermore, this study set out to investigate which was the best GM to use in the critical seed germination stage, utilizing the in vitro MS0 medium as the reference GM since it is considered one of the most efficient germination substrates [24,32]. It was compared with filter paper (GM-B), mainly used in germinability tests of different species (according to ISTA protocols and/or in general for seed viability tests [30]), and with peat characterized by species-specific pH and Electro conductivity and a fine grain size (GM-C) which allows for close contact between the seed surface and peat particles. From the results obtained, it was noted that seed scarification treatment with H_2SO_4 positively influenced germination rate; indeed, scarified seeds of all three genotypes expressed a high germination rate in all three GM without showing statistically significant differences between the GM or among the genotypes (Figure 2). The influence of the GM and genotypes has been observed in seeds not subjected to the scarification pretreatment; indeed, it was possible to visualize statistically different germination efficiency among the three distinct GM and the three genotypes.

The GM that led to the best average germination rate and fastest seedling emergence among the not-scarified seeds of all three genotypes was substrate C, corresponding to the peat mixture. In contrast, the best-performing genotype among the not-scarified seeds was 68 regardless of the GM used; this genotype corresponds to an advanced June-bearing selection belonging to the D3A breeding program, UNIVPM.

At the germination stage, it was confirmed that a temperature set at 24 ± 1 °C was optimal for seed germination with all three GM exploited [28], with a 16 h photoperiod at an intensity of 70 µmol/m^2/s. These conditions promoted the rapid germination of the different seed pools in all three GM (Figure 6); therefore, it has been shown that environmental conditions must be optimal to achieve a high germination rate in a short time [28].

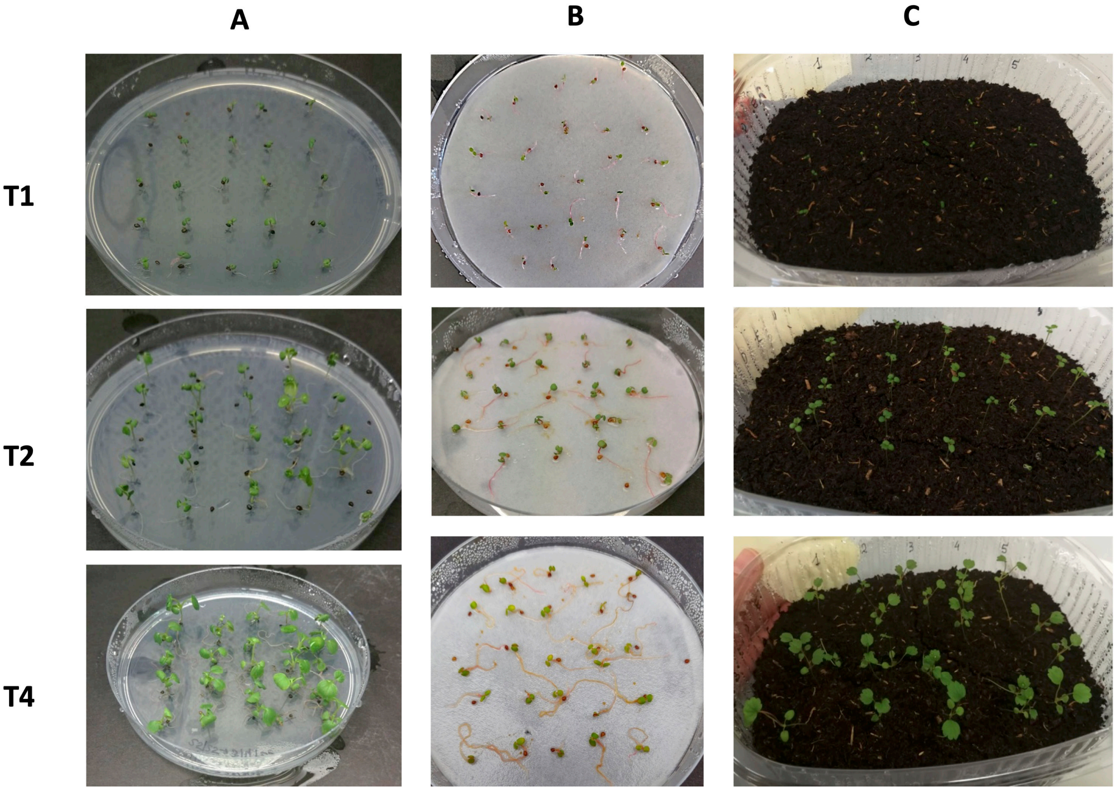

Figure 6. Germination of a scarified seed pool belonging to genotype 71 at three different time intervals and on the three different GM. T1 = 7 days after sowing; T2 = 14 days after sowing; T4 = 28 days after sowing. A = MS in vitro medium; B = Whatman® filter paper; C = peat.

Therefore, the present scarification and germination protocol, developed by modifying the protocol described in Pergolotti et al. [41], can contribute to the efficiency of the seed germination process in small and large breeding programs. Furthermore, it can be exploited for other purposes, such as the production of strawberry plants from seeds [42], avoiding the use of expensive facilities and/or equipment, and consequently also avoiding the use of specialized manpower, as is the case for micropropagation laboratories. Although the in vitro substrate led to a more rapid germination response, we observed that placing scarified seeds to germinate under favorable environmental conditions resulted in a timely and efficient response with no significant difference among the three genotypes and GM tested.

Problems were found in the post-germination stages for seeds sowed on filter paper (GM-B) for both the scarified and control seeds. This response is probably due to the absence of a real substrate, which is necessary for root support during seedling germination and growth, and the lack of nutrients, as there was an absence of micro and macro elements that are essential for the development of plantlets. In fact, this type of GM is usually exploited for germinability tests on seed stocks and not for the germination and development of seedlings [30]. Finally, it was observed that the genotype influenced germination success rates only when seeds were not scarified, as already reported in previous studies [14,25,29,38]. Among not-scarified seeds of the three genotypes, 68 was found to have the highest performance in all the GM, followed by genotype 71. Not-scarified seeds from genotype 97 showed the poorest performance of all three GM; this could be due to the different genetic bases, which could somehow affect the physiological processes during germination or achene frigo-storage pretreatment.

All seedlings obtained were acclimatized in tunnels at constant temperature and saturation humidity. Following this stage, measurements of plant height were carried out and showed significant differences based on the GM used for seed germination. The GM that led to the worst results, both in terms of survival rate and height of the plant, was substrate B, corresponding to filter paper, probably due to the reasons mentioned above. The other two GM produced significantly better results, with the best performance being obtained from seedlings germinated on substrate C, corresponding to peat, indicating a better vegetative development of the seedlings.

In conclusion, the scarification and germination protocol developed in this trial effectively maximized the germination efficiency of strawberry seeds, mitigating the influences of genotype and germination media. However, for optimal results aimed at high uniformity and vigor in seedlings, it is essential to utilize germination media capable of sustaining germination and subsequent vegetative growth. To achieve this goal without incurring in excessive costs associated with specialized facilities, the most effective approach is employing the scarification protocol followed by in vivo germination using a peat-based germination medium in a controlled environment.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae10121345/s1, Figure S1: Acclimatisation of plantlets derived from seeds germinated on Whatman® filter paper.

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

Funding: We gratefully acknowledge financial support by Geoplant s.r.l. and BreedingValue project—European Union's Horizon 2020 research and innovation program under grant agreement No. 101000747.

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding authors.

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 potential conflicts of interest.

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