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Application of Biotechnology for Sunflower Inbred Line Development: Immature Embryo Rescue

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Abstract: Immature embryos rescued in sunflower breeding programs represent one of the important procedures for creating a competitive research program. The development of inbred lines in a short period offers a significant advantage, enabling faster adaptation of sunflower hybrids to farmers' needs, including new herbicide technologies, such as existing sulfonylurea- and imidazoline-based treatments or the introgression of new disease-resistant genes. In our research, we initiated immature embryo rescue by harvesting at 168 h, 216 h, 264 h, and 312 h after fecundations. The media used were based on MS (Murashige and Skoog) and supplemented with different concentrations of BAP, NAA, and GA_3. For the experiments, three types of genotypes were used: inbred lines, populations, and sunflower hybrids. Our results demonstrated a significant connection between harvest timing, the medium used, and the genotype. The highest survival rates were recorded as up to 81% for hybrids and 71% for inbred lines. For immature embryo rescue, they were obtained at 312 h on MS5—2.0:0.4:0.4 (BAP:NAA:GA_3). The genotype, culture medium composition, and harvesting time are essential factors for optimizing the in vitro rescue technique of immature sunflower embryos. The survival rates of immature embryos were significantly influenced by the interaction of these factors.

Keywords: sunflower breeding; phytohormones; immature embryo rescue; in vitro

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1. Introduction

Sunflower breeding has advanced significantly with the introduction of biotechnology, initially focusing on developing new varieties and later creating hybrids that ensure high productivity and resilience to soil and climatic stress factors [1–3]. Over the years, sunflower oil has gained recognition for its health benefits and the ease with which it can be extracted from seeds [4,5]. Its ability to manage water stress and adapt to variable climatic conditions also contributes positively to crop rotation systems [6]. In agriculture, sunflower is one of the most important crops in the world, with approximately 30 million hectares cultivated globally, being highly valued both for the significance of its oil-rich seeds and for its usefulness in industry and food production [7]. Sunflower oil is used both in food

production and for industrial purposes [8,9]. According to FAOSTAT, global production exceeded 50 million tons in 2022 and 2023, while national production reached over 1.5 million tons in 2022 and more than 20 million tons in 2023 [10]. Additionally, sunflowers have significant value as animal feed, with sunflower meals being widely used for livestock nutrition [11].

In Romania, the development of sunflower hybrids began in 1971, with the creation of the first two hybrids in the world, Romsun 52 and Romsun 53 [12], which were based on nuclear male sterility. This discovery marked the beginning of the "hybrid sunflower" era in the world [13]. Their success was followed by the creation of sunflower hybrids utilizing cytoplasmic male sterility (CMS-PET1) [14], further advancing hybrid breeding programs [15]. This source of the cytoplasmic male sterility (CMS) is the result of an interspecific cross between *Helianthus petiolaris* and *H. annuus* (PET1), and it is the one currently used worldwide to create sunflower hybrids [16].

The development of sunflower hybrids has led to high yields and uniform production [5]. Sunflower breeding relies on the cytoplasmic male sterility (CMS) system, which involves the development of three types of inbred lines: the CMS line (A line), the maintainer line (B or HA line), and the restorer inbred line (Rf or RHA line). In this system, the female parent (CMS line) is male-sterile, due to cytoplasmic genes that inhibit fertility. The CMS line's maintenance is ensured by a maintainer line (B line), which is fertile but does not possess genes for cytoplasmic fertility restoration (rfrf) [17]. On the other hand, the male parent of the hybrid is a restorer line that carries at least one dominant gene (Rf1) [18,19] responsible for restoring fertility [20]. New methodologies of sunflower breeding introduce a new system by using inbred line B-PET1-rf1Rf1, reducing the inbred lines' development down to two inbred lines [21,22]. The use of inbred lines provides several advantages, including genetic stability and improving F1 seed production [8]. A major challenge in modern sunflower breeding involves overcoming difficulties related to the development of inbred lines, which require a minimum of 6–8 generations before being introduced into breeding programs. One solution to address these challenges is the application of biotechnology in sunflower improvement through the optimization of more efficient technologies for generating new sunflower genotypes [23].

Biotechnology has significantly influenced sunflower breeding by enabling advanced genetic improvement techniques, including the use of molecular markers that allow for the rapid identification of target genes [24,25], genomic selection that estimates genetic value based on whole-genome analysis [26], and the introduction of in vitro cultures in breeding programs [27–29]. The use of biotechnological techniques for plant regeneration from immature embryos has a significant benefit in sunflower breeding, enabling the recovery of viable genotypes from interspecific crosses to enhance genetics [28,29].

Immature embryo rescue is one of the methods used for obtaining inbred lines in a short period, offering the advantage of seed harvesting at physiological maturity and the continuation of the line development process. The applicability of immature embryo rescue also extends to interspecific crosses, being utilized for obtaining tolerance to abiotic and biotic factors [30]. Immature embryo rescue technology has facilitated genetic diversity by enabling the recovery of viable embryos from interspecific crosses involving *Helianthus annuus* and *Helianthus decapetalus*, *H. originalis*, *H. angustifolius*, *H. resinosus*, *H. nuttallii*, *H. debilis*, *H. argophyllus*, and many others [31]. Pre-zygotic barriers primarily involve reproductive incompatibilities that prevent fertilization, whereas post-zygotic barriers can restrict or impair embryo development. Immature embryo rescue procedures help the sunflower breeding programs to not only enhance the success rate of hybridization, but also to enable the generation of haploids and doubled haploids [32]. According to the present studies, 15-day-old immature embryos enabled the development of drought-tolerant genotypes de-

rived from the wild species *H. argophyllus* by supplementing MS media with hormones [33]. Other reports of using immature embryo rescue methodologies reveal the successful rescue of immature embryos up to the flowering stage, managing the development of imidazoline sunflowers [34]. Additionally, in vitro cultures have been applied in various fields, such as plant regeneration through organogenesis and embryogenesis in culture media [35], the induction of genetic variability through mutation induction [36–38], and the production of haploids through anther and pollen culture to obtain haploid lines for breeding programs [39,40]. In vitro technology relies on sterile conditions and nutrient-rich media to enhance plant survival rates. By placing plants in culture media, nutrients—including macroelements, microelements, and vitamins—necessary for growth and development are supplied. Murashige and Skoog (MS) established a widely used culture medium containing macroelements, microelements, vitamins, and growth regulators including auxins, cytokinins, and gibberellins [41], and agar as a gelling agent in the optimal pH for plant growth ranges between 5.0 and 6.0 [42,43].

Among the most widely used and important methods in sunflower breeding are in vitro cultures. Providing an optimal balance of hormones can significantly improve the embryo rescue rate. Additionally, accurately identifying the stage of the embryos is essential to maximize the efficiency of this technique, as the reduced germination of immature embryos is often caused by dormancy or an insufficiently developed embryo. Furthermore, the use of different types of hormones can significantly enhance the success of this technique, especially through the application of gibberellin that could potentially reduce the dormancy effect [44].

In this study, we aimed to (i) demonstrate and optimize an efficient in vitro method for rescuing immature embryos using in vitro cultures; (ii) evaluate the influence of genotype type, whether it is an inbred line, population, or hybrid; (iii) determine the effect of optimal concentrations of key phytohormones—NAA, BAP, and GA_3; and (iv) establish the optimal timing for harvesting immature embryos.

2. Materials and Methods

2.1. Chemicals

The in vitro rescue of immature sunflower embryos was performed using Murashige and Skoog (MS), Basal Medium with Vitamins and PPM (Preservative for Plant Tissue Culture), ordered from Plant Cell Technology (Washington, DC, USA). Growth hormones—BAP (6-Benzylaminopurine), NAA (Naphthaleneacetic Acid), and GA_3 (Gibberellic Acid)—used to supplement the MS medium were obtained from Sigma-Aldrich (St. Louis, MO, USA and Burlington, MA, USA).

2.2. Germplasm

For the experiment, three types of sunflower genotypes were used: inbred lines, populations (F2), and hybrids.

Inbred lines include SURES-1 and SURES-2, ordered in 2023 from North Dakota Foundation Seedstocks (Fargo, ND, USA).

SURES-1 (G1) is an F3-derived F4 oilseed maintainer genetic stock obtained from the cross HA 424/3/HA 406//HA 89/SU Res. wild *H. annuus*.

SURES-2 (G2) is an F3-derived F4 oilseed restorer genetic stock obtained from the cross RHA 377/3/RHA 392//RHA 376/SU Res. wild *H. annuus*.

Population 1—F_2 (G3) was obtained from the cross between SURES-1 and AH1104, an *Orobanche cumana*-resistant line developed through interspecific crossing with *H. divaricatus* [45].

Population 2—F_2 (G4) was obtained from the cross between SURES-2 and RHA419 (PI 619204), an inbred line ordered from the USDA-ARS Germplasm Resources Information Network (GRIN) [46]. RHA-419 is a fertility restorer line selected from the cross RHA 373/ARG 1575-2. RHA 373 was released by the USDA and the North Dakota Agricultural Experiment Station in 1990 [47].

Hybrid—F1 (G5) is a hybrid obtained from the cross between CMS (HA 89) and SURES-2.

2.3. Pre-Embryonic Stage Growth Conditions

The sunflower genotypes were sown under different field conditions in the middle of March in the year 2024, with the objective of obtaining two generations in the off-season. One generation was grown in the field and the other in the greenhouse. The genotypes were sown at a distance of three meters apart, with 30 cm spacing between rows and 30 cm spacing between plants. The plants were watered regularly, once every two days until germination, and every four days after germination. Before flowering, plants were isolated to prevent pollen contamination (Figure 1).



Figure 1. Germplasm flowering stage: pre-embryonic stage.

To evaluate the optimal timing for rescuing immature embryos, the fecundation appearance of the row was considered on the first day of fecundation (Figure 2A), after which the immature embryos were rescued in several intervals: 168 h, 216 h, 264 h, and 312 h. The selection of time intervals was determined pre-experimentally, and the specific time frames were chosen in which all media's successfully rescued embryos were present (168 h), up to the maximum interval where the survival rate of immature embryos exceeded 80% (312 h). After a 312 h period of time, embryo maturity and the role of hormones no longer significantly contributed to improving the survival rate compared to the control. For an accurate evaluation of the fertilization period and embryo maturation, during the flowering phase, the capitula were isolated using protective isolators to ensure controlled conditions and preserve germplasm. For each genotype, both fertilized and unfertilized flowers were monitored. (Figure 2B,C).



Figure 2. Tubular flowers at different stages in field: (**A**) first row of fertilized flowers, (**B**) fecundated flowers, and (**C**) unfecundated flowers, anthers with pollen.

For each time interval of each genotype, plants were selected. In total, 100 immature embryos were harvested, with 50 immature embryos' repetition. Immature embryos were then processed under in vitro conditions for plant regeneration. The immature embryos were harvested at the torpedo stage of development, removing other stages of embryo development [48].

2.4. Preparation of MS Medium and Hormonal Supplements

The culture medium used in this study was prepared following the standard Murashige and Skoog (MS) medium, supplemented with growth hormones according to the experimental design. The medium composition included macronutrients, micronutrients, and sucrose (20 g/L) [40,49].

The pH of the medium was adjusted to 5.7 using 1N NaOH or HCl solutions. The medium was solidified with 0.8% agar and sterilized by autoclaving at 121 °C for 20 min. After sterilization, the medium was distributed into sterile cylindrical tubes of 25 × 95 mm and allowed to solidify at room temperature.

In vitro medium variants were designed to promote shoot formation, shoot elongation, and rooting.

For this to occur, the MS medium was supplemented with different combinations of plant growth regulators to create four types of experimental media, according to other results from different combinations of BAP, NAA [42,50], and GA_3 [51].

- Medium I: MS basal medium without plant growth regulators (Control);
- Medium II: MS + 0.5 mg/L benzylaminopurine (BAP) [42,52] + 0.05 mg/L naphthalene acetic acid (NAA) + 0.05 mg/l gibberellic acid (GA_3);
- Medium III: MS + 1.0 mg/L benzylaminopurine (BAP) + 0.1 mg/L NAA + 0.1 mg/L gibberellic acid (GA_3);
- Medium IV: MS + 2 mg/L + benzylaminopurine (BAP) + 0.2 mg/L NAA + 0.2 mg/L gibberellic acid (GA_3);
- Medium V: MS + 2 mg/L benzylaminopurine (BAP) + 0.4 mg/L NAA + 0.4 mg/L gibberellic acid (GA_3) [53].

This supplementation was added prior to autoclaving to the cooled medium (~45–50 °C), and the prepared media were used for explant inoculation.

To increase the success of immature embryo rescue by reducing embryo infection, PPM was used at a concentration of 0.2%, added after autoclaving to the cooled medium (~45–50 °C).

2.5. Immature Embryo Saving in In Vitro Conditions

After fecundation, immature embryos were harvested at 168 h, 216 h, 264 h, and 312 h.

Immature embryos were sterilized using a 0.2% mercuric chloride ($HgCl_2$) solution in in vitro conditions.

A total of 100 immature embryos (2 repetition/50 embryos) per genotype and per each period point was harvested and processed for inoculation in in vitro conditions.

Following sterilization, the embryos were transferred one by one in five washes of sterile distilled water, with each wash lasting five minutes.

The immature embryos were placed with the tip facing downward in the medium to mimic their natural orientation within the capitulum in sterile individual cylindrical tubes of 25 × 95 mm. In vitro conditions for embryo rescue involved placing the embryos in an experimental medium (MS1–MS5) in growing conditions at a temperature of 24 °C, with a 14/10 h light/dark photoperiod [49]. The immature embryos were kept in the medium for 15 days and were evaluated for their survival rate based on their normal development and maturation of cotyledons.

2.6. Immature Embryo Saving—Survival Rate Evaluation

For the evaluation of the survival rate, embryos that either died or developed abnormally were excluded. The embryo survival rate was calculated as the percentage of embryos that developed normally, with a minimum root length of 3 cm during the 15-day period [53] (Figure 3). The survival rate was calculated as the number of normal embryos rescued divided by the total number of embryos harvested (50), multiplied by 100. This calculation was performed for the two repetitions.

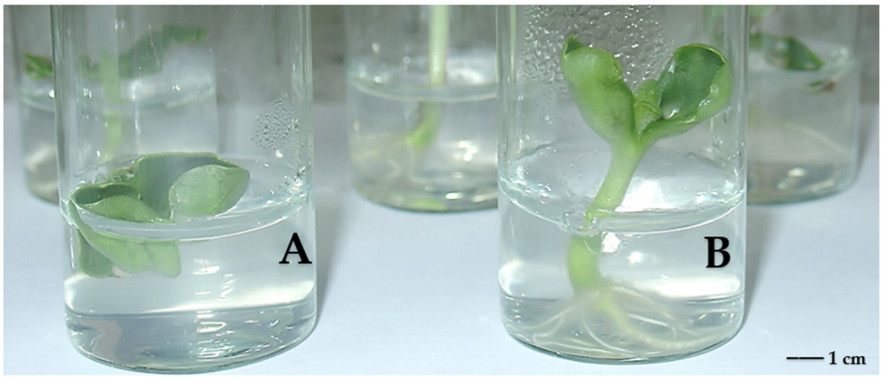


Figure 3. Immature embryo rescue transfer in cylindrical tubes containing Murashige and Skoog (MS) medium under in vitro conditions. (**A**) Embryos abnormally developed and (**B**) embryo with a minimum root length of 3 cm during the 15-day period.

The successfully rescued embryos that developed normally in in vitro conditions were then acclimatized in the growth chamber until the emergence of the first true leaves to ensure and verify whether the embryo survival rate changed. Throughout the experiment, no reductions in the number of surviving plants were recorded during the acclimatization process [29]. Acclimatization and advancement to the next generation were carried out in the growth chamber.

2.7. Experimental Design—Variants and Notations

This study aimed to optimize an in vitro embryo rescue method using two replications, each with 50 embryos. Five different genotypes were used in the experiment: G1: Inbred line—SURES-1, G2: Inbred line—SURES-2, G3: Population 1—F_2, G4: Population 2—F2, and G5: Hybrid—F1.

The experiment utilized the Murashige and Skoog (MS) medium (20 g/L sucrose), supplemented with various concentrations of BAP (benzylaminopurine), NAA (naphthalene acetic acid), and GA_3 (gibberellic acid) (BAP:NAA:GA_3). Four intervals of harvesting were used after fecundations of 168 h, 216 h, 264 h, and 312 h (Table 1).

Table 1. Experimental design of the immature embryo rescue optimization study.

Medium Type	BAP:NAA:GA_3 Concentration (mg/L)	Tested Genotypes	Immature Embryos per Replication	Repetitions	Harvesting Intervals (hours)
MS1	0:0:0 (Control)	G1, G2, G3, G4, G5	50	2	168 h, 216 h, 264 h, 312 h
MS2	0.5:0.05:0.05	G1, G2, G3, G4, G5	50	2	168 h, 216 h, 264 h, 312 h
MS3	1.0:0.1:0.1	G1, G2, G3, G4, G5	50	2	168 h, 216 h, 264 h, 312 h
MS4	2.0:0.2:0.2	G1, G2, G3, G4, G5	50	2	168 h, 216 h, 264 h, 312 h
MS5	2.0:0.4:0.4	G1, G2, G3, G4, G5	50	2	168 h, 216 h, 264 h, 312 h

For each experimental variant, statistical calculations were performed to compare the effects of the three factors studied on the rescue of immature embryos: genetic factor (inbred lines, populations, and hybrids), medium factor (supplementation with different concentrations of BAP:NAA:GA_3), and immature embryo harvesting interval factor (168–312 h) (Table 1). Data were collected for each replication and subjected to statistical analysis to evaluate the results obtained for each experimental variant.

2.8. Statistical Analysis

Statistical calculations were performed, including the one-way ANOVA test and Tukey's test, to evaluate the significance levels between experimental variants regarding genotypes, medium types used, and harvesting intervals. The mean and standard error were calculated between replications of each experimental variant, and the immature embryo rescue rate (%) was determined. A multifactorial ANOVA test was also performed to assess the cumulative impact of the genotype, medium type, and harvesting interval on the immature embryo rescue rate. All statistical analyses and graphs were performed and considered significant at $p < 0.05$. Statistical analyses were conducted using RStudio software v4.4.2.

3. Results

3.1. Survival Rate of Immature Embryos—Genotype Factor

The rescue of immature embryos was successfully achieved for all genotypes, including inbred lines (G1 and G2), populations (G3 and G4), and the hybrids. Regarding the influence of the genetic factor, a significant effect ($p < 0.01$) was observed on the regeneration capacity of plants from immature embryos (Table 2).

Table 2. ANOVA survival rate (%) of immature embryos—genotype factor.

Source	Df	Sum Sq	Mean Sq	*F* Value	Pr (>F)
Genotype	4	11,530	2882.4	4.228	0.00264 **
Residuals	195	132,947	681.8		

Significance levels: ** $p < 0.01$.

Regarding the survival rates of immature embryos, the lowest values were recorded for the inbred lines (G1 and G2). Intermediate values were observed for the populations derived from the F2 generation (G3 and G4). In contrast, the highest values were obtained for the hybrid genotype, showing a significant difference compared to the survival rates of the other genotypes (Figure 4).

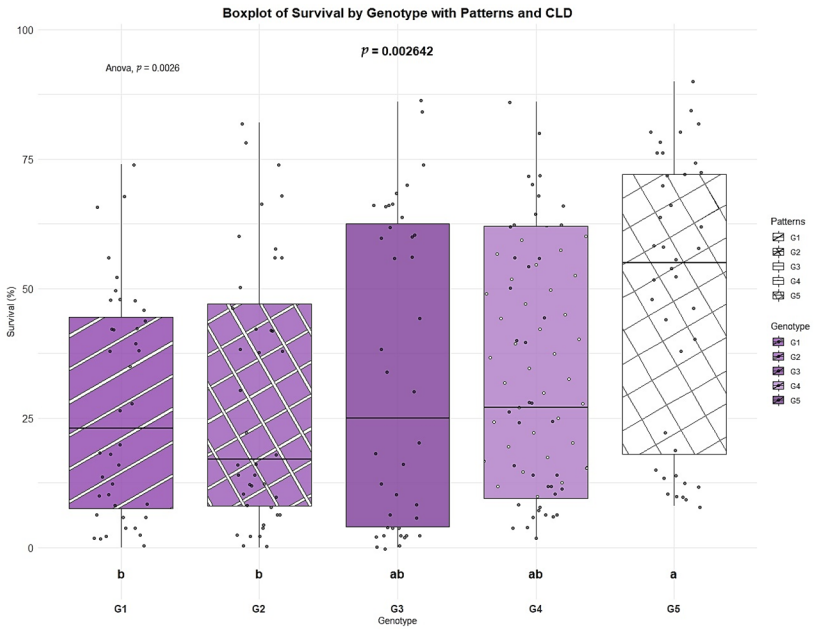


Figure 4. Results of the rescue rate of immature embryos—genotype factor: inbred lines (G1 and G2), populations (G3 and G4), and hybrids (G5). Tukey test was performed on all five genotypes The highest values are denoted starting with the letter "a". Similarity between superscript letters indicates non-significant differences between the values obtained ($p > 0.05$): LSD0.05 = 11.51, LSD0.01 = 15.13, and LSD0.001 = 19.33.

The impact of genetic variability on the rate of immature embryo rescue highlights the importance of the genotypic factor in optimizing protocols for immature embryo rescue. The lowest survival rates observed in the inbred lines could be attributed to their higher levels of homozygosity, which are associated with a reduction in vigor and, consequently, a decrease in survival rate (Figure 4). Furthermore, hybrids exhibited a higher survival rate, most likely due to the heterosis effect, which enhances growth, vigor, and tolerance to various stresses during the in vitro culture procedures (Figure 4).

Additionally, partial heterozygosity provides a higher survival rate compared to inbred lines, which are affected by the inbreeding effect. This could pose challenges in selection, as embryos with higher heterozygosity—and thus greater vigor—are more likely to be promoted to the next generation, potentially conflicting with the objective of inbreeding and the development of pure lines. However, the influence of the F_2 population was not significantly higher than that of the inbred lines, nor significantly lower than the F_1 hybrids, suggesting that this may not represent a major issue, but rather an aspect to consider when applying embryo culture techniques at different stages of sunflower breeding programs. To mitigate risks, it may be more advantageous to apply embryo culture techniques in later generations, such as F_3 or F_4, where uniformity, flowering period, and other morphological traits can be further evaluated under field conditions, ensuring a correlation with homozygous progenies. Nonetheless, a safer and more direct approach with minimal risk in sunflower breeding programs could be the application of embryo rescue techniques specifically for CMS conversion of B-lines.

3.2. Survival Rate of Immature Embryos—Medium Type Factor

The in vitro medium used, along with the hormonal concentrations, significantly influenced the rate of immature embryo rescue. Upon evaluating the results across all genotype types, a significant effect of the medium type was observed, regardless of inbred lines, populations, or hybrids (Table 3) ($p < 0.01$).

Table 3. ANOVA survival rate (%) of immature embryos—medium type factor.

Source	Df	Sum Sq	Mean Sq	*F* Value	Pr (>F)
Medium Type	4	10,210	2552.5	3.707	0.00622 **
Residuals	195	134,267	688.5		

Significance levels: ** $p < 0.01$.

The results demonstrate the importance of using phytohormones in the immature embryo rescue procedures, as significantly lower survival rates were observed in the MS1—0:0:0 (Control) medium, without phytohormone supplementation (Figure 5).

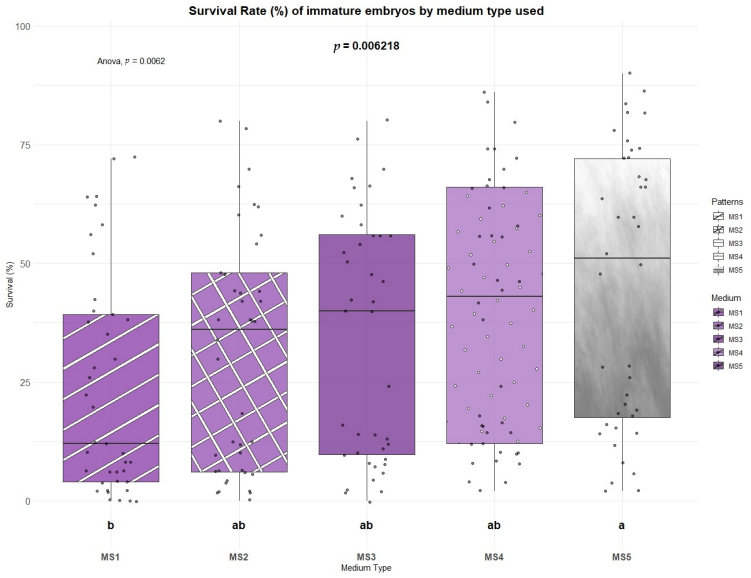


Figure 5. Results of the rescue rate of immature embryos—medium type factor (BAP:NAA:GA_3-(mg/L): MS1—0:0:0 (Control), MS2—0.5:0.05:0.05, MS3—1.0:0.1:0.1, MS4—2.0:0.2:0.2, and MS5—2.0:0.4:0.4. Tukey test was performed on all five types of medium. The highest values are denoted starting with the letter "a". Similarity between superscript letters indicates non-significant differences between the values obtained ($p > 0.05$): $LSD_{0.05}$ = 11.57, $LSD_{0.01}$ = 15.20, and $LSD_{0.001}$ = 19.42.

The average survival rate of embryos increased progressively with the addition of phytohormones in different combinations and concentrations (BAP:NAA:GA_3).

All tested media demonstrated the potential for rescuing immature embryos with varying success rates. However, according to the results, the media MS2, MS3, and MS4 showed statistically similar outcomes, whereas the highest survival rates were observed in MS5—2.0:0.4:0.4 (BAP:NAA:GA_3-mg/L) (Figure 5).

3.3. Survival Rate of Immature Embryos—Time of Maturation After Fecundation Factor

A key factor in the rescue of immature embryos is their developmental stage or level of maturity. The timing of harvesting after fertilization has a significant impact on the survival rate of immature embryos. ($p < 0.001$) (Table 4).

Table 4. ANOVA survival rate (%) of immature embryos—time of maturation after fecundation factor.

Source	Df	Sum Sq	Mean Sq	*F* Value	Pr (>F)
Time Influence	3	109,268	36,423	202.8	$<2 \times 10^{-16}$ ***
Residuals	196	35,209	180		

Significance levels: *** $p < 0.001$.

Significant influences were observed for all genotype types based on the harvesting time of the embryos after fertilization, with a high level of significance ($p < 0.05$). The lowest survival rates (%) were recorded at 168 h (1–15%), followed by an increase at 216 h (3–55%). Survival rates continued to rise at 264 h, reaching values between 11 and 79%, while the highest values, ranging between 37 and 81%, were observed at 312 h.

Additionally, the survival rate was significantly influenced by the type of medium used, emphasizing the importance of optimizing both the harvesting time and culture conditions (Figure 6).

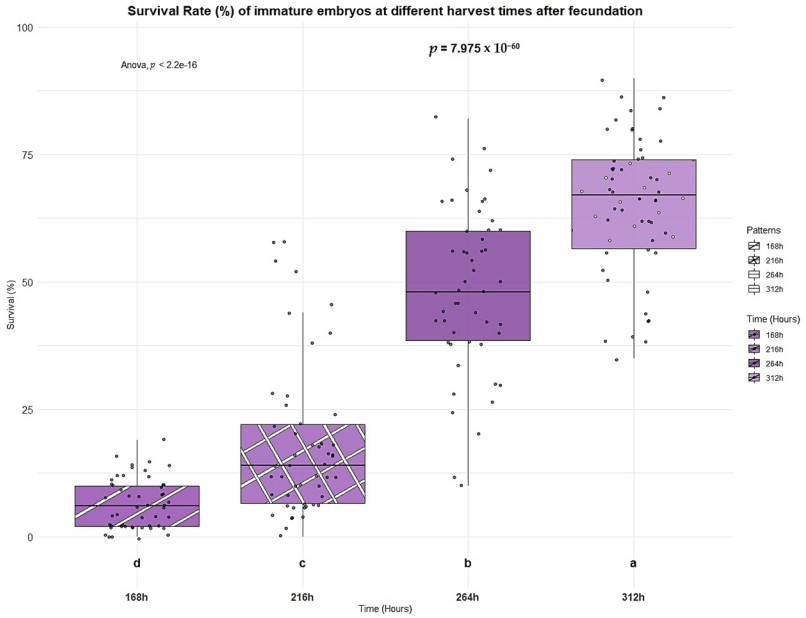


Figure 6. The rescue rate of immature embryos across three genotype categories: inbred lines (G1 and G2), populations (G3 and G4), and hybrids (G5). Tukey test was performed on all intervals used. The highest values are denoted starting with the letter "a". Similarity between superscript letters indicates non-significant differences between the values obtained ($p > 0.05$): $LSD_{0.05} = 5.28$, $LSD_{0.01} = 6.94$, and $LSD_{0.001} = 8.87$.

The results indicate that embryo survival rates are strongly influenced by their developmental stage and harvest timing, with mature embryos (312 h) showing the highest survival rates. This suggests that longer maturation periods allow embryos to accumulate nutrients and develop structural integrity, enhancing their ability to withstand in vitro culture conditions.

However, the lower survival rates observed at 168 h highlight the vulnerability of immature embryos during the early developmental stages. These findings emphasize the importance of optimizing harvest time to balance developmental maturity with regeneration potential, particularly for genotypes requiring early rescue to preserve genetic material. These insights are critical for advancing embryo rescue techniques and improving the efficiency of in vitro breeding programs, especially for genotypes prone to reproductive barriers or delayed maturation.

3.4. Survival Rate of Immature Embryos—Multiple Factors Influence

Multifactor ANOVA analysis demonstrated the significant influence of the three factors considered in the study—the type of genotype, the type of medium used, and the harvesting period of immature embryos (Table 5).

Table 5. ANOVA survival rate (%) of immature embryos—multiple factors influence.

Source	Df	Sum Sq	Mean Sq	*F* Value	Pr (>F)
Genotype	4	11,530	2882	91.158	$<2 \times 10^{-16}$ ***
Time	3	109,268	36,423	1151.883	$<2 \times 10^{-16}$ ***
Medium	4	10,210	2553	80.725	$<2 \times 10^{-16}$ ***
Genotype–Time	12	4491	374	11.836	1.66×10^{-14} ***
Genotype–Medium	16	957	60	1.892	0.02966 *
Time–Medium	12	2049	171	5.4	5.44×10^{-7} ***
Genotype–Time–Medium	48	2810	59	1.851	0.00503 **
Residuals	100	3162	32		

Significance levels: *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$.

According to the results, all these three factors showed a significant effect ($p < 0.001$), emphasizing the importance of optimizing the techniques for saving immature embryos based on these three factors. The interactions between the factors were significant, which shows that the variability of the embryo rescue rate is influenced by these three factors ($p < 0.01$) (Table 5).

The results demonstrate the fact that the medium without phytohormones (MS1—0:0:0) had the lowest rescue rate of immature embryos. On the other hand, the most effective results were evidenced for the MS5—2.0:0.4:0.4 medium, demonstrating that a balanced combination of phytohormones leads to improved rescue rates of immature embryos. This aspect was observed for all analyzed time intervals, even at the 168 h interval.

The interval of 168 h (7 days) after fertilization demonstrated a period with the lowest salvage rates of immature embryos, reflecting the fact that this period is not optimal for the salvage of immature embryos based on the structure and protocol presented in this study (Table 6).

Table 6. Results of the in vitro conditions regarding the survival rate of immature embryos.

Genotype	Time **	MS1 *	MS2 *	MS3 *	MS4 *	MS5 *
	hours	%	%	%	%	%
G1—line	168 h	1 ± 0.5^{d}	4 ± 1.0^{cd}	5 ± 1.5^{cd}	4 ± 1.0^{cd}	6 ± 1.0^{bcd}
G2—line	168 h	1 ± 0.5^{d}	1 ± 0.5^{d}	3 ± 0.5^{cd}	9 ± 0.5^{abcd}	10 ± 1.0^{abcd}
G3—Pop	168 h	1 ± 0.5^{d}	3 ± 0.5^{cd}	1 ± 0.5^{d}	3 ± 0.5^{cd}	3 ± 0.5^{cd}
G4—Pop	168 h	4 ± 1.0^{cd}	9 ± 1.5^{abcd}	9 ± 1.0^{abcd}	12 ± 1.0^{abc}	15 ± 0.5^{ab}
G5—Hib	168 h	9 ± 0.5^{abcd}	11 ± 0.5^{abc}	11 ± 1.0^{abc}	12 ± 1.0^{abc}	17 ± 1.0^{a}
G1—line	216 h	5 ± 0.5^{e}	14 ± 2.0^{de}	12 ± 1.0^{de}	14 ± 1.0^{de}	23 ± 2.5^{cde}
G2—line	216 h	5 ± 0.5^{e}	9 ± 1.5^{de}	15 ± 0.5^{de}	17 ± 0.5^{de}	18 ± 2.0^{de}
G3—Pop	216 h	3 ± 1.5^{e}	4 ± 1.0^{e}	9 ± 0.5^{de}	14 ± 1.0^{de}	19 ± 0.5^{de}
G4—Pop	216 h	6 ± 1.0^{e}	5 ± 0.5^{e}	9 ± 1.5^{de}	16 ± 4.0^{de}	27 ± 0.5^{bcd}
G5—Hib	216 h	17 ± 2.5^{de}	41 ± 1.5^{abc}	47 ± 3.5^{ab}	52 ± 3.0^{a}	55 ± 1.5^{a}
G1—line	264 h	23 ± 1.5^{hi}	40 ± 1.0^{defgh}	45 ± 1.5^{cdefgh}	44 ± 1.0^{cdefgh}	49 ± 0.5^{bcdefg}

Table 6. *Cont.*

Genotype	Time **	MS1 *	MS2 *	MS3 *	MS4 *	MS5 *
	hours	%	%	%	%	%
G2—line	264 h	11 ± 0.5^{i}	34 ± 2.0^{fghi}	44 ± 1.0^{cdefgh}	47 ± 4.5^{cdefgh}	63 ± 1.5^{abcde}
G3—Pop	264 h	34 ± 2.0^{fghi}	39 ± 2.5^{efgh}	58 ± 1.0^{abcdef}	61 ± 2.5^{abcde}	64 ± 2.0^{abcd}
G4—Pop	264 h	34 ± 3.0^{fghi}	49 ± 2.5^{bcdefg}	32 ± 4.0^{ghi}	53 ± 1.5^{bcdefg}	65 ± 0.5^{abc}
G5—Hib	264 h	55 ± 1.5^{abcdefg}	55 ± 3.5^{abcdefg}	61 ± 2.5^{abcde}	73 ± 0.5^{abc}	79 ± 1.5^{a}
G1—line	312 h	37 ± 1.0^{d}	43 ± 2.5^{cd}	54 ± 1.0^{abcd}	55 ± 5.5^{abcd}	71 ± 1.5^{abc}
G2—line	312 h	40 ± 1.0^{cd}	49 ± 3.5^{bcd}	54 ± 2.0^{abcd}	71 ± 1.5^{abc}	80 ± 1.0^{ab}
G3—Pop	312 h	63 ± 0.5^{abcd}	63 ± 1.5^{abcd}	68 ± 1.0^{abcd}	76 ± 5.0^{ab}	79 ± 2.5^{ab}
G4—Pop	312 h	64 ± 4.0^{abcd}	66 ± 2.0^{abcd}	65 ± 1.5^{abcd}	71 ± 4.5^{abc}	79 ± 3.5^{ab}
G5—Hib	312 h	68 ± 2.0^{abcd}	79 ± 0.5^{ab}	78 ± 1.0^{ab}	77 ± 3.5^{ab}	81 ± 4.5^{a}

* MS1—0:0:0 (Control), MS2—0.5:0.05:0.05, MS3—1.0:0.1:0.1, MS4—2.0:0.2:0.2, and MS5—2.0:0.4:0.4—(BAP:NAA:GA_3). ** The results in each column represent the survival rate (%), mean, and standard error of sunflower embryos harvested at different time intervals after fertilization. The Tukey test was performed on all five genotypes for each interval (168 h, 216 h, 264 h, and 312 h) separately. The highest values are denoted starting with the letter "a". Similarity between superscript letters indicates non-significant differences between the values obtained ($p > 0.05$).

The interval of 216 h (9 days) after fertilization demonstrated moderate results and effects on the survival rate of immature embryos. However, for the hybrid genotype (G5), the impact of MS5 resulted in a 55% survival rate. Comparable results were also observed for MS2, MS3, and MS4, with survival rates ranging between 41% and 52%. In contrast, the control medium (MS1), which lacked phytohormones, produced a low survival rate of only 17%, even for the hybrid genotype. These findings highlight that genotype plays a crucial role in rescuing immature embryos during this stage. Inbred lines (G1 and G2) achieved a maximum survival rate of 23%, indicating a lower response compared to hybrids.

The interval of 264 h (11 days) after fertilization revealed an optimal period for rescuing immature sunflower embryos and advancing them into the next generation. The MS5 medium demonstrated a survival rate above 50% for most genotypes. During this period, the number of genotypes or plants progressing to the next generation increased significantly. However, it is essential to ensure that the chosen medium supports this transition effectively.

The interval of 312 h (13 days) after fertilization recorded the highest survival rates for immature embryos. Nevertheless, inbred lines (G1 and G2) did not demonstrate a 50% survival rate potential in MS1 medium (0:0:0). On the other hand, when using MS5 medium (2.0:0.4:0.4), most results showed low variability in the embryo saving rate (%), indicating a stable and optimal period for embryo rescue using a single-stage in vitro procedure. According to the results, most genotypes achieved survival rates above 71%, with the highest rate observed for hybrids (81%).

These results demonstrate that genotype is a critical factor to consider in immature embryo rescue. The heterosis effect (hybrid vigor) could influence the selection of embryos in the population, favoring the development of more vigorous embryos. Therefore, it is recommended to use optimal conditions that exhibit low variability in terms of the survival rate. Low variability, which was not dependent on the genotype, was observed when using the MS5 medium at the 312 h interval (13 days) after fertilization.

Additionally, the time interval is a key factor in improving the survival rate of immature embryos. If a specific period is targeted for embryo rescue, it is necessary to consider the number of embryos harvested to ensure success.

The medium used also significantly influenced the stability of the survival rate. All tested media supplemented with phytohormones outperformed the MS1 control medium without hormones. However, the highest survival rate was recorded at the concentration of MS5—2.0:0.4:0.4 (BAP:NAA:GA_3).

4. Discussion

Immature embryo saving offers substantial advantages for sunflower breeding programs and indirectly to farmers who have use of hybrids adapted to both abiotic and biotic conditions. Enhancing the combinatory ability and exploiting heterosis through the introduction of inbred lines represent a crucial aspect of sunflower breeding programs [54,55]. However, given the increasing number of new lines and populations in the breeding programs, sustaining them economically while ensuring feasibility has become increasingly challenging. Whenever a new technology is introduced into breeding programs, it is essential to evaluate whether it can be scaled up effectively or if it should remain a specific tool for improving the breeding process [56].

Our results regarding the rate of survival of the immature embryos show a higher survival rate and root generation rate compared to the hormone-free MS1 medium (free of phytohormones). However, medium MS5—2.0:0.4:0.4 (BAP:NAA:GA_3) demonstrated a stable rate of embryo saving for all the genotypes at the 312 h (13 days) harvesting interval. Thus, differences were identified both in terms of the phenotypic characteristics of the medium used and based on the time since pollination and the maturation stage of the immature embryos.

Although the embryo rescue rate at 168 h (7 days) after fertilization was not significant in our study, other studies achieved immature embryo saving at 5 days using the following two-stage approach: a growth medium for the immature embryo followed by a germination medium achieving an overall rate of 32.6%, depending on the genotype [49]. Immature embryos at 5 days after fertilization were first cultured on a solid growth medium (B5 macronutrients, vitamins, amino acids, NAA, sucrose, and agar) for complete maturation. In the second step, after 1–2 weeks, embryos were transferred to a germination medium with modified sucrose (10 g/L) and agar. After 3–5 weeks, the germinated embryos with well-developed roots were acclimatized under controlled conditions [49]. However, our results indicate that it is possible to have significant results that can compensate for the elaborate procedure in only one stage, only saving embryos at 13 (312 h) days at MS5—2.0:0.4:0.4 (BAP:NAA:GA_3). Other reports also show a significant result in a one stage embryo rescue procedure, in embryos of 10 days [29,34].

Other studies by other authors demonstrate that the rescue of immature embryos was possible 10 days after fertilization in the MS medium supplemented with 20 g/L sucrose, at a pH range of 5.6–5.7, achieving notable results in inbred lines during the CMS conversion process [40]. Additionally, herbicide evaluation and immature embryo rescue were demonstrated in the MS medium, free of hormones, with 20 g/L sucrose and a pH of 6.00, according to Breccia, G. et al. [34].

Genotypically, the results indicate that the phenomena of inbreeding (such as G1 and G2) and heterosis (such as G5) are expressed in the ability to influence the immature embryos saving rate and their normal development. This aspect may influence the selection process of immature embryos within the population (such as G3 and G4), as genotypes with a high degree of inbreeding, closer to the final breeding objectives in developing inbred lines, may sometimes be excluded in favor of better-developed embryos or more vigorously growing immature embryos. However, more vigorously growing immature embryos could also exhibit a relatively higher level of heterosis than homozygosity. This approach could drive selection over time, considering the risk of excessive heterosis in F_2 generations.

Although there is minimal risk, this highlights the need to alternate embryo rescue in the greenhouse with field evaluation when developing populations of new B-lines and Rf-lines. This approach ensures a better understanding of the stage of inbred line development, as well as the uniformity in flowering and the overall morphology of the plants. Minimizing risks in developing inbred lines emphasizes the importance of incorporating immature embryo rescue technology into the breeding program. Converting maintainer lines (B-lines) into male-sterile lines (A-CMS) does not pose a significant risk. Therefore, the application of CMS conversion through embryo rescue could be considered and optimized in the greenhouse.

In vitro conditions and harvesting intervals influence the rate of the embryos saved, in combination with the stage of the development of embryos, reaching a significative increase in the 264 h and 312 h harvest intervals for all genotypes. Our results revealed that the rescue of immature embryos was also possible without the addition of phytohormones, with notable results after 264 h and 312 h of plant fecundation. However, the research shows a decrease in the rate of embryo rescue in the MS medium supplemented with BAP and NAA when the embryo age reached 18–30 days, with the success rate dropping below 20% for embryos at 30 days after fertilization [49]. Therefore, dormancy played a crucial role in embryo germination at this stage. However, our results indicate an increasing rescue rate, suggesting that potential dormancy can be effectively overcome through the incorporation of GA_3 into the MS medium [49,56,57]. This finding underscores the significance of GA_3 in enhancing embryo rescue efficiency in sunflowers [56,57].

The combination of cytokinins, auxins, and gibberellins significantly influenced the embryo rescue rate compared to the control MS medium in our experiments. On the other hand, the embryo rescue rate at 168 h (7 days) after fecundation did not demonstrate a significant success rate, with rates below 20% in all genotypes used. However, studies reflect the procedure for rescuing five-day-old immature embryos at different stages of embryonic development, successfully achieved by initially placing them in growth medium (high sucrose content—120 g/L, amino acids, vitamins, and NAA for growth stimulation, using Gamborg's B5 macronutrients), followed by transfer to germination medium (lower sucrose content—10–20 g/L, B5 salts, pH buffer (MES), and solidified with agar), resulting in a germination rate of 50% in some cases [49,56]. Differences in sucrose concentrations at various stages of embryo development were also successfully managed in a medium containing 90 g/L sucrose for immature embryos aged 4–21 days [39]. Also, the source of carbohydrates influences the embryo rescue process. As reported in scientific studies, the best regeneration results were achieved with 120 g/L sucrose (46.8%), followed by 90–120 g/L glucose (33.1%). Medium supplementation with maltose was found to be inhibitory, and embryos did not respond positively [53].

Our results show that the rescue of immature embryos can be successfully implemented in breeding programs for the development of inbred lines. This approach represents faster technology, with better-established protocols, which can be effectively integrated into sunflower breeding programs with results of a 71–81% rate of immature embryo saving, allowing for 4 to 5 cycles depending on the maturity of the genotype [58].

Other biotechnological methods used in developing complete homozygous sunflower inbred lines for breeders include gynogenesis and androgenesis, which involve the production of haploid plants with a single set of chromosomes (n) [59]. Consequently, although these technologies are functional, their limited economic benefits have hindered their large-scale application in sunflower breeding. Research shows that androgenesis was achieved using a simple technique based on an MS medium supplemented with 0.5 mg/L IAA and 0.5 mg/L BAP for callus induction from anthers. Shoots were obtained using the L_4M culture medium containing 30 g/L sucrose and a pH of 5.7. Callus formation was successful in

over 80% of cases. For shoot formation, the calluses derived from pollen were transferred to the MS medium without hormones [59,60]. However, the plant regeneration rate was very low, below 10%. Also, research reporting on gynogenesis shows that plant regeneration was achieved in only one genotype out of the nine genotypes studied following pollination with irradiated pollen [61]. Therefore, there is still not a clearly established high rate of success for gynogenesis and androgenesis, also known as a genotype dependent technology, that should be included in specific parts of the breeding programs. Our future studies should concentrate on developing inbred lines using gynogenesis and androgenesis.

This study contributes to the development and implementation of biotechnologies in sunflower breeding programs, highlighting the synergistic impact of genotype, media composition, harvesting time, and the importance of balancing cytokinins, auxins, and gibberellins in increasing the immature embryo rescue rate.

5. Conclusions

The implementation of immature embryo rescue under in vitro conditions in sunflower breeding programs has proven to be effective in accelerating the breeding cycle in developing inbred lines. By using MS5 medium (2.0:0.4:0.4 BAP:NAA:GA_3), the results demonstrated the highest rate of success (%) across all types of genotypes—inbred lines, populations, and hybrids. Additionally, the most favorable period for rescuing immature embryos was within 312 h (13 days) after fertilization, when the highest survival rates were recorded as up to 81% for hybrids and 71% for inbred lines. The higher survival rates of immature embryos in hybrids compared to inbred lines may be attributed to heterosis and inbreeding effects. Considering the results, it is expected and essential to consider selection in sunflower breeding programs, particularly in F_2 populations, where the effects of heterosis and inbreeding have an important role in the selection of the saved embryos. The medium with phytohormones at a low concentration (MS2—1:0.05:0.05) revealed a higher survival rate than the control medium (MS1—0:0:0). Therefore, optimizing combinations of cytokinins, auxins, and gibberellins and selecting suitable genotypes can significantly improve the efficiency and success of embryo rescue rates.

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