International Journal of Agriculture, Environment and Food Sciences
e-ISSN : 2618-5946
www.jaefs.com
JAEFS
edit PUBLISHING

Research Article
Int J Agric Environ Food Sci 6 (3): 471-479 (2022)

Application of multiple strategies to efficiently break seed dormancy of permanently odd pentaploid rose hip (*Rosa canina* l.) under in vitro conditions

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Citation
Hajyzadeh, M. (2022). Application of multiple strategies to efficiently break seed dormancy of permanently odd pentaploid rose hip (*Rosa canina* l.) under in vitro conditions. International Journal of Agriculture, Environment and Food Sciences, 6 (3), 471-479
Doi: https://doi.org/10.31015/jaefs.2022.3.17

Received: 14 July 2022
Accepted: 22 July 2022
Published Online: 17 August 2022

Year: 2022
Volume: 6
Issue: 3 (September)
Pages: 471-479

Available online
http://www.jaefs.com
https://dergipark.org.tr/jaefs

Abstract

Rosehip (*Rosa canina* L.) is an important medicinal, and ornamental plant species with high commercial value. Its sugars, phenolics, organic acids, water-soluble vitamins, and mineral contents composition varies depending on environmental conditions and genetics. The plant is also used as a perfect rootstock for many rose cultivars. Seed proliferation is extremely difficult because of multiple endogenous and exogenous dormancy factors. There is a need to breed standard rosehip cultivars rootstock developments with desired characteristics and outperforming yields in fields. The study aimed to break the seed dormancy of rosehip under *in vitro* conditions by application of multiple strategies in an efficient manner. The seeds were treated with different doses of GA_3, scarified mechanically, stratified on agar solidified MS medium containing GA_3 singly or in combinations of the two each or three treatments, and subjecting them to the regulated physiological treatment of alternating warm/chilling and cold/dark treatments in parallel for 21 d ensued by 18 d warm/light treatments. It was noted that the rosehip seeds could be germinated variably if the scarified seeds were stratified on agar solidified MS medium with or without GA_3. Optimum seed germination (80.00- 85.00%) was noted when the three treatments were combined and the seeds were subjected to regulated and alternating warm and chilling treatments for 21 d leaving them for 18 d in warm/light. These results are very important and could be utilized in breeding and multiplication programs to develop new rosehip fruit and rootstock cultivars.

Keywords

Breeding, Diversity, Germination, Pericarp, Scarification, Stratification

Introduction

Rosehips, dog rose or Rosa canina L. Brier 2n=5x=35, genus Rosa is anorthoploids or permanently odd polyploids. They produce seven bivalents leaving the remaining 21 unpaired chromosomes as univalent during meiosis that are included in egg cells only and fail to recombine (Lim et al., 2005). These multiple hybridization events (Ritz et al., 2005) overcame sterility and develop a distinct meiosis mechanism to reproduce sexually (Täckholm, 1920; Blackburn and Harrison, 1921; Blackburn, 1925). It grows under wild and cultivated conditions throughout Turkey, Eastern Europe, West Asia, and some parts of North African countries (Zeven et al., 1982; Nilsson, 1997; Kim, et al., 2021). It is propagated both through vegetative and generative propagation techniques.

Its red to orange-colored fruits also called pseudo fruits are highly rich in vitamin C, Ca, Mg, Cu, and Mn. The fruit also contains amino acids, essential oils, bioflavonoids, organic acids, pectin, polyphenolics, tannins, tocopherol, carotenoids, and sugars (Nilsson, 1997; Ercisli, 2005; Quave et al., 2008; Christensen et al., 2014; Nagatomo et al., 2015; Selahvarzian et al., 2018) that makes rosehip nectar as a highly valuable beverage, in industry. It is mostly used for the prevention and treatment of the common cold, gastrointestinal disorders, diabetes, kidney disorders, and other infections (Duru et al., 2012). Rosehip oil is also used in various cosmetic products and aromatherapy (Ercisli, 2007; Kazaz et al., 2010; Mármol et al., 2017).

R. Canina also acts as an ideal and efficient rootstock for the multiplication of many rose cultivars (Grbic et al., 1996). They are well known for their multiple resistance against several abiotic (heat, chilling cold, drought, and water stress, etc.) and biotic (viral, bacterial and fungal

diseases, etc.) stresses that, increase their economic value in trade and economics (Nilsson, 1997; Ercisli, 2005; Quave et al., 2008; Pugazhendhi et al., 2021).

Growing plants through seeds could also serve in the maintenance of genetic diversity among local populations, however, rosehip seeds have high dormancy (Haouala et al., 2013; Venkatesha et al., 2022). Uniform seed propagation is very difficult due to combined endogenous (physiological and/or morphological dormancy) and exogenous (mechanical and/or physical dormancy) factors that make it difficult to germinate (Werlemark et al., 1995; Zhou et al., 2009; Iakovoglou and Radoglou, 2015). These include physiological inhibition like accumulation of abscisic acid in testa, pericarp, and embryos that block successful seed germination (Hosafci et al., 2005; Werlemarket al., 2009; Zhou et al., 2009). Koornneef et al. (2002) report the crucial role of gibberellins (GAs) in breaking seed dormancy and germination. The level of seed dormancy differs among species, cultivars, varieties, seed lots, and even hips of a single bush on a plant (Meyer, 2008). Grbic et al. (1996), reports the germination of rosehip seeds by prolonged stratification in polythene bags for four to six months. All of these studies suggest prolonged treatment times with low efficiency to break seed dormancy.

Seed germination behavior could vary even among different collections or populations of the same species collected from different places (Acharya et al., 2006; Albrecht and Penzagos, 2012), due to odd ploidy levels and physical genetic determinants. Good seed germination is very difficult from rosehips.

Standard rosehip cultivars have been developed by selection in many countries (Balta and Cam, 1996, Mármol et al., 2017). High genetic diversity in Turkish rosehips is not new. This diversity could contribute to breeding programs of the plant with the introduction and improvement of desired plant traits like improvement in yield, quality of plant and fruit along with resistance against various types of biotic and abiotic stresses. Another aim of breeding could be the development of rootstock cultivars for grafting (Balta and Cam, 1996; Demir and Ozcan, 2001; Davoudi et al., 2019).

Despite a large population of rosehip all over Turkey, their standard cultivars for any desired purpose have not been developed so far. Local rosehip populations could participate in breeding of new promising rosehip cultivars with improved resistance against biotic and abiotic stress and out-performance in the fields (Nair, 2019). These rootstock lines or cultivars with known compatibility for various rose species and cultivars could be used ideally for commercial exploitation (Ouyang et al., 2019).

This study aimed to investigate the possibility of germinating pentaploid seeds of local rosehip germplasm for increased synchronous seed germination percentage; for use in breeding programs having desirable fruit and/or rootstock properties.

Materials and Methods

Plant Material

The yellowish orange ripe fruits of the pentaploid *R. canina* were collected from the faculty of Agriculture Usak Province (Usak, Turkey, 38°40′24″ N latitude, 29°24′20″ E longitude, and 3000 m Elevation above sea level) in October 2017. Their voucher samples are deposited in the Herbarium of the Faculty of Science, Gazi University, Ankara, Turkey (GUE 2380).

Viability or Tetrazolium Test

Five replicates of 30 seeds each were hydro stratified using filter papers in between two filter papers moistened to around 3 times their dry weight for 4 h at 24-±1°C before carrying out the tetrazolium test. Thereafter, the treated seeds were horizontally cut through their hard seed coats close to the micropyle end and were incubated in polystyrene dishes (100 × 10 mm). These were dipped in 1 mg ml^{-1}concentration of 2, 3, 5 triphenyl tetrazolium chloride solution. The seeds were incubated at room temperature at 24±1°C overnight in dark. All seeds were checked carefully under 40 × magnification of the microscope to distinguish viable and unviable seeds.

The viable seeds' tissues with live cells attain red stain due to formazan formation. Whereas, the seeds with dead tissues do not take red stains. The formazan formation was expected to evaluate the seed viability. All viable seeds were expected to take light and dark red colors; whereas, semi-viable and unviable embryos were expected to take mosaic/light red and white color on radicles and plumules or their tips respectively. The seed germination percentage was computed using visible observations (Ista, 2019).

Seed Moisture Content and 1000 Seed Weight

After collection of the *R. canina* hips, they were manually cut with a sharp knife to extract seeds followed by cleaning them from rosehip flesh. The seeds were washed under running tap water to remove any traces of foreign material if any. The initial viability of the seeds was checked by soaking them in the water at room temperature. All floating seeds were counted and treated as dead, hollow, or empty and were removed for discarding. The seeds were subjected to sensitive balance to take the ratio of flesh to seeds, fresh 1000 seeds weight (Zhou *et al*. 2009). These seeds were dried in a cool and dry place for 48 hours at room temperature. After complete drying, the seeds were zip packed in transparent polyethylene bags for 24 h.

Physical characteristics of Fruit (hip) and seeds like, fruit weight, average 1000 seed weight, average moisture content, average fruit width, fruit length, and flesh to seed ratio were also measured. The seed moisture for four replications of 100 seeds each was measured at 103°C for 17 h and 1000 seeds weight based on 10 replicates of 100 seeds (10 x 100 seeds) (ISTA 1993).

Surface Sterilization

Fresh and healthy rosehip seeds were cleaned with a commercial detergent (Hacı Sakir, Turkey) on a seed sieve to minimize *in vitro* contamination. These were surface sterilized using 5% (v/v) sodium hypochlorite (NaOCl) for 20 min followed by 3 × 5 min rinsing and stirring in sterile distilled water. Any dead and floating seeds were discarded at this step. These were divided into 5 lots – the seeds in the first lot were subjected to Tetrazolium test after cutting each seed into two using a sharp knife. The tetrazolium test was carried out by dipping these freshly cut seeds into 1 mg ml^{-1} Tetrazolium solution for 12 hours under dark conditions (Porter *et al*. 1947).

The seeds belonging to 2nd and 3rdlot were stratified on MS medium (*used as control 1*), or MS medium containing 3 and 5 mg/l GA_3 (control 2 and 3).

The seeds belonging to mechanically scarified 4th and 5th lot were stratified on MS medium, or MS medium containing 5 mg/l GA_3 in the same order.

Care was taken to add heat-labile GA_3 supplements to respective treatments after filter sterilization at 44-45 °C.

Thereafter, each of the above 3 stratified and 2 scarified +stratified treatments were subjected to 14 different alternative and regulated chilling (4 °C under dark conditions) + warm (24 °C under 16 h light photoperiod) treatments such that the first treatment of seeds received 0 days chilling cold and 39 d warm 16h-light photoperiod (hereinafter called regulated chilling in dark + warm 16h- light photoperiod stratification). The 2nd treatment consisted of 3 days of chilling cold dark and 36 days- of warm-light stratification treatment. Each of the next treatments received a regulated decrease of 3 days chilling in dark + increased 3 d warm 16h- light photoperiod stratification treatment until the equilibrium was achieved for the 14th treatment that received 39 days chilling dark and no warm light stratification treatment (Table 1).

Table 1. Effects of alternative and regulated cold and warm temperature treatments to seeds stratified on MS medium and MS medium with or with out containing GA_3

Serial No.	Cold and warm temperature treatments		Experiment 1 (control) Seeds stratified on MS medium		Experiment 2 Seeds stratified on MS medium containing 3 and 5 mg/l GA_3			
					Experiment 2.1		Experiment 2.2	
	4°C	24°C	MS medium	Physical Change in seeds	MS containing 3 mg/l GA_3	Physical change in seeds	MS containing 5 mg/l GA_3	Physical change in seeds
1.	0	39	0.00	-	0.00	-	0.00	-
2.	3	36	0.00	-	0.00	-	0.00	-
3.	6	33	0.00	-	0.00	-	0.00	-
4.	9	30	0.00	-	0.00	-	0.00	-
5.	12	27	0.00	-	0.00	-	0.00	-
6.	15	24	0.00	swellings	0.00	swellings	0.00	Swellings and cracking
7.	18	21	0.00	-	0.00	swellings	0.00	Swellings and cracking
8.	21	18	0.00	-	0.00	swellings	0.00	Swellings and cracking
9.	24	15	0.00	-	0.00	-	0.00	-
10.	27	12	0.00	-	0.00	-	0.00	-
11.	30	9	0.00	-	0.00	-	0.00	-
12.	33	6	0.00	-	0.00	-	0.00	-
13.	36	3	0.00	-	0.00	-	0.00	-
14.	39	0	0.00	-	0.00	-	0.00	-

All values in a single column represented by different letters are significantly different using Tukeys test at 0.05 level of significance

All the experiment was conducted in triplicates using a total number of 60 seeds per treatment with 20 seeds in each replication.

Statistical Analysis

One-way ANOVA was used to evaluate the germination percentage of the seeds for each of the 4 different types of treatments using IBM SPSS 24. Means values were compared by Tukey's test at the 0.05 probability level. The percentage of data were Arcsine transformed before subjecting them to statistical analysis

Results

Physical Characteristics of Fruit (hip) and Seeds

Fruit weight ranged 59.69 g to 71.26 g. The average 1000 seed weight of Rosehip seeds remained 23.61 g with an average moisture content of 12.67 %. The average fruit width was 1,74 cm with a length of 2.31 cm with a flesh ratio of 65.26%.

Water Soaking Viability Test

The water soaking test showed 78.50 % seed viability. The rest of the seeds floated and were not viable.

Tetrazolium Test

The seed viability was interpreted by the pattern of staining and the intensity of red color. Tetrazolium test results showed that 86.75 % of seeds were vigorously viable showing sparkling light red color on the cotyledons and the embryos. Around 2 % of seeds showed non vigorous viable cotyledons. The results indicated that only a minor quantity of the seeds (11.5 %) showed no staining and nonviability.

The procedure allowed prompt and precise discrimination among the percentage of viable seeds and determined the physiological basis essential for checking the quality of seeds before the actual sprouting of the seeds.

Non-scarified Seeds Stratified on MS Medium (used as control)

The results showed swellings on a few seeds without germination using any of the regulated 14 chilling - warm dormancy breaking treatments (Table 1-Fig. 1a).

Non-Scarified Seeds stratified on MS medium Containing 3 and 5 Mg/L GA3 (used as control 2)

Although the results showed swellings on a few treatments using 3 mg/l GA3, no splits or cracks were

noted on the seeds (Table 1). Splitting and sprouting after swellings on a few "14 d chilling dark and 12 d warm light stratified treatments was noted after 14 days (the data not given - Fig. 1 b) using 5 mg/l GA3. These sprouts had difficulty to grow.

Mechanically Scarified Seeds Stratified on MS Medium (used as control 3)

The seeds were cultured on (i) MS medium for 39 days warm d (ii) 3 d chilling (4°C) + 36 d warm (iii) 6 d chilling (4°C) + 33 d warm (iv) 9 d chilling (4°C) + 30 d warm and (v) 12 d chilling (4°C) + 27 d warm treatments (Table 2) showed the maximum seed germination in range of 48.33 - 55% in the same order. The seed germination on all treatments was statistically similar (Table 2 - Fig. 1c). Rest of the seed treatments showed significant differences among them that had non-consistent and nonlinear but reduced seed germination with a range of 13.33 – 41.67%.

Mechanically Scarified Seeds Stratified on MS Medium Containing 5 Mg/L GA_3

Seven statistically significant and different groups for germination percentage were noted on regulated 5mg/l GA_3 treatments for different durations of chilling (4°C) + dark and warm periods in days (Table 2). Maximum and statistically similar seed germination was noted on 3 regulated alternate temperatures of 24 d chilling (4°C) dark+ 15 d warm; 21 d chilling (4°C) dark+18 d warm and 18 d chilling (4°C) dark+ 21 d warm treatments using 5mg/l GA_3 on MS medium stratified seeds in a range of 80- 85% (Fig. 1d).

It was followed by statistically different germination percentage ranges of 23.33- 66.67% on the rest of the treatments.

All of these studies approve that longer chilling (4°C) dark temperature induced negative effects on seed germination of Rosehip. Moreover, it was known beyond doubt that chilling in dark + warm 16h- light photoperiod shocks are critical and improve seed germination.

Table 2. Effects of alternative and regulated cold and warm temperature treatments to mechanically scarified seeds stratified on MS medium with or without containing GA_3

Serial No.	Cold and warm temperature treatments		Mechanical scarification followed by stratifications on MS medium	Mechanical scarification followed by stratifications on MS medium containing 5 mg/l GA_3
	4°C	24°C		
1	0	39	55.00a	45.00c
2	3	36	55.00a	60.00c
3	6	33	55.00a	51.66bc
4	9	30	48.33ab	62.66b
5	12	27	51.67a	66.67b
6	15	24	35.00cde	80.00a
7	18	21	38.33cd	85.00a
8	21	18	41.67bc	81.66a
9	24	15	36.67cde	66.67b
10	27	12	31.67de	40.00d
11	30	9	28.33ef	46.67d
12	33	6	36.67cde	36.66e
13	36	3	23.33f	25.00f
14	39	0	13.33g	23.33g

All values in a single column represented by different letters are significantly different using Tukeys test at 0.05 level of significance

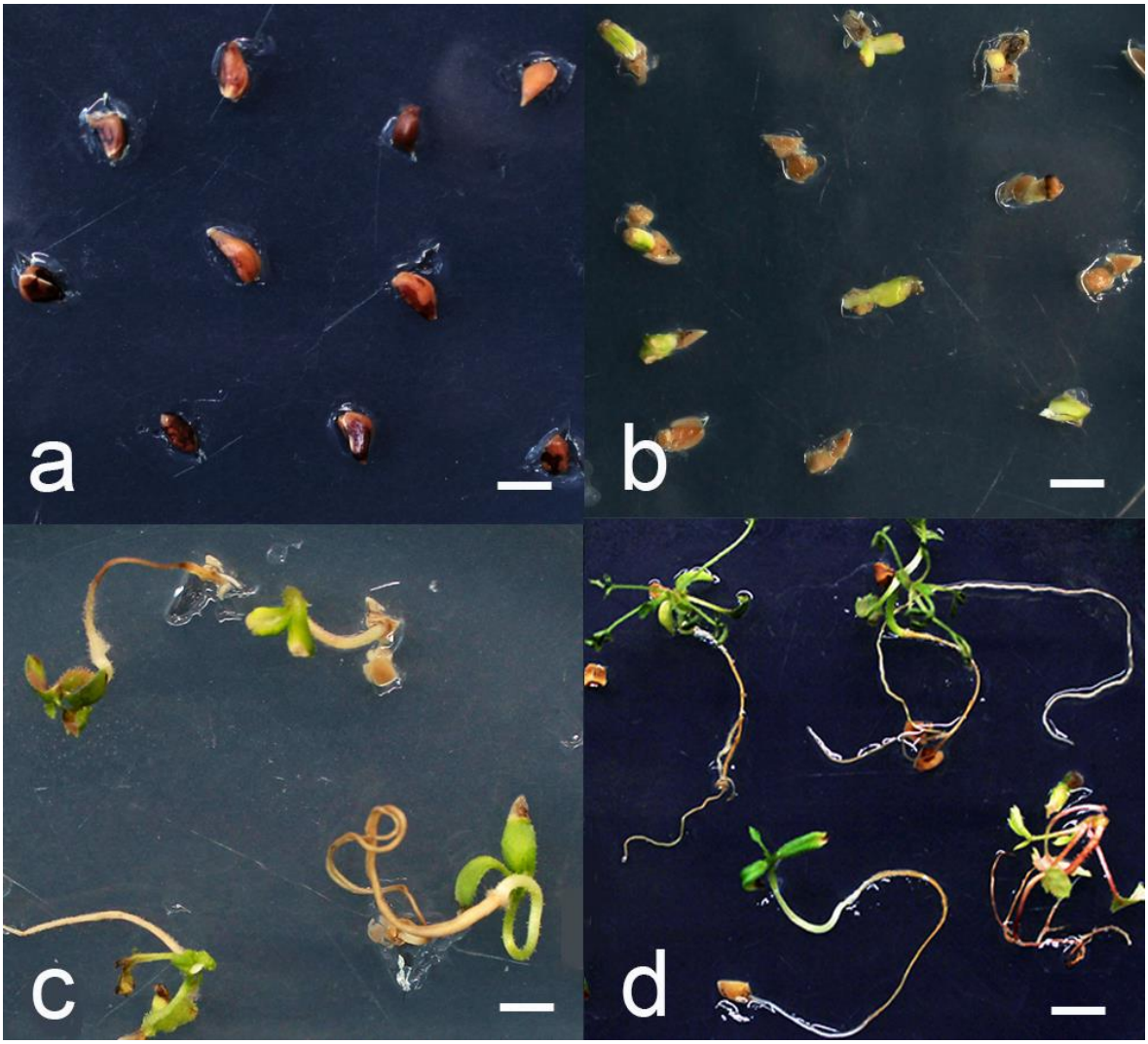

Figure 1. Breaking seed dormancy of rosehip. (a) Swellings on a few seeds without germination using any of the regulated 14 chilling cold- warm dormancy breaking treatments (b) swellings with rare germination on few seeds with limited germination using GA_3 treated seeds on regulated 14 chilling dark- warm dormancy breaking treatments (c) seeds germination on MS medium (d) and 5 mg/l GA_3 treated 14 chilling dark- warm dormancy breaking treatments Bar Fig 1a, b, c =0.5 cm, d=0.9 cm

Discussion

Rosehip achenes are very small (3-6 mm) and the structure of achenes plays an important role in seed germination. Rosehip achenes are covered by a thin testa and a hard pericarp that contain many inhibitors. Therefore, the structure of achenes or thickness of endocarp in pericarp plays an important role in inducing seed dormancy and limiting germination in a significant manner as reported by Nadeem et al. (2013) in Rosa × hybrida. Their achene structure is fully under the genetic control of, number of environmental factors including coldness, temperature, humidity at the time of seed maturity, and photoperiod (Gudin et al., 1990). The Rosehip seeds are covered by testa that covers embryos.

Water sinking test is widely used to test seed viability but the test is not fully reliable and has certain limitations. If the seeds are collected under dry conditions or are stored under dry conditions, or they have air pockets; the good seeds could also float like other dead seeds (Daneshvar et al., 2017; Kochanek, 2018). Therefore, it is desirable to imbibe seeds for a longer time by stirring them manually or magnetic stirrer. Due to these reasons, the water sinking method is generally evaluated not evaluated as the desired method of seed testing.

Tetrazolium test involves rapid understanding and interpretation of seed viability levels of seeds by staining pattern (McDonald et al., 1998; Soyler et al., 2012). It is possible to estimate seed viability through the intensity of cellular dehydrogenase enzyme activity; where deep red colored formazan is formed by hydrogen transfer catalyzing reaction on all respiring cells/tissues that helps to avoid erroneous results (Sumlu et al., 2010). The absence of this catalyzing reaction on dead cells inhibits formazan conversion which results in their nonstaining.

The rosehip seed sprouting percentage results reported in this study were different from the observations noted during water soaking/sinking and tetrazolium seed viability tests.

Seeds of many roses including rosehips undergo dormancy under unfavorable environmental conditions by developing hard pericarps around them to prevent precocious germination of the seeds. The seeds fail to germinate without water penetration in them (Semeniuk and Stewart, 1970; Svejda, 1972), therefore softening or breaking of pericarp or removal of physical barriers is a condition to let water penetrate in seeds.

It was determined that the rosehip seeds could only germinate when physical hard coat seed-based primary seed dormancy is removed. This study reports primary seed dormancy break after mechanical damaging of pericarp and supplying alternate cold-high temperature shocks in a medium containing GA3. Nadjafi et al. (2006) and also support the idea and used H2SO4 scarification ensued by GA3 treatment for 48 hands chilling (5°C) for 14 days to break seed dormancy of T. polium and F. gummosa Seeds. Nadeem et al. (2013), treated seeds to warm temperature for 30 days ensued by scarification and cold treatment for 60 days to induce seed germination in Rosa × hybrida. Cold-high temperature shocks could vary during different seasons due to a number of reasons like stratification treatment period, habitat of plants, and the species. These are likely responsible for fluctuation in rosehip seed germination under natural conditions (Stewart and Semeniuk, 1965; Gudin et al., 1990; Werlemark et al., 1995; Claessens, 2012; Rakhimova et al., 2020). In line with this, the present seed germination scheme describes a systematic protocol to break both primary (hard seed coat dormancy) and secondary embryo-based physiological dormancy in the seeds to avoid reinduction of previously existing or nonexisting new types of dormancy/dormancy. As defined by Semeniuk and Stewart (1970). It was also displayed that the seeds behaved variably towards germination on hydrated and GA3 stratified seeds after their culture in regulated chilling - dark + warm - 16 h light stratification shocks. The results of this study displayed a significantly varied behavior of scarified seeds compared to noncertified seeds; hydrated and GA3 stratified seeds and regulated light-dark photoperiod period along with varied temperature treatments in agreement with Semeniuk and Stewart (1966), Svejda (1968), Koornneef et al. (1984); Karssen et al. (1983); Prevost and Le Page-Degivry (1985); Walker-Simmons (1987); Groot and Karssen (1992) and Bewley and Black (1994), Zhou et al. (2009), Werlemark et al. (1995), Leubner (2010).

Generally, such types of seeds need 'winter rest' before germination (Arora et al., 2003). This results in irregular, slow or insufficient germination among Rosehip seeds depending on winter harshness. Regardless of the type of treatment, the results of this study clearly and precisely demonstrate the effects of longer periods of chilling cold treatments that continuously negate seed germination (Nitsch, 1957; Heide, 2008; Sonsteby and Heide, 2010). The results of this study are in full agreement with the abovementioned observations. The maximum speed germination was achieved on MS medium containing GA3 with regulated chilling and dark + warm and 16 h light shocks in 39 days. All previously reported methodologies display long and unreproducible seed germination protocols (Stewart and Semeniuk, 1965; Hajian and Khosh-Khui, 2000; Gudin, 2001; Zlesak, 2007; Zhou et al., 2009). The most-reported strategy to break seed dormancy of rosa species is stratification under cold conditions (Zlesak, 2007; Zhou et al., 2009).

The researchers report seed dormancy break of R. setigera and R. multiflora after 30 days of cold stratification. Furthermore, they report 45 d cold stratifications for R. wichuraiana for maximum germination. Hajian and Khosh-Khui (2000) have proposed chemical scarification with sulphuric acid followed by 150-180 d cold stratification. The current study reports 83.50% percent viability in watersoaked seeds and 86.75% viability in tetrazolium-treated seeds. The average seed germination percentages in the seeds were 80-85%. The variation in the percentage of viable seeds percentage and germination percentage could be due to the random selection of seeds for each of the tests.

The results of this study approve that seed dormancy is under the control of multiple factors that are effective and established depending on the types of treatments given to seeds (Thomas and Vince-Prue, 1997; Nishimoto and McCarty, 1997; Tavşanoğlu et al., 2021).

Conclusion

This study explains a step-by-step detailed, powerful, and useful protocol to enhance rosehip seed germination with a comparison to other potential seed germination techniques. The results suggest the role of pericarp cracking or softening, regulated chilling+darkand warm +16 h light treatments mimicking differences in natural day and night conditions to break seed dormancy in the presence of GA3. The dormancy break results of the current study are of special interest in the context of the potential future impacts of ongoing climatic warming and could be helpful to enhance in vitro rosehip seed germination under adverse conditions. The present results could contribute to governing the selection of new rosehip fruit and rootstock cultivars through breeding and seed multiplication programs. A study on molecular and the precise genetic mechanisms controlling temperature and light sensing to regulate dormancy would be beneficial and advantageous.

Compliance with Ethical Standards

Conflict of interest

The author declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author contribution

The author read and approved the final manuscript. The author verifies that the Text, Figures, and Tables are original and that they have not been published before.

Ethical approval

Ethics committee approval is not required.

Funding

No financial support was received for this study.

Data availability

Not applicable.

Consent for publication

Not applicable.

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