

The Effects of Cold Stratification, Cold Storage and Gibberellic acid (GA₃) on Seed Germination of Bilberry (*Vaccinium myrtillus* L.)

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Abstract

Aim of study: The effects of cold stratification, cold storage and gibberellic acid (GA₃) on seed germination of bilberry (*Vaccinium myrtillus* L.) were investigated.

Area of study: Ripe berries of *V.myrtillus* were collected from the Kaz Mountains (İda Mount) National Park-Balıkesir province in Turkey.

Material and methods: In the study five levels of cold moist stratification period, cold dry storage period (control, 15, 30, 60, 90 days) at +4 °C and three dose of GA₃ (control, 500, 1000, 1500 mg/L) treatments were applied. Data were tested independently by ANOVA according to three factors with three replications. 50 seeds were used for each treatment for each replication.

Main results: Cold stratifications, cold storage and GA₃ treatments did not increase the germination percentage of bilberry seeds. Germination was found between 23-40% in all experiments.

Highlights: Different pretreatments are advised for increasing germination percentage of *V. myrtillus* L. seeds

Keywords: *Vaccinium myrtillus* L., Cold Stratification, Cold Storage, GA₃, Seed Germination

Çoban Üzümü (*Vaccinium myrtillus* L.) Tohumlarının Çimlendirilmesinde Soğuk Katlama, Soğuk Depolama ve Gibberellik asit (GA₃) Uygulamalarının Etkisi

Öz

Çalışmanın amacı: Soğuk saklama, soğuk depolama ve gibberellik asidin (GA₃) çoban üzümü (*Vaccinium myrtillus* L.) tohumlarının çimlenmesi üzerine etkileri araştırılmıştır.

Çalışma alanı: *V. myrtillus* L.'un olgun meyveleri Türkiye de Balıkesir-Kaz dağları Milli Parkı içerisinde toplanmıştır.

Materyal ve yöntem: Bu çalışmada +4 °C'de beş seviyeli soğuk nemli saklama, soğuk kuru depolama periyodu (kontrol, 15, 30, 60, 90 günler) ve üç doz (kontrol, 500, 1000, 1500 mg/L) GA₃ işlemleri uygulanmıştır. Elde edilen veriler, üç tekerrürlü üç faktöre göre birbirinden bağımsız olarak ANOVA ile test edilmiştir. Her bir işlem ve tekerrür için 50 adet tohum kullanılmıştır.

Temel sonuçlar: Soğuk katlama, soğuk depolama ve GA₃ işlemleri çoban üzümü tohumlarının çimlenme yüzdesini artırmamıştır. Tüm denemelerde çimlenme oranları %23-40 arasında bulunmuştur.

Araştırma vurguları: *V. myrtillus* L. tohumlarının çimlenme yüzdesini arttırmak için farklı ön uygulamaların yapılması önerilmektedir.

Anahtar Kelimeler: *Vaccinium myrtillus* L., Soğuk Saklama, Soğuk Depolama, GA₃, Tohum Çimlenmesi



Introduction

Genus *Vaccinium* L. belonging to the Ericaceae family has nearly 450 species, which are divided into two subgenera *Oxycoccus* (Hill.) A. Gray and *Vaccinium* (Eminağaoğlu, 2018). “*Vaccinium myrtillus* (bilberry) is a deciduous shrub” (PFAF, 2020) with rhizome growing to 30 cm (Eminağaoğlu, 2018). It is stem angled, sparsely puberulent or not. (Davis, 1988). It flowers in late spring and fruit is ripe between July and September (Kemper, 1999). Bilberry is naturally distributed in Bursa-Uludağ, Kastamonu (İlgaz Mount), Ordu, Gümüşhane, Trabzon, Artvin, Balıkesir (Kaz Mount) provinces at altitude between 1280 and 2700 m in among *Rhododendron caucasicum*, *Pinus* L. or *Juniperus* L. in Turkey (Davis, 1988).

Fruits and leaves of *V. myrtillus* have been traditionally used in folk medicine of several European countries (Woodward, 1986). Bilberry has been used in the treatment of various diseases such as diarrhea, dysentery and diabetes (Grieve, 1971; Bone & Morgan, 1997). The clinical actions of bilberry have been determined as antiedematous, antioxidant, vasoprotective, anti-inflammatory and astringent (Bone & Morgan, 1997). Total phenol contents of bilberry leaves and berries (138.53; 47.05 mgGAE/g, respectively), radical scavenging activities and capacities to retard oxidation caused by the oil/water emulsion reveal the fact that bilberry leaves and berries are potential resources to be utilized in industry (Bilgin et al., 2011). It is useful to human health in terms of the antioxidant and other compounds of the berries. Bilberry is collected for commercial purposes from the wild in the countries where it grows. Bilberry berries are used in the various food industry (jellies, tarts, fruit and wine making etc.) (Turner, 1997).

Seed dormancy is defined as the inability to complete germination of mature seed under suitable conditions (Bewley, 1997). Some studies have been carried out for the germination and breaking the dormancy of the seeds of some *Vaccinium* species (Baskin & Baskin, 2014). According to academic data, it is not easy to decide if viable seeds are dormant or nondormant. Seeds of *V. myrtillus* L. are conditionally dormant at maturity (Baskin et al., 2000). Cold moist stratification and gibberellic acid (GA) are generally applied to break seed dormancy and obtain maximum germination (Wang & Berjak, 2000; Taiz & Zeiger, 2002). Some seed germination studies using cold stratification and gibberellic acid treatments have been stated for bilberry (*V. myrtillus* L.) (Baskin et al., 2000; Ciordia et al., 2006; Milbau et al., 2009; Ranwala & Naylor, 2010; Karabulut & Çelik, 2013).

Bilberry is used in industries such as medicine, pharmacy and food since it has antioxidants and other compounds beneficial to human health. Therefore, cultivation and breeding studies are very important for bilberry. Bilberry seed germination studies have not been done enough in Turkey. Germination characteristics seeds of bilberry from İda Mount has not been studied. This study aims to determine effects of cold stratification, cold storage and gibberellic acid on seed germination of İda Mount origin bilberry (*V. myrtillus* L.).

Material and Methods

Material

Ripe berries of *V. myrtillus* were collected in 17 August 2010 at 1453 m a.s.l. from the Kaz Mountains (İda Mount) National Park-Balıkesir province in Tozlu çeşme location (39° 39' 49" N, 26° 56' 43" E) in Turkey (Figure 1). Seed samples of *V. myrtillus* were investigated in Aegean Forestry Research Institute Laboratory.



Figure 1. A view from the natural distribution area of *V. myrtillus* L., *Mont Ida* (left). *V. myrtillus*, L. (right)

Methods

Berries were kept in plastic bags in a refrigerator (+4°C) until experiments were established. Berries were crushed gently to release the seeds, which were washed free of fruit material. Seeds were floated in distilled water (Figure 2, left). Sunken seeds were

dried in an open container at room temperature (20-22°C) for 24 h. Seeds were placed in glass petri dishes (Figure 2, right) on 25.08.2010 and three replications of 50 seeds each were used for each test condition. The seeds were not surface sterilized.



Figure 2. Application of the water floatation technique to seeds of *V. myrtillus* L. (left). Seeds of *V. myrtillus* L. (right)

Cold Moist Stratification

Seeds were placed in glass petri dishes on Whatman No. 1 filter paper moistened with 3 ml of distilled water in a refrigerator at +4 °C for five levels of stratification period (control, 15, 30, 60, 90 days).

Cold Dry Storage

Seeds were placed glass petri dishes on Whatman No 1 dry filter paper. Then petri dishes were sealed with parafilm and placed in a refrigerator at +4 °C for five levels of storage period (control, 15, 30, 60, 90 days).

Gibberellic Acid Treatment

Seeds were soaked in GA₃ (0, 500, 1000, 1500 mg/L) for 24 h in the dark condition. Before the applications, 2000 mL 1500 mg/L GA₃ stock solution was prepared. For this purpose, 3000 mg of GA₃ (Sigma-G7645) in pure powder form was weighed into a 2000 mL measuring cylinder on precision scales and 5 mL of ethyl alcohol (96%) was added to dissolve. After the powder dissolved, the solution was completed to 2000 mL with distilled water. For each application 500, 1000, 1500 mg/L GA₃ was prepared from the stock solution. In the control application, distilled water was applied.

Seeds were placed in 10 cm diameter petri dishes on a single layer of Whatman No. 1 filter paper moistened with distilled water. Distilled water was added to each treatment group during the test period when necessary. Then seeds were placed in a growth cabinet (Nuve ID 501) at 60-70 % humidity and at 18-25 °C with a photoperiod of 12 h light (40 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 400-700 nm cool white fluorescent light) and 12 h darkness after the different treatments. The number of germinated seeds (defined as emergence of the radicle to at least 2 mm) was counted on certain days (7st, 14st, 21st, 28st, 40st day) (Figure 3, left). Final germination counting was done on 40st day. 1000 seed weight (g) was calculated according to ISTA (1996) with 100x8 sampling.

Statistical Analysis

The significance of treatment means was tested by one-way analysis of variance

(ANOVA) on the transformed arcin square root of germination percentages. Data were evaluated independently by ANOVA according to three factors with three replications. Where significant differences were found ($p < 0.05$) treatment means were compared using Tukey's HSD test. ANOVA and Tukey's analyses were performed using SPSS for windows.

Results and Discussion

1000 seed weight of *V. myrtillus* was as 0.36 g. Shahram (2007) found that average weight of *V. arctostaphylos* seed was 0.27 g. Ranwala and Naylor (2010) found that *V. myrtillus* berries contained on average 71 (SE=3) seeds and the total seed weight per berry was 0.106 mg (SE= 0.0021). Germination percentages of *V. myrtillus* seeds under cold stratification, cold storage and gibberellic acid treatments are given in Table 1.

Table 1. Germination percentages (mean \pm SE) of *V. myrtillus* seeds under cold stratification, cold storage and gibberellic acid treatments

Dry Storge Periods (Days)	Germination Percentages (%)	Moist Stratification Periods (Days)	Germination Percentages (%)	GA ₃ Concentration (Mg L ⁻¹)	Germination Percentages (%)
0	28.67 \pm 2.67a*	0	37.33 \pm 6.57a	0	37.33 \pm 6.57a
15	40.00 \pm 3.46a	15	38.00 \pm 4.16a	500	32.67 \pm 1.76a
30	30.00 \pm 5.77a	30	37.33 \pm 5.70a	1000	25.33 \pm 4.37a
60	35.33 \pm 2.40a	60	24.67 \pm 2.91a	1500	29.33 \pm 3.71a
90	29.33 \pm 4.81a	90	23.33 \pm 2.67a		

mg: milligram; L:liter; *Means in the same column followed by the same letter are not significantly at $p < 0.05$



Figure 3. Germinating *V. myrtillus* seeds (left). Infected *V. myrtillus* seeds (right)

Germination was found between 23-38% in the cold moist stratification experiments (Table 1). Cold moist stratification experiments did not increase the germination percentage of bilberry seeds in our study. Milbau et al. (2009) found that in general cold stratification treatments for 20 weeks in 23 subarctic species, the percentage of germination under the thick snow cover treatment (43%) was higher than the germination percentage under the thin snow cover treatment (39%). After stratification, *V. myrtillus* germination percentage (67%) was found under the optimum (germination temperature 20/10 °C) incubation treatment (Milbau et al., 2009). Additionally, Baskin et al. (2000) recorded 62-100% germination of seeds of *V. myrtillus* and *V. vitis-idaea* in light at 20:10°C and 25:15°C. Barney et al. (2001) reported that the maximum germination percentage (70%) of *V. membranaceum* seeds was achieved by 28 days stratification at 1-3°C. Besides, Ranwala and Naylor (2010) reported that above 80% germination in fresh large *V. myrtillus* seeds and the final number of germinating seeds of *V. myrtillus* in moist at 2°C decreased by about 2.2% per week of storage and zero after 43 weeks. Germination percentage decreased arithmetically as the stratification time increased also in our study.

Germination was found between 28-40% in the cold dry storage (Table 1). Griffin & Blazich (2002) stated that seeds of some *Vaccinium* species do not need any pretreatment for adequate germination. Aalders & Hall (1979) reported that the fresh seeds of *V. angustifolium* Ait. will germinate if sown immediately. Likewise, Vander Kloet & Hall (1981); Smreciu et al. (2008) stated that seed of *V. myrtilloides* Michx. do not need cold stratification. Smreciu et al. (2008) compared germination after of 4 weeks of cold stratification with a control (no stratification) and found no significant difference between them. The cold requirements of seeds vary from species to species. Thakur & Rathore (1991) recommended 1 month of cold stratification at 3-4°C for seeds of *Vaccinium* spp. Shahram, (2007) reported that dry stratification was been better than wet stratification for Qare-Qat (*V. arctostaphlos* L.) seeds; they needed 15-90 days of dry chilling to remove the

dormancy and the best germination (55%) was after 90 days dry chilling. In our study, germination percentage did not increase as the cold dry storage period increased.

Germination was found between 25-37% in the GA₃ treatments (Table 1). According to some studies, it has been reported that gibberellic acid (GA₃, GA₄₊₇, or gibberellin A₃) treatment can increase the germination percentage (Devlin & Karczmarczyk; 1975; Dweikat & Lyrene; 1989; Giba et al., 1993). Dweikat & Lyrene (1988) reported that highbush blueberry (*V. corymbosum* L.) seeds treated with 900 ppm GA₃ had 50% germination while the untreated seeds had 4% germination and higher concentrations did not increase the germination percentage. Devlin & Karczmarczyk (1977) found that the cranberry (*V. macrocarpon* Ait.) seeds treated with 500 ppm GA after 20 days dark treatment had 69% germination. Lopez et al. (2008) explain that red huckleberry seeds treated with the gibberellic acid potassium salt (GA-K) (1000 mg L⁻¹ and 1500 mg L⁻¹ treatments) had higher germination percentage in a shorter time compared to control seeds. For instance, Ciordia et al. (2006) found that 58.9% germination of bilberry (*V. myrtillus* L.) seeds treated with 1 mg GA₃ I⁻¹ and 84.4% germination of bilberry seeds treated with 0.5 mg GA₃ I⁻¹. Additionally, Karabulut & Çelik (2013) reported that *V. myrtillus* seeds cold dry storage for 180 days and then applied with 1000 ppm GA₃ gave the highest cumulative germination rate (91%). In contrast, Ballington et al. (1976) reported that gibberellic acid treatments did not enhanced the final germination percentage of seeds of *V. ashei* Reade. In our study, GA₃ treatments did not increase the germination percentage of *V. myrtillus* seeds (Table 1). It can be said that the effect of stratification, storage and gibberellic acid treatments on seed germination in some studies are effective and not in others. It is thought that this effect can vary under influences such as species, seed source, dormancy state and degree.

The germination percentages of cold stratification, cold storage and gibberellic acid experiments were found to be low in the study. The low germination percentages think that the seeds can be dormant. Germination was between 23-40% in all experiments

(Table 1). It has been found that non-germinating seeds are infected (Figure 3, right). The low germination percentage could be due to not applying surface sterilization to the seeds. Barney (2003) found that surface sterilization with 0.5% sodium hypochlorite solution eliminated contamination by microorganisms without reducing germination percentage of black huckleberry (*Vaccinium membranaceum* Douglas ex Hooker) seeds. Barney et al. (2001) reported that surface sterilization of black huckleberry seeds for 20 min in 0.5% sodium hypochlorite had little effect on maximum cumulative germination, as compared to nonsterilized controls.

Conclusion

In this study, cold stratifications, cold storage and GA₃ treatments did not increase the germination percentage of bilberry seeds. Germination was between 23-40% in all experiments. The low germination percentages think that the seeds can be dormant. Different pretreatments and more extensive studies can be applied to increase germination percentage for İda Mount origin bilberry.

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Ethics Committee Approval

N/A

Peer-review

Externally peer-reviewed.

Author Contributions

Conceptualization: S.B.; Investigation: S.B., E.A.; Material and Methodology: S.B., E.A.; Supervision: S.B., E.A.; Visualization: S.B., E.A.; Writing-Original Draft: S.B.; Writing-review & Editing: S.B., E.A.; Other: All authors have read and agreed to the published version of manuscript.

Conflict of Interest

The authors have no conflicts of interest to declare.

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