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## EFFECTS OF OZONE TREATMENTS ON IN VITRO SEED GERMINATION OF *Ruscus aculeatus*, *Ruscus hypoglossum* AND *Danae racemosa*

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**Abstract:** *Ruscus aculeatus*, *Ruscus hypoglossum*, and *Danae racemosa* are cut foliage and valuable for ornamental plants; they are also very valuable in terms of their medicinal aromatic properties. Propagating these plants using their seeds is very challenging due to deep dormancy and required pre-treatments before sowing. Ozone is a colorless gas with a pungent odor, made up of three oxygen atoms, and it offers an eco-friendly solution to break dormancy in seeds. In this study, stored seeds for four years were treated to ozone gas for 0, 15, and 30 minutes using an ozone generator that has the capacity to produce 6 grams of ozone per hour. Then, the seeds were cultured in Petri dishes containing Murashige and Skoog medium without plant growth regulators. *In vitro* seed germination rates were recorded 30, 40, 50, and 60 days after culture initiation. According to statistical analysis, the effects of species, duration of ozone treatments, and interaction of species and duration of ozone treatments on *in vitro* germination rates of seeds were statistically significant. The highest *in vitro* germination rates of 42%, 28%, and 24% were recorded at 30 min ozone treatment in *R. aculeatus*, *R. hypoglossum*, and *D. racemosa*, respectively. These results indicate that ozone application positively affects the germination of seeds.


**Keywords:** Dormancy, Germination, In vitro, Ozone, Seed


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### 1. Introduction

*Ruscus* species are used as cut foliage in floral arrangements such as baskets, bouquets, wreaths, and arrangements to enrich the texture, create a green background, and highlight the cut flowers they are used with. Additionally, after it is dried and painted in different colors, it can be used in decorative indoor designs or as green vases. The *Ruscus* genus belongs to the Asparagaceae family. However, the genus fluctuated between families and by turns in Liliaceae, Convallariaceae and Ruscaceae (Veronese, 2014). The genus is represented by four species and totally five taxa in Türkiye, including. *R. aculeatus* var. *aculeatus*, *R. aculeatus* var. *angustifolius* Boiss., *R. hypoglossum* L., *R. colchicus* P. F. Yeo, *R. hypophyllum* (Güvenç et al., 2011). *R. aculeatus* originating from Mediterranean region is spread in western and southern Europe and Africa (Manole and Banciu, 2015). *R. hypoglossum* is native to the Euro-Mediterranean, North Italy to Austria and Slovakia, and east to Türkiye and Crimea (Ivanova et al., 2013). Plants belonging to the genus *Ruscus* are

evergreen, shrub-like perennials that can grow up to approximately one meter tall. They form branched stem structures with numerous cladodes (flat, leaf-like stem tissue known as phylloclade). Cladodes can reach 2 to 18 cm long and 1 to 8 cm in width. The true leaves are small, scale-like structures and are not photosynthetic. The flowers are small, white with dark violet centers, and in the middle of the cladodes. The fruits are red and 5 to 10 mm in diameter. Some species are monoecious, while others are dioecious. They reproduce by seeds or underground rhizomes (Kebeli, 2021).

*D. racemosa* (L.) Moench (Syn. *R. racemosus*) commonly known as Alexandrian laurel or poet's laurel, belongs to the Liliaceae/Ruscaceae/Asparagaceae family. It is the only species in the genus *Danae* (Shen et al., 2013). *D. racemosa* is distributed in a narrow area between Istanbul and Adapazarı and in Mersin, Adana, Osmaniye and Hatay provinces in the Mediterranean part of the Türkiye (Kebeli, 2021). This species is also found in Iran, Syria and Italy. *D. racemosa*, an erect, evergreen shrub, thrives under the shade of forest trees. It features green



shoots, glossy leaves, thick unarmed alternate cladophylls, and terminal racemes of white-yellow flowers that are followed by red berries. This plant is frequently used for its decorative green foliage in fresh flower arrangements (Masoudi et al., 2022).

These species are not only valuable as ornamental plants, but studies have reported that they are also precious in terms of their medicinal aromatic properties and contain active ingredients used to treat many diseases. Additionally, while the aerial parts of *Ruscus* species are edible, their underground organs, including rhizomes and root structures, are utilized as phytotherapeutic products in traditional medicine (Kebeli and Çelikel, 2024). For instance, *R. aculeatus* was used to treat skin diseases (Ali-Shtayeh et al. 1998), and its decoction was used to treat eczema, diarrhea, and nephritis (Tuzlaci and Aymaz, 2001). Hadžifejzović et al. (2013) reported that the fruits of *R. hypoglossum* species are boiled and used to treat skin disorders such as chilblains and warts. *D. racemosa*, abundant in powerful natural flavonoids such as quercetin and kaempferol, could be an attractive option as a valuable source of natural antioxidants. It can potentially replace synthetic antioxidants and neutralize stress-induced free radicals (Fathiazad and Hamedeyazdan, 2014).

In Türkiye, these species are traditionally harvested from the wild due to the ease and low cost of collection. However, cultivating and rapidly propagating these species from seeds is crucial. This approach protects their natural populations and satisfies sector demand (Özden et al., 2016). The production of these species can be done using vegetative and generative methods. Generative production using seeds obtained from plants is not at the desired level due to the deep dormancy seen in the seeds and harms the germination process (Halada and Erdelská, 2005). Therefore, various seed priming methods were applied previously to break seed dormancy. However, Ozone provides an eco-friendly technological method to improve seed germination and disinfestation, leaving minimal residual impact (Pandiselvam et al., 2020).

Ozone is a colorless gas with a pungent odor made up of three oxygen atoms. Unlike the stable oxygen molecule ( $O_2$ ), ozone ( $O_3$ ) is unstable. It was first used for

disinfecting water treatment plants (Bocci, 2004; Uslu et al., 2022). Unlike other oxidizing agents, ozone breaks down into molecular and atomic oxygen and limited oxides during chemical reactions. These byproducts do not pollute the environment or form carcinogenic substances, unlike chlorine or fluorine oxidation products (Normov et al., 2019).

Despite the considerable amount of research on ozone, its stimulating properties on germination of dormant seeds remain insufficiently understood. Therefore, our study aimed to investigate the effect of ozone on the seed germination rates of *R. aculeatus*, *R. hypoglossum* and *D. racemosa*.

## 2. Materials and Methods

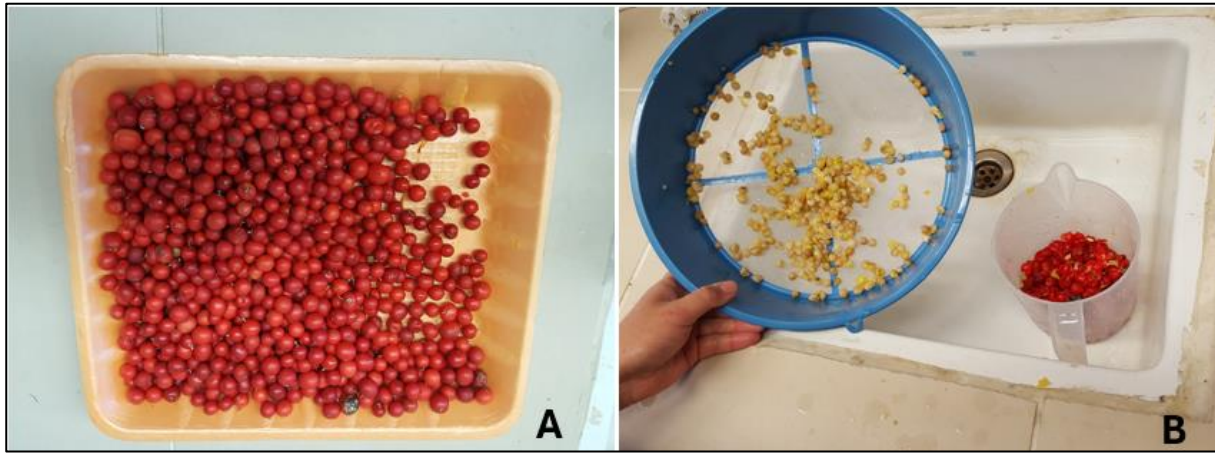
Kebeli (2021) gathered the plant materials of *R. aculeatus*, *R. hypoglossum*, and *D. racemosa* (Figure 1) from their natural habitats around the Beykoz district of Istanbul. The samples were then planted in a greenhouse at the Agriculture Faculty of Ondokuz Mayıs University in 2018.

In 2019, the harvested seeds were first separated from the fruit flesh under laboratory conditions and then cleaned using tap water. This step was taken to prevent any fungal agent formation that could arise from fruit flesh residues during drying. The washed seeds were laid out on blotting paper to remove excess water (Figure 2). Then, the dried seeds were stored in the dark in a glass jar at room temperature for four years.

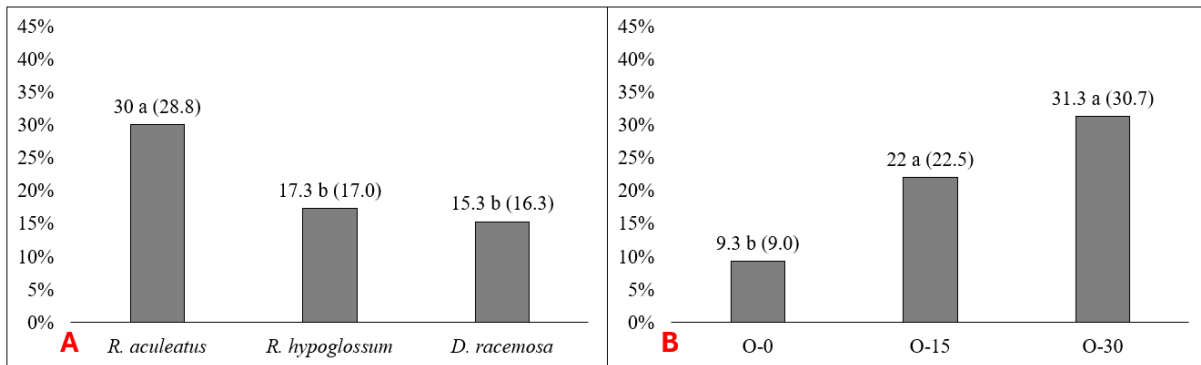
The stored seeds were pre-treated with ozone, and then *in vitro* germination rates were assessed. The ozone generator used in the study can produce 6 grams of ozone per hour. The ozone concentration varies between 3% and 10%. The reactor pressure ranges from 6.5 to 8.5 PSI, and the gas inlet flow rate averages between 0 and 4 liters per minute. The ozone production technique used is the Corona Discharge System. Cooling is achieved with air. The generator operates at 220 volts and 800 Hz, with an energy consumption of 250 watts (Hacı, 2015). The seeds were soaked in water overnight before application. Then, they were placed in a beaker containing 500 ml of pure water and ozone gas was applied to the water for 15 and 30 minutes. No application was made to the control group.



**Figure 1.** The visual appearance of the species in their natural habitats.



**Figure 2.** A) harvested fruits, B) separating fruit flesh from the seeds.



**Figure 3.** A: seeds germination rates of species, B: effects of ozone treatment time on seed germination. Levels not connected to same letter are significantly different ( $P < 0.05$ ), LSDspecies and LSDozone: 5.294, Arc-sin transformed values shown in parentheses.

The seeds were submerged in a 70% ethanol solution for 1-2 minutes and rinsed several times with sterile water to eliminate any remaining ethanol. They were soaked in a 25% commercial bleach solution (Domestos®, 4.5% v/v) for 15 minutes. The final step consisted of four additional washes with sterile distilled water. Seeds were cultured in Petri dishes (90 × 15 mm) containing 25 ml of full-strength MS medium (Murashige and Skoog, 1962), supplemented with 3% sucrose, and did not include plant growth regulators. The medium was solidified with 7 g/L agar, and its pH was adjusted to 5.6–5.8 before being autoclaved at 121°C and 15 psi for 15 minutes. All explants were maintained at  $23 \pm 2^\circ\text{C}$  in complete darkness for 2 months and were subcultured every 4 weeks. The germination rates of the seeds were recorded. Data from the last observation were used to calculate *in vitro* germination rates of the species.

*In vitro* germination tests with ten replicates (Petri dishes) and each replicate containing five seeds were established based on a completely randomized design. Means of all data were separated by variance analysis, and significant differences ( $p < 0.05$ ) were evaluated with an LSD test using the JMP® program (SAS Institute, Cary, NC). The percentage values were arc-sin transformed before the variance analysis. *In vitro* germination of seeds was recorded 30 (D-30), 40 (D-40), 50 (D-50), and 60 (D-60) days after the culture initiation.

### 3. Results and Discussion

According to statistical analysis, the effects of species, duration of ozone treatments, and interaction of species and duration of ozone treatments on *in vitro* germination rates of seeds were statistically significant. The highest *in vitro* germination rate with 30% was recorded in *R. aculeatus*, while the lowest with 15.3% in *D. racemosa* (Figure 3A). A significant increase in the germination rate of seeds is observed as the duration of ozone application increases. Compared to the control group, a 15-minute ozone application increases the germination rate by approximately 2.4 times, while a 30-minute ozone application increases the germination rate by approximately 3.4 times. These results indicate that ozone application positively affects the germination of plant seeds (Figure 3B).

Table 1 provides significant information on the interactions between species and ozone treatments. The germination rates generally increased with longer ozone treatment durations and germination rates of untreated seeds were the lowest for all species. *R. hypoglossum* shown that the lowest seed germination rate with 2% in control group, while *R. aculeatus* had the highest seed germination rate with 42% at 30 minutes ozone treatment. *R. hypoglossum* has the lowest germination rate without ozone treatment but shows substantial improvement with increased ozone exposure. *D.*

*racemosa* shows moderate improvement with longer ozone treatments, but the changes are not as pronounced as in *R. aculeatus* and *R. hypoglossum* (Table 1).

**Table 1.** Effects of ozone treatment and species interaction on seed germination

Species	Ozone treatment (min.)	Germination rate (%)
<i>R. aculeatus</i>	0	16.0 bcd (15.58)
	15	32.0 ab (32.44)
	30	42.0 a (38.65)
<i>R. hypoglossum</i>	0	2.0 d (2.65)
	15	16.0 bcd (17.08)
	30	28.0 ab (31.52)
<i>D. racemosa</i>	0	10.0 cd (9.0)
	15	18.0 bcd (18.23)
	30	24.0 abc (21.92)

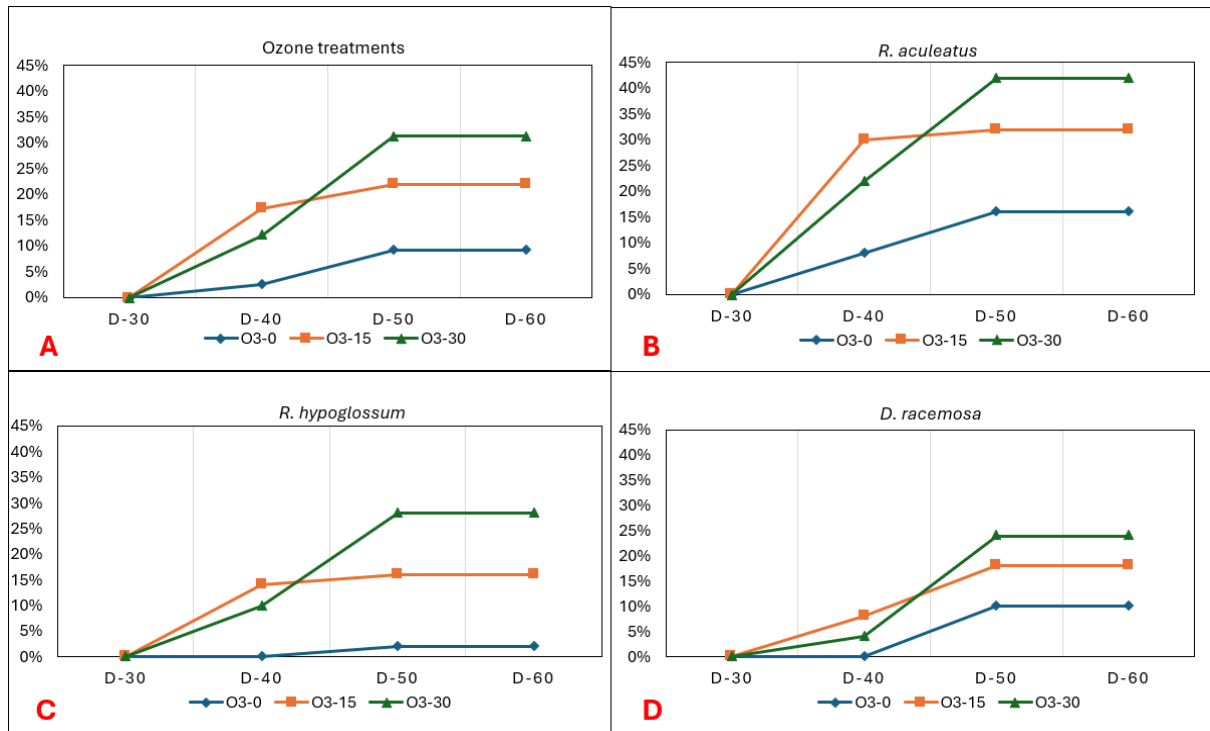
Levels not connected to same letter are significantly different ( $P < 0.05$ ), LSDspecies\*ozone: 7.487 and Arc-sin transformed values shown in parentheses.

Overall, ozone treatments showed that the germination rates increase for all treatments by the D-60. The highest germination rate is observed with the O<sub>3</sub>-30 treatment, followed by O<sub>3</sub>-15, while O<sub>3</sub>-0 remains the lowest. At day 50, the germination rates continued to rise, with O<sub>3</sub>-30 showing the highest increase, followed by O<sub>3</sub>-15, and O<sub>3</sub>-0 showing the least increase. By day 60, the germination

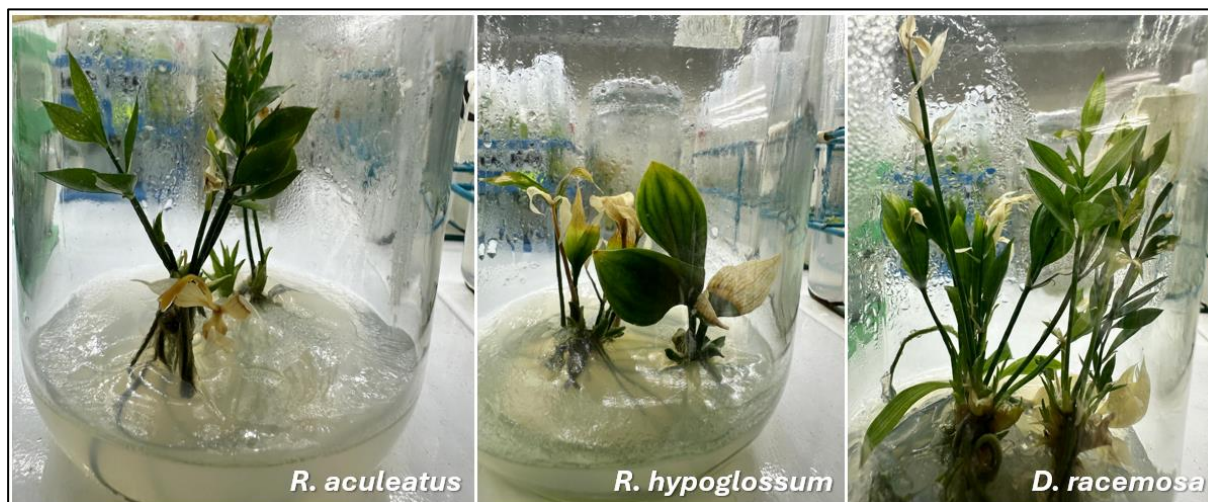
rates reached plateau. O<sub>3</sub>-30 maintains the highest germination rate, followed by O<sub>3</sub>-15, and O<sub>3</sub>-0 shows the lowest rate. It was found that the longer ozone treatments lead to higher germination rates over time, with the most significant increases observed for the 30-minute treatment (O<sub>3</sub>-30) (Figure 4A).

In germination rates of *R. aculeatus*, significant increases were observed by the D-40, with O<sub>3</sub>-30 showing the highest rate, followed by O<sub>3</sub>-15, and O<sub>3</sub>-0 showing the least increase. At day 50, O<sub>3</sub>-30 continues to show the highest germination rate, followed closely by O<sub>3</sub>-15, with O<sub>3</sub>-0 lagging. By day 60, the germination rates for O<sub>3</sub>-30 and O<sub>3</sub>-15 remain high, while O<sub>3</sub>-0 shows minimal increase (Figure 4B). Similar results were recorded for *R. hypoglossum* (Figure 4C) and *D. racemosa* (Figure 4D).

*R. aculeatus* and *R. hypoglossum* exhibit significant improvements in germination rates with increased ozone treatment durations. *D. racemosa* shows a moderate response to ozone treatments, with the highest rates at 60 days. These findings highlight the positive impact of ozone treatments on seed germination rates, particularly for *R. aculeatus* and *R. hypoglossum*. The data suggests that ozone exposure can be a beneficial treatment to enhance germination, with optimal durations varying slightly among different species. After seed germination, the shoots continued to be cultured in the same nutrient medium and rooting and tillering were observed in the shoots at 8 months after seed germination (Figure 5).



**Figure 4.** Effects of ozone treatments in *in vitro* germination 30, 40, 50, and 60 days after culture initiation, A) overall effects of ozone, B) seed germination by the time in *R. aculeatus*, C) in *R. hypoglossum*, D) in *D. racemosa*.



**Figure 5.** *In vitro* grown seedlings from left to right *R. aculeatus*, *R. hypoglossum* and *D. racemosa*.

The effects of different chemicals on seed germination of *Ruscus* species have been previously studied by many researchers. Research conducted by Kebeli (2021) reported that the germination rate, which was 50% in control, increased to 68% with 8 mg/L  $\text{KNO}_3$  pre-application, to 65% with 100  $\mu\text{M}$  SNP pre-application and to 64% with 48 h 1500 ppm permalink, immersion pre-application in *R. aculeatus*. The first germination was reported at 50<sup>th</sup> day after seed sown. Özden et al. (2016) reported that the highest germination rate of 65% was obtained from seeds kept in  $\text{KNO}_3$  (4 mg) solution for 6 hours in *R. aculeatus*, while in *R. hypoglossum*, the highest germination rate of 58.7% was obtained from 1000 ppm  $\text{GA}_3$  application. It was determined that there was no germination in both control and  $\text{H}_2\text{SO}_4$  (2 and 4 minutes) applications. The researchers indicated that the first germinations were observed on the 50<sup>th</sup> day in *R. aculeatus* and on the 40<sup>th</sup> day in *R. hypoglossum*. Banciu and Aiftimie-Păunescu (2012), indicated that *in vitro* seed germination of *R. aculeatus* was notably slow, taking six months to occur in dark conditions. On MS medium without hormones, 60% of seeds germinated after this six-month period. However, when the MS medium was supplemented with  $\text{GA}_3$ , the germination rate increased to 85% over the same timeframe. These studies show that the germination rates and germination times of the species vary according to treatments. Halada and Erdelská (2005) reported that the dormancy of *R. hypoglossum* seeds lasts one year. However, in our study, germination rates were lower than in previous studies. This was probably due to some of the seeds losing their viability during the storage for 4 years. On the other hand, these findings highlight the positive impact of ozone treatments on seed germination rates, particularly for *R. aculeatus* and *R. hypoglossum*.

The beneficial effect of ozone treatment on seed germination were also reported in different species such as cyclamen (Tütüncü, 2022), barley (Dong et al., 2022), winter wheat (Avdeeva et al., 2018), corn (Violleau et al., 2007) and tomato (Sudhakar et al., 2011). It is

hypothesized that the application of  $\text{O}_3$  plays a crucial role in speeding up seed germination by prematurely breaking dormancy, which is linked to a decreased level of ABA in seeds treated with  $\text{O}_3$  (Sudhakar et al., 2011). However, it is reported that ozone treatments can have negative effects depending on concentration in species such as vetch (Uslu et al., 2022) and alpine plants (Abeli et al., 2017). Therefore, it is crucial to determine optimum concentration and duration.

#### 4. Conclusion

In this study, we assessed the effects of different durations of ozone treatments on stored seeds of *R. aculeatus*, *R. hypoglossum*, and *D. racemosa* for four years. The results of the study show that ozone treatments lasting 30 minutes result in increased germination rates for all species over time, while the control group ( $\text{O}_3\text{-0}$ ) consistently shows the lowest germination rates. The data suggests that ozone exposure can be a beneficial treatment to enhance germination, with optimal durations varying slightly among different species. In conclusion, ozone treatments can be suggested as environmentally friendly and inexpensive methods to break dormancy and enhance germination in the seed germination process.

# Author Contributions

The percentage of the author(s) contributions is presented below. All authors reviewed and approved the final version of the manuscript.

	M.T.	M.A.	F.K.	F.G.Ç.	Ö.Ş.
C	40	20	10	10	20
D	50				50
S	40			30	30
DCP	40	30	10	10	10
DAI	50				50
L	20	20	20	20	20
W	50				50
CR	20	20	20	20	20
SR	20	20	20	20	20
PM	20	20	20	20	20
FA	20	20	20	20	20

C=Concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

# Conflict of Interest

The authors declared that there is no conflict of interest.

# Ethical Consideration

Ethics committee approval was not required for this study because of there was no study on animals or humans.

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