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# Reproductive biology of *Centaurea lydia* Boiss. (Asteraceae)

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**Abstract**

*Centaurea lydia*, an endemic species belonging to the Asteraceae family, has a limited distribution in Turkey and is currently listed in the LR/cd category in the Red Data Book of Turkish Plants. This study, conducted in 2024, examined the impacts of wildfires in Bornova, İzmir, on the species and its reproductive biology. Key factors such as flower morphology, pollen viability, stigma receptivity, seed production success, and germination rates were evaluated.

The results showed that the species exhibits protandry and relies on insect pollinators for effective reproduction. It has a self-incompatible pollination system and a fertile seed ratio of 40.86%. The high seedling establishment rate of fertile seeds is a significant advantage that enhances the reproductive capacity of the species. However, despite the high seedling success, the wildfires in İzmir have caused severe habitat loss, placing the *Centaurea lydia* population at considerable risk. Therefore, it is suggested that the conservation status of the species should be elevated to "Vulnerable".

**Keywords:** *Centaurea*, secondary pollen presentation, conservation, reproduction

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## *Centaurea lydia* Boiss'in (Asteraceae) üreme biyolojisi

**Özet**

Asteraceae ailesine ait endemik bir tür olan *Centaurea lydia*, Türkiye'de dar bir yayılış alanına sahiptir ve IUCN'e göre LR/cd kategorisinde yer almaktadır. Bu çalışma, 2024 yılında İzmir Bornova'daki yangınların tür üzerindeki etkilerini ve üreme biyolojisini incelemiştir. Çiçek morfolojisi, polen canlılığı, stigma alıcılığı, tohum üretim başarısı ve çimlenme oranları gibi faktörler değerlendirilmiştir.

Elde edilen sonuçlara göre, tür protandrik bir yapıya sahip olup etkin tozlaşma için böceklere bağımlıdır. Kendine uyumsuz bir tozlaşma sistemi sergileyen türde fertil tohum oranı %40.86 olarak belirlenmiştir. Fertil tohumların fide oluşturma oranının oldukça yüksek olması, türün üreme kapasitesini artıran önemli bir avantajdır. Ancak, yüksek fide başarısına rağmen, İzmir'deki yangınların türün habitatında ciddi kayıplara yol açması, *Centaurea lydia* popülasyonunun risk altında olduğunu göstermektedir. Bu nedenle, türün mevcut koruma statüsünün "Vulnerable" seviyesine yükseltilmesi gerektiği düşünülmektedir.

**Anahtar kelimeler:** *Centaurea*, ikincil polen sunumu, koruma, üreme

## 1. Introduction

The Asteraceae Bercht. & J.Presl family is the largest plant family in the world, comprising 1,707 accepted genera and over 25,000 species. Among these, *Centaurea* L., the fourth largest genus in the family, consists of 771 species distributed across the Palearctic, Afrotropical, and Oriental biogeographic regions [1]. Approximately 23% of all *Centaurea* species are naturally distributed in Turkey, where the genus is represented by 221 species. Of these taxa, 134 are endemic and endemism rate of these genus for Türkiye is 60.63% [2,26].

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Approximately 40% of endemic plant species in the Mediterranean Basin are confined to highly restricted areas [3]. Species with restricted distributions often exhibit low genetic diversity at the population level due to genetic drift, reduced individual fitness, and inbreeding depression [4]. Understanding the reproductive biology of endemic species is a critical step in elucidating the causes of their narrow distribution. Studies have demonstrated that insights into floral morphology and biology can enhance our understanding of plant pollination ecology [5-7]. Floral biology represents adaptations to various modes of pollination and is closely associated with reproductive systems [8].

Unraveling the physiological and molecular processes occurring during pollen-stigma interactions is a key goal for researchers working with plants, as it provides insights into their reproductive systems. Ultimately, this fundamental biological process is essential for food production, upon which we all depend either directly or indirectly [9]. Pollen viability refers to the ability of pollen grains to germinate and produce a growing pollen tube on a receptive stigma or pistil while maintaining survival in the environment [10]. Pollination, as an outcome of reproductive system studies, has been highlighted for its significance in understanding seed germination and seedling development, which are critical for the conservation of endangered species. This is particularly important for endemic species, as their potential for germination and seedling establishment provides key insights into their future survival [11].

In Turkey, 111 *Centaurea* taxa are reported to be under threat according to the Red Data Book of Turkish Plants. Based on IUCN (International Union for Conservation of Nature) threat categories, six species are classified as Critically Endangered (CR), 23 as Endangered (EN), 28 as Vulnerable (VU), 10 as Lower Risk/Near Threatened (LR(nt)), 11 as Lower Risk/Conservation Dependent (LR(cd)), 23 as Lower Risk/Least Concern (LR(lc)), and 10 as Data Deficient (DD) [12]. Research on the reproductive biology and systems of these taxa, a critical stage in their life cycle, has been limited to a few species, such as *Centaurea tchihatcheffii* Fisch. & C.A.Mey., *C. amanea* Boiss. & Balansa, and *C. kilaea* Boiss. [13-15].

The western and southwestern regions of Turkey, under the influence of the Mediterranean climate, are particularly vulnerable to forest fire events. Moreover, large areas of vegetation are destroyed by high rates of forest fires in these regions every year [24]. In Izmir, one of the most important areas where *Centaurea lydia* is distributed, 13,547 hectares of forest and maquis have been burned in the last 10 years due to forest fires. A total of 1,481 forest fires broke out between January and July 2024 alone, and 6,433 hectares of forest were damaged. In 2024, during the vegetation period of the species, where the *C. lydia* population is most dense, 3 fires occurred in Yamanlar Mountain, 3 in the Homeros Valley, and 1 in the Sabuncubeli Pass. Although we cannot access current forest fire data, we know that a large portion of the populations in these regions were damaged.

This study focuses on the endemic *C. lydia*, which is categorized as LR(cd) and suffered extensive habitat loss due to wildfires in 2024. The study aims to evaluate the species' current status by presenting its functional floral morphology and phenology, comprehensive assessments of pollen viability and stigma receptivity, reproductive success, seed germination studies, and the main factors threatening the species.

## 2. Materials and methods

### Materials

Specimens of *Centaurea lydia* were chosen from the Homeros Valley in İzmir, Bornova, Türkiye. The flower and fruit samples to be studied were carefully selected, labeled and their flowers photographed, and collected for testing at various stages of development throughout May and June 2024. (accesion numbers Ege 44318, 38°30’04.88’’N, 27°13’11.16’’E WGS84; approximately 825 m a.s.l). The study areas have a warm and temperate climate; The average annual temperature, sunshine duration, rainy day and rainfall amount are 18 °C (max: 22.7 °C, min: 13.6 °C) , 8.1 hours, 77.8 days and 712.1 mm, respectively [16].

### Methods

#### Functional floral morphology and Phenology

Flower samples were randomly selected from different plants homogeneously from the plant's distribution area, and a total of 50 flower samples from each flower stage were collected and preserved in Formalin Acetic Alcohol (FAA: acetic acid 5%, formaldehyde 5%, and ethanol 90%) solution and transported to the laboratory. To examine the structural features of the flowers, the samples were examined under an Olymus SZ61 stereomicroscope and measurements were made with a Dino-eye AM7025X camera. To understand the functional characteristics of flowers, phenological stages were observed in their natural environment. The changes in the flowers from bud to full bloom were carefully monitored. In particular, protandry characteristics, pollen presentation and stigma receptivity were examined. Measurements of flower parts were made at each stage in laboratory.

#### Stigma Receptivity and Pollen Viability Tests

All experiments were carried out in a laboratory where temperature, light and humidity values were constant in order to obtain standard data. The temperature of the laboratory where the experiment was carried out was fixed at 25°C,

the illumination amount was 1500 lumens and the humidity rate was 50%. In each of the tests, 30 anther and 30 stigma samples were taken for each flower stage.

***p-NPA Test for Detection of Esterase Activity***

To detect esterase activity in pistils, p-Nitrophenyl Acetate (Sigma, N8130) was dissolved in methanol and stored in the refrigerator. Prior to use, the p-NPA solution was diluted with ultra-pure water (at a ratio of 1:100) to obtain a working solution with a concentration of 3.5 mM [17,18]. Pistils were incubated in the working solution for an average of 5 minutes and then photographed under a microscope. Stigmas showing yellow or dark coloration were considered positive for esterase activity.

***Evans Blue Test for Control of Plasma Memrane Permeability***

To determine stigma permeability, a solution was prepared by dissolving Evans blue (Sigma E2129) in distilled water at a rate of 1%. The stigmas were kept in the solution for 5 min, then washed with 3 series of distilled water and photographed. It was shown that there was no plasma membrane permeability in the cells in the blue-colored areas of the tissues [36].

***MTT Test for Dehydrogenase Detection***

MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, Sigma M2128, 10mg) was dissolved in a 5% sucrose solution and stored as a stock solution in a dark environment at 2-8°C. The stigma sample was directly immersed in the reagent within an Eppendorf tube. For pollen samples, 5-10 µl of reagent droplets were placed on a slide, allowed to dry, and the process was repeated. After 10 minutes, pollen grains stained dark purple were considered viable, and stigmas were considered receptive [19].

***TMB Test for ROS/$H_2O_2$ Detection***

ROS/$H_2O_2$ localization was performed by immersing stigmas and pollen in a solution containing the ROS indicator dye TMB (3,39,5,59-tetramethylbenzidine-HCl Sigma T-8768, TRIS-acetate (Tris(hydroxymethyl)-aminomethane Acetate) TCI T3294 at 0.1 mg/ml, pH 5.0) until a blue color developed [20]. Blue staining indicated $H_2O_2$ production in stigmas and pollen.

***DAB Test for Peroxidase Detection***

The DAB test was prepared by mixing 1% DAB (3,3′-Diaminobenzidine Sigma D12384), 3% $H_2O_2$, and water in 60% ethanol in a V:V:V ratio of 4:11:22. The stigma sample was directly immersed in the reagent within an Eppendorf tube, while the pollen sample was placed in 5-10 µl reagent droplets on a slide, mixed thoroughly to prevent clumping, allowed to dry, and the process was repeated [21]. Pollen grains stained dark brown were considered viable.

**Seed production and TTC Test for Seed Viability**

To calculate the total number of seeds produced, capitula were counted from 30 random individuals. Two weeks after the flowers opened, the total number of tubular and marginal flowers were counted from one capitulum from each individual and the number of developing and non-developing achenes were counted. We determined seed viability via staining with TTC (2,3,5 triphenyltetrazolium chloride Sigma 1.08380) was measured in all seeds before experiments after desiccation and storage. Seeds were imbibed for 12 h, and immersed in 1% aqueous (w/v) TTC at 30 °C for one hour, and viability was assessed by intensity and location of staining [22]. We stained 400 seeds from tubulate and marginal seed. Results were expressed as the percent of seeds that positively reacted to the TTC.

**Seed Germination Succes**

Mature seed samples were collected from 30 randomly selected individuals in June 2024. Before starting the germination tests, 0.5% sodium hypochlorite solution was applied to the seeds for surface sterilization. Seeds were placed on moistened Whatman No. 1 filter paper in a single layer in glass petri dishes and subjected to germination tests at 25°C and 12 h light conditions. Petri dishes were wrapped with aluminum foil for continuous dark period application. 10 seeds were used in each petri dish and the experiments were carried out with 10 replicates. Petri dishes were checked daily and seeds with radicle emergence were accepted as germinated [23]. After 7 days, germinated seeds were transferred to soil vials. Germination Rate (GR) were calculated using the following equations (Bewley and Black, 1994). Where, G: Number of germinated seeds, T: Total number of seeds used. GR (%)= (G /T) x 100

**Reproductive succes**

Thirty capitula were randomly selected to determine the number of seeds and ovules under natural conditions. One month after flowering, all mature capitula were collected and the number of mature and immature fruits in each capitulum was counted. Reproductive success was calculated from the average number of mature fruits and immature fruits produced per capitulum.

**Statistical Analysis**

Comparisons between the bud stage and anthesis stage of the flower parts were performed using Multiple t test analysis in the Graphpad prism 8 program.

**3. Results**

**Functional flower morphology and phenology**

In the field study, capitula are observed in three different forms according to their development. The first form is the bud stage. In capitula at this stage, the upper phyllaries have not yet opened and the petals are not visible (Figure 1a). The second form is the pollination stage, where the terminal phyllaries open and the tubular and marginal flowers open (Figure 1b). The third form is the fruit development stage, where pollination is over and the corollas dry and fall off. (Figure 1c).

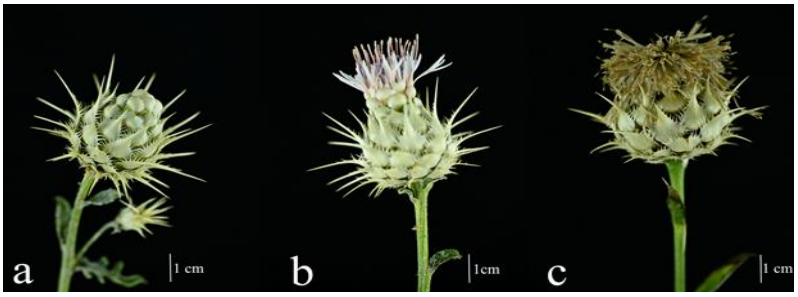


Figure 1. Capitula developmental stages **a)** bud stage **b)** pollination stage **c)** fruit development stage

The capitulum consists of marginal sterile flowers and centrally located tubular actinomorphic fertile flowers. Four key developmental stages were identified in the protandrous tubular fertile flowers, which play a critical role in reproduction. Bud Phase: During this initial stage, introrse anthers tightly surround the style and dehisce along longitudinal slits. As the style elongates, unicellular hairs located at the tip of the style collect pollen from the anthers and carry it upward (Figure 2a, f, k). Pre-anthesis Phase: In this stage, although the corolla tube opens, the rigid, pollen-free apical portion of the anther remains tightly closed, preventing contact between the pollen-laden style and the external environment. The corolla tube and style continue to elongate during this stage (Figure 2b, g). Secondary Pollen Presentation Phase: This stage is characterized by the opening of the rigid apical portion of the anther. The style extends beyond this point, presenting pollen to pollinators via the hairs on the style. Since pollen is presented through the style hairs rather than directly from the anthers, this phenomenon is referred to as secondary pollen presentation (Figure 2c, h). Female Phase: The final stage is the female phase, during which the remaining pollen on the style hairs is shed, and pollen viability decreases significantly. At this stage, the corolla begins to wither (Figure 2d, i, j).

The marginal sterile florets undergo continuous development starting from the bud stage, open their corollas with the blooming of the first tubular florets, and begin to wither during the final stages of the tubular florets (Figure 2e). Measurements associated with the flower developmental stages are presented in Table 2.

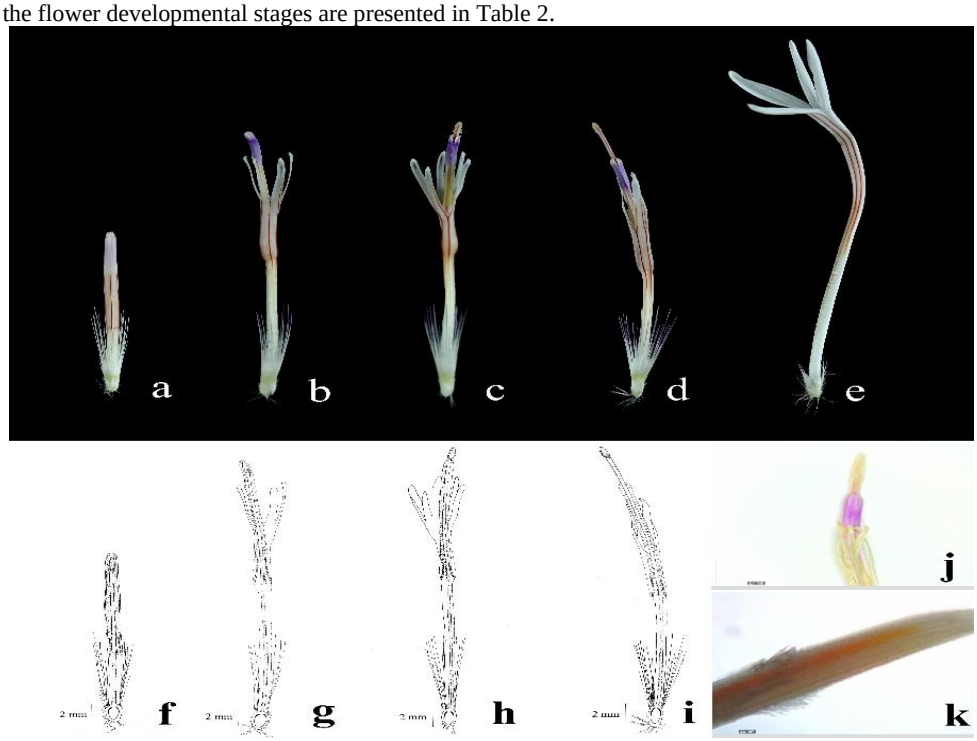


Figure 2. Flower development phases of *Centaurea lydia* **a,f)** 1st stage - bud phase **b,g)** 2nd stage pre-anthesis phase **c,h)** 3rd stage - post anthesis phase (secondary pollen presentation phase) **d,i)** 4th stage - mature pahase (female phase) **e)** marginal sterile tubular flower **j)** female phase **k)** pollen-collecting hairs on style

One of the most remarkable aspects of floral development is the increase in the length of the corolla tube and style. The corolla tube elongates rapidly during the transition from the bud stage to the anthesis stage. This rapid elongation of the corolla tube occurs simultaneously with the rapid elongation of the style (Table 2). The rapid elongation of these two structures prior to anthesis is critical for the loading of pollen grains from the anthers onto the style hairs. During the secondary pollen presentation stage, when the elongation of the corolla tube slows down, the style continues to elongate, surpassing the corolla and presenting the pollen adhered to the style to pollinators (Figure 3, Table 1).

Table 1. Flower measurements depending on development stages

| | Flower development stages | | | |
|---|---|---|---|---|
| Character | 1st | 2nd | 3rd | 4th |
| Corolla length (mm) | 20.95 ± 0.64 | 37.05 ± 0.44 | 38.55 ± 0.44 | 39 ± 0 |
| Corolla lobe (mm) | 7 ± 0 | 7 ± 0 | 7 ± 0 | 7 ± 0 |
| Corolla tube (mm) | 10.45 ± 0.64 | 10.55 ± 0.44 | 10 ± 0 | 10 ± 0 |
| Corolla throat (mm) | 3,5 ± 0 | 20 ± 0 | 21.55 ± 0.44 | 22 ± 0 |
| Papus length (mm) | 10.6 ± 0.7 | 10.75 ± 0.79 | 12.45 ± 0.44 | 13.05 ± 0.5 |
| Achene length (mm) | 2 ± 0 | 3 ± 0 | 3 ± 0 | 3.7 ± 0.26 |
| Anther length (mm) | 12.45 ± 0.37 | 13 ± 0 | 13 ± 0 | 13 ± 0 |
| Anther pollen-free part length (mm) | 5.25 ± 0.42 | 6 ± 0 | 6 ± 0 | 6 ± 0 |
| Anther pollen part length (mm) | 7 ± 0 | 7 ± 0 | 7 ± 0 | 7 ± 0 |
| Filament length (mm) | 3.5 ± 0 | 7 ± 0 | 7 ± 0 | 7 ± 0 |
| Stamen length (mm) | 15.95 ± 0.37 | 20 ± 0 | 20 ± 0 | 20 ± 0 |
| Style length (mm) | 16.2 ± 0.59 | 33.55 ± 0.44 | 40.65 ± 0.41 | 42.2 ± 0.42 |
| Stigma length (mm) | 3 ± 0 | 3 ± 0 | 3 ± 0 | 3 ± 0 |
| Pistil length (mm) | 21.2 ± 0.59 | 39.55 ± 0.44 | 46.65 ± 0.41 | 48.9 ± 0.61 |

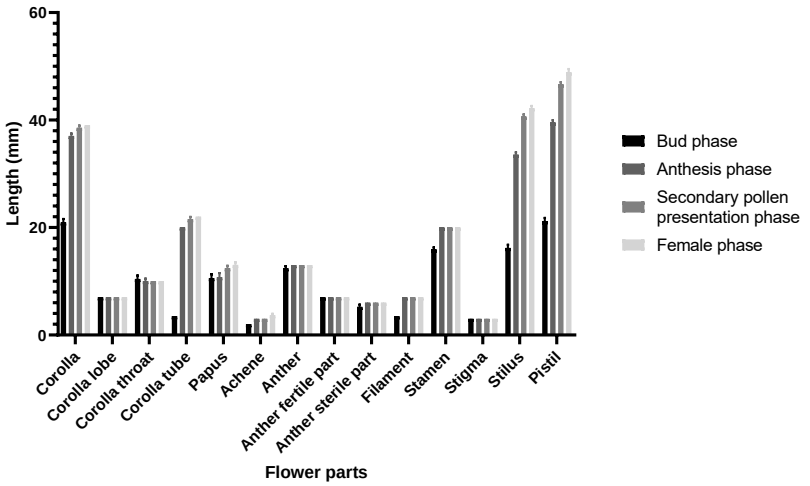


Figure 3. The sizes of flower parts according to flower development stages

Table 2. Comparison of the development of flower parts between the flower bud stage and anthesis stage in C. lydia using Multiple T tests (P value <0.05 is significant)

| Flower parts | Discovery? | P value | Mean of bud phase (mm) | Mean of anhesis phase (mm) | Difference | SE of differerence | t ratio | q value |
|---|---|---|---|---|---|---|---|---|
| Corolla | Yes | <0,000001 | 20,95 | 37,05 | -16,10 | 0,2461 | 65,43 | <0,000001 |
| Anther | Yes | 0,000173 | 12,45 | 13,00 | -0,5500 | 0,1167 | 4,714 | 0,000058 |
| Anther sterile part | Yes | 0,000027 | 5,250 | 6,000 | -0,7500 | 0,1344 | 5,582 | 0,000011 |
| Stamen | Yes | <0,000001 | 15,95 | 20,00 | -4,050 | 0,1167 | 34,71 | <0,000001 |
| Stilus | Yes | <0,000001 | 16,20 | 33,55 | -17,35 | 0,2315 | 74,93 | <0,000001 |
| Pistil | Yes | <0,000001 | 21,20 | 39,55 | -18,35 | 0,2315 | 79,25 | <0,000001 |

Capitula buds are observed from the last week of April to June and the capitula in bud form mature in about 1 week and begin the anthesis process. The pollination phase of the species takes place in a 1.5-month period from the beginning of May to the middle of June, and fruit development lasts 2 weeks after pollination. The matured seeds are dispersed into nature 1 week after fruit development (Table 3).

Table 3. Flower phenology observations of *Centaurea Lydia*

| | April | | | | May | | | | June | | | | July | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Weeks | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
| Bud stage | | | | | | | | | | | | | | | | |
| Pollination stage | | | | | | | | | | | | | | | | |
| Fruit development stage | | | | | | | | | | | | | | | | |
| Seed dispersal | | | | | | | | | | | | | | | | |

**Stigma Receptivity and Pollen Viability Tests**

Esterase activity begins in the stigmas at the bud stage and continues slowly until anthesis. After anthesis, an increase in esterase activity is observed. As esterase activity increases, the structure of the plasma membrane of stigma cells degenerates and cell permeability decreases. NADPH-dependent dehydrogenase activity of stigmata, which is directly related to respiration, is not seen at the bud stage. It shows a gradual increase in respiration in stigmas before anthesis. The activity of peroxidases is also observed in the flower bud stage to eliminate toxic effects by scavenging reactive oxygen species (ROS) formed as a result of respiration and increases with anthesis. ROS-$H_2O_2$, which is an important signal molecule for pollen germination in the stigma, shows a sudden increase before anthesis, reaches the highest level with the anthesis of the flower, and then declines again with the maturity stage. All tests indicate that stigma receptivity occurs at stages after the bud stage and that stigma receptivity is highest at the anthesis stage. The results of the tests are given in Table 4 and Figure 4.

It was determined that pollen grains were viable from the bud stage to the maturity stage. It was determined that pollen viability was highest in the pre-anthesis stage (dehydrogenase 78.17% and Peroxidase 82.75) and lowest in the bud stage. The observation of ROS-$H_2O_2$ in pollen at all stages except the bud stage shows that even if the pollen is alive, it has the ability to germinate only at the pre-anthesis and post-anthesis stages. The germination ability of pollen grains was also determined to be at the post-anthesis stage (71.83%). The results of the tests are given in Table 5 and Figure 4.

Table 4. Chromogenic enzyme activity test results applied to stigma depending on flower development stages

| Flower development stage | Esterase activity | Permeability | Dehydrogenase activity | Peroxidase activity | Peroxidase activity and endogen $H_2O_2$ |
|---|---|---|---|---|---|
| (1) Bud | + | + | - | + | - |
| (2) Pre-anthesis | + | + | + | + | ++ |
| (3) Post-anthesis | ++ | ++ | ++ | ++ | +++ |
| (4) Maturity | ++ | +++ | +++ | ++ | ++ |

(-) no reaction; (+) weak reaction; (++) medium reaction; (+++) strong reaction.

Table 5. Chromogenic enzyme activity test results applied to pollen grains depending on flower development stages

| Flower development stage | Dehydrogenase activity | Peroxidase activity | Peroxidase activity and endogen $H_2O_2$ |
|---|---|---|---|
| (1) Bud | 37,23 ±5,56 | 49 ±3,34 | 0 |
| (2) Pre-anthesis | 78,17 ±7,56 | 82,70 ±5,09 | 71,83 ±4,18 |
| (3) Post-anthesis | 71,57 ±5,49 | 75,27 ±4,58 | 28,57 ±3,5 |
| (4) Maturity | All the pollen has spread | | |

**Seed production and TTC Test for Seed Viability**

An individual produces approximately 42.6 capitula and approximately 84.01% of the flowers produced in these capitulums consist of tubular flowers and 15.99% consists of infundibular flowers. It was determined that 81.61% of the achenes of tubular flowers and 97.38% of the achenes of marginal flowers developed approximately 1 week after flowering (Figure 5, Table 6). In the seed viability test (TTC) carried out on developed achenes, it was determined that the seeds in the infundibular flower achenes were completely sterile and 59.5% of the seeds in the tubular flowers were viable (Figure 6, Table 7).

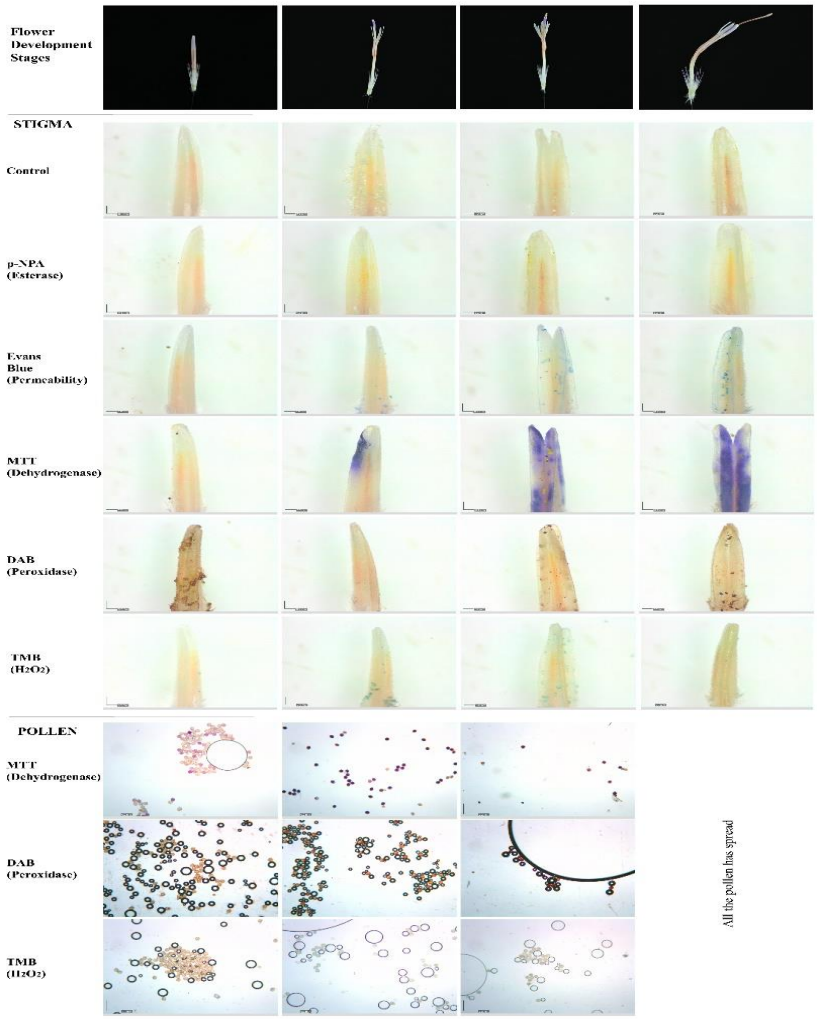


Figure 4. Patterns of chromogenic enzyme activity tests in stigma and pollen depending on flower development stages in *Centaurea lydia*.


Figure 5. *C. lydia* flowers and achenes. a) tubular b) marginal

Table 6. Flower and seed production of *C. lydia*

| Character | Mean | Min-Max |
|---|---|---|
| Capitula in a plant (C) | 42.6 ± 24.18 | 16–92 |
| Total flowers in a capitula (CF) | 128.03 ± 20.77 | 101–171 |
| Tubular flowers in a capitula (T) | 107.86 ± 18.76 | 85–148 |
| Infundibular flowers in a capitula (I) | 20.17 ± 2.15 | 16–24 |
| Developing tubular flower achenes per capitula (TD) | 88.03 ± 15.78 | 69–121 |
| Non-developing tubular flower achenes per capitula (TN) | 19.83 ± 3.05 | 16–26 |
| Developing infundibular flower achenes per capitula (ID) | 19.67 ± 1.75 | 17–22 |
| Non-developing infundibular flower achenes per capitula (IN) | 0.53 ± 0.73 | 0–2 |

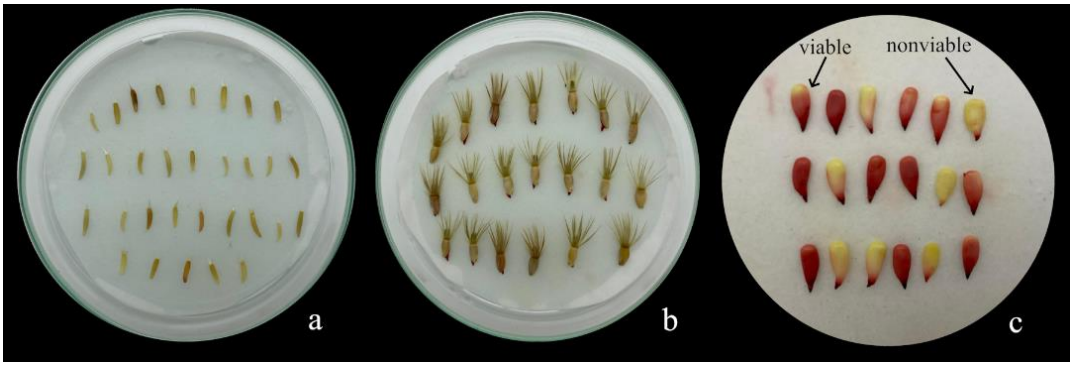


Figure 6. *Centaurea lydia* seed viability tests a) achenes of marginally sterile flowers b) achenes of tubular flowers c) seeds of tubular flowers

Table 7. *Centaurea lydia* seed viability test results

| Seed source | Number of seeds analyzed | Number of viable seeds | Viable seeds (%) | Nonviable seeds (%) |
|---|---|---|---|---|
| Tubular flower achenes (VT) | 400 | 238 | 59,5 | 40,5 |
| Infundibular flower achenes (VI) | 400 | 0 | 0 | 100 |

**Seed Germination Succes**

It was observed that the seeds planted in the petri dish germinated within 2-5 days. At the end of the 5th day, the seed germination rate was determined to be 66% (±15.1). The seeds germinated and began to show their radicles 2 days after the experiment was established. In addition to radicle elongation, hypocotyl region elongation was observed from the 3rd to the 7th day. At the end of the 7th day, there was no loss in the seeds transferred to soil vials. On the 14th day, the seeds that photosynthesize with their cotyledons begin to develop their first leaf primodia and on the 21st day, they begin to develop their second leaf (Figure 7).

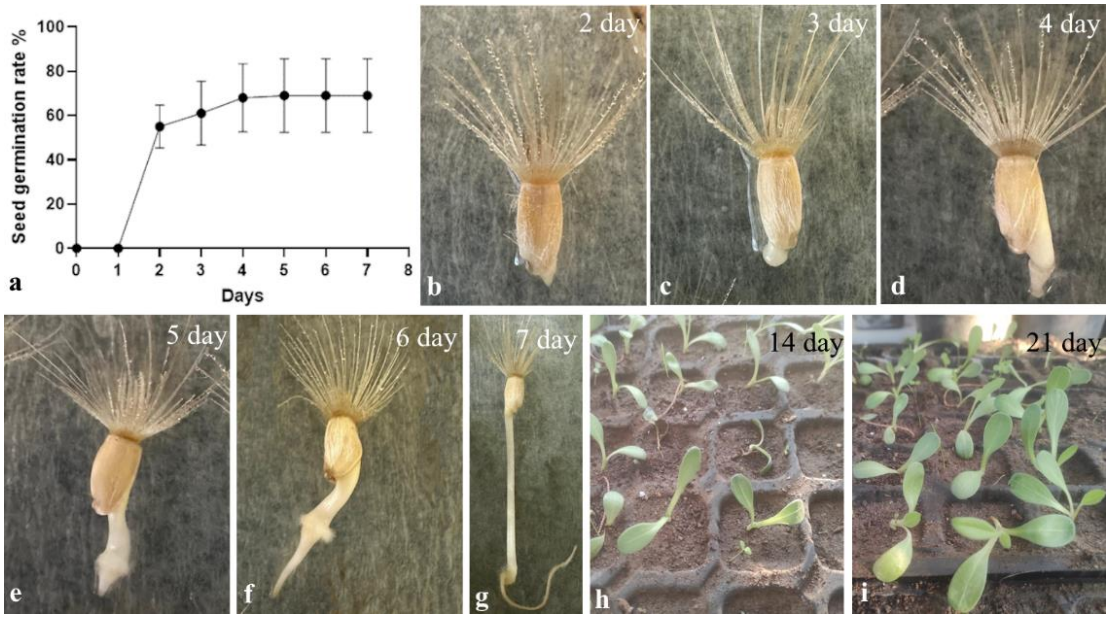


Figure 7. The observation of seed germination **a)** day by day seed germination rate **b)** 2 day **c)** 3 day **d)** 4 day **e)** 5 day **f)** 6 day **g)** 7 day **h)** 14 day **i)** 21 day

**Reproductive succes**

An individual produces approximately 5456 seeds. 40.86% of the seeds produced are fertile. 15.9% of the 59.14% loss in seed development is due to negativities in the pre-zygotic stage, and 43.24% is due to loss of vitality due to negativities in the use of maternal resources after fertilization (Table 8). Despite these failures, no loss was experienced during the transfer of germinated seeds from petri dishes to vials and from there to soil.

Table 8. Reproductive succes of *Centaurea lydia*

| Reproductive success criteria | Calculation formula | Results |
|---|---|---|
| Total Number of Seeds Produced in a Plant | C×(TD+TN+ID+IN) | 5456,36 |
| Number of Fertile Seeds Produced in a Plant | C×((TD×VT/100)+(ID×VI/100)) | 2228,82 |
| Rate of Fertile Seeds Produced in a Plant (%) | (Fertile Seeds Produced in a Plant/Total seeds in a plant) × 100 | 40,86 |
| Number of Sterile Seeds Produced in a Plant | Total seeds in a plant-Fertile Seeds Produced in a Plant | 3227,54 |
| Rate of Sterile Seeds Produced in a Plant (%) | (Sterile Seeds in a Plant/Total seeds in a plant) × 100 | 59,14 |
| Failure Rate Due to Non-Developing Seeds (%) | ((TN+IN)/(T+I))x100 | 15,90 |
| Failure Rate Due to non-viability (%) | (C×(TD×(1-VT/100)+ID×(1-VI/100)))/Total seeds in a plant | 43,24 |

C: Average number of capitula on a plant; T: Average number of tubular flowers per capitula; I: Average number of infundibular flowers per capitula; TD: Average number of developing tubular flower achenes per capitula; TN: Average number of non-developing tubular flower achenes per capitula; ID: Average number of developing infundibular flower achenes per capitula; IN: Average number of non-developing infundibular flower achenes per capitula; VT: Viability rate of developing tubular flower achenes; VI: Viability rate of developing marginal flower achenes

## 4. Conclusions and discussion

In our research on the reproductive system of *C. lydia*, we examined the stigma receptivity and pollen viability tests in detail with multiple chromogenic enzyme activity tests at 4 different stages of flower development. It has often been emphasised that understanding pollen-stigma interactions requires the use of multiple assays [29,30,31,32]. Therefore, in our study, we used multiple assays to investigate pollen viability and stigma receptivity. Among the tests we used, MTT indicated the presence of respiration through dehydrogenase activity, DAB revealed whether ROS produced as a result of respiration were scavenged by peroxidases, TMB detected the presence of $H_2O_2$, which acts as a signalling molecule in pollen germination on stigmas, TMB detected the presence of $H_2O_2$, which acts as a signalling molecule in pollen germination, in stigmas, p-NPA showed at which flower stage the stigma tissue was softened with esterase activity and provided a suitable environment for pollen germination, and Evans Blue showed at which stage the permeability of the stigma cells was reduced and stigma secretions were released for pollen germination. Recent studies have shown that higher levels of $H_2O_2$ than other reactive oxygen species (ROS) are required for pollen grains to germinate on stigmas [33,34,35]. We observed that dehydrogenase activity in *C. lydia* stigmas gradually increased after the second stage of flowering and that peroxidase activity, which functions to scavenge ROS produced during respiration, increased in parallel. The high levels of $H_2O_2$ required for pollen germination in stigmas at all flowering stages except the bud stage indicated that the stigmas were receptive at these stages and that both esterase and permeability in the stigmas were impaired at these stages, suggesting that the stigmas provided a suitable environment for pollen germination. The fact that pollen is viable and the stigma is receptive in the pre-anthesis and post-anthesis stages of the flower suggests that the species is open to both autogamy and allogamy. It was determined that pollination can occur before the flowers start to open and during the secondary pollen presentation. In addition to Hildebrand's [25] secondary pollen presentation mechanism in genus *Centaurea*, measurements were made in detail at 4 flower phases in the species we studied, and showed that the coral tube and style elongation are of critical importance in presentation. Additionally, in this study, which we initiated for ex-situ conservation purposes at the Ege University Botanical Garden with seed samples collected from pre-fire populations, positive results were obtained and the grown seedlings were transferred back to the natural population. In addition, within the scope of the "Collection and Ex Situ Conservation of Rare, Endemic and Threatened Plant Species in İzmir Province" project carried out by the Aegean Agricultural Research Institute, seed samples taken from the mentioned regions were transferred to the National Gene Bank. According to our studies, the fact that the fertile seeds produced by the species with a potential of 40.86% can be obtained as seedlings without any loss shows the high success of the species in reproduction. However, the fact that the Izmir population of *C. lydia* is located very close to the city is the biggest threat to the species, as seen in the fires that occurred in 2024 and before.

The close proximity of the population of *C. lydia* in Izmir to urban areas has resulted in the occurrence of several human-induced fires within its distribution range in 2024 and in previous years. The response of *Centaurea* species to fire and post-fire conditions varies among taxa. In the United States, high-temperature summer fires have been shown to be effective in controlling the invasive species *Centaurea stoebe* by reducing its dominance and decreasing soil levels of the allelopathic compound catechin [27]. A similar outcome was reported in a study by Riba et al. [28], who exposed seeds from 20 *Centaurea* taxa to heat treatments ranging from 70–110°C. While eight taxa exhibited increased germination rates with rising temperatures, the remainder showed decreased germination, with most of these being rare

species. These findings suggest that rare taxa may have limited capacity for post-fire recruitment and establishment. Within this context, fire represents a significant threat to *C. lydia*, a narrow endemic species. A substantial proportion of its population in Izmir has already been affected by fire, and the majority of extant populations are located in close proximity to residential areas, leaving the species continuously vulnerable to future fire events. Given this ongoing threat, it is estimated that at least 10% of the population may be lost within the next 100 years, highlighting the urgent need for a comprehensive population study to realistically assess its conservation status. A comparable reassessment was observed in *Centaurea amanea*, whose conservation status was elevated from Endangered (EN) to Critically Endangered (CR) due to anthropogenic pressures [13]. Although *C. lydia* exhibits relatively high seed germination rates, the inability to protect its habitat from human disturbance undermines the effectiveness of all conservation actions undertaken to date.

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