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AUTECOLOGY AND CONSERVATION BIOLOGY OF *CENTAUREA AMAENA* (ASTERACEAE)

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ABSTRACT

Centaurea amaena is an endemic plant species that is under critical risk of extinction and was known from only one population in Kayseri, Turkey. During our research another locality was found at about 15 km distance from the first known population. In this study, the population pattern of this species and its distribution, the reasons for its narrow range, its pollen viability, stigma receptivity, and the breeding system have been identified, and practical efforts have been carried out for conservation. Pollination experiments showed that *C. amaena* pollination mechanism allows first for allogamy and then secondary pollen presentation and that the timing pollen viability and stigma receptivity are asynchronous. The seed-to-ovule ratio was calculated as 35%. According the tetrazolium staining viability test, the seed viability also was determined as 82.6%. The main factor threatening the species were identified to be anthropogenic-related (including construction, over-grazing, tourism), and some strategies have been developed for the purpose of conservation it.

Keywords: *Centaurea amaena*, Conservation Biology, Endemic, Kayseri, Turkey

INTRODUCTION

Turkey has a unique and rich flora in the neighboring countries because of its geographic location at the crossroads of three phytogeographical regions (Euro-Siberian, Irano-Turanian and Mediterranean), as a bridge between Europe and Asia, its geological history, different climatic conditions, and different topographic conditions (Erik and Tarikahya, 2004). The Mediterranean and Irano-Turanian regions are major gene centers in Western Asia and have contributed greatly to the origin of many cultivated plant species (Ekim, 2006). Additionally, Anatolia is a speciation center of many plant genera and sections. Turkey has nearly 12,000 natural vascular plant taxa, almost 32% of which are endemic (Güner *et al.,* 2012). High percentages (95%) of these are categorized as endangered, rare or threatened. Exacting studies on the conservation biology of such species are required to prevent extinction (Gücel and Seçmen, 2009).

Some studies have been conducted on the reproductive biology and conservation biology in Turkey. Some of these are studies as follows. Gücel and Seçmen (2008) investigated reproductive biology of subalpin endemic *Minuartia nifensis* Mc Neill. Gücel and Seçmen (2009) studied that conservation biology of *Asperula daphneola* O. Schwarz. Seçmen *et al.* (2010) investigated pollination behaviour of *Linum aretioides* Boiss. (Linaceae) and its relations with air temperature and humidity. Subaşı and Güvensen (2011) investigated the reproductive ecology, reproductive system, and reproductive success of the *Salvia smyrnaea* Boiss. Güvensen *et al.* (2013) determined the heterostyly in *Linum aretioides.* Oskay (2017) studied that reproductive biology of the critically endangered endemic plant *Erodium somanum* Peşmen.

The *Centaurea amaena* Boiss. & Balansa growing in Turkey, is one of the ten members that belong to the *Phalolepis* section of the *Centaurea* genus. The *C. amaena* was first collected by Balansa in the year 1856, and then collected by Skřivanek in Kayseri in 1939. *C. amaena* is endemic to the Irano-Turanian region and was known from a single locality at Kayseri (Davis, 1975). Ekim *et al.* (2000) reported the conservation status of the species as EN (endangered). We also updated the threat category of *C. amaena* as CR B2ab(i,iii) (Atasagun *et al.,* 2013).

The reproductive ecology of flowering plants is crucial for conservation, for understanding pollination and breeding systems that regulate the genetic structure of populations, especially with small populations (Tandon *et al.,* 2003). In this study;

a. A geographic distribution map of *C. amaena* was determined.
b. Pollen viability and stigma receptivity, breeding system and seed viability was investigated.
c. Within the framework of practices for conservation studies, efforts were made to make the species live ex-situ and in-situ. The main factors threatening the species were identified.

MATERIALS AND METHODS

Study species and distribution area: The material consisted of *C. amaena* (Sect. *Phalolepis*). It is an endemic, perennial and radiant capitula plant species. The species occurs on the stony slopes of Yılanlı Mountain, located between 38º 38'- 38º 41' N longitudes and 35º 30'- 35º 35' S latitudes to the west of Kayseri. *C. amaena* is grown in an arid environment at a height of 1.170–2.300 m. and is an important member of rock vegetation (Figure 1). In order to determine whether other populations existed in the area, additional suitable habitats for this species were visited. It was determined that *C. amaena* grew in slightly acidic soil including high organic matter and $CaCO_3$ (Atasagun *et al.,* 2013).

During the area investigations, distributions of plant specimens were recorded using GPS. The distribution map of plants obtained from GPS data was drawn using the program ArcGIS 9.3. The number of individuals in the populations was estimated by using long line transect, which was repeated 10 times.

Pollen viability and stigma receptivity: In order to evaluate stigma receptivity and pollen viability, five plants were selected while their flowers were still in the bud stage. One capitulum was cut from these plants every day, and two flowers on the cut capitula were evaluated for each one of the procedures described below. a) three days prior to flower opening; b) two days prior to flower opening; c) one day prior to flower opening; d) day of flower opening; e) one day after flower opening; f) two days after flower opening; g) three days after flower opening; h) four days after flower opening; i) five days after flower opening; and j) six days after flower opening. A total of 100 flowers were used in each experiment.

Each collected flower was separately placed in tube, stored in a cool box and transported to the laboratory within a few hours following collection. In the laboratory during examination each flower was dissected and the anthers and stigmas were removed (Subaşı and Güvensen, 2011).

For the pollen viability tests, the MTT (2.5-diphenyl tetrazolium bromide – also known as thiazolyl blue) method was used. This test detects the presence of dehydrogenase. In the presence of dehydrogenase, the color of pollen grains turns dark purple or black. The grains that were stained were considered as viable; non-colored grains were accepted as unviable. A 1% solution of MTT was mixed with 5% sucrose solution, then used for pollen staining. Pollen preparations were incubated for 30 minutes at 35-37°C. The preparations were examined under a Leica DM750 light microscope by randomly selecting 500 pollen particles per anther (Firmage and Dafni, 2001).

In order to determine stigma receptivity, Macherey-Nagel Peroxtesmo Ko peroxidase test paper was used. One 15×15 mm Peroxtesmo Ko paper was soaked in 1 ml distilled water. A droplet of the fresh solution was applied directly onto the stigma. In the presence of peroxidase, the color of stigma was very dark or blue coloration and those stigmas were considered receptive (Dafni and Maues, 1998). The stigma preparations were examined under stereo microscope. Each experiment was replicated three times, therefore a total of 30 stigmas were used in experiments.

Breeding System: The *C. amaena* pollination type was monitored in June and July of 2011 and 2012. The plants were randomly chosen in the fields. For autogamy, 20 flower heads from different plants were bagged with nylon mesh prior to anthesis without pollination. Free pollination (control) – 20 flower heads were exposed to the natural agents of pollination. The treated and control flowers were monitored and investigated for 4 weeks. The flower heads were harvested at maturation and investigated.

Seed/Ovule ratio: Twenty-five plants were sampled in the site, just before opening and release of both seed and senesced flowers. One capitulum was collected per plant. In each mature capitulum, the number of disk flowers (*nd*) and the number of filled achenes (*na*) were counted. Seed/ovule ratios (*S/O*, equal to fruit/flower ratios) were calculated as *S/O= na/nd* (Colas *et al.,* 2001).

Seed viability: In order to determine seed viability, the tetrazolium test was used. A total of 75 randomly selected achenes were soaked in water overnight. Then, the testas were removed and embryos were placed in groups of 25 into petri dishes and treated with 0.1% tetrazolium chloride. Stained embryos were categorized as viable, and those that had not been stained were considered not viable (Moore, 1985).

Conservation studies: Conservation applications, including ex-situ and in-situ was carried out in two phases. Within the scope of ex-situ conservation, a total of 100 seeds were germinated (April-2012); the germinated seeds were then transferred into containers with perlite, and allowed to grow in a growth cabinet until the seedlings reached a certain size. Once the seedlings reached a certain size, they were transferred into pots and taken to our university's greenhouse. Efforts were then made to keep the plants viable.

Within the scope of in-situ conservation, 200 seeds were planted to the distribution areas of the populations, in October 2011. The germination of the planted seeds was observed and monitored during field studies performed in different periods.

RESULTS

Distribution area: With regard to records in the Flora of Turkey and GAZI Herbarium, the *C. amaena* is a known population on Yılanlı Mountain. The distribution area of *C. amaena* on Yılanlı Mountain covers approximately 0.25 km^2 and the number of individuals are 1347 (Figure 2). During field investigations, a second locality was found at about 15 km distance from *C. amaena* known first locality. This new locality covers about 0.3 km^2 and includes 4325 individuals (Figure 3).

Pollen viability and stigma receptivity: It was determined that the flowers of *C. amaena* fully opened at 7 days, after formation of the capitula. Pollen viability test was assigned as: 46% ± 6.5 three days before the flowers opened, 57% ± 6.2 two days before the flowers opened, 74% ± 5.3 one day before the flowers opened, 86% ± 6 the day of flower fully opened, 78% ± 6 one day after the flowers opened, 64% ± 3 two days after the flowers opened, 50% ± 3.5 three days after the flowers opened, 43% ± 1.8 four days after the flowers opened, 37% ± 2.2 five days after the flowers opened, and 31% ± 1.1 six days after the flowers opened (Figure 4).

The results of stigma receptivity test showed that stigmas were not receptive during the bud stage, but receptivity started at the time of flower opening 1-2 days later and the receptivity period lasted about 5-6 days. In addition, the evaluation of style length along with the stigma receptivity revealed that styli less than 1.5 cm in length displayed no enzymatic activity, while styli longer 1.5 cm displayed enzymatic activity (Figure 5).

Breeding system: The results from pollination experiments showed that fruit set was observed at 15 of 20 bagged capitula for self-pollination and 18 of 20 untouched capitula for control. When the flowering period, the flowers were visited by several insects. Two insect orders including Diptera, Hymenoptera were displayed in the pollinator investigation.

Seed/Ovule ratio: To determine the S/O rate in the collected 25 capitulum, the mean number of disk flowers and filled achenes per capitulum were reported as 33.1 and 11.7, respectively. When the S/O ratio was calculated using these data, the mean of seed fertilization of each capitulum was determined as 35%. According to these results, *C. amaena* seed fertilization rate was low.

Seed viability: With regard to the tetrazolium staining viability test, 62 embryos were stained dark red, while 13 embryos were not stained. According to Handbook on Tetrazolium Testing evaluated, dark red stained embryos were evaluated as viable, while unstained embryos were evaluated as dead. Using these results, seed viability was determined as 82.6 % ± 2.08.

Conservation implications: A total of 100 seeds germinated within the scope of the ex-situ conservation activities were transferred into containers with perlite, and efforts were made to keep the seedlings viable within the growth cabinet. A total of 80 seedlings were transferred into soil brought from the seedlings' natural environments. The seedlings were then taken to the greenhouse within our university, and efforts were made to keep the plants viable. Half of the pots were placed inside the enclosed greenhouse, while the remaining portion was placed in the garden (Figure 6). The growth studies in both environments were unsuccessful, resulting in the death and loss of all plants.

Within the scope of in-situ conservation activities, 200 seeds were planted in the natural environments of the plant (100 in Yılanlı Mountain, and 100 in Perikartın) within the month of October 2011. No germination was observed in the seeds planted during field studies performed on different periods.

Figure 1. General view and habitat of the *C. amaena*.

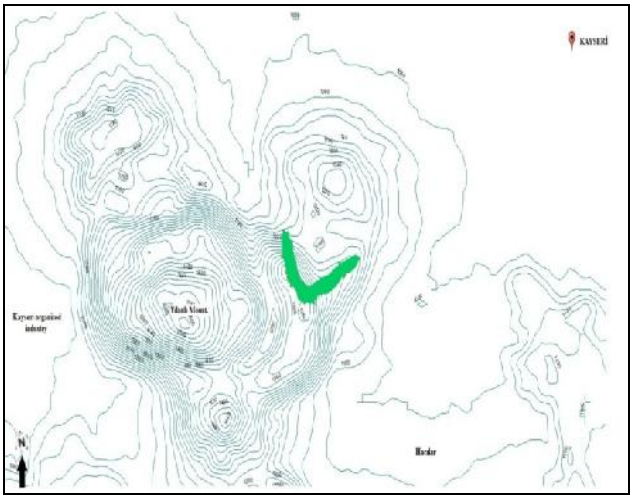

Figure 2. Distribution map of the *C. amaena* at Yılanlı Mount.

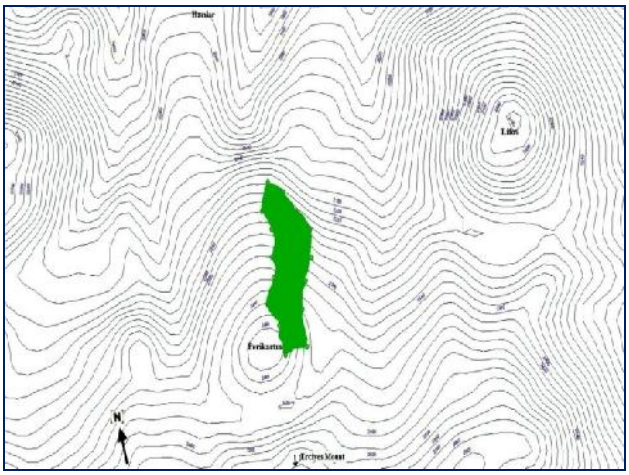

Figure 3. Distribution map of the *C. amaena* at Perikartın region.

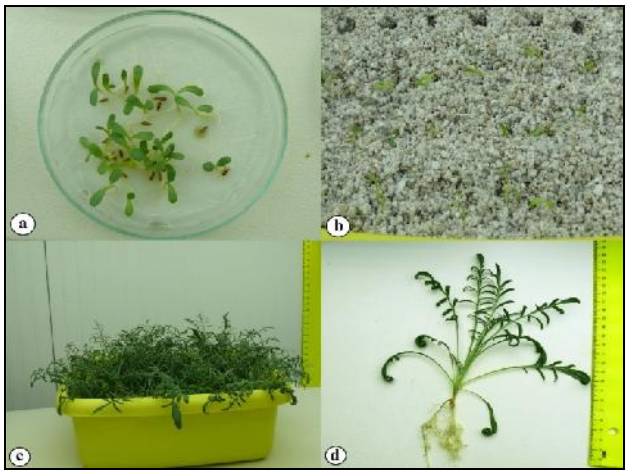

Figure 6. Germination of *C. amaena* seed b-d. Developmental stage of the seedlings.

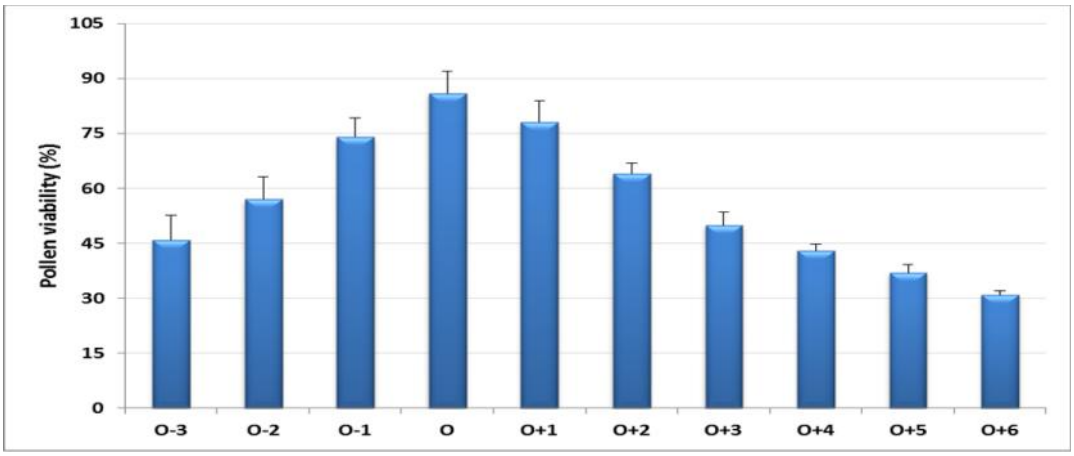

Figure 4. Percent pollen viability of *C. amaena* flowers (O: fully opened, O-3: Three days before opening, O-2: Two days before opening, O-1: One day before opening, O+1: One day after opening, O+2: Two days after opening, O+3: Three days after opening, O+4: Four days after opening, O+5: Five days after opening, O+6: Six days after opening).

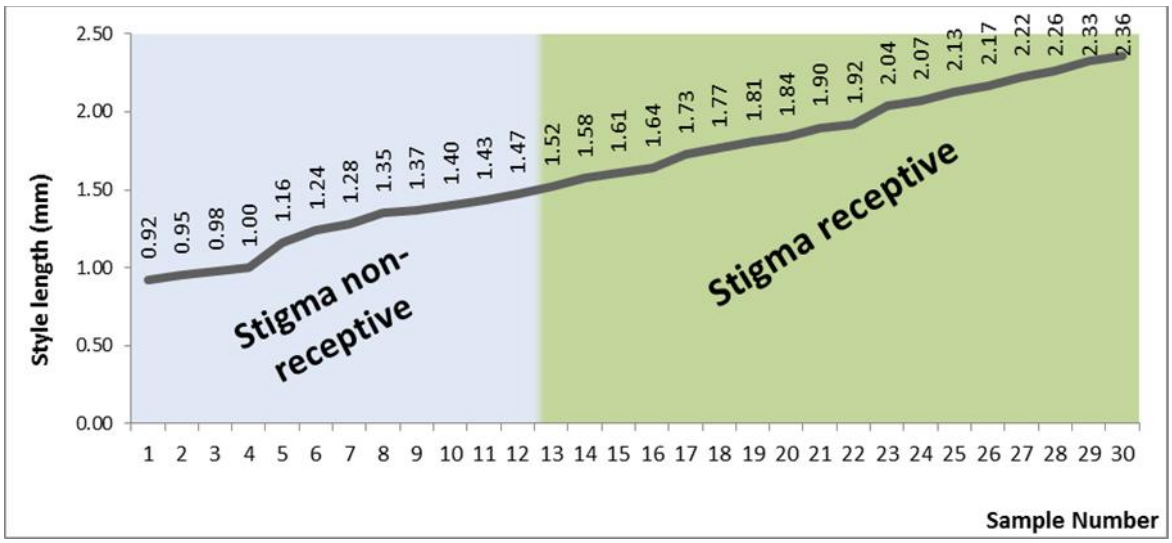

Figure 5. Style length and stigma receptivity.

DISCUSSION

For many endangered plants, rarity may be due to habitat loss, fragmentation or degradation and to the resulting genetic consequences via inbreeding depression, population obstacles and genetic drifts (Weekley and Race, 2001). Environmental and demographic stochasticity have the greatest importance in small populations and can therefore contribute to the risk of extinction of endangered species (Menges, 1992). Hence, to recover of an endangered plant species requires a demographic assessment biological status, the identification of life story, breeding biology, genetics, spatio-temporal distribution patterns, the factors affecting seeds (Schemske *et al.,* 1994). Determining monitoring programs and building quantitative databases for conservation programs are essential to assess future success in maintaining biodiversity (Kaya and Raynal, 2001).

In this study, pollen viability, stigma receptivity, breeding system, S/O ratio and seed viability of the *C. amaena*, an endemic species in Turkey, were investigated. The results presented here indicate that the pollen viability of *C. amaena* reached its peak when the flowers fully opened, then it gradually decreased. Similar to our results, Hong *et al.* (2008) reported that the pollen viability of *Mikania micrantha* H.B.K. began to gradually decrease after the flowers opened. Hiscock (2000) stressed that pollen viability post anthesis in *Senecio squalidus* L. ranged from 16.5 to 53%, with a mean value of 29.6%.

The stigma receptivity test indicated that the receptivity of *C. amaena* begins in 1-2 days after blooming and lasts approximately 5-6 days**.** The stigma surface was not receptive although the pollen viability became the highets. Contrary to our findings, Gücel and Seçmen (2008) stressed that stigma receptivity in *Minuartia nifensis* Mc Meill starts to develop 2 days before the blooming and reached its peak when the flowers fully opened. If the androecium matures before the gynoecium and produces pollens in a hermaphrodite flower, this called protandry (Percival, 1965). In the current study, it was determined that timing pollen viability and stigma receptivity are asynchronous. Anthesis is obviously asynchronous from plant to plant, capitulum to capitulum and floret to floret. It is stressed that pollen viability decreases over time, whereas stigma receptivity increases in protandric species (Rodriguez-Riano and Dafni, 2007). Similar results of protandrous have been observed in previous studies (Young *et al.,* 2002; Wani *et al.,* 2006).

Our pollination experiments with bagged *C. amaena* flowers indicate that the species is allogamous. One study on the pollination biology of *Grindelia covasii* Bartoli et Tortosa (Asteraceae) stated that *G. covasii* exhibited strong xenogamy (Roitman, 1999). The presence of obligate xenogamy has also been reported in other genera of the Asteraceae, for instance, *Senecio* (Abbott and Irwin, 1988) and *Cirsium* (Michaux, 1989). However, it has previously been showed that *Mikania micrantha* was protandrous and had a secondary pollen presentation system which characterizes the family Asteraceae (Hong *et al.,* 2008). Furthermore, Shabir *et al.* (2013) reported that *Inula racemose* Hook was protandrous and the pollen was shed from the anthers onto the sweeping hairs of the style in the bud stage. Howell *et al.* (1993) reported that secondary pollen presentation is a widespread phenomenon that characterizes the Asteraceae family nearly throughout, and it was usually the terminal style that acts as the pollen presenter with active pollen placement. In our pollination experiments seed formation at bagged capitula could be attributed to secondary pollen presentation. Based on the family of the plant, pollination mechanism allowed first for allogamy and then secondary pollen presentation opening. Wyatt (1982) stressed that the protracted asynchronous mode of pollen presentation by the species guarantees the long term availability of the pollen to ensure effective pollination.

One of the factors that threaten *C. amaena* is anthropogenic activities. On Yılanlı Mountain, the main factors threatening the species were determined as to remain in the center of population, be zoned for housing, habitat fragmentation, construction of bond house, over-grazing, and the rock pit activity approximately 500 m from the population.

For the Perikartın population, factors that limited the spread and distribution of this species included the fact that the region figured within the Erciyes master plan; the fact that a ropeway for ski tourism was built immediately next to this population; the fact that flowering took place in August in this region due to the increase in altitude; and the fact that the seeds could not fully mature due to seasonal conditions that became unfavorable while the plant seeds were in germination. Furthermore, it was determined that the seeds of both populations were attacked and eaten by *Oxycarenus* sp. from the Heteroptera suborder. According to Ananthakrishnan *et al.* (1982), adults and nymphs of this bug suck oil from mature seeds and fluid from leaves of young stems to obtain moisture. Slater and Baranowski (1994) stressed that the bug feeds on plants belonging to family Malvaceae, also in Tiliaceae and Sterculiaceae. The fact that seeds were consumed by these insects decreased the chances for new individual plants growing and developing, which in turn negatively affects the development of the population.

In order to conserve the future of the populations, we suggested conservation status of *C. amaena* as CR B2ab(i,iii) according to the IUCN (2001) in our previous study (Atasagun *et al.,* 2013). Moreover, there is an immediate need to reduce anthropogenic

activities on the distribution area of the species. Measures can be taken to prevent the extinction of the species by the following: construction of new buildings around the population should be prohibited, quarry activity should be terminated, ski tourism should be implemented with great care, over-grazing should be controlled, and the species should be protected as in-situ and ex-situ.

This study represents one of the few studies in Turkey on conversation biology. Most of the endemic species are categorized as endangered or threatened in Turkey. For this reason, It has been believed that this study and other similar studies will greatly contribute to the conservation of biodiversity in Turkey.

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