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FLOWER TYPE AFFECTS THE TIMING OF EMBRYO DEVELOPMENT IN NATIVE PRIMULA VULGARIS

Mehmet TÜTÜNCÜ[1]*, Şenay KARABIYIK[2], Başar SEVINDIK[3], Neriman BEYHAN[1]

[1]*Ondokuz Mayıs University, Faculty of Agriculture, Department of Horticulture, 55200, Samsun, Türkiye*
[2]*Çukurova University, Faculty of Agriculture, Department of Horticulture, 01330, Adana, Türkiye*
[3]*Izmir Demokrasi University, Vocational School, 35140, İzmir, Türkiye*

Abstract: In this study, pin and thrum flowered wild primrose (*Primula vulgaris= Syn: Primula acaulis*) populations, which are naturally distributed in Black Sea Region in Türkiye, were cultivated and their pollen viability, pollen tube growth and embryo development stages were investigated. As a result of the study, pollen viability in pollen of the pin flower type was 83.10%, while the *in vitro* germination rate was 69.43%. The viability and *in vitro* germination rates of pollen of thrum flower type were 84.91% and 67.92%, respectively. As a result of the squash preparation examinations, it was observed that the pollen tubes of both types started to germinate on the stigma on the 1 DAP (Day after pollination). On 4 DAP, it was determined that pollen tubes penetrated to the ovule. Embryo developmental stages examinations showed that, zygote formation occurred on 6 DAP in the pin flower type; while on the 20 DAP, the embryo was in the heart stage and finally on the 40 DAP, it was seen that it formed a mature cotyledonary stage embryo. Endosperm in the thrum type started to develop faster than the pin type and formed on the 6 and 7 DAP. In the thrum flower type, the proembryo formed on 10 DAP, but embryo formation could only be seen on 30 DAP. In the ovary examinations performed on the 40 DAP, some embryos were in the cotyledon stage while some were still in the globular or heart stage. It was also determined that no embryos were found in some ovules.

Keywords: Fertilization, Germination, Histology, Primrose, Pollen viability

***Corresponding author:** Ondokuz Mayıs University, Faculty of Agriculture, Department of Horticulture, 55200, Samsun, Türkiye
E mail: mtutuncu.tr01@gmail.com (M. TÜTÜNCÜ)
Mehmet TÜTÜNCÜ https://orcid.org/0000-0003-4354-6620
Şenay KARABIYIK https://orcid.org/0000-0001-8579-6228
Başar SEVINDIK https://orcid.org/0000-0002-1448-6617
Neriman BEYHAN https://orcid.org/0000-0002-1268-197X

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1. Introduction

The genus *Primula* is taxonomically classified in the family Primulaceae and is naturally grown in the temperate climate zone of Europe, South America, Asia and North Africa. (Smith and Forrest, 1929; Jia et al., 2014). The genus is the largest genus of the family but there is no consensus about the number of the species. It is indicated that there are about 400 or more than 500 species in the genus (Zhang and Kadereit, 2004; Yankova-Tsvetkova et al., 2021). Additionally, several new species have been identified in the last decade (Xu et al., 2019). Many species have become popular ornamental plants because attractive flowers bloom in early spring (Jia et al., 2014). Native primroses grown in high altitudes are used by the local people for the treatment of skin wounds as well as their visual beauty (Uce and Tunçtürk, 2014). Prapajati et al. (2003) reported that extracts were obtained from the leaves and rhizomes of *P. veris* L., *P. vulgaris* Huds. and *P. elatior* (L.) Hill widely used since ancient times as a diuretic, antispasmodic, analgesic, antipyretic, expectorant, cough suppressant, sedative, relieving insomnia and in the treatment of colds, acute and chronic bronchitis.

P. vulgaris (Syn: *P. acaulis*) is one of the common primroses and its chromosome number is 22 (2n =22) (Cocker et al., 2018). The species is represented by two subspecies, namely *P. vulgaris* subsp. *vulgaris* (Syn: *P. acaulis* subsp. *acaulis*) and *P. vulgaris* subsp. *sibthorpii* (*P. acaulis* ssp. *rubra*) in Türkiye. The yellow and white flower colors appear in *P. vulgaris* subsp. *vulgaris*, while white flowers and dark purple to pink flower color appear in *P. vulgaris* subsp. *sibthorpii*. Both subspeciesshare the same habitat at altitudes from 500 to 850 m along the eastern Black Sea coast of the country (Gündoğan et al., 2019). *P. vulgaris* exhibits heteromorphic flower types that prevent self-fertilization. The sporophytic self-incompatibility with heteromorphic flower development originating from S alleles is common in primroses (Li et al., 2011; Li et al., 2015; Keller et al., 2016), making these species interesting for researchers. Although observations on the existence of heteromorphic flowers date back, Charles Darwin was the first to realize the importance of this reproductive system. The distylous primroses have been studied since then (Cocker et al., 2018). Distylous primroses have two different flower structures called

'Pin' and 'Thrum'. In the pin flower structures, the stigma is positioned at the mouth of the corolla tube, while the stigma in the thrum flower structure is located close to the flower base and the anthers are located at a higher level (Cahalan and Gliddon, 1985; Li et al., 2011). Long-styled flowered (pin) plants have homozygous s allele (ss), whereas short-styled flowered (thrum) plants have heterozygous dominant S allele (Ss) (Bateson and Gregory, 1905). When the flower structure is observed from outside, it can be understood which flower structure the plant has. The fertilization does not occur between flowers with the same flower structure. However, thrum plants with homozygous S allele (SS) can be rarely obtained from crossing thrumxthrum (Webster and Gilmartin, 2006).

Researchers have conducted many studies on the members of the genus *Primula* to understand this heteromorphic flower structure and its genetic structure. However, a limited number of studies have been carried out on embryo development in pin and thrum flower of primroses. Therefore, it was aimed to reveal whether there is an effect of flower structure on embryo development in primrose after reciprocally crossing pin and thrum flowers.

2. Materials and Methods

2.1. Plant Material

Wild *P. vulgaris* plants were collected from the campus area of Ondokuz Mayıs University, Samsun, Türkiye in the autumn of 2019 and cultivated in pots (2.5 L) containing peat: perlite: sand in the ratio (1:1:1 v/v/v) in an unheated greenhouse. Two different populations were created with at least 20 plants considering flower structure and populations were labeled as pin and thrum. The plants were irrigated once a week in the cool season and twice a week in summer. The cultivated plants started to bloom in January 2020. At the end of January, with the increase of flowering in the plants, crossing studies were started for histological analysis.

2.2. Pollen Collection and Hand Pollination

The emasculation process was carried out in flowering plants within the population. Unopened flower buds were carefully emasculated with forceps to avoid any injury of the stigma just prior to their opening. All previously opened flowers and small immature buds were removed and the emasculated flowers were covered with cotton bags to avoid free pollination. Anthers separated from flower buds and they were placed in separate Petri dishes according to flower type (pin or thrum) (Figure 1a). Anthers brought to the laboratory environment were kept overnight at room temperature for dehischence (Figure 1b). One day later, at anthesis, the emasculated flowers were pollinated with fresh pollen by hand using a small brush and covered with cotton bags immediately (Karabıyık and Eti, 2020). During hand pollination (Figure 1c), crossing was carried out with pollen obtained from thrum flowers in cases where the pin flower type was used as the mother (i.e. pinxthrum) and with pollen obtained from plants with pin flower type when the thrum flower type was the mother (i.e. thrumxpin).

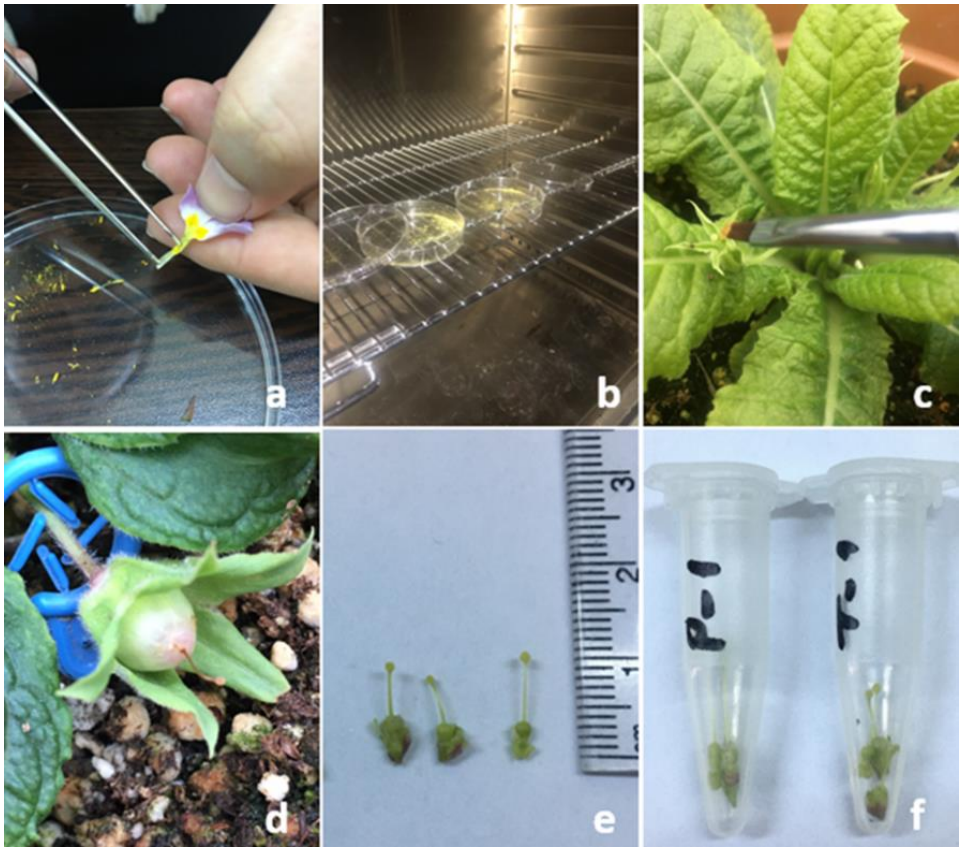

Figure 1. Hand pollination and sample collection in primroses (a: isolation of anthers, b: dehiscence of anthers, c: hand

pollinations, d: pollinated pistils at 40 DAP, e-f: harvesting samples).

2.3. Pollen Viability and *in vitro* Pollen Germination

To obtain fresh pollens, 30 flowers were collected one day before anthesis from each type and brought immediately into the laboratory. The anthers were removed and left to dehiscence at room temperature throughout the night. Pollen viability rates were tested with 1% 2,3,5 Triphenyltetrazolium Chloride. Fresh pollen grains were dispersed homogeneously on a TTC drop with a brush. Then, the top of the drop was covered with a coverglass. In the test, dark red colored pollens were recorded as 'viable' and colorless or very light pinkies as 'non-viable'. The pollen viability rate was obtained by calculating the total value of viable pollens. For each flower type, pollen viability was recorded in 4 slide replications by counting at least 100 tetrads for each replication.

Pollen germination rates were tested with "agar in petri" method with medium consisting 10% sucrose, 1.0 mM $CaCl_2$, 0.16 mM boric acid, and 7g/L agar (Grouh et al., 2015). Pollens which has a pollen tube greater than its diameter was recorded as germinated. Germinated pollens were used for calculating the germination percentage. For each type, germination was recorded in 3 petri dish replications by counting at least 100 tetrads for each replication.

2.4. Experimental Design and Statistical Analysis

Pollen viability and *in vitro* pollen germination tests were designed according toa randomized plot design. TTC test was carried out with 4 replications for each flower type. The germination test was carried out in 3 replications, one repetition for each petri dish. The arc-sine transformation was applied to the percentage values obtained before statistical analysis. All data were subjected to analysis of variance with the JMP (version 8.00) and the significance levels of the means were compared with the LSD ($P<0.01$) test (Genç and Soysal, 2018).

2.5. Histological analysis

Pollen tube growth and embryo developmental stages were investigated after reciprocal crossing between pin and thrum flowering plants according to Tütüncü and Mendi (2020). Emasculated flower buds were pollinated and collected in 24 h intervals until 7th day after pollination (DAP) to scan pollen germination on the stigma, pollen tube growth through the style and penetrating to the ovule. In addition, for paraffin sectioning studies, pollinated pistils were harvested on 10, 20, 30 and 40 DAP to determine zygote/embryo developmental stages. The samples (Figure 1d-e) were fixed immediately in FPA-70 (formaldehyde-propionic acid-alcohol) until examination (Figure 1f).

Pollen tube germination on the stigma, polen tube growth through the pistil and polen tube penetration to the ovules were monitored on squash preparations of pistils, previously softened in 8N sodium hydroxide for 5-7 h, stained with 0.1% aniline blue in 0.1 N K_3PO_4 and observed under a fluorescence microscope (Olympus BX51, Tokyo, Japan) equipped with a U-MWU filter (Olympus, Tokyo, Japan). Pollen tube growth rate was determined as percentage of the style traversed by the longest pollen tube in each pistil by a digital micrograph system (Olympus DP72 camera, Tokyo, Japan) (Karabiyik, 2022).

Paraffin sectioning method was used for determining embryo formation in pistils. Five samples from all fixed ovaries of flower buds and pollinated pistils were dehydrated in ethanol and tert-butanol series and embedded into paraffin (Johansen, 1940). Then the samples were blocked on a wood block and sectioned with a rotary microtome (Leica RM2135, Leica, Wetzlar, Germany) at 10µ. All preparations were stained with 0.125% hematoxylin buffered with $KMnO_4$ and mounted in Entellan (Karabıyık and Eti, 2020). Preparations were observed with fluorescence microscope (Olympus BX51, Tokyo, Japan) equipped with a U-MWU filter (Olympus, Tokyo, Japan) and photos were obtained by a digital micrograph system (Olympus DP72, Tokyo, Japan).

3. Results and Discussion

3.1. Viability and Germination Rates of Pollens

The results showed that, there was no statistically significant difference between pollen viability and *in vitro* pollen germination rates in terms of flower types. According to this, pollen viability in pollen of pin flower type was 83.10%, while *in vitro* germination rate was 69.43%. Pollen viability and in vitro pollen germination rates in the thrum flower type were 84.91% and 67.92%, respectively (Figure 2).

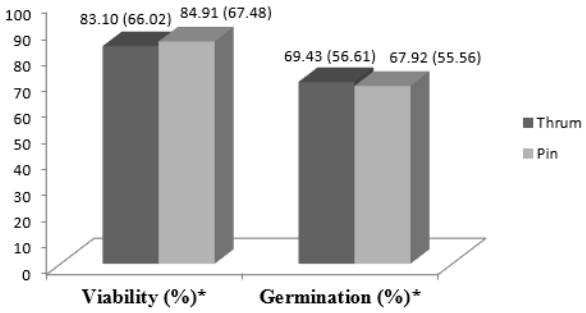

Figure 2. Percentage of pollen viability and *in vitro* pollen germination (*the difference between means was not statistically significant. Arc-sine transformed values are indicated in the parentheses).

Determining pollen viability and *in vitro* pollen germination are significant parameters for the reproductive biology of species. However, they are affected by genotype and environmental factors. In the present study, *in vitro* pollen germination was lower than pollen viability and no statistical differences were found between viability and germination rates of thrum and pin flower structures. In contrast, Aronne et al. (2021) reported that the pollen viability rate of thrum flower was significantly higher than that of the pin flower type

in *P. paliurni*. However, Yankova-Tsvetkova et al. (2021) reported that pollen sterility (unviability) in pin flower structure in *P. bayernii* was found by Gachechiladze (1993), while fertile (viable) pollen grains in both pin and thrum flower formed in their study.

3.2. From Pollen Tube Growth to Embryo Development

Flower samples were taken daily from first to the seventh day after pollination (DAP) on the primrose plant. As a result of the aniline blue staining, it was observed that the pollen tubes of both types started to germinate on the stigma on the 1 DAP (Figure 3a). In the examinations made on the samples of 2 DAP, it was determined that the pollen tubes progressed in style (Figure 3b). Pollen tubes in pin flower type reached to the ovary on the 3 DAP, and some of them penetrated the ovules. The thrum type determined that the pollen tube continued to elongate in style on 3 DAP, with a maximum elongation of 72% and entered the ovules on 4 DAP (Figure 3c).

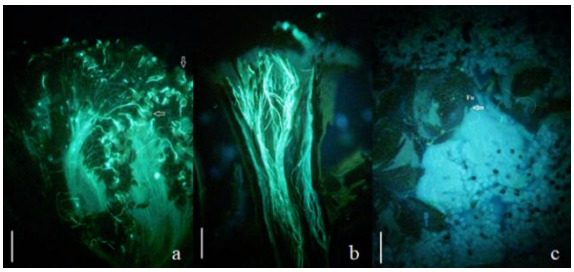

Figure 3. Pollen tube development and penetrate to the ovule. a: in the samples of 1 DAP, pollen tubes start to germinate on the stigma (white arrows) and enter the style (scale bar: 100µm), b: progress of pollen tube in style (scale bar: 100µm), c: entrance of pollen tube into ovule (white arrow) (Fu: Funiculus, scale bar: 50µm).

It was observed that many ovules were tied with a short funiculus by wrapping the placenta. This structure is called free central placentation, also observed in previous studies in *P. veris* (Webster and Gilmartin, 2003; Yankova-Tsvetkova et al., 2021) and *P. vulgaris* (Webster and Gilmartin, 2003). As a result of the examinations, when the sections of the first and second days of both flower types were examined, the ovule was seen to have an anatropous type. The embryo sac has 8 nuclei and it has been determined that antipodes are located in the chalaza part, and synergids and egg cells are located in the micropylar part (Figure 4a). These specialized nuclei could not be imaged together due to the cross-section direction and depth difference. In this context, it can be stated that the embryo sac is polygonum type. Most flowering plants have polygonum type embryo sacs, a common feature of the Primulales (Johri et al., 1992). In ovule imaging, unlike many other species, the nucellus did not develop and there were two well-developed integuments. The undeveloped nucellus, which is common in gamosepalous species, is called 'tenuinucellate'. Tenuinucellate ovule structure was also reported in *P. algida* and *P. amoena* by Akhalkatsi et al. (1998). This structure results from the failure of the differentiated archespore cell to divide into two during the initial formation of the ovule and it is a general feature of the Primulales (Johri et al., 1992). Since tapetum cells do not form, nucellus tissue does not form, and a rather large embryo sac is formed in the middle of the ovule (Figure 4b). In addition, the ovules were surrounded by the epidermis structure rich with oxalate crystals, and a similar structure was found between the inner integument and the embryo sac (Figure 4b).

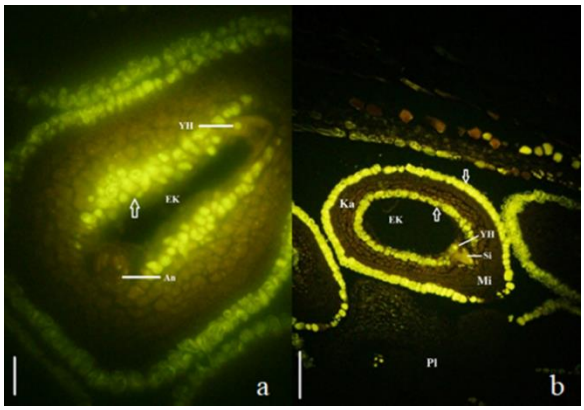

Figure 4. Ovule structure of the pin flower type. a: egg cell and antipodes (Scale bar: 20µm). b: the epidermis structure surrounding the integuments in the ovule of the seed (white arrows) (Scale bar: 50µm) An: Antipodes, EK: Embryo Sac, Ka: Chalaza, Mi: Micropil, Pl: Placenta, Si: Synergids, YH: Egg cell).

It was determined that the first pollen tube reached the ovule on 3 DAP in pin flower. Additionally, residues of pollen tubes were found in ovule samples belonging to 3 and 4 DAP in sectioning examinations (Figure 5a). Again, on the third and fourth days, the embryo sac was highly developed and even the micropylar part of some ovules began to elongate. It was observed that synergids and egg cells showed fluorescence inside ovules at this period which means that the pollen tube penetrated to the ovule via synergids and fertilized the egg cell (Figure 5b).

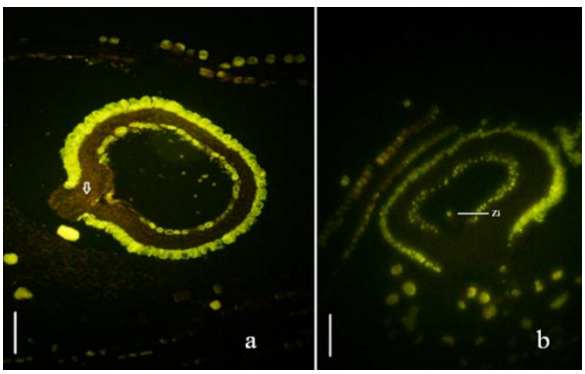

Figure 5. The ovule of the post-fertilization stage. a: pollen tube residue (white arrow) displayed on 3 DAP (Scale bar: 50µm), b: On the fourth day, the zygote (Scale bar: 50µm). Zi: Zygote.

In the samples obtained on the sixth day, a proembryo was formed where the structure thought to be a zygote was located. This cell clump was determined to proliferate adherent to the integuments (Figure 6a). In the examinations made on the 10th-day samples, it was observed that the proembryo became rounded (Figure 6b and Figure 6c), and the endosperm structure began to develop in the lower part of this structure (Figure 6b).

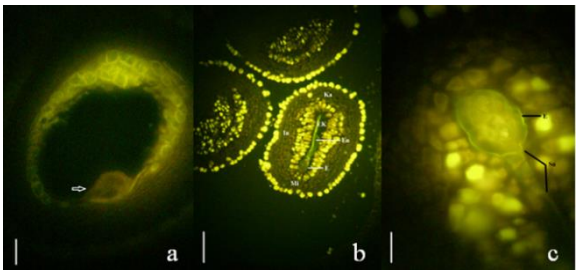

Figure 6. Embryo formed on the 6 and 10 DAP of the pin type and the developing endosperm. a: proembryo (white arrow) formed in the embryo sac on 6 DAP (Scale bar: 20 µm). b: close-up view of embryo and endosperm (Scale bar: 10 µm) c: suspensor, embryo and endosperm were developing on 10 DAP (Scale bar: 50 µm). E: Embryo, EK: Embryo Sac; En: Endosperm; In: Integuments; Ka: Chalaza; Mi: Micropyle; Su: Suspensor.

After this stage, the embryo continued to develop and that the secondary nuclei of the endosperm were also formed. It was then observed that the embryo, which was at the heart stage by 20 DAP (Figure 7a), formed a mature embryo that reached the cotyledon stage at 40 DAP. In addition, it was determined that embryo development was not the same size in all ovules on this stage. In this context, while some embryos did not cover half of the ovules (Figure 7b), some of them were quite larger (Figure 7c). It was also observed that the endosperm was not completely consumed and some of it remained during the period when the embryo gained its full size.

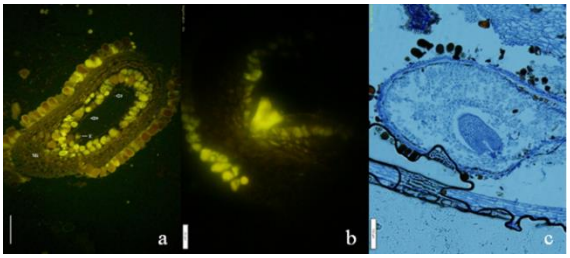

Figure 7. Embryo and endosperm development in pin flower type. a: embryo and endosperm secondary nuclei formed on day 20 (Scale bar: 50µ). b: heart stage embryo from day 20 (scale bar: 20µ). c: embryo and endosperm at the cotyledonous stage at day 40 Scale bar: 100µ). E: Embryo; Mi: Micropyle.

In the thrum flower type, it was determined that fertilization took place on the 4 and 5 DAP (Figure 8a), and pollen tube residues were also formed on the 5 DAP in some ovules. Endosperm in the thrum flower type started to develop faster than the pin type and formed on the 6 and 7 DAP and endosperm tissue was formed on the 10 DAP (Figure 8b). However, embryo development was not as fast as in the pin type; the proembryo started to form on the 10 DAP, but the embryo formation could only be seen on the 30 DAP. In the ovary examinations performed on the 40 DAP, it was determined that some embryos were in the cotyledon stage while some were still in the globular or heart stage and no embryos were found in some ovules. Woodell (1960) reported that inhomogeneous embryo developmental stages and the final size of the seeds arose from environmental differences in legitimate reciprocal crosses between *P. vulgaris* and *P. veris*. This unhomogeneous embryo development is thought to be due to pollenizer quality. As a result of the examinations, there were developing and non-developing ovules in both types and the size difference between them is quite high. When the spare pin flower samples on 20 DAP were examined, the ovules were closer to each other, while the difference between the sizes of the ovules was higher in the other samples on 20 DAP. This may have resulted from either the amount of pollen during pollination or the pollination period. The same situation was also observed in the thrum type.

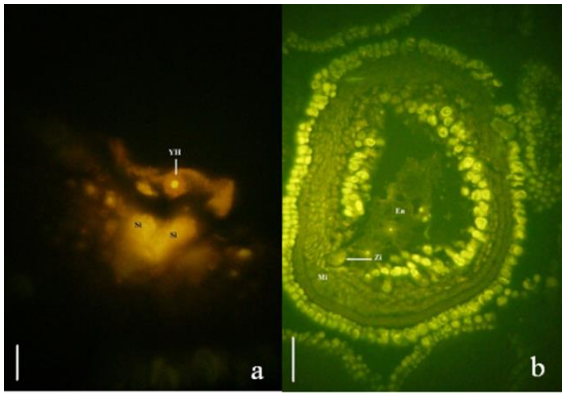

Figure 8. Fertilization and endosperm development in Thrum type. a: fluorescence of synergids during fertilization and prominence of the egg cell. (Scale bar: 20 µ). b: zygote and endosperm formation on day 10 (Scale bar 50 µ). En: Endosperm, Mi: Micropyle, Si: Synergids, YH: Egg cell.

4. Conclusion

At present study, pollen viability, in vitro pollen germination, in vivo pollen tube growth, fertilization and embryo developmental stages were illustrated by histological analysis after legitimate reciprocal crosses between pin and thrum flowers in wild *P. vulgaris* plants. No differences were found in pollen viability and *in vitro* pollen germination rates between flower structures and general pattern of the reproductive biology of *P. vulgaris* were similar with the members of the genus. However, timing of fertilization and embryo developmental stages in the pin and thrum flowers were slightly varied that may have resulted from the amount of pollen during

pollination, the pollination period or environmental stress during flowering period. Unhomogeneous development of the embryo will most likely result with different sizes of seeds and different seed germination abilities. Therefore, whether asynchronous development of embryos in inter-morph flowers affects sustainability of fragmented *P. vulgaris* populations over the years and seasons should be investigated.

Author Contributions

Concept: M.T. (35%), Ş.K. (35%) and B.S. (30%), Design: M.T. (50%) and Ş.K. (50%), Supervision: M.T. (100%), Data collection and/or processing: M.T. (25%), Ş.K. (25%), N.B. (25%) and B.S. (25%), Data analysis and/or interpretation: M.T. (25%), Ş.K. (25%), N.B. (25%) and B.S. (25%), Literature search: M.T. (25%), Ş.K. (25%), N.B. (25%) and B.S. (25%), Writing: M.T. (50%) and Ş.K. (50%), Critical review: M.T. (35%), Ş.K. (35%) and N.B. (30%). Submission and revision. All authors reviewed and approved final version of the manuscript.

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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