

Article

Pollination Strategies and Reproductive Biology of *Fritillaria imperialis* L. (Liliaceae): Insights from Erzincan, Türkiye

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Abstract: *Fritillaria* spp., comprising perennial bulbous plants of significant medicinal and ornamental value, face high endangerment in their natural habitats. Despite their importance, the reproductive characteristics and adaptive evolution mechanisms of these species remain incompletely understood. This study focused on the pollination strategies and reproductive biology of *Fritillaria imperialis* L. The research was conducted at the Erzincan Horticultural Research Institute in Türkiye. Our investigation categorized the flowering process of *F. imperialis* into nine distinct phases. Through comprehensive assessments of the pollen/ovule ratio, self-incompatibility index (SII), and ex situ pollination experiments, we observed high levels of self-incompatibility and allogamy in *F. imperialis*. Our findings revealed that pollination of *F. imperialis* primarily relied on pollen vectors, with *Apis mellifera* and *Bombus terrestris* identified as the most effective pollinators. Furthermore, average seed set rate, seed production, and seed viability were quantified at 80.5%, 228 seeds, and 86.3%, respectively. The average numbers of pollen viability and pollen grains were measured at 93% and 702,000, respectively. This comprehensive analysis of the reproductive biology of *F. imperialis* provides crucial insights for the conservation and genetic management of this highly valuable species. The findings contribute to a deeper understanding of the reproductive strategies employed by *Fritillaria* spp., which may inform future conservation efforts and breeding programs for these endangered plants.

Keywords: *Apis mellifera*; cultural and ornamental plants; flowering dynamics; phenology

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1. Introduction

Global climate change, a prominent issue over the past decade, is widely acknowledged as the most critical environmental challenge affecting the survival and reproduction of numerous plant species [1,2]. Recent scientific investigations have extensively explored the adaptation mechanisms of plant reproductive processes in response to climate change, examining aspects such as flowering phenology [3], plant–pollinator interactions [4], and mating systems [5]. Reproduction in plants, a vital life process essential for species perpetuation, generates genetic diversity primarily through recombination processes during sexual reproduction, thus playing a crucial role in species and population biology [6,7]. In essence, reproduction not only forms the core of plants' evolutionary process but also represents a relatively vulnerable stage in their life cycle [8]. Consequently, a comprehensive understanding of their reproductive biology is imperative for successful cultivation and conservation efforts, and studies focusing on the reproductive characteristics of a species are instrumental in elucidating the mechanisms underlying their extinction risk status [8,9]. The field of plant reproductive biology primarily encompasses the study of

pollen–pollinator interactions, floral biology, flowering phenology, gene flow, and breeding systems through seeds and pollen [10]. Previous research has focused on observing and recording plants' regular growth stages, as well as investigating how environmental factors influence their yearly cycles. Thus, these steps help us better understand plant development and responses to their surroundings over time. Phenological events in plants, including fertilization, bud burst, flowering, leaf expansion, fruiting, leaf abscission, seed germination, seed dispersal, and seed set follow a sequential pattern within a given season. Numerous studies have examined plant phenological events and breeding systems, encompassing a spectrum from total self-incompatibility to complete self-compatibility [11–13]. Autogamy in species eliminates mate constraints by enabling self-fertilization, whereas being self-sterile in certain plant species mitigates the risk of inbreeding depression by limiting the number of compatible mating pairs [7]. A phenomenon often observed in hermaphroditic plants with self-incompatibility is that open-pollinated flowers exhibit a very low fruit set. Generally, low fruit set may be attributed to a high level of autogamy and a high incidence of self-fertility in natural settings; however, other factors such as the location of fruit within inflorescences and resource limitations may also contribute to this outcome [14,15].

Although approximately 600 species are documented to have become extinct in the past 250 years worldwide, current estimates suggest that between 20 and 39% of plant diversity is at risk of extinction [16]. Considering plants specifically, only 10% of these species have been stated to be globally at risk of extinction and have been included in the IUCN Red List [17]. The genus *Fritillaria* L., a widely distributed member of the Liliaceae family, comprises about 163 species occurring in various regions of the northern hemisphere, with centers of speciation in East Asia, Greece, Iran, Türkiye, and western North America [6,18–25]. *Fritillaria* spp. are found in diverse habitats and climatic regions, spanning a wide latitudinal range from riparian zones to coasts, deserts, steppes, woodlands, alpine zones, meadows, and mountain screes. Some *Fritillaria* species are classified as critically endangered according to IUCN Red List Categories and Criteria [26]. The natural regeneration of these species is relatively poor, with few seedlings observed in each population [27]. It is crucial to emphasize that the remaining partial populations are severely impacted by human activities, rendering their status a cause for serious concern and necessitating urgent protection measures for these wild resources. These species are bulbiferous, flowering in spring after snowmelt, with an erect flowering stem producing either a single flower or multi-flowered racemes [21]. The flowers of *Fritillaria* species are generally actinomorphic, featuring a typical campanulate perianth that is tulip-like and trimerous, with flowers facing downward after blooming [28,29]. In Türkiye, which holds a significant position in terms of the genus's distribution, 48 species belonging to the genus *Fritillaria* exhibit natural habitats. *Fritillaria imperialis* L., one of the first *Fritillaria* spp. introduced to the scientific world by Linnaeus in 1753, is the sole species of its genus in Türkiye characterized by its umbellate inflorescence and tufted apex [30–32]. It possesses numerous leaves and bulbs reaching up to 8 cm in diameter, stems extending up to 125 cm, numerous glossy leaves, and circular nectaries, thus making it readily distinguishable from other species. *F. imperialis* exhibits conspicuous flowers and is extensively cultivated for bulb export, particularly to the Netherlands and various other European countries. The natural distribution of *F. imperialis* across Türkiye, including regions such as Erzincan, Hakkari, Van, Kahramanmaraş, Şırnak, Bitlis, Siirt, and Muş, underscores its national significance [18]. The species' limited range within these areas highlights its importance to Türkiye's biodiversity and ecological heritage. Conservation of *F. imperialis* is crucial not only for maintaining local ecosystems but also for preserving Türkiye's unique floral diversity. Its potential extinction would represent a significant loss to the country's natural patrimony and could have cascading effects on associated wildlife and ecosystem services. According to Adıgüzel et al. [33], some *F. imperialis* such as *Fritillaria gentneri* Gilkey, endemic to Oregon, USA, and *Fritillaria ehrhartii* Boiss. & Orph., native to Greece, are classified as Vulnerable (VU) in the IUCN Red List, indicating a high risk of extinction

in their natural habitat. These species are easily cultivated and are highly prized for their exotic and attractive flowers. The flowers of *F. imperialis* are typically orange to red, rarely yellow, with a rather broad corolla [18,30].

Given these characteristics, the color and flower size, which are involved in attraction and protection, could also serve as signals informing potential floral visitors regarding the characteristics of the present reward. In natural conditions, the most likely pollinators for *F. imperialis* are thought to be passerine birds and *Bombus* spp. [34–36]. This hypothesis is supported by the presence of a landing platform for *F. imperialis* with large pendulous and showy flowers. Though data are limited, flower pollinators are attracted by a combination of nectars and visual cues, and one might assume this is true for *F. imperialis*. However, the relationship between visual pollination and nectar strategies and their impact on the breeding systems within *F. imperialis* remains obscure, with no information currently available. In this framework, the current research represents the first article on the reproductive strategies of *F. imperialis*, and therefore, the objectives of our work were to (1) define the seed and pollen characteristics, (2) detect flowering phenology; (3) determine reproductive strategies, and (4) verify pollinator identities.

2. Materials and Methods

2.1. Study Site and Plant Material

Fritillaria imperialis exhibits a natural distribution encompassing the Middle East and Türkiye, specifically across regions including Erzincan, Hakkari, Van, Kahramanmaraş, Şırnak, Bitlis, Siirt, and Muş [18] (Figure 1). (<https://www.google.com/maps/place/T%C3%BCrkiye/@39.0579139,32.4911301,7z/data=!3m1!4b1!4m6!3m5!1s0x14b0155c964f2671:0x40d9dbd42a625f2a!8m2!3d38.963745!4d35.243322!16zL20vMDF6bmNf?entry=tту>, accessed on 11 June 2024).



Figure 1. Distribution of *Fritillaria imperialis* in the world and in Türkiye (Red stars: *F. imperialis* in Türkiye; Green countries: *F. imperialis* in world; Purple countries: *F. imperialis* moved to an area outside their native range) (world map by Plants of the World Online <https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:535166-1> accessed on 12 June 2024).

Data pertaining to the reproductive biology of *F. imperialis* were systematically collected during the flowering seasons of 2018/2019, 2019/2020, and 2020/2021, based on the protocol of Tekşen and Aytac [18]. The plant samples used for the reproductive biology studies were sourced from *F. imperialis* specimens cultivated ex situ at the Ornamental Plant Breeding and Agronomy Section of the Horticultural Research Institute in Erzincan, Türkiye, derived from the private collections of co-author Meral Aslay (Figure 2). Plant samples were kept under natural conditions at the research facility.



Figure 2. Field studies and pollination treatments (photo by M. Aslay, F. Yildiz).

Certain morphological characteristics of *F. imperialis*, including leaf dimensions, vegetation period, flowering patterns, tepal attributes (size, quantity, color, and odor), and overall plant dimensions (height and width), have been previously documented in the literature [28,29]. In ex situ conditions, *F. imperialis* typically produces between 3 and 21 flowers per plant, characterized by a perigonal broad sack morphology, predominantly red with orange coloration, and yellowish-orange internal hues. The tepals are distinctly lanceolate and pointed [18,30] (Figures 2–4). The phenological phases of the species, including the initiation and duration of nectar secretion, pistil fertility stages and their correspondence to anther development, and the location of nectaries, were examined in accordance with methodologies reported by previous researchers [28,29]. We systematically observed and recorded the phenological stages of *F. imperialis* over time. This involved regular monitoring of the plants to document key developmental stages such as bud formation, flowering onset, nectar production, anther dehiscence, and pistil maturation. We used visual observations, microscopy, and histological techniques to examine nectary locations and structure. We carefully noted the timing and duration of each phase, along with environmental factors that might influence these processes. Our comprehensive approach allowed us to gather detailed data on the reproductive biology of *F. imperialis* throughout its growth cycle. Specifically, we also employed digital calipers and high-precision rulers for quantitative assessments of the morphological features.

2.2. Detection of Pollination Strategies

To investigate the pollination strategies of the *F. imperialis* population under examination, this study randomly selected and marked 240 individual plants at the tepal-unopened phase. These specimens were subjected to seven distinct experimental procedures (Table 1): (C) Natural pollination (control group): flowers were left unmanipulated and exposed to natural conditions (n: 50); (P1) Spontaneous self-pollination: flowers were enclosed in mesh bags to prevent external pollination (n: 40); (P2) Induced self-pollination: flowers were emasculated, enclosed in mesh bags, and manually self-pollinated 2–6 d post-anthesis (n: 30); (P3) Geitonogamy: emasculated flowers were enclosed in mesh bags and artificially pollinated with pollen from different flowers of the same individual plant 2–6 d post-anthesis (n: 30); (P4) Spontaneous cross-pollination: flowers were emasculated and left exposed to natural conditions (n: 30); (P5) Xenogamous pollination: emasculated flower buds were enclosed in mesh bags and manually pollinated using pollen from plants situated at least 5–7 m apart 2–6 d post-anthesis (n: 30); (P6) Apomixis: flowers were emasculated and enclosed in mesh bags to test for asexual seed production (n: 30). The apomixis treatment also served to evaluate the possibility of wind pollination. For manual

pollination procedures, following the methodology of Dafni [37], pollen was sourced either from a dehiscent anther of the same plant (self-pollination) or from a flower of a plant approximately 3–4 m away (cross-pollination).



Figure 3. Flowering dynamics of ex-situ-cultivated *Fritillaria imperialis*: (1) bud burst; (2) initial flowering; (3) pre-dehiscence stage; (4) first dehiscence stage; (5) full dehiscence; (6) end-dehiscence stage; (7) fresh perianth stage; (8) perianth-wilting stage; (9) fruit/seed development stage (photos by M. Aslay and F. Yildiz).

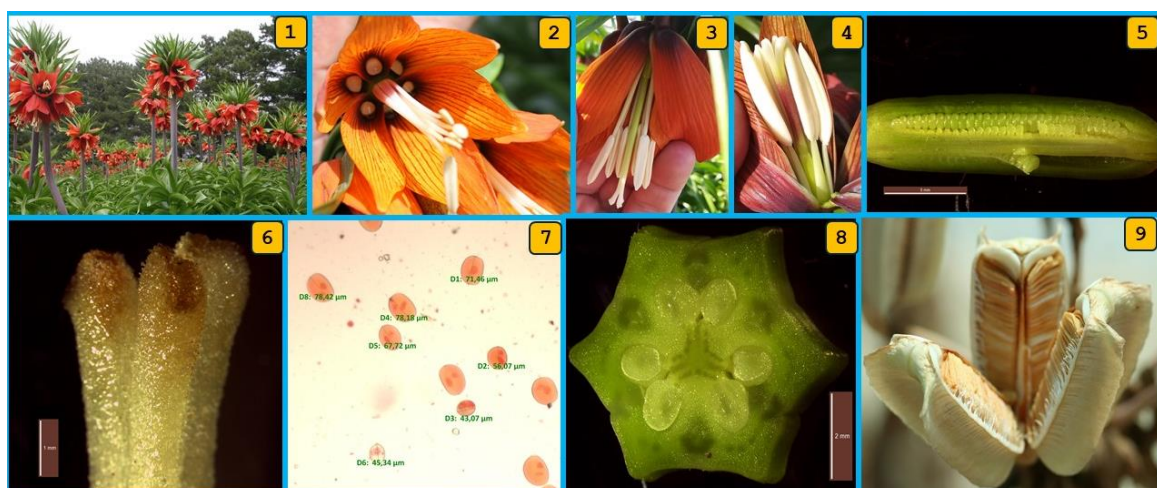


Figure 4. Floral features and reproductive phenology of full *F. imperialis*: (1) *F. imperialis* in full flowering; (2) nectary sacs; (3) pistil; (4) stamens; (5) ovules; (6) stigma surface; (7) pollen viability; (8) young seed after capsule; (9) capsule dehiscence (photos by F. Yildiz and M. Aslay).

Table 1. Description of procedures utilized to examine reproductive biology of *Fritillaria imperialis*.

Procedure Code	Procedure	Measured Strategy	Possibility of Self-Pollination	Possibility of Cross-Pollination	Fruit/Seed-Setting Status
C	Free exposure (control group)	Natural Pollination	Likely	Likely	+
P1	Bagged	Spontaneous autogamy	Likely	No	—
P2	Bagged, hand self-pollinated	Assisted autogamy	Likely	No	—
P3	Emasculated, bagged, hand self-pollinated	Geitonogamy	Likely	No	—
P4	Emasculated, free exposure	Spontaneous allogamy	No	Likely	+
P5	Emasculated, bagged, hand cross-pollinated	Assisted allogamy	No	Likely	+
P6	Emasculated, bagged	Apomixis	No	No	—

“+”: seed formation observed “—”: no seed formation observed.

To ensure the comprehensive assessment of reproductive strategies in induced self-pollination, the pollination experiments were repeated daily throughout the flowering period, aiming to capture peak periods of stigma receptivity and pollen viability. Experimental flowers were identified with tags, and the fruit set was recorded in May upon seed maturation (Figure 4). Fruit and seed set were quantified using the following formulas [38]:

$$\text{Fruit set rate (\%)} = \frac{\text{the number of fruit sets (mean capsules)}}{\text{the number of processed single flowers}} \times 100\%$$

$$\text{Seed set rate (\%)} = \frac{\text{the number of seed sets (mean mature seeds)}}{\text{the number of the ovules}} \times 100\%$$

2.3. Detection of Flowering Dynamics

This study presents the first detailed study of the flowering dynamics of this species, which occurs from March to April. We have delineated the flowering process into nine distinct stages (Figure 3): (1) Bud burst: the initial emergence of the floral bud; (2) Flowering initiation: the commencement of the flowering phase; (3) Pre-dehiscence stage: the period immediately preceding anther dehiscence; (4) First dehiscence stage: the initial release of pollen from the anthers; (5) Full dehiscence: the period of maximum pollen release; (6) End-dehiscence stage: the final phase of pollen release; (7) Fresh perianth stage: the period when the floral parts remain turgid and vibrant; (8) Perianth-wilting stage: the onset of senescence in the floral parts; (9) Fruit/seed stage: the development and maturation of seeds and fruits. These stages are visually represented in Figure 3, providing a comprehensive illustration of the reproductive phenology of *F. imperialis*.

2.4. Detection of Pollen Viability and Stigma Receptivity

To assess the stigma receptivity and pollen viability of *F. imperialis*, we employed a systematic sampling approach. Ten flowers were randomly selected and marked at each of six distinct phenological phases: initial flowering, pre-dehiscence phase, first dehiscence phase, full dehiscence, end-dehiscence phase, and the fresh perianth phase. Stigma receptivity was evaluated using a $\text{C}_{12}\text{H}_{12}\text{N}_2\text{-H}_2\text{O}_2$ (benzidine–hydrogen peroxide) reaction solution, following the methodology outlined by Dafni and Maués [39]. The reaction solution was prepared in a ratio of water: 3% H_2O_2 : 1% $\text{C}_{12}\text{H}_{12}\text{N}_2$ (22:11:4). Stigmas from marked *F. imperialis* specimens were placed on Petri dishes and treated with an appropriate volume of the $\text{C}_{12}\text{H}_{12}\text{N}_2\text{-H}_2\text{O}_2$ solution. The resultant bubble formation on the stigma surface was observed and quantified. Discoloration and bubbling were examined under a stereomicroscope (Leica M165C), with images captured after one minute of exposure. Stigma receptivity was categorized based on the observed bubble density,

as per Dafni and Maués [39]: (i) weak receptivity (5–10% bubble coverage or very low bubble density), (ii) moderate receptivity (45–50% bubble coverage or moderate bubble density), and (iii) strong receptivity (85–90% bubble coverage or high bubble density). Pollen viability was assessed using the acetocarmine staining method [40]. Ten *F. imperialis* flowers at the pre-dehiscence stage were randomly selected for this analysis. Anther sacs were placed on microscope slides and treated with 1% acetocarmine solution to disperse the pollen grains. The preparations were then heated at 50 °C for 1 min to facilitate dye diffusion. Pollen grain viability was evaluated based on the observed staining patterns under a light microscope (Olympus BX50, Tokyo, Japan) at 10× magnification. Unstained grains were classified as non-viable, light red grains as semi-viable, and dark red grains as fully viable [40].

2.5. Detection of the Pollen/Ovule Ratio (P/O) and Self-Incompatibility Index (SII)

To assess the pollen and ovule production of *F. imperialis*, a systematic sampling approach was employed. Ten distinct individuals cultivated in pans were randomly selected for analysis. At the pre-dehiscence stage, one anther was excised from each of these specimens. The pollen from these anthers was dispersed onto microscope slides equipped with a 10 × 10 mm counting scale. Pollen grain quantification was subsequently performed under stereomicroscopic examination. For ovule enumeration, ten different plants were randomly chosen at the onset of flowering. Young ovules were carefully extracted and bisected using a scalpel. These prepared ovules were then subjected to microscopic examination and counted. The ratio of pollen grains produced per ovule within a single flower was detected following the methodology described by Cruden [41]. To evaluate the self-incompatibility index (SII) of *F. imperialis* flowers, seed set success was recorded post-flowering for both self-pollinated and cross-pollinated plants. The SII was calculated utilizing the method outlined by Zapata and Arroyo [42], which compares the relative success of self-pollination to cross-pollination.

$$SII = \frac{\text{Percentage (\%) of seed sets in selfing flowers}}{\text{Percentage (\%) of seed sets in crossed flowers}}$$

The interpretation of SII values follows this framework: fully self-compatible: SII value = 1 or SII > 1; partially self-compatible: 0.2 < SII < 1; self-incompatible: SII < 0.2 or SII = 0.

2.6. Detection of Germination and Seed Viability

Approximately 25–30 d following the perianth-wilting phase, a random selection of ten *F. imperialis* fruits was made to assess seed viability. The evaluation was conducted using the TTC (2,3,5-triphenyl tetrazolium chloride test), as described by Dafni [37]. A sample of 300 seeds extracted from these fruits was subjected to the TTC assay. The protocol involved an initial 24 h imbibition period, during which the seeds were submerged in distilled water to activate the embryos. Subsequently, the seeds were exposed to a 0.1% TTC solution and incubated at 22–24 °C for an additional 24 h. Following this incubation period, the seeds were bisected to expose the embryos for examination. Stereomicroscopic analysis was employed to evaluate the staining patterns of the embryos. The seeds exhibiting no color change were classified as non-viable, while embryos that developed a red coloration were deemed viable.

2.7. Detection of Floral Visitors

To elucidate the pollinator behavior of *F. imperialis*, a comprehensive observational study was conducted over three consecutive years (2019–2021). Observations were carried out during the full bloom period for twenty consecutive days, exclusively on sunny days to ensure consistent environmental conditions. The observation protocol encompassed hourly recordings from 08:00 to 16:00 each day. The data collection process was structured into three distinct stages: (i) Video documentation: insect activity was captured using a Canon 5D Mark IV camera (5D Mark IV, Canon, Japan), with each recording session

lasting approximately 30 min; (ii) Flower selection: random flowers were chosen based on observed insect-visitation patterns; (iii) Insect collection: visiting insects were captured using specialized traps for subsequent identification. Effective floral visitors were defined according to the criteria established by Stout [12], which stipulate that pollinators must not only deposit pollen on receptive stigmas but also actively collect pollen from the flowers. This definition ensures the identification of insects that contribute substantially to the pollination process. Insects meeting these criteria were systematically collected, with representative samples preserved for taxonomic identification. The visiting behaviors of these effective pollinators were meticulously documented through both photographic and video recordings.

3. Results

3.1. Floral Features and Reproductive Phenology of *F. imperialis*

This study revealed novel findings regarding the floral characteristics of *F. imperialis*, as summarized in Table 2. The inflorescence exhibited a broad umbellate structure (see stage 4 in Figure 3), comprising 3–21 flowers. It featured 1–13 branches with similar morphological traits, with flowers arranged in a campanulate and bell-shaped succession. The hermaphroditic flowers displayed weak zygomorphy and were predominantly orange to red, with rare instances of yellow coloration. They were odorless, with an average length ranging from 28 to 122 cm. The nectar sacs (see stage 2 in Figure 4) were white, droplet-shaped, and dense. Anther dehiscence occurred through inward-facing slits, averaging 11–22 mm in length. The corolla measured 35–75 mm on average. The androecium consisted of six stamens arranged in a ring surrounding the carpel, positioned above the stigma level (see stage 4 in Figure 4). Quantitative floral characteristics included an average of 702,000 pollen grains per flower, six ovary capsule lobes, 228 ovules per ovary, and filament, anther, and style lengths of 41 mm, 8 mm, and 36 mm, respectively. The filaments were short and greenish-creamy, while the basifixed anthers were yellowish. Anther dehiscence occurred between 09:30 am and 13:30 pm through vertical slits (see stage 3 in Figure 4), typically 40–50 min post-anthesis, releasing approximately 75% of pollen within 3–4 h. Non-fruiting flowers abscised after 7–8 d, followed by tepal wilting and stamen desiccation from the ovary base (see stage 8 in Figure 3). Post-pollination, the ovary remained attached to the pedicel, while the style and stigma dehydrated. Pollen characteristics included a granulate surface, subprolate shape, 2-celled structure, 42–79 μm size, and 93% viability. The stigma was trifid or trilobate with a wet surface. The ovary averaged 3.4 mm in diameter, with axile placentation and hypogynous insertion. The androecium exhibited syngensis, and the perianth formed a campanulate perigon (Table 2).

Table 2. Measured floral features of *F. imperialis*.

Character	Unit	Observations	Mean Value
Flowering period	month	March–April	-
Inflorescence	term	Umbella	-
Bulb	term	Ovoid, perennial	-
Perigon	term	Broadly campanulate	-
Plant length	cm	28–122 (min and max)	58 ± 0.9
Flower	term	Hermaphrodite, weakly zygomorphic	-
Flower color	term	orange to red, rarely yellow	-
Flower number	number	3–21 (min and max)	8 ± 1.2
Flower length	mm	36–68 (min and max)	52 ± 3.4
Odor	term	Nil	-
Nectar	term	White, drop-like, dense	-
Anther dehiscence	term	Bursting inward by slits, extrorse	-
Anthers per flower	number	6	6 ± 0.5
Pollen grains per flower	number	612,000–775,000 (min–max)	702.000 ± 12.36
Ovules/ovary per flower	number	168–240 (min and max)	228 ± 5.3

Table 2. Cont.

Character	Unit	Observations	Mean Value
Length of petal	mm	35–75 (min and max)	54 ± 1.5
Length of filament	mm	18–64 (min and max)	41 ± 2.4
Length of anther	mm	11–22 (min and max)	18 ± 1.6
Length of style	mm	16–46 (min and max)	36 ± 1.3
Pollen surface	term	Granulate	-
Pollen shape	term	Subprolate	-
Pollen size	µm	42–74 (min and max)	56 ± 4.3
Pollen cell	number	2-celled	2 ± 0.4
Pollen viability	%	83–97 (min and max)	93%
Stigma shape	term	Trifid or trilobate	-
Pollen–ovule ratio	number	2898–3220 (min and max)	3080 ± 10.9
Seed viability	%	71–92 (min and max)	86.3 ± 1.2
Stigma surface	term	Wet	-
Diameter of ovary	mm	1.8–4.2 (min and max)	3.4 ± 0.5
Ovary placentation type	term	Axile	-
Ovary capsule	number	6-lobed	6 ± 1.1
Ovary status	term	Hypogin	-
Stamen type	term	Singenesia stamen	-
Filament type	term	Trichomes at the base	-
Perianth type	term	Campanulate perigon tepal	-
Perianth estivation	term	Imbricate-alternate	-
Anther type	term	Basifix	-
Corolla type	term	Campanulate, bell-shaped	-

3.2. Flowering Phenology of *F. imperialis*

According to our comprehensive observations, the flowering period of the *F. imperialis* population extended over approximately three weeks. The ex situ reproductive phenology of *F. imperialis* commences in early March with the formation of primordial flowers within dormant underground buds. These floral structures emerge concurrently with vegetative growth from late March to mid-April (see stage 1 in Figure 3). Floral development from bud to anthesis spans approximately 8 d, with pollen presentation persisting for 7–9 d on average. Peak flowering occurs between the second week of March and the first week of April. The complete flowering process of *F. imperialis* extends over a period of 17–21 d, encompassing the following discrete stages: (i) Subterranean bud phase (two–three days); (ii) Initial flowering period (six–nine days); (iii) Pre-dehiscence tepal pigmentation (three–four days); (iv) Full anther dehiscence (three–five days); (v) Terminal dehiscence phase (four–five days); (vi) End-dehiscence stage (six–eight days); (vii) Tepal senescence (four–five days); (viii) Post tepal desiccation and seed capsule initiation (two days); (ix) Seed capsule maturation (see stage 1–8 in Figure 3). A gradual decline in flowering is observed from the final week of March, with complete cessation of floral activity by the third week of April.

3.3. Pollen/Ovule (P/O) Ratio and Self-Incompatibility Index (SII) of *F. imperialis*

In the analysis of the pollination strategies of *F. imperialis*, the self-incompatibility index (SII) was not quantified as zero (0) due to the absence of self-pollination. The number of ovules per flower over a three-year period (2019–2021) exhibited a range of 168 to 240, with a mean of 228. Total pollen production per flower was quantified at an average of 702,000 grains. Based on these measurements, the P/O ratio for *F. imperialis* was determined to be 3080 (Table 2).

3.4. Stigma Receptivity and Pollen Viability of *F. imperialis*

The pollen viability of *F. imperialis* was assessed at 93% during anther dehiscence utilizing acetocarmine staining (see stage 7 in Figure 4 and Table 2). Throughout the flowering phases, pollen viability ranged from 83% to 97%. In ex situ conditions, pollen viability demonstrated a steady increase during flower development, declining after the

tepal-wilting stage (Figure 5). Stigma receptivity was quantified at various developmental stages: 2% at bud burst, 13% at flowering initiation, 38% at pre-dehiscence, 56% at first dehiscence, 79% at full dehiscence, 83% at end-dehiscence, 68% at the fresh tepal stage, and 11% during tepal wilting. The $C_{12}H_{12}N_2 \cdot H_2O_2$ reaction test revealed that stigmas were non-receptive on the first day of anthesis and during the sympetalous period. Stigma secretions increased 2–7 d post-anthesis, with peak receptivity observed at 5–7 d, followed by a rapid decline (Figure 5).

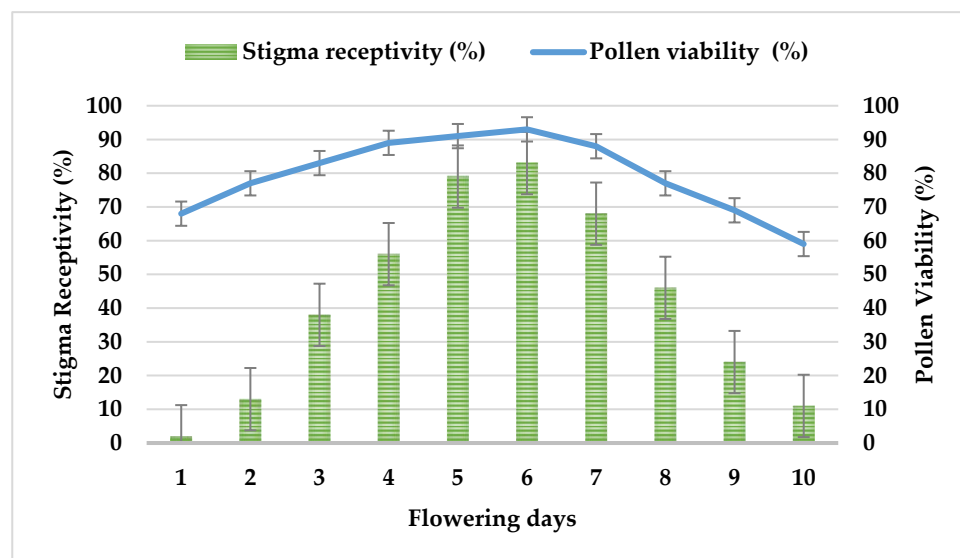


Figure 5. Changes in stigma receptivity and pollen viability during anthesis of *Fritillaria imperialis*.

3.5. Pollination Experiments of *F. imperialis*

Six pollination types were examined in *F. imperialis*, yielding varying fruit set rates ranked as follows: spontaneous allogamy (77.7%) > assisted allogamy (69.5%) > natural pollination (63.6%) > spontaneous autogamy (0) = assisted autogamy (0) = geitonogamy (0) = apomixis (0) (Table 3). Emasculated and bagged flowers, as well as those bagged without castration, failed to set fruit. Seed length ranged from ca. 8 to 12 mm. The average number of mature seeds per flower was 75.2%, 81.4%, and 85.0% in natural pollination, assisted cross-pollination, and spontaneous allogamy, respectively. The ovoid capsule dehiscence into six equal parts (see stage 8 in Figure 4), containing 28–40 seeds per capsule, with an average of 228 seeds per flower (see stage 5 in Figure 4).

Table 3. Seed and fruit setting success of tested *F. imperialis* individuals according to breeding procedures.

Code	No. of Flowers	No. to Recycle *	Fruit Set Rate %	Seed Set Rate %	Seed Viability (%)
C	50	44	63.6 ± 3.2	75.2 ± 1.9	89.3 ± 2.3
P1	40	36	0	0	-
P2	30	25	0	0	-
P3	30	26	0	0	-
P4	30	27	77.7 ± 1.5	85.0 ± 1.5	83.4 ± 1.5
P5	30	23	69.5 ± 2.4	81.4 ± 1.8	86.1 ± 0.9
P6	30	26	0	0	-

Average ± standard deviation (SD). * Number to recycle refers to the number of individuals unharmed by insects or animals in each application.

3.6. Seed Viability of *F. imperialis*

Seed viability was observed only in spontaneous allogamy, assisted allogamy, and natural pollination treatments. The tetrazolium staining test revealed viable seed ratios of 83.4%, 86.1%, and 89.3% for spontaneous allogamy, assisted allogamy, and natural pollinations, respectively (Table 3).

3.7. Pollinator Observations of *F. imperialis*

Fritillaria imperialis flowers were visited by *Apis mellifera* L. and *Bombus terrestris* L. (Hymenoptera) (Figure 6). These pollinators were observed visiting flowers in the morning (9:00–12:00 h) and afternoon (13:00–16:00 h) post-anthesis. Both species played crucial roles in spontaneous cross-pollination and natural pollination. The average number of visitors during peak flowering for 20 different plants is presented in Figure 7. Flies (Diptera) and moths (Lepidoptera) were observed infrequently and briefly (one or three min), and thus were not considered effective pollinators.

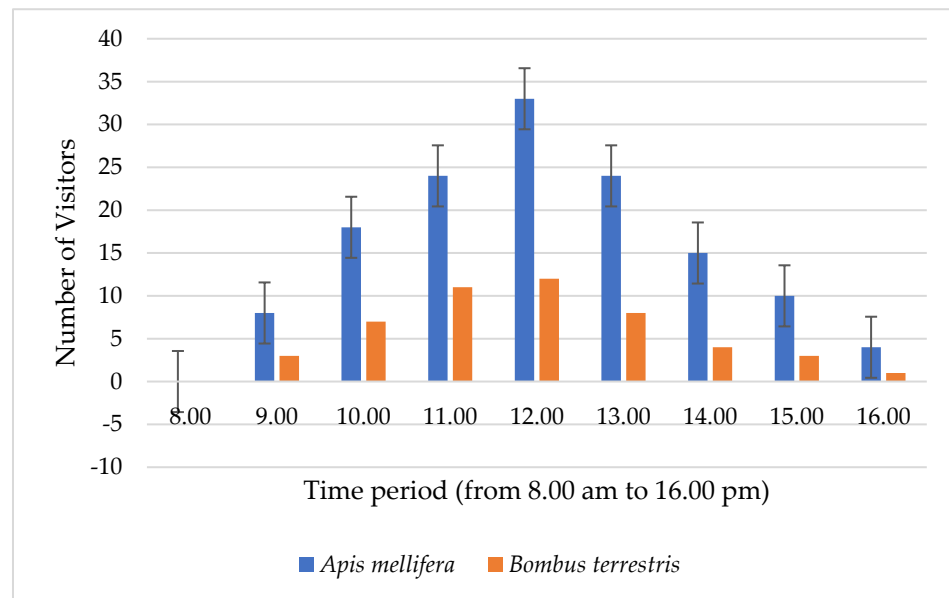


Figure 6. The average number of floral visitors in twenty different plants studied during the peak flowering period.



Figure 7. *Apis mellifera*, key pollinator species of *Fritillaria Imperialis* (photo by M. Aslay).

4. Discussion

While significant progress has been made in elucidating the reproductive biology and floral traits of several *Fritillaria* spp. [6,10,43,44], a critical question persists among researchers: What factors influence the reproductive ecology and floral characteristics of endemic and threatened plants with limited populations such as the herein studied *F. imperialis*? Despite existing studies on the floral attributes of certain *Fritillaria* spp. [6,31,43,45,46], the description of *F. imperialis* floral morphology remains insufficiently delineated. Consequently, this study provides a comprehensive report on the morphological phenology of the reproductive organs of *F. imperialis*, incorporating established plant terminology along with quantitative and qualitative characteristics (Table 2), thus distinguishing it from previous investigations. Our findings indicate that certain floral characteristics of *F. imperialis*, including anther dehiscence, anther number per flower, pistil type, ovary capsule, stigma morphology, ovary placentation, stamen type, ovary status, perianth type, and anther type, align with those of many *Fritillaria* species [6,28,38,47–49]. However, other attributes such as flowering period, inflorescence structure, floral dimensions, color, odor, nectar production, pollen and ovule quantities, tepal length, filament length, stamen length, anther length, pollen size, style length, pollen viability, pollen surface morphology, pollen–ovule ratio, pollen cell structure, pollen shape, stigma surface characteristics, ovary diameter, and corolla type differ from those reported in other *Fritillaria* species [6,28,48–53]. *F. imperialis*, for instance, produces large, early-blooming flowers in umbel-like clusters with a strong odor, while *F. meleagris* L. typically bears solitary, checkered flowers later in spring. *Fritillaria pudica* (Pursh) Spreng is characterized by smaller, yellow flowers. These interspecific differences reflect adaptations to diverse ecological niches and pollination syndromes within the *Fritillaria* genus. Notably, this study presents the first documentation of the specific floral characteristics of *F. imperialis*, including stamen type, ovary status, ovary placentation type, anther type, anther dehiscence direction, average pollen grain count per flower, ovule number per flower, and corolla type (Table 2), rendering a comparative analysis with the existing literature challenging.

A comprehensive understanding of plant reproductive biology is crucial not only for systematic and evolutionary studies [54] but also for developing effective conservation strategies [55] for endemic and threatened plants with limited populations, like *F. imperialis*. Given that reproduction represents a critical stage in a plant's life cycle, the current lack of data on germination, seed dispersal, and seedling establishment in *F. imperialis* populations is noteworthy. Information on reproductive stages is particularly significant in the context of resource allocation within a plant's life cycle, as assimilates previously allocated to vegetative growth are redirected toward reproductive purposes [56]. Although this research represents the first comprehensive investigation of the reproductive biology of *F. imperialis*, it reveals that the flowers are generally nodding and bell-shaped, a characteristic shared with many other *Fritillaria* species [44]. Consequently, anthers and nectaries are concealed from approaching pollinators, a finding supported by the observed fruit set results (Table 3). Table 3 illustrates the fruit set, seed set, and seed viability rates of *F. imperialis* across various pollination syndromes. These findings suggest a weak pollen limitation in *F. imperialis* populations, contrasting with the significant pollen limitation reported in some *Fritillaria* species, including *F. maximowiczii* Freyn, *F. meleagris*, and *F. delavayi* Franch, [48–50]. However, our results align with the studies of self-incompatibility in closely related species such as *F. camtschaticensis* L., *F. aurea* Schott, *F. cirrhosa* D. Don, and *F. michailovskyi* Fomin [6,38,57,58]. This context invites further exploration of the evolution of incompatibility systems in these species and other perennials.

In our results, fruit sets were not observed in the induced self-pollination, spontaneous self-pollination, geitonogamy, or apomixis treatments. The absence of seed sets in the apomixis treatments also indicates a lack of wind pollination. These results from four pollination treatments without fruit sets suggest that *F. imperialis* exhibits pollination limitations and relies exclusively on obligate outcrossing for reproduction. Furthermore, neither herkogamy nor dichogamy events were observed in the floral life cycle of this species. Despite the temporal overlap between female and male fertility and the positioning of stamens and pistils in the flower, self-pollination did not occur, contrary to expectations

based on the absence of hercogamy. The predominant self-incompatibility of *F. imperialis* likely serves to protect and maintain genetic inheritance and variability. This aligns with observations that the degree of allogamy can vary significantly within a single plant species, depending on factors such as dichogamy degree, pollinator abundance, and pollen viability duration [59]. Insect pollinators appear to play a crucial role in facilitating xenogamy in *F. imperialis*. These results suggest that *F. imperialis* may be considered functionally heterostylous or characterized by a self-incompatibility system. This hypothesis is consistent with the understanding that avoidance of inbreeding is essential for efficient pollen exchange in evolutionarily disadvantageous mating scenarios [60,61].

Based on our pollen/ovule ratio (P/O), self-incompatibility index (SII), and natural pollination experiments, we have determined that *F. imperialis* exhibits high levels of self-sterility in its reproductive biology. This finding indicates that wind or insect vectors are essential for pollination in this species, which is consistent with the findings reported for other *Fritillaria* species [6,58]. The observation that plants lacking flower covers and possessing brown or green flowers generally do not rely on wind pollination supports our conclusion that *F. imperialis* is not dependent on anemophily [62,63]. Furthermore, the structurally reverse-growing perianth, which prevents pollen from reaching the stigma through selfing or wind dispersal, suggests that *F. imperialis* is incapable of self-pollination. This aligns with the general trend of cross-pollination observed in the Liliaceae family, including members of the genus *Fritillaria* [21]. A previous study identified blue tits (*Parus caeruleus* L.) as the primary pollinator of *F. imperialis*, noting that the species is self-sterile [34]. Additional research by Roguz et al. [35] and Peters et al. [36] emphasized the importance of passerine birds as critical pollinators for *F. imperialis*. However, our observations did not include bird visits. The potential alteration of pollinator bird behavior in ex situ environments may impact the ecological validity of *F. imperialis* pollination dynamics observations, necessitating a cautious interpretation of results. Future research should incorporate comparative analyses between artificial and natural settings to elucidate the effects of man-made environments on pollinator bird behavior and provide a more comprehensive understanding of *F. imperialis* pollination ecology. Nevertheless, *F. imperialis* demonstrates remarkable self-infertility, which does not ensure sexual reproduction in the absence of pollinators. These findings contrast with the reports on self-pollinating *Fritillaria* species, including *F. meleagris* [48], *F. maximowiczii* [49], *F. delavayi* [51], *F. cirrhosa* [38], *F. persica* L. [64], *F. koidzumiana* Ohwi [65], and *Fritillaria montana* Hoppe ex W.D.J. Koch [66]. It should be noted that our study did not specifically examine the factors determining self-incompatibility in *F. imperialis*. To further our understanding of *Fritillaria* reproductive dynamics, expanding our knowledge of germination fraction and seed viability in *F. maximowiczii* would enhance our ability to model effective population sizes and describe demographic patterns more accurately. To effectively model population sizes and accurately describe demographic patterns, it is essential to study closely related species such as *F. maximowiczii* within the subgenus *Petilium*, which shares a similar morphology and biology with *F. imperialis* and *F. raddeana*.

Fritillaria imperialis is a remarkable ornamental species, distinguished by its showy flowers, umbrella-shaped structure, size, color, and noble stance. Among the *Fritillaria* species in Türkiye, *F. imperialis* L. boasts the most striking and attractive flowers, followed by *F. persica* L., *F. michailovskyi*, and *F. aurea*. The floral cover of *F. imperialis* exhibits abundant blooms in vibrant hues of yellow, red, and orange, with a diverse range of corolla forms. These characteristics facilitate the easy differentiation of *F. imperialis* from other *Fritillaria* species during the fruiting period. Our flowering dynamics findings indicate that *F. imperialis* blossoms remain open from approximately March to April annually (Figure 3), with pollen presentation lasting 7–9 d, consistent with previous studies on *Fritillaria* species [6,48,49]. In contrast to the perianth color and shape of many *Fritillaria* species described in the literature [38,48–50], our findings reveal that *F. imperialis* flowers possess a relatively large appearance when open, with a strikingly distinct perianth coloration (Figure 2, see stage 4 in Figure 3, see stage 1 in Figure 4). The flowering process spans 17–21 d, with variations likely attributable to temperature fluctuations in March and

April. The partial similarities and slight asynchrony observed among previously reported *Fritillaria* spp. from flowering to fruiting can be explained by microhabitat and climatic differences, as reported in the literature [6,49,51].

Pollen viability and stigma receptivity are prerequisites for seed formation and successful pollination in flowering plants, playing a crucial role in understanding species reproductive performance and implementing effective breeding programs [67,68]. Our results demonstrate that stigma receptivity in *F. imperialis* was highest at the full dehiscence stage (see stage 5 in Figure 3), lowest at the fresh perianth phase (see stage 7 in Figure 3), and gradually reduced throughout the perianth-wilting period (see stage 8 in Figure 3). The pattern of stigma receptivity in *F. imperialis*, which initially increased and subsequently decreased with the flowering stage, aligns with other reports [38,50]. The species exhibited peak stigma receptivity for a duration of 5–7 days (Figure 5), consistent with previously reported results for other *Fritillaria* species [67,68]. Pollen viability in *F. imperialis* initially increased and then decreased as the life cycle progressed. Pollen grains displayed high viability during the pre-dehiscence stage (see stage 3 in Figure 3), first dehiscence stage (see stage 4 in Figure 3), and full dehiscence stage (Figure 3, stage 5), subsequently declining to 46% during the perianth-wilting period (see stage 8 in Figure 3). The observed variation in pollen viability rates compared to other *Fritillaria* species is an expected feature, likely reflecting species-specific characteristics. Indeed, pollen production in plants is known to depend on various factors, including pollen grain size, anther length, season, and anther separation mode [69]. Recent studies have emphasized the significance of pollen quantity and quality in pollination and fertilization success, highlighting their key role in plant conservation [70–73]. These factors are closely associated with demographic and genetic variables that influence population decline and extinction.

Our pollinator studies revealed that *A. mellifera* was the primary pollinator of *F. imperialis*, followed by *B. terrestris* (Figure 7). These species are recognized as more effective pollinators compared to other bee species due to their generalist nature and ability to collect pollen from diverse sources, resulting in heterospecific pollen loads on the stigmatic surface [74]. Pollinators were observed visiting flowers at various times throughout the day after flower opening, playing a crucial role in spontaneous cross-pollination. The floral structure of *F. imperialis* facilitates pollinator access, with nectar located in a spacious area within the bell-shaped corolla and at the base of the tepals (see stage 2 in Figure 4). Moths and flies, rarely observed during daylight hours, are considered nectar thieves rather than pollinators. These insects may feed on nectar by piercing the perianth base, potentially contributing to pollination deficits and higher yields in this species. We hypothesize that bees preferentially visit *F. imperialis* flowers due to their nectar composition, which exhibits a balance between glucose and fructose, and normal nectar volumes. This hypothesis aligns with observations by Yıldız et al. [6], Zych and Stpiczynska [48], Knuth [75], Rix and Rast [76], Burquez [34], and Huang et al. [77], who reported visits by *A. mellifera* and/or *B. terrestris* to various *Fritillaria* spp. Our results also indicate differences in both the frequency and duration of flower visits, with insects readily foraging on the pendulous flowers (Figure 7). Previous studies have reported that *Lasioglossum* spp. and *Andrena* spp. individuals typically spend more time on flowers compared to bumblebees, which corroborates our findings [48]. Moreover, honeybees (*A. mellifera*) exhibited more erratic movement patterns within *F. imperialis* flowers, occasionally covering the stigma entirely with pollen, resulting in increased pollen loads on similar stigmas. These observations partially elucidate the processes underlying the dependent shifts in pollination behaviors of *F. imperialis*. Our findings are comparable to those reported for *F. aurea* and *F. michailovskyi* pollinators [6,58]. However, they differ from studies on other *Fritillaria* spp. such as *F. meleagris*, *F. maximowiczii*, and *F. delavayi*, which were found to be pollinated by spiders, bumblebees, and bumblebee queens [48,49,51]. Given the lower abundance of other pollinators in the study area compared to the honeybee population, we propose that honeybees visit *F. imperialis* flowers more frequently than other bee species. However, we acknowledge that this hypothesis requires further investigation to be fully substantiated. This assertion

must be supported by data and observations from wild habitats to ensure its validity. Additionally, understanding the foraging behaviors and preferences of other bee species in these habitats will provide a more comprehensive view of the pollination dynamics.

5. Conclusions

The current study provided a comprehensive examination of various aspects of the reproductive biology of *F. imperialis*, an Irano-Turanian endemic species. Our investigation encompassed the pollen/ovule (P/O) ratio, self-incompatibility index (SII), flower biology, breeding systems, phenology, and flower visitors. *F. imperialis* flowers were characteristically bell-shaped and nodding, with anthers and nectaries concealed from approaching pollinators. Our findings revealed the presence of specific flower visitors, primarily *A. mellifera* and *B. terrestris*, and demonstrated high levels of self-incompatibility and xenogamy in *F. imperialis*. Given that the evolutionary success and survival of *F. imperialis* are largely dependent on the efficiency of its reproductive performance, these detailed findings on its reproductive biology are significant. The valuable information presented regarding the reproductive biology of this range-restricted species may have crucial implications for the management and conservation of threatened populations. Furthermore, our results are expected to prove beneficial for breeding system and pollination ecology research in other threatened *Fritillaria* spp. This research should be perceived as a contribution to a broader understanding of the reproductive strategies in rare and range-restricted *Fritillaria* spp., potentially informing future conservation efforts for other threatened members of the genus *Fritillaria*.

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