



Cooler Is Better: The Role of Temperature in the Reproductive Biology of Persian Walnut (*Juglans regia* L.) Studied in a Hungarian-Bred Cultivar

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Abstract

The aim was to determine the growth of pollen tubes throughout the transmitting tissue and the process of fertilization in open, optimal and over pollination on the 3rd, 6th and 9th day after pollination of pistillate flowers, together with apomixis. The experiment was carried out over 2 years in Lovasberény, Fejér district, Hungary, which differed in temperature conditions. The average number of pollen tubes in the locule of the ovary was significantly higher in 2017 (1.3%) than in 2019 (1%). A higher percentage of fruit set was recorded in 2017 compared to 2019. The percentage of apomictic fruits from variant of unpollination was higher in 2017 (10%) than in 2019 (3.7%). Based on these results it can be concluded that pollen tube growth and post-fertilization processes together with apomixis of cultivar ‘AK’ are better adaptable to lower temperatures. These findings may help us to move walnut production to the north or to higher altitudes, where the climate is more continental.

Keywords Embryo sac · Fertilization success · Fruit set · Pollen tube growth · Pollination

Introduction

The walnut (*Juglans* spp.) is a nut species whose use dates back to 7000 BC in Persia (Dreher et al. 1996). Its gene center is in Persia (as the name implies), from where it was distributed eastward to China, and westwards to Europe

and by colonists to the Americas. The walnut is cultivated in temperate and semi-arid regions (30–55° N and from 30–40° S). The Persian (English) walnut (*Juglans regia* L.) is well-known in the Carpathian Basin, as this fruit species could also be native there (Bujdosó et al. 2020). China is the largest walnut producer in the world (with a production share of 46%), while Iran is in the second place, followed by the USA, and Turkey (FAOSTAT 2023). Hungarian walnut production has increased sharply in the last two to three decades, and its cultivars are grown in many European countries (Bujdosó et al. 2005, 2016, 2019a, 2020).

In the 2022–2023 harvest year, almonds and walnuts accounted for 27% and 22% of global nut production, respectively. According to International Nut and Dried Fruit Council (INC 2021/2022), almonds and walnuts were the most consumed tree nuts in 2021, accounting for 31% and 21% of the world share, respectively. Walnuts owe their status as one of the most widely consumed nuts to their high proportion of fatty acids (mainly linoleic and oleic acid), proteins (globulins, albumins and prolamins), carbohydrates, phenolic compounds (phenolic acids and flavanols), tannins, minerals (P, Ca, Mg, K and Fe), vitamins (B1, B2, B6), tocopherols, carotenoids, melatonin, serotonin, saponins, glycosides, alkaloids, and steroids (Özcan 2009; Matthaus and Özcan 2021; Özcan and Lemiasheuski 2020; Kashif

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Ghafoor et al. 2020). Walnut consumption is also associated with antioxidant, atherogenic, anti-inflammatory, antimutagenic, antihelminthic, hepatoprotective and anticancer properties, prevention of neurological disorders and inhibition of the growth of some bacteria (Ruijun et al. 2015; Vinson and Cai 2012). Due to their high nutritional profiles, walnut kernels, as other nuts, are mostly consumed as snacks and in confectionary, while a liqueur is made from green walnut fruits (Karaosmanoğlu and Şule Üstün 2022). In addition to the kernels, other parts of the fruit are also used in the cosmetic and pharmaceutical sector (Ghiravani et al. 2020; Rabrenović et al. 2021), while the tree is used for timber and landscaping. The walnut is a wind-pollinated, monoecious and dichogamous self-fertile and cross-compatible fruit species. No evidence of infertility or incompatibility problems have been found in walnut trees (Kelles, 2021). This fruit species is characterized by a complex sexual reproductive process that differs from that of most other fruit species, and shows a phenotypic dimorphism of protandrous and protogynous mating types. The male flowers, catkins, can produce up to several million pollen grains (Milatović et al. 2020). Pistillate flowers lack the visible sepals and petals of the perianth, and the entire basal part of the flower is surrounded by a hairy, sticky bract that is fused with four sepals (Janković et al. 2021). The stigma is designed to catches and recognizes wind-borne pollen and exclude pollen of other species (Pollegioni et al. 2010). The longevity of the embryo sac is limited to 3–5 days in some walnut cultivars (Pintea 2014). In some walnut genotypes, a high correspondance was found between the outer phase of pistillate flower development and the stages of megasporogenesis and megagametogenesis (Janković et al. 2021). The pollen tubes can reach the walnut ovule within 24 h after pollination, and the process of syngamy usually occurs 5–7 days after pollination, with an eight-celled proembryo forming 1 week after fertilization (Janković et al. 2021). However, some walnut cultivars show a tendency towards apomictic fruit development (Abedi and Parvaneh 2016; San and Dumanoglu 2006). In some experiments involving walnut cultivars, apomixis ranged from 7.86% to 81.2% (Cosmulescu et al. 2012; Solar et al. 1995). Based on the recent studies by Solhjoo et al. (2023) on some Iranian walnut genotypes with cytometric and genotyping analyses, apomeiosis could not be confirmed, while cytohistological studies indicated that adventitious embryony occurs very rarely in these genotypes.

On the other hand, some walnut cultivar showed pistillate flower abortion (PFA), about 2–3 weeks after flowering. In some cultivars of Persian (*J. regia*) and black walnut (*J. nigra*), pistillate flower abortion is caused by excessive pollen load, leading to a reduction in final fruit set (Pollegioni et al. 2010). The incidence of pistillate flower abortion is influenced by hereditary and environmental factors and varies in

different years. It is generally believed that abundant self-pollination with pollen from the same tree or cultivar at the beginning of stigma receptivity can increase ethylene synthesis, causing pistillate flower abortion (Polito et al. 2005a, b).

The most important climatic factor for the flowering process of the walnut is the average air temperature. Temperature influences the onset of flowering, the duration of flowering, the distance between the maximum receptivity of female flowers and pollen release, and the flower type (Cosmulescu et al. 2010). Lee and Sumner (2016) showed that climate change can affect some walnut cultivars, especially in years with warm weather, seriously impacting their productivity. The most favorable conditions for this species are sites with mean annual temperatures between 10.5 °C up to 15 °C. *J. regia* is tolerant to low winter temperatures when the tree is fully cold acclimated, but one of the main limitations is its sensitivity to off-season frost events in autumn or spring (Guàrdia et al. 2013).

To date, numerous studies have been conducted to investigate pollen germination on the stigma, pollen tube growth and fertilization, and determine the best pollination combination in numerous fruit and nut species and cultivars to provide recommendations for the fruit industry (Milatović et al. 2010; Radičević et al. 2018; Cerović et al. 2020, 2021; Guastella et al. 2020; Bhat et al. 2016; Solhjoo et al. 2023).

Knowledge of their reproductive biology, which encompasses the process from the attachment of the pollen grain to the sticky stigma, to the growth of the pollen tube through the transmitting tissue, to the release of sperm cells into the immediate vicinity of the egg apparatus, is a necessary prerequisite for their successful commercial production and economically accepted yields. For this reason, the aim of the present work was to investigate progamic phase of fertilization and fertilization success using fluorescent microscopy (to the best of our knowledge, for the first time), as well as the fruit set of the new and perspective walnut cultivar ‘AK’ under Hungarian climate conditions, which have problems with its productivity in some years. The findings of this study would allow a better understanding of how factors (especially temperature) influence pollination, and thus, provide information to optimize pollination and increase fruit set. In addition, a possible recommendation for cultivation in other locations with similar agroecological conditions and/or use in some breeding programs can be withdrawn from this study.

Materials and Methods

Plant Material

In 2017 and 2019, the progamic phase of fertilization, fertilization success, and fruit set were investigated in four pollination variants on pistillate flowers of the cultivar 'AK' ('Alsószentiváni 117' × 'Pedro'). This genotype, which derives from the Carpathian breed, is characterized by an early ripening period (in September at the place of origin) and large fruit size (Ebrahimi et al. 2017; Bujdosó 2019b). For the artificial pollination of the cultivar 'AK', pollen from the walnut cultivars 'Chandler' and 'Bonifác' was used as a mixture (50:50). These cultivars showed an overlap of the pollen shading with the receptivity of the pistillate flower of the cultivar 'AK'. The trial was conducted in a 2-ha orchard in the Lovasberény region of the Fejér district (Hungary) located at 47°19'41" N, 18°35'47" E, 213 m above sea level. The observed trees were grafted on *J. regia* seedlings, planted in fall of 2001. Planting spacing was 10 × 12 m, with a central leader canopy. The orchard contained the main cultivar 'AK' and 1% of the pollinizers.

Floral Phenology and Climate Conditions

The UPOV descriptor (UPOV 2017) was used to determine the flowering stages of the female and male flowers in the walnut cultivars tested. The flowering period of pistillate flowers in walnut was recorded from the stage of the beginning of stigma opening (Ff1) (anthesis) to the stage of the beginning of stigma drying (Ff3). The period of pollen shedding was recorded from the beginning of another opening stage (Fm) to the complete anthers dehiscence stage (Gm).

The average, maximal and minimal daily temperatures (°C) and precipitation (mm) were recorded by an automatic weather station in the experimental years 2017 and 2019 and presented in supplementary Figs. 9 and 10. The Lovasberény region has a typical continental climate with hot, dry summers and mild, cold and snowy winters. An average annual temperature of 11.8 °C and annual precipitation of 551 mm are typical recordings for this part of Hungary.

In the experimental years, the warmest months were June and August and the coldest January (Weather and Climate 2023). The average annual number of rainy days is 134. During the experiment, the distribution of precipitation was different: in 2017, September and October were the wettest months, while in 2019 it was May (Weather and Climate 2023).

Pollen Germination *in Vitro* and Pollination Treatments

For the pollen analysis, catkins were collected from numerous trees within the orchard in the early morning prior the high daytime temperatures, when the first staminate flowers of the catkins had begun to shed their pollens. The catkins were taken to the laboratory and placed in Petri dish. After 1 h, the dishes were sealed and moved by hand left and right, in circles, and up and down for 2–3 min to vibrate the entire area to break the anthers and release pollen. Sowing of pollen on artificial media was done immediately after the cooled down, but before the pollens lost its viability (Mert 2009; Fotirić Akšić et al. 2019).

The pollen for *in vitro* study of pollen germination was collected from the catkins of the cultivars 'Chandler' and 'Bonifác' from the beginning of anther dehiscence. The shaded pollen from these two cultivars were mixed (50:50 ratio) and tested *in vitro* in a medium of 1% agar +15% sucrose, and stored at room temperature (±20 °C) for 24 h. More than 500 germinated/ungenerated pollen grains were counted.

The fresh catkins of the cultivars 'Chandler' and 'Bonifác' were collected immediately before artificial pollination. As soon as the stigmas were receptive (stigmas at an angle of 45° and the red color of the tips), the pistillate flowers of 'AK' were hand-pollinated with a prepared pollen mixture. For optimal pollination, a 1% pollen mixture of 'Chandler' and 'Bonifác' was combined with 99% talcum powder. For over pollination, 5% of pollen mixture was mixed with 95% of talcum powder. Both mixtures were shaken well before use in the experiment. Cotton bags were used to isolate the pollinated pistillate flowers. To test the tendency to apomixis, the pistillate flowers were covered with cotton during flowering (third pollination variant). Finally, the fourth variant was open pollination as a control treatment. For the study, about 240 pistillate flowers were prepared (isolated and labeled) for each pollination variant in each year.

Pollen Tube Growth in Pistillate Flowers

Samples of the pistillate flowers (30 flowers) were taken from all pollination combinations on the 3rd, 6th, and 9th day after anthesis (three replicates × 10 pistils). The fixation, preparation and examination of the pistillate flowers were carried out as previously described in Cerović et al. (2021). The number of pollen tubes in the basal part of the pistil and in the locule of the ovary was counted for optimal, over (excessive) and open pollination and for each fixation time (3, 6 and 9 day after anthesis). Based on the percentage of pistils where pollen tubes can be observed,

the dynamics of pollen tube growth in the different parts of the pistil and the ovary were determined.

Fertilization Success

To investigate the success of pollination, samples of pistillate flowers from all pollinations were taken 3, 6 and 9 days after anthesis (three replicates $\times$ five pistils). The permanent histological preparations from the ovaries of the pistillate flowers were prepared according to the method already described in Cerović et al. (2021).

Fruit Set

For the evaluation of fruit set, a group of 150–180 (three replicates $\times$ 50–60 pistils) pistillate flowers were randomly selected for each of pollination variant. The percentage of fruit set was counted shortly before harvest (number of fruits/total number of pistillate flowers $\times$ 100).

Statistical Analysis

Statistical analyses were performed using the software package STATISTICA for Windows 11.0 (StatSoft Inc., Tulsa, OK, USA). Fischer model of two-factor analyses of variance (ANOVA) was used to process the data obtained. When necessary, the least significant difference (LSD 0.05 = 95% confidence) was used.

Results

Effect of Climate Conditions on Flowering Phenology

Temperature exhibits great interannual variability and thus has the greatest influence on the quantitative variation in biological processes (Luedeling and Gassner 2012). Phenology, daily air temperatures (average, max and min) and precipitation (mm) during pistillate flower opening and pollen release in 2017 and 2019 are shown in Fig. 1. The walnut cultivar ‘AK’ showed pronounced protogyny. In 2017, the pistillate flowers began to bloom on April 30, in 2019 on April 25. The interval of stigma receptivity, between the peak of stigma receptivity (50% of flowers are open) and the end of stigma receptivity was 6 days in both years.

The catkins of cultivar ‘AK’ started pollen shading two days after the pistillate flowers finished flowering, on May 7 in 2017 and May 3 in 2019. The pollen shading of pollinizer ‘Chandler’ overlapped with the receptivity of pistillate flowers of ‘AK’ 4 days in 2017 and 6 days in 2019. In contrast, for overlap of the pollinizer ‘Bonifác’ with the female flowers ‘AK’ was 6 days in 2017 and 4 days in 2019. The average temperature recorded in April (just before flowering)

was 10.4 °C in 2017, while 13.3 °C in 2019. During this period, precipitation was lower in 2017 (10.2 mm) compared to 2019 (13.7 mm).

The flowering time of the cultivar ‘AK’ in 2017 was cooler (13.6 °C) compared to 2019 (15.3 °C). The amount of precipitations during flowering was 5.1 mm (2017) and 2.2 mm (2019).

Pollen Tube Growth in Pistillate Flowers

The percentage of germination of the pollen grains (mixture of two different pollinizers) tested *in vitro* on the artificial medium was 30% (Fig. 2).

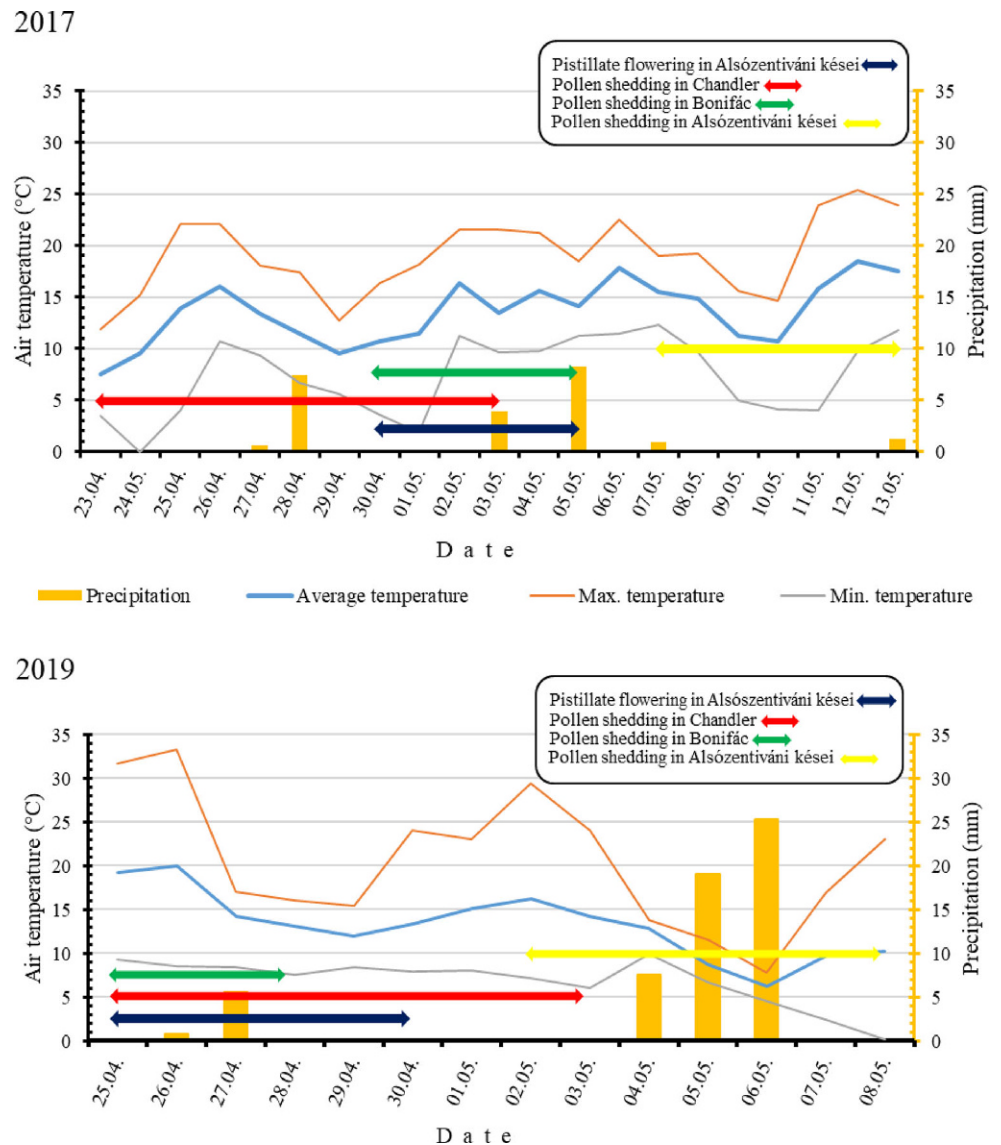
The pollen tube growth *in vivo* started with the germination of the pollen on the stigma surface (Fig. 3a). It was found that the distribution of germinated pollen grains on stigma lobes was uneven in all pollination variants. After penetrating the stigma surface, the pollen tubes grew throughout the entire transmitting tissue toward the base of the stigma (Fig. 3b). The pollen tubes grew along the entire length of the short, sessile pistil, to a narrow canal, along the surface cells of this canal (Fig. 3c). The pollen tubes clearly grew into the ovary after leaving the ovary canal. During pollen growth in the different tissues of the pistil and the ovary, the number of pollen tubes decreased sharply. In the ovary, the pollen tubes were observed near the funiculus (Fig. 3d), and later continued through the nucellus tissue until they entered the embryo sac (Fig. 3e, f). The chalazogamic pathway of pollen tube growth to the embryo sac was also observed.

The base of style and the locule of ovary are the most important regions for evaluating the quantitative efficiency of pollen tube growth (Fig. 4). The percentage of pollen tubes in these parts of the pistil varied depending on the pollination type.

The average number of pollen tubes in style and locule of ovary showed a similar trend in both years. During open pollination on 9th day after anthesis, the percentage of pollen tubes in the style was highest in both years (48.5% in 2017 and 35.8% in 2019). The lowest average number of pollen tubes was recorded in the style during optimal pollination (1% of pollen mixture), which was 22.1% in 2017 and 30.5% in 2019. The average number of pollen tubes in the locule of ovary was significantly higher in 2017 (1.3%) than in 2019 (1%) for all three types of pollination types (optimum, over and open pollination). In optimal pollination and over pollination, the average number of pollen tubes in the ovary was the highest in 2017 (1.4%). The dynamics of pollen tube growth in all pollination variants of the cultivar ‘AK’ are shown in Fig. 5.

The highest percentage of pistils (70.0%) with pollen tubes penetrating the ovary of ‘AK’ was recorded in 2017 on the 9th day after optimal pollination. In 2019, the highest

Fig. 1 Phenology, daily temperatures and precipitation during flowering of pistillate flowers in cultivar 'AK' and pollen shedding period of cultivars 'AK', 'Chandler' and 'Bonifác' in 2017 and 2019



percentage of pistils (66.7%) with pollen tubes in the locule was also observed on the 9th day but with over pollination. Open pollination (control) on the 9th day after anthesis was different in both years (50% in 2017 and 66.7% in 2019). The growth of pollen tubes through certain parts of the pistil (style and locule of ovary) also showed uniform dynamics in the first 9 days after pollination in optimal and over pollination and as well as in open pollination (control), except in the variant of optimal pollination on the 3rd day after anthesis in 2019.

Development of Embryo Sac and Fertilization Success

During anthesis, the histological structure and shape of the ovule change rapidly, and the process of megagametogenesis begins in the pistillate flowers immediately before the

flower opens. Sequences of this process (three mitotic cycles) were observed at the beginning of pistillate flower opening (stages between the beginning of stigma opening [Ff1] and the peak stigma receptivity –50% open flowers [Ff2]) (Fig. 6a). The embryo sac development process ended with the formation of monospore-like bipolar embryo sacs with eight nuclei (eight-nucleate embryo sac). It contained two synergids, one egg cell, and one central cell with two polar nuclei and three antipodes (Fig. 6b–d). Irregularities and partially or completely degenerated embryo sac were also observed (Fig. 6d). The described irregular arrangement of the individual elements and the degenerative changes in the embryo sacs were usually followed by degeneration of the ovule tissue (integument and nucellus). Sometimes supernumerary embryo sacs were also observed (Fig. 6e). In the later stage of full flowering, the fertilized egg cell and the process of endosperm formation occurred

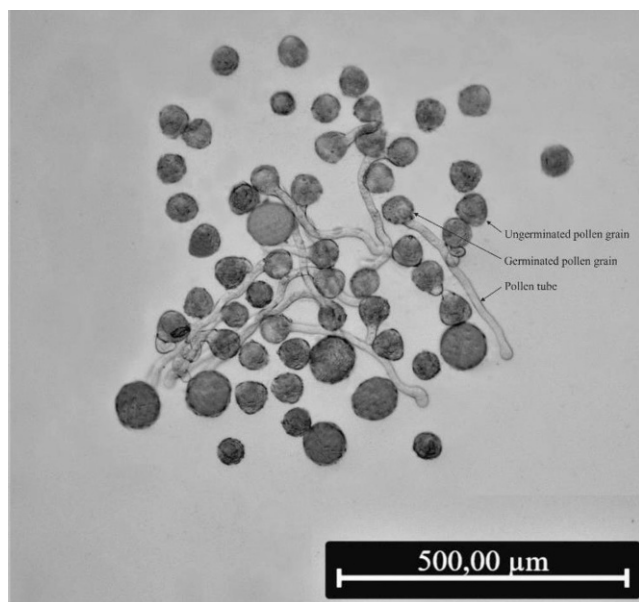


Fig. 2 Germination of the ‘Chandler’ and ‘Bonifác’ pollen mixture (50:50)

(Fig. 6f). In the unpollinated variant (apomixis checking), the occurrence of early embryogenesis was only observed in the unfertilized egg cell.

The percentage of functional embryo sac stages (eight- and five-nucleated) was recorded in all pollination variants during full flowering of pistillate flowers in both years (Fig. 7). As expected, their percentage decreased from the 3rd to the 9th day thereafter. In 2019, the highest percentage of functional stages of embryo sacs was 33.3% (five-nucleated embryo sacs) on the 6th day of pistillate flower anthesis.

In 2017, the highest percentage (66.7%) of embryo sacs with early embryogenesis was recorded with optimum pollination after the 6th day (Fig. 7). With this pollination variant, this percentage remained the same after the 9th day after anthesis. Over pollination led to the better results than open pollination: 50% of the embryo sacs were in early embryogenesis, compared to 33.3% on the 6th day after anthesis of the pistillate flower. In the variant with unpollinated flowers, 50% of embryo sacs showed signs of early embryogenesis on the 9th day after the onset. In the following year, embryo sacs with early embryogenesis were not detected until shortly after the 9th day after the pistillate flowers began to be receptive (Fig. 7).

Fruit Set

The final fruit set values for all pollination variants are shown in Fig. 8. In 2017, the final fruit set values were higher than in 2019 for all variants of pollination. The higher percentage of fruit set was recorded in 2017 with op-

timal pollination (30%), and with over pollination (12.5%) compared to 2019 (7.8% and 6.7%, respectively). The percentage of apomictic fruits in the variant without pollination was higher in 2017 (10%) than in 2019 (3.7%).

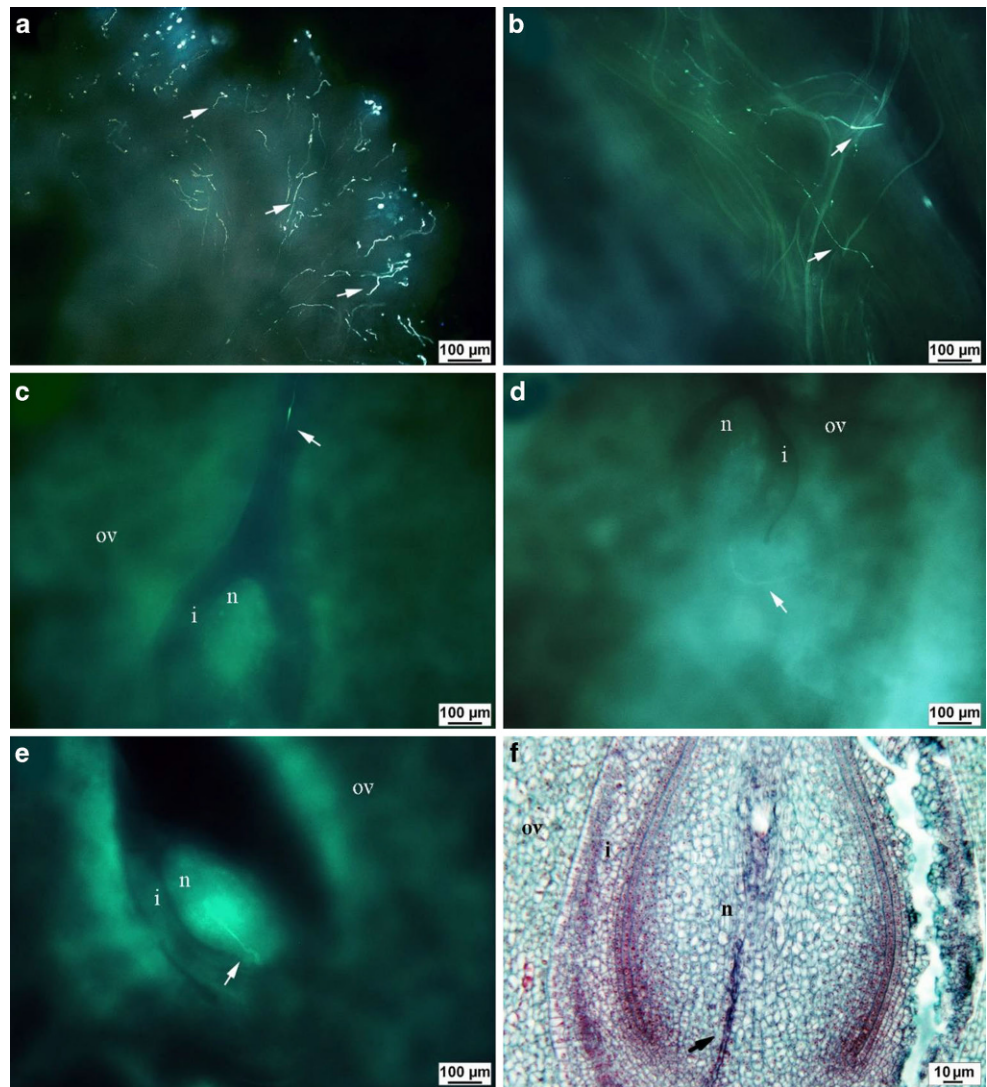
Discussion

Phenology of Flowering

The ecological factors during the flowering and pollination period influence a higher percentage of pollination, which increases productivity. Temperature and relative humidity control the opening of the anthers and the release of pollen, which includes active water extraction from the anthers and swelling of the pollen grains. When pollen shedding starts, ecological factors (air temperature and relative humidity) influence the speed of pollen flight and have a decisive influence on the availability of pollen (Cosmulescu et al. 2010; Ionescu and Cosmulescu 2017). Relatively cool springs with late frosts can cause pollen anomalies, especially those associated with tapetum degeneration and abnormal tetrad formation, all leading to pollen sterility (Kvaliashvili et al. 2006).

Elevated temperatures often occur in combination with high solar radiation and drought which can be harmful for the plant and especially for the pollination process (Monteiro et al. 2015). According to Pollegioni et al. (2010), warm weather increases protandry in walnuts, while cool weather after a warm winter increases protogynous tendency and modifies protandric tendencies. Protogynous cultivars can even be protandrous in some locations and seasons, and likewise a protandrous cultivar may occasionally appear protogynous. In the cultivar ‘AK’, the receptivity period of the female flowers was 6 days in both years, which is somewhat shorter than the 8-day receptivity of some newly bred Hungarian walnut genotypes (Bujdosó et al. 2019b). A shorter receptivity period of female flowers (4 days) was also observed in some Iranian walnut genotypes (Mahmoodi et al. 2016). In the study of Janković et al. (2021), the flowering of female flowers ranged from 11 (‘Elit’) up to 20 days (‘MS-1’ seedling). This depends on the cultivar and the weather conditions during flowering. The earlier onset of pistillate flowers receptivity in the cultivar ‘AK’ in 2019 (April 25) compared to 2017 (April 30) could be related to the higher average temperature (13.3 °C) and precipitation (13.7 mm) in 2019, compared to 2017 (10.4 °C and 10.3 mm, respectively). A study by Lee and Sumner (2016) proved that walnut is one of the most weather-susceptible crops by showing that in some years with warm winters and springs, some genotypes can have very low productivity, which can be influenced by its origin. Cosmulescu et al. (2010) showed that the flowering time is shorter when tem-

Fig. 3 The growth of the pollen tubes in the pistil of cultivar ‘AK’. Growth of pollen tubes on the surface of the stigma (a); the penetration of the pollen tubes into the tissues of the style (b); pollen tubes entering the narrow canal of style base (c); the penetration of the pollen tube near the funiculus (d); penetration of the pollen tube throughout the tissue of the nucellus (e, f). *ov* Ovary tissue, *i* Integument, *n* Nucellus, *arrows*: Pollen tubes in different regions of pistil



peratures are higher, while Yates and Sparks 1989 demonstrated that at a temperature of $<10^{\circ}\text{C}$ with high relative humidity ($>85\%$) anther opening can be delayed or already opened anthers can be closed again. Mahmoodi et al. (2016) found a difference of almost 1 month between the flowering time of female flowers in years with different ecological conditions. The cultivars ‘Chandler’ and ‘Bonifác’ shared the different overlap time with the receptivity of pistillate flowers of the cultivar ‘AK’ (6 days in 2017 and 3 days in 2019). This indicates that the higher average temperature in 2019 influenced the shortening of the flowering time of the male flowers of these cultivars. Environmental factors, especially temperature, are very important for the opening of male flowers, pollination and the fertilization processes (Cosmulescu et al. 2010; Çetinbaş-Genç et al. 2019).

Pollen Tube Growth in Pistil In this study, pollen germination on the stigma surface was not uniform. Some parts

of the stigmatic lobe showed more and others less germinated pollen grains. The pollen tube grew intercellularly through the short style canal toward the ovary. Finally, the pollen tube penetrated into the chalazal end of the nucellus of the ovule and further into the embryo sac. These results indicate that chalazogamous growth of the pollen tube is characteristic for cultivar ‘AK’. Chalazogamy is generally considered characteristic of *Juglandaceae*, although the occurrence of chalazogamy/porogamy is correlated with the developmental stage of pistillate flower. In our results, the presence of pollen tubes in the ovary on the 3rd day after pollination was also noted.

Due to the presence of dichogamy, the effectiveness of the progamic phase of walnut fertilization is influenced by many factors, including environmental conditions during flowering, pollinizer selection, the quantitative characteristics of pollen tubes growth in the pistil and ovary, and the overlap of the main cultivar and pollinizers during full flow-

Fig. 4 The average number (bars) and $\pm$ standard deviation of pollen tubes in the style and in the locule ovary of the cultivar ‘AK’ in different variants of pollination in 2017 and 2019. Different letters below the bars denote a significant difference according to LSD test ($p < 0.05$). *p* Pollen grain, *pt* Pollen tube; *lo* Locule of ovary, *m* Micropyle, *i* Integument, *ec* Embryo sac, *n* Nucellus, *f* Funiculus, *ov* Ovary tissue

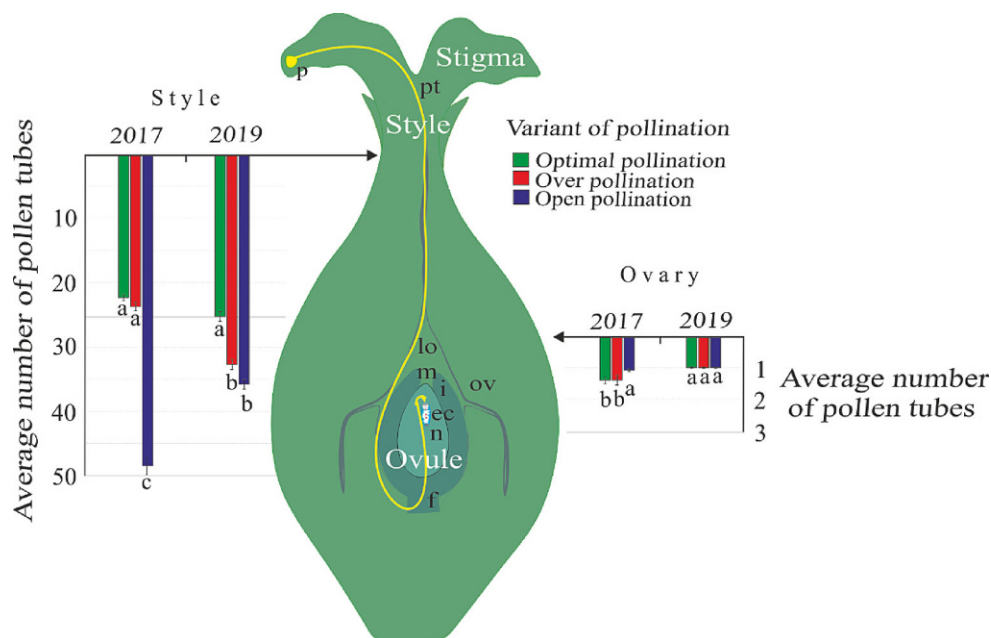
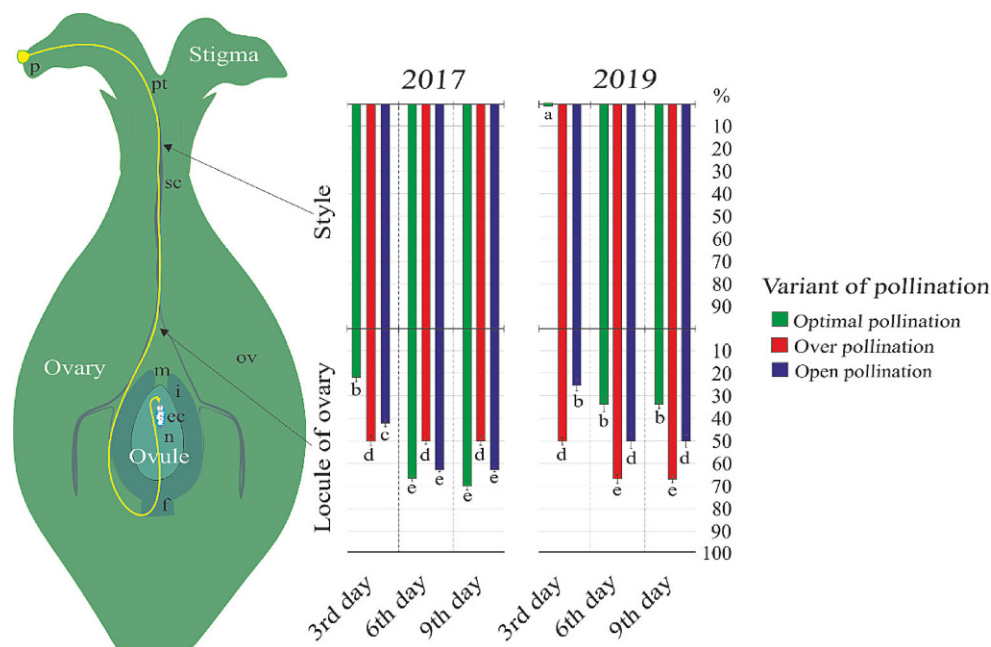


Fig. 5 The dynamic (bars) and $\pm$ standard deviation of pollen tube growth through certain pistil parts (style and locule of the ovary) 3, 6 and 9 days after pollination (variants optimal and over pollination) and open pollination of walnut cultivar ‘AK’ in 2017 and 2019. Different letters below the bars denote a significant difference according to LSD test ($p < 0.05$). *p* Pollen grain, *pt* Pollen tube; *lo* Locule of ovary, *m* Micropyle, *i* Integument, *ec* Embryo sac, *n* Nucellus, *f* Funiculus, *ov* Ovary tissue

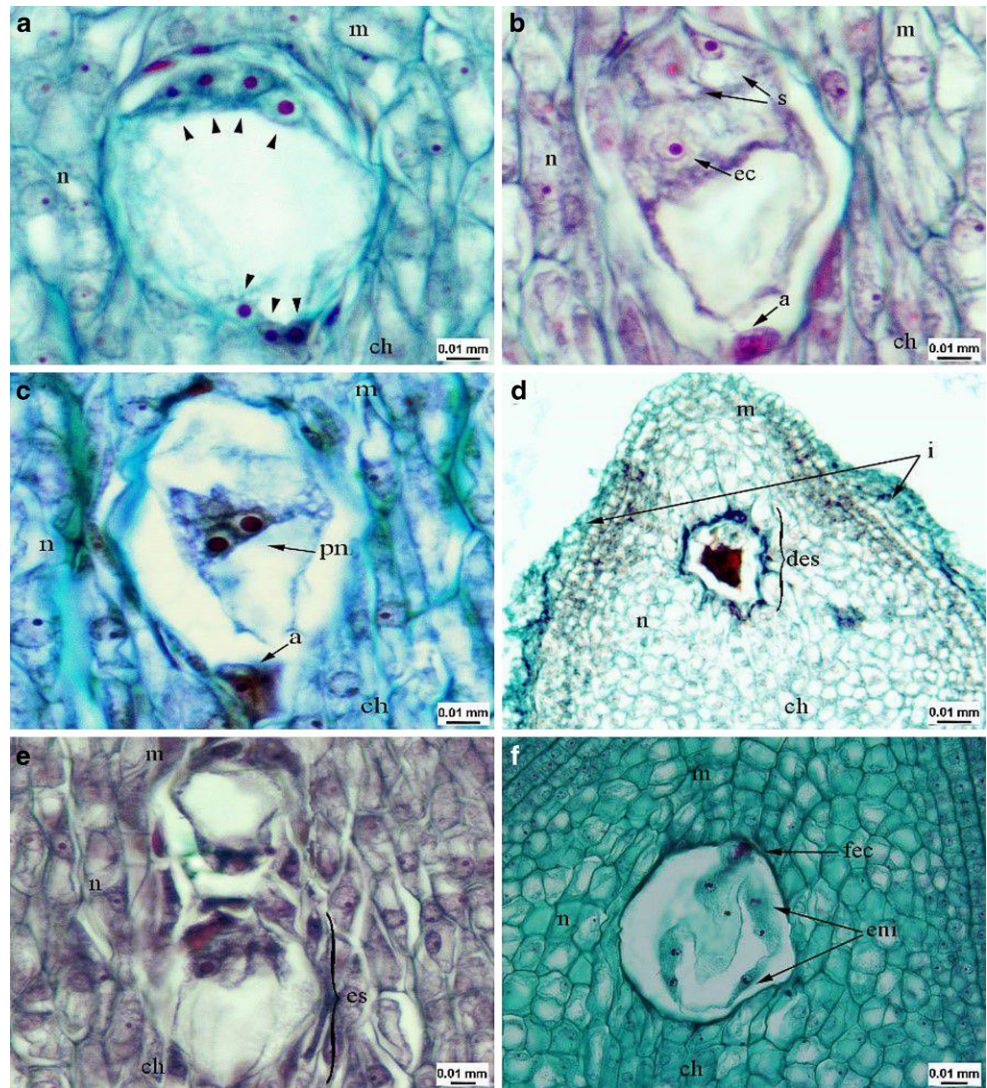


ering (Heslop-Harrison 1987). The lifespan of walnut pollen can be very short under higher temperature and relative humidity due to desiccation which can also jeopardize the fertilization process (Mert 2009; Simpson et al. 2021). Lang and Simpson (2018) treated shedding walnut catkins with a range of temperatures (23–40 °C) and reported that pollen viability decreased with increasing temperature, with less than 0.5% of pollen grains viable at temperatures ≥ 36 °C.

In this study, the average number of pollen tubes in the style was significantly higher in over pollination in 2019 than in 2017, but exactly opposite in open pollination, being higher in 2017 than in 2019. In the ovary, the average

number of pollen tubes was significantly higher in 2017 than in 2019. On the other hand, the growth of pollen tube in the pistillate flowers was better in 2017 (cooler year) compared to 2019 (warmer year) in all pollination variants. The results obtained clearly indicate a specific response of the ‘AK’ genotype to temperature in all pollination variants. It is assumed that the cultivar has a better adaptability to lower temperatures during the progamic phase of fertilization. According to Huo et al. (2014) mild temperatures and high humidity are required for the pollen tube to grow rapidly and complete the process successfully in *Corylus kweichowensis*.

Fig. 6 Embryo sac in cultivar 'AK'. Megagametogenesis after third mitotic division (four cells on the micropylar pole and three visible cells on the chalazal pole of the embryo sac (a); normal position of synergid cells and egg cell in the embryo sac (b); two polar nuclei in the central cell (c); degeneration of whole embryo sac (d); super-numerary embryo sac (e); early embryogenesis (f). *m* Micropylar pole, *ch* Chalazal pole, *arrow-heads* Nuclei, *s* Synergid cells, *ec* Egg cell, *pn* Polar nuclei, *a* Antipodal cells, *des* Degenerated egg apparatus, *n* Nucellus, *i* Integument, *es* Supernumerary embryo sacs, *fec* Fertilized egg cell, *eni* Endosperm nuclei



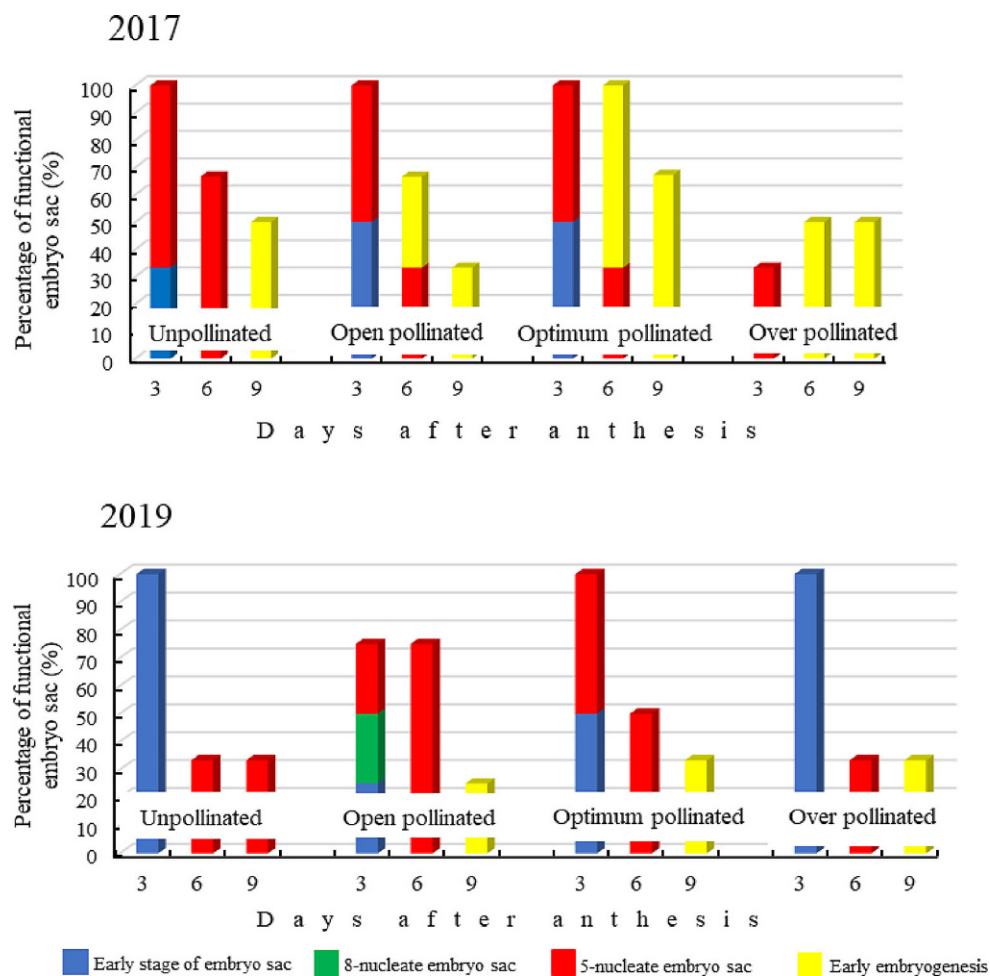
Fertilization Success and Fruit Set

The embryo sac of walnut cultivar 'AK' belongs to monosporous, bipolar, eight-nucleated *Polygonum*-type (Solhjoo et al. 2023). Although the pistil of the walnut consists of two carpels, only one ovule is formed in the ovarian cavity (Janković et al. 2021).

This stage of the eight-nucleate embryo sac was only observed in open pollination in 2019, while five-nucleate embryo sac was present in all pollination variants in 2017 and in the open and optimal pollination variants in 2019. In 2019, the five-nucleate embryo sac was only present 6th day after the pistillate flowers bloomed. In our study, only one pollen tube was observed entering the embryo sac, whereas Langdon (1934) found that it was not uncommon for two tubes to enter the micropylar canal, with both synergids being destroyed by tubes in *J. mandshurica*.

On the 9th day after pollination, no functional embryo sacs could be observed except in the unpollinated variant in 2019. Tadeo et al. (1994) found that the egg cell and the two synergids appeared to be normal until the 5th day after pollination. However, in the period after the 6th day after pollination, embryo sacs can be observed in the early stages of embryogenesis. The occurrence of this stage coincides with the period in which fertilization takes place in walnuts. In walnut, the fertilization occurs 5–7 days after pollination (Pollegioni et al. 2010). In our study, the fertilized egg cell was still unicellular and surrounded by discolored material originated from the collapsed synergid. The endosperm remained coenocytic at this stage. Division of the fertilized egg cell begins 1 week after fertilization, followed by the process of cellularization of the endosperm, which fills most of the apical portion of the embryo sac. In this study, the occurrence of early embryogenesis was also observed in the unfertilized egg cell of the unpollinated variant. In

Fig. 7 Percentage of functional embryo sacs in early stages of the development, eight- and five-nucleate embryo sac and early embryogenesis in cultivar 'AK' in four variants of pollination during 2017 and 2019



our study, the difference in early embryogenesis between the unpollinated variant and other pollinated variants was not noticed.

Our results showed that the average percentage of embryo sacs in early embryogenesis was higher in 2017 (50.2%) compared to 2019 (30.5%) for all pollination variants. This could be due to the fact that the exudates of the stigmas persist longer under cool and humid conditions and prolong the receptivity of the stigmas (Lora et al. 2011). In 2017, the optimal pollination variant had the highest percentage of embryo sacs in early embryogenesis (66.7%). In 2019, the optimal and over pollination variants had the same percentage of embryo sacs in early embryogenesis (33.3%). A higher percentage of embryo sacs in early embryogenesis therefore had an effect on a higher percentage of fruit set.

The average fruit set percentage calculated from all pollination types was higher in 2017 (17.2%) compared to 2019 (7.7%). With optimal pollination, the fruit set was higher in 2017 (30%). In 2019, the highest percentage of fruit set was recorded with over pollination (12.5%), followed by optimal pollination (7.8%). In fact, only fruit set after

over pollination did not differ statistically between years, but for all other pollination options, fruit set was significantly higher in 2017 than in 2019. Regardless of the fact that female reproductive organs in walnuts are surrounded by several layers of plant tissue and are more stress-resistant than the male reproductive structures (Lin et al. 2016), these results suggest that the fertilization and post-fertil-

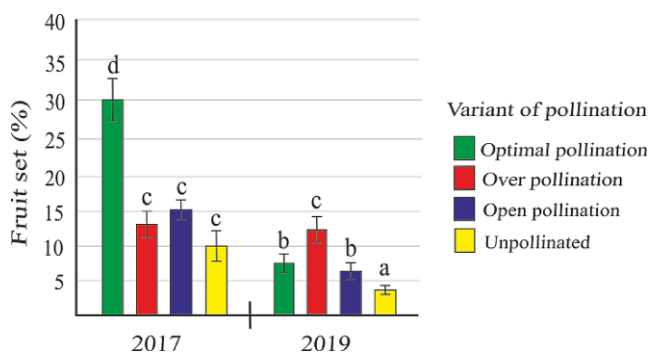


Fig. 8 Fruit set in cultivar 'AK' in four variants of pollination during 2017 and 2019. Different letters above the bars denote a significant difference according to LSD test ($p < 0.05$)

ization processes of the cultivar 'AK' are better adapted to lower temperatures, as was also found for pollen tube growth through the pistil. The values of fruit set in cultivar 'AK' after optimal, over and open pollination were significantly lower in both years than the values of 70–80% fruit set reported by Rovira and Aleta (2006) in some walnut varieties, which can be explained by different cultivars and unfavorable ecological factors.

Excessive pollen tubes growing down the style of the female flower produce higher amounts of ethylene, which is associated with flower senescence (Beede and Polito 2003). When pistillate flowers were collected in this study, no signs of necrosis at the stigma tips or necrosis around the integument, which are signs of pistillate flower abortion, were observed in the histological preparations in any cross combination. In this study, the over-pollinated variant had no effect on pistillate flowers abortion and abscission especially when compared to open pollination fruit set. According to the study by Bhat et al. (2016), walnut cultivars respond differently to high pollen load, while Rovira and Aleta (2006) found that climatic conditions may also have an influence on pistillate flower abortion (PFA). Polito (1998) found that more than 85 dead or live pollen grains on the stigma surface of a walnut produced the amount of ethylene that can cause abortion. McGranahan et al. (1994) concluded in their study that PFA was first observed in walnut orchards with low pollen diversity when either self-pollination occurred or a single pollinizer cultivar was used. According to the literature, PFA ranges from 4.29% to 100% in walnut cultivars from Chile, Iran, India, and Turkey, Gonzalez et al. 2008; Lemus 2010).

The frequency of apomixis in walnut is highly variable and depends on genetic control, environmental influences, the ploidy effect, hormone levels, and mineral nutrition. Due to the floral variation and limited pollen production of *J. regia*, apomixis is common (Bernard et al. 2018). Chen et al. (2020) reported the development of apomictic embryos from the egg cell in different walnut cultivars. Apomixis testing showed a fruit set of 10% in 2017 and 3.7% in 2019, which is not sufficient for an economically viable yield. In some Turkish walnut genotypes, the percentage of apomictic fruits was only 0.5–1.6% (San and Dumanoğlu 2006). Cosmulescu et al. (2012) determined 7.86–12.46% apomictic fruits in some Romanian walnut

genotypes, while Solar et al. (1995) found 31.5% apomictic fruits under Slovenian conditions. In addition, Solhjoo et al. (2023) reported that adventitious embryony are very rare in frequency in some commercial walnut cultivars.

Conclusions

In order to increase walnut production, it is necessary to better understand the process of pollination, fertilization, and fruit set. For this reason, the pattern of fertilization biology in walnut cultivar 'AK' was observed and thoroughly investigated in this study. The overall reproductive process of the 'AK' along with apomixis was more pronounced in the year with cooler temperature. In addition, it is very important to emphasize that none of the signs of pistillate flower abortion could be observed in over pollinated pistillate flowers, which means that this cultivar is not susceptible to this physiological disorder. For this reason, 'AK' could also serve as good material for walnut improvement, as plant breeders usually have no control over the amount of pollen that lands on a stigma in controlled crosses. As this cultivar prefers a cooler climate, it has the potential to extend the walnut growing area northward, or to higher altitudes where the climate is more continental. Clearly, further research is needed to evaluate the behavior of this cultivar under different management practices, different combinations of pollenizers and different climatic conditions. Since flowering and pollination in walnut is under the strong influence of ecological factors, producers should be recommended to use more than one cultivar-pollenizer in walnut orchards in order to provide regular yield and positive economic turnover.

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Appendix

Fig. 9 Average temperatures (°C) during the experimental period

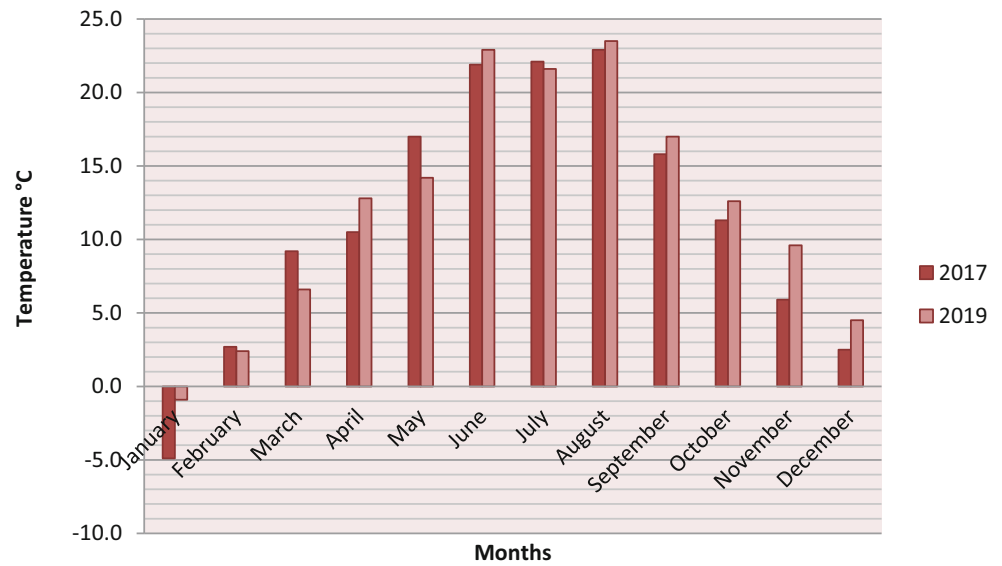
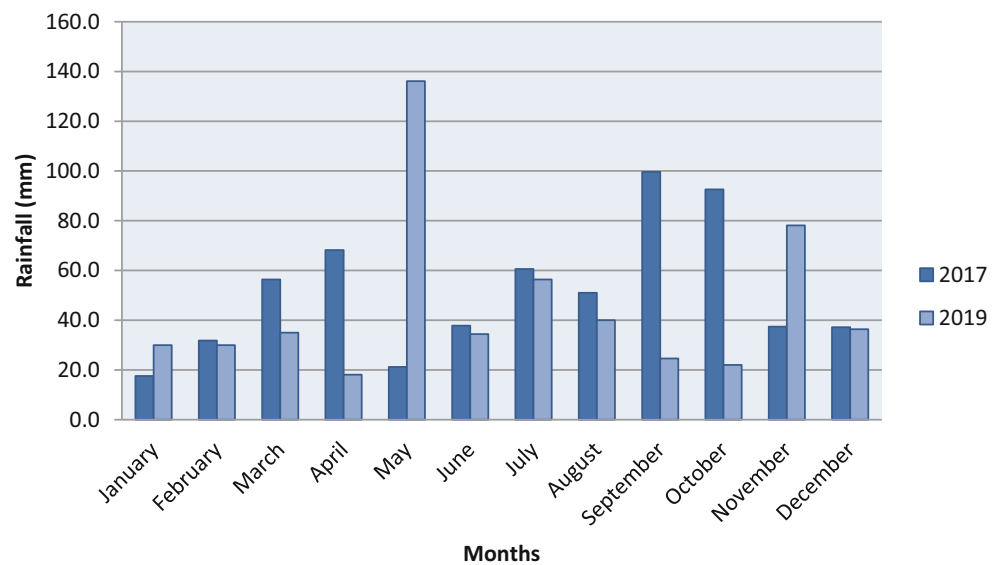


Fig. 10 Average rainfall (mm) during the experimental period



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Data Availability Data will be made available on request.

Conflict of interest M. Fotirić Akšić, S. Ercisli, M. Meland and G. Bujdosó declare that they have no competing interests.

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