ISSN 2075-5457 (print), ISSN 2220-9697 (online)

••• ФІЗІОЛОГІЯ ТА БІОХІМІЯ РОСЛИН ••• PLANT PHYSIOLOGY AND BIOCHEMISTRY •••

DOI: https://doi.org/10.26565/2075-5457-2026-46-7
UDC: 635.656:631.527:57

The effectiveness of artificial pollination in various representatives of the genus *Pisum* L.

A.O. Vasylenko, N.O. Vus, L.M. Shevchenko, O.M. Bezuhla,
A.V. Hlyantsev, V.O. Skydan, Yu.O. Zimohlyad

Wild relatives of cultivated pea (*Pisum sativum* L.) represent an important reservoir of alleles associated with resistance to biotic and abiotic stressors; however, their use in breeding programs is constrained by limited knowledge of their diversity and by difficulties in obtaining hybrids under field conditions. In this study, we evaluated the efficiency of interspecific hybridization among *P. sativum*, *P. elatius* and *P. fulvum* under field conditions, focusing on the effects of flower emasculation timing, parental genotype, and direction of the cross. Morphological characteristics of the parental accessions were used to facilitate hybrid identification based on seed-coat coloration in direct and reciprocal crosses. Two pollination approaches were compared: immediate pollination following flower emasculation and delayed pollination conducted 24 hours after emasculation.

Across combinations involving *P. sativum*, hybrid seed set (S/F index) was consistently higher when pollination was performed immediately after emasculation. Delayed pollination resulted in a marked reduction of hybrid seed production, likely reflecting both physiological constraints and high temperature stress during flowering. Reciprocal crosses revealed low and relatively uniform hybridization efficiency in combinations with *P. fulvum*, whereas crosses involving *P. elatius* produced substantially higher seed set, consistent with its close genetic affinity to *P. sativum*. The stability of hybridization efficiency across years (2020 – 2021) was confirmed for several cross combinations. Evaluation of the F_1 and F_2 seed lots demonstrated the successful production of true interspecific hybrids, with diagnostic segregation observed in combinations where the wild species served as the maternal parent.

These results confirm that viable hybrids among representatives of the genus *Pisum* can be reliably obtained under field conditions and highlight that immediate post- emasculation pollination is the most effective strategy for maximizing hybrid seed production. The findings support the broader use of wild *Pisum* germplasm in pre-breeding and genetic research and provide practical recommendations for improving interspecific hybridization success in breeding programs.

Key words*: Pisum sativum L., Pisum elatius, Pisum fulvum, hybridisation*

Cite this article: *Vasylenko A.O., Vus N.O., Shevchenko L.M., Bezuhla O.M., Hlyantsev A.V., Skydan V.O., Zimohlyad Yu.O. The effectiveness of artificial pollination in various representatives of the genus Pisum L. The Journal of V. N. Karazin Kharkiv National University. Series Biology, 46, 2026, p. 82-92.https://doi.org/10.26565/2075-5457-2026-46-7*

About the authors:
Vasylenko A.O. – Yuriev Plant Production Institute of National Academy of Agrarian Science (NAAS), 142 Heroiv Kharkova av, Kharkiv, Ukraine, 61060, avase2015@gmail.com, https://orcid.org/0000-0003-0081-5894
Vus N.O. – Agroécologie, INRAE, Institute Agro, University Bourgogne Franche-Comté, 17 Sully st, Dijon, France, Yuriev Plant Production Institute of National Academy of Agrarian Science (NAAS), 142 Heroiv Kharkova av, Kharkiv, Ukraine, 61060vus.nadezhda@gmail.com, https://orcid.org/0000-0001-7098-9158
Shevchenko L.M. – Yuriev Plant Production Institute of National Academy of Agrarian Science (NAAS), 142 Heroiv Kharkova av, Kharkiv, Ukraine, 61060, larashevchenko667@gmail.com, https://orcid.org/0009-0000-5213-4527
Bezuhla O.M. – Yuriev Plant Production Institute of National Academy of Agrarian Science (NAAS), 142 Heroiv Kharkova av, Kharkiv, Ukraine, 61060, olgabezuglaya61@gmail.com https://orcid.org/0000-0002-1458-1630
Hlyantsev A.V. – Yuriev Plant Production Institute of National Academy of Agrarian Science (NAAS), 142 Heroiv Kharkova av, Kharkiv, Ukraine, 61060, avglyancev@gmail.com, https://orcid.org/0009-0007-6331-1286
Skydan V.O. – Yuriev Plant Production Institute of National Academy of Agrarian Science (NAAS), 142 Heroiv Kharkova av, Kharkiv, Ukraine, 61060, VScydan@gmail.com, https://orcid.org/0000-0001-5592-2107
Zimohlyad Yu.O. – Yuriev Plant Production Institute of National Academy of Agrarian Science (NAAS), 142 Heroiv Kharkova av, Kharkiv, Ukraine, 61060, zimogladyulia25@gmail.com, https://orcid.org/0009-0003-1808-5727

Received: 27.11.2025 / Revised: 02.01.2026 / Accepted: 09.05.2026 / Published: 31.05.2026

Introduction

The use of genetic diversity of crops, including wild species, in breeding work is hampered by limited knowledge of their diversity and by difficulties in processing hybrid material. Yet, in the context of climate change, individual populations may contain important adaptive traits (Smýkal et al., 2018). Wild representatives of the genus *Pisum* L. may serve as promising germplasm sources for breeding for resistance to diseases and pests (Nami-Feil et al., 2017; Warkentin et al., 2024). These accessions also show high resistance to various abiotic stressors, such as drought and extreme temperatures (Nami-Feil et al., 2017). Several traits in which they differ from the field pea *Pisum sativum* subsp. *sativum* (hereafter *P. sativum*) include quality parameters and agronomic properties (branching pattern, winter hardiness, rapid root growth and rooting depth, etc.). Hybrids obtained between *P. sativum* and *Pisum fulvum* L. exhibit a significantly shorter growing season compared to each parent, which may enable them to “escape” drought and thereby increase drought tolerance (Sari et al., 2021).

P. fulvum is reported to be resistant to pea weevil (*Bruchus pisorum* L.) (Yan, 2023; Byrne et al., 2008), powdery mildew (full resistance) (Fondevilla et al., 2007), rust (Barilli et al., 2010), and Ascochyta blight (Jha et al., 2016; Jha et al., 2017). Pods of *Pisum sativum* subsp. *elatius* (hereafter *P. elatius*), as well as most landraces originating from the Balkans and Western Asia, respond to pea weevil oviposition by callus-like epidermal growth, which sometimes causes beetle eggs to detach (Fernàndez et al., 2018). Unlike *P. elatius*, *P. fulvum* does not show this response and its resistance is based on different mechanisms. *P. elatius* is also resistant to the nematode *Heterodera goettingiana* Liebscher (Vito et al., 1978; Valderrama et al., 2004), to the broomrape *Orobanche crenata* Forsk. (Valderrama et al., 2004), to powdery mildew (Devi, 2022), to fusarium and Ascochyta blight, and to white mould caused by *Sclerotinia sclerotiorum* (Lib.) de Bary (Chen et al., 2024). Some representatives *P. abyssinicum* A. Braun shows resistance to the nematode *H. goettingiana* (Vito et al., 1978) and to stem rot caused by *Pseudomonas syringae* (Martin-Sanz et al., 2012).

Thus, various authors have used *P. fulvum* and other wild *Pisum* representatives as sources of alleles for important breeding traits, including resistance to fungal diseases (Barilli et al., 2010; Fondevilla et al., 2011; Jha et al., 2012) and to *Bruchus pisorum* L. (Clement et al., 2009; Tayeh et al., 2015).

Although pea is a predominantly self-pollinating crop, heterogeneity has been observed within crop populations, including in self-fertilising species (Leimu et al., 2006). This highlights the importance of appropriate sampling strategies for germplasm collections (Hoban et al., 2015) to ensure the preservation of genetic diversity. Currently, wild pea accessions preserved ex situ originate from a limited number of individuals (Smýkal et al., 2015) and are therefore prone to genetic erosion (Kilian et al., 2012).

Some researchers have reported difficulties in obtaining hybrid seeds from crosses between *P. sativum* and *P. fulvum* or *P. elatius*. However, according to modern nomenclature, *P. elatius* is confirmed to belong to the species *P. sativum*, and no barriers to hybrid seed formation have been detected (Smýkal et al., 2012; Sari et al., 2023; Ellis et al., 2024). This discrepancy is explained by Yang et al. (2022), who showed that *P. elatius* exhibits a high level of introgression, suggesting that it may be of hybrid origin between cultivated and wild peas. This hybrid origin has also been supported by recent analyses of admixture among wild *P. sativum* groups, including northern *Pisum humile* Boiss. & Noë, southern *P. humile* and *P. elatius*.

Therefore, the aim of our study was to demonstrate the feasibility of obtaining hybrids under field conditions and to assess the effectiveness of pollination methods for representatives of the genus *Pisum* L.

Objects and methods of research. The taxonomic description of representatives of the genus *Pisum* L. was provided according to the GRIN Taxonomy database (USDA, accessed 30.06.2025). Detailed passport data for all genotypes are available via their catalogue numbers (noted in parentheses) at the National Center for Plant Genetic Resources of Ukraine or through the international Plant Genetic Resources for Food and Agriculture (PGRFA) information platform, Genesys-PGR (Genesys-PGR, 2025).

The components selected for crossing represented a range of agronomic characteristics. All *P. sativum* accessions used in the study have white corolla coloration. The breeding line DTR 94-120 is characterized by determinate growth (the shoot apex terminates in a double peduncle) and multifloration. The variety Kharkivskyi yantarnyi (UD0101296) has orange cotyledons, while Tsarevich (UD0102105) exhibits a leafless phenotype and seeds with resistance to lodging. The varieties Oplot (UD0102351) and Haiduk (UD0102726), like Tsarevich, are leafless, but lack the lodging-resistant phenotype while showing high productivity potential. The Oplot variety is used as a resistance standard for leafless varieties against scochyta blight (Vasylenko et al., 2021).

P. fulvum L. (UD0101508) displays typical wild-type characteristics: thin stems, high branching tendency, small leaves and seeds, yellow-brown corolla color and brown-green seeds with dark mottling.

P. elatius L. (UD0101506), in contrast to *P. fulvum*, has a thicker medium-length stem, a large leaf petiole with well-developed leaflets, and flowers of medium size. Its corolla is light purple with darker venation. Seed coloration is similar to *P. fulvum*, although seeds are larger.

Thus, in the crossing variants where *P. sativum* acts as the maternal component and *P. elatius* or *P. fulvum* act as the paternal component, if pollination occurs, the F_1 hybrid seeds will exhibit the seed-coat coloration of the paternal component, and there is no doubt that the resulting seeds are hybrid. In the reciprocal variant (*P. elatius* or *P. fulvum* as the maternal component × *P. sativum*), doubts may arise regarding the hybridity of the F_1 seeds, and in this case it is necessary to obtain the second generation of seeds (F_2) to confirm the authenticity of the hybrids.

Plant placemented for crossing according to the author’s patented scheme (Chekrygin at al., 2008).), which ensures gradual flowering of plants (due to different sowing dates): 4 blocks were sown every 4 days; The first block was sown at the optimal time, subsequent blocks were sown every 4 days. Each block consists of 3 plots with three rows. The length of the row is 1 m, row spacing – 0.15 m, the distance between three-row plots is 0,3 m. Ten seeds were planted in each row, the distance between them was 0.10 m. Sowing was carried out using manual planters.

In 2020, to determine the efficiency of hybridization procedures in obtaining hybrids among *P. fulvum* L., *P. elatius* L. and *P. sativum*, the following experiment was conducted. In the first treatment, pollination was performed 15 minutes after emasculation; in the second treatment, pollination was performed 24 hours after emasculation (“waiting”). Emasculation was performed by cutting the keel along its sagittal suture and removing the anthers. The flower was then pollinated with pollen from the paternal component and tagged with colored threads.

To ensure hybrid formation, emasculation of flowers was carried out prior to pollination. This procedure typically lasted from 5:00 a.m. to 4:00 p.m, but it is most convenient to carry out this procedure before 1 p.m.

Statistical analysis and data visualization

To assess the efficiency of the crosses, we calculated ratio the number of seeds obtained per emasculated (artificially pollinated) flower (S/F). All statistical analyses and data visualization were performed using R software version 4.2.2 (R Core Team, 2023). Data preprocessing and basic statistical calculations were conducted using the ‘openxlsx’ (Schauberger & Walker, 2022), ‘tidyverse’, and ‘rlang’ packages (Henry & Wickham, 2023). Graphical visualization was carried out using ‘ggplot2’ (Wickham, 2016), with figure composition supported by ‘patchwork’ (Pedersen, 2024) and label optimization by ‘ggrepel’ (Slowikowski, 2024).

Statistical support for was assessed using non-parametric bootstrap (5000 resamples) (Davison & Hinkley, 1997) using ‘boot’ (Canty & Ripley, 2024) and ‘purrr’ (Wickham & Henry, 2023) packages. Differences were considered significant when 95% confidence intervals of percentage differences did not overlap zero.

Results and Discussion

In 2020, the crossing was carried out from June 17 to June 21, and in 2021 from June 14 to June 23. Usually, this period is characterized by sufficient rainfall and optimal temperature conditions. However, the conditions of the 2020 growing season turned out to be hotter with a long period of no precipitation (Table 1).

Table 1. Weather conditions of the growing season of peas from 20.05. to 04.07. in 2020–2021

Year	Temperature, °C			∑ of precipitation, mm	N of days without precipitation	Maximum duration of the rainless period	Number of days of extreme heat		
	Mean	Max	Min				>25°C	>28°C	>30°C
2020	21.18	33	5	131.6	35	12	27	15	6
2021	20.66	33	5	94.1	27	7	17	11	7
mean	20.28	32.06	6.19	97.49	30	9.78	22.4	11.13	6.06

If in 2021 the maximum period without precipitation was 7 days, then in 2020 it was 12. There were also a greater number of days with extreme temperatures in 2020. It should be noted that the rainless period in 2020 fell just before the pea flowering and crossing. Thus, just from the middle of the first decade of June 2020, when pea plants were entering a state of active formation and growth of generative organs, a significant increase in temperature began and for 12 days there was no precipitation of any kind (Fig. 1).

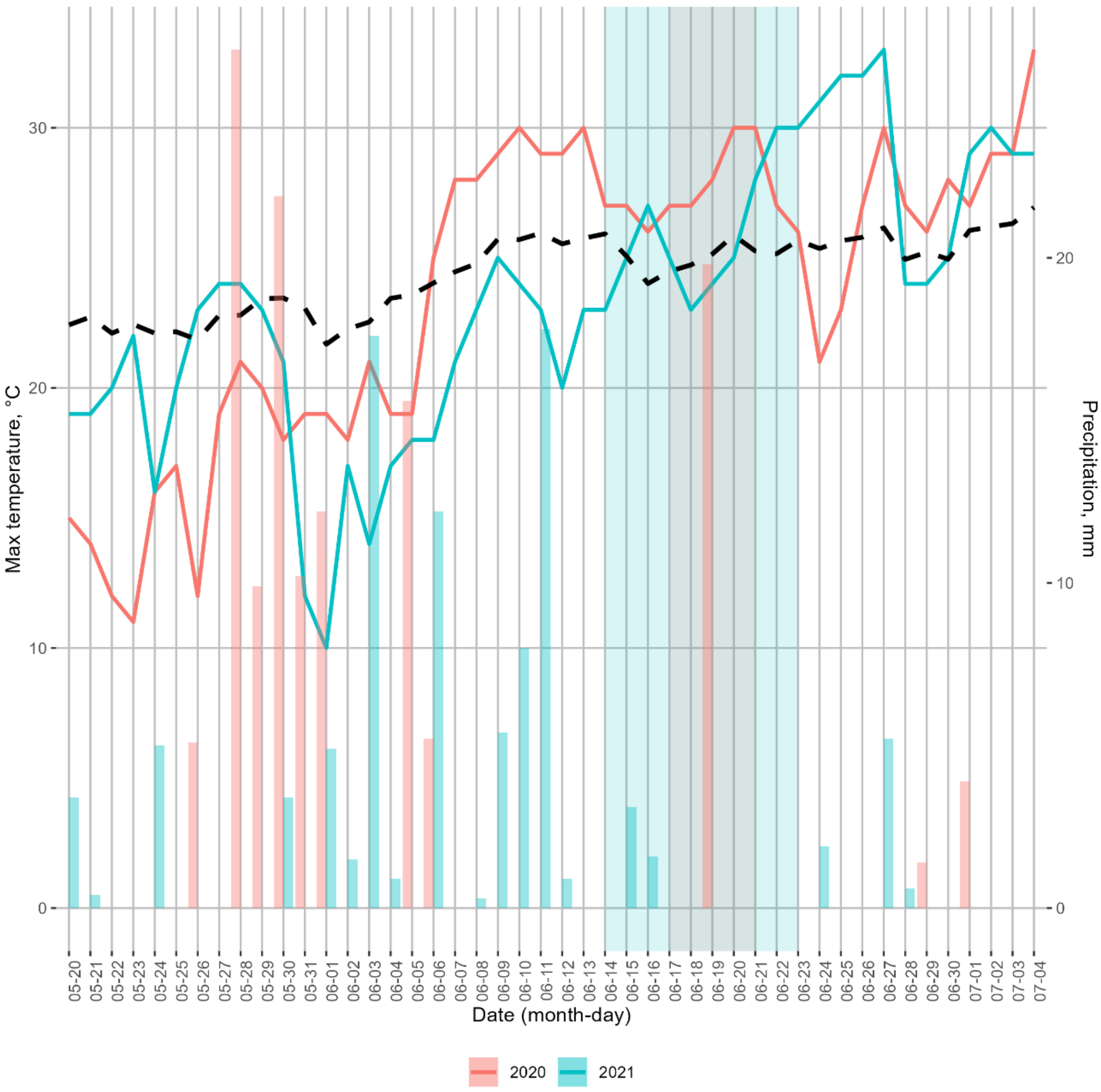

Fig. 1. Daily maximum of temperature and sum of precipitation. Main lines – years of research, dashed line – multi-year average. Shaded area - crossing period for each year

An increase in temperature from 24 to 36°C during the pollination period of pea has a significant negative effect on pollen germination, ovary formation, seed formation in the pod, and pod development, and is also genotype-dependent (Jiang et al., 2015). Our studies allowed us to assess the possibility of obtaining productive hybrid seeds under extreme field conditions.

Given that in pea the gynoecium matures earlier than the androecium, some researchers do not perform emasculation but pollinate the flowers directly. In cultivated varieties, self-pollination occurs in flower buds at a more advanced developmental stage (stage 4), whereas in wild plants pollination may occur already in buds at stages 2–3 (Knott, 2008)

Additional insect-mediated pollination cannot be excluded, and ovaries that lag behind in development may also become pollinated during the open-flower stage, because the stigma remains receptive for 8–10 days, although the highest pollination rate occurs during the first three days after emasculation.

The process of emasculation in the field, in our opinion, the most convenient is the manual method with complete removal of anthers. For different crops, the process itself and its effectiveness for obtaining hybrids are very specific. In the study by Sekiguchi et al. (2023), male sterility was effectively induced in cowpea and two dicotyledonous model species (*Arabidopsis thaliana* (L.) Heynh. and *Nicotiana benthamiana* Domin) using trifluoromethanesulfonamide (TFMSA). Pollen-viability assays using Alexander staining showed that 30 ml of 1000 mg/L TFMSA, applied twice at a one-week interval at the early stage of the reproductive phase under field or greenhouse conditions, induced 99% pollen sterility in cowpea.

Guerra et al. (2008) identified flower emasculation as the cause of premature ovule degeneration and its implications for subsequent fruit set. There is no consensus regarding the time of emasculation and pollination. Thus, Saha (2021) suggests carrying out emasculation on the eve of pollination at 4-6 am.

The results of assessing hybridization efficiency depending on the time between emasculation and pollination with bootstrap analysis showed a significant difference in both the crossing probability (percentage of productive pods per pollinated flower) and pollination efficiency (number of seeds formed per pollinated flower) of immediate pollination after emasculation versus delayed pollination regardless of the type of maternal component (Table 2).

Differences in crossing probability and pollination efficiency between immediate and delayed pollination were assessed using bootstrap resampling (5000 iterations), with hybrid combinations treated as independent experimental units. Immediate pollination increased pod set by 0.28 - 0.70 (absolute proportion units) compared to delayed pollination. Seed production per pollinated flower was significantly higher under immediate pollination (Δ = 1.12 seeds per flower, 95% CI: 0.74–1.56).

Table 2. Effect of pollination timing on hybridization success in *Pisum* (bootstrap analysis), 2020

Response variable	Time after emasculation, h	Mean	Δ (0 h – 24 h)	95% CI
Crossing probability (Pod per flower)	0	0.61	0.43	0.28 - 0.70*
	24	0.18		
Pollination efficiency (Seeds per flower)	0	1.44	1.11	0.74 - 1.56*
	24	0.33		

Values represent means across maternal genotypes.
Δ = difference between immediate and delayed pollination.
* Significant difference inferred from bootstrap 95% confidence interval (does not include zero)

Although the numbers of emasculated flowers differed between the immediate-pollination variant and the “waiting” (24-hour delay) variant, this did not influence the overall interpretation of pollination efficiency. For example, in the combination Kharkivskyi yantarnyi × *P. fulvum*, the numbers of emasculated flowers were nearly identical in both treatments, yet the pollination efficiency after one day was extremely low, as it was in the combination Tsarevich × *P. fulvum*. In the combination DTR 94-120 × *P. fulvum*, the number of emasculated flowers in the immediate-pollination variant was twice lower than in the 24-hour variant, but both treatments produced the same number of pods. At the same time, the number of seeds in the first variant was considerably higher: the S/F index reached 1.63, compared with only 0.60 in the “waiting” treatment. In our experience, combinations in which both parental components belong to *P. sativum* usually show a high ratio of seeds per emasculated flower – typically two or more seeds per flower, depending on the conditions of the year (Fig. 2).

Although pea is considered a predominantly self-pollinating species, cross-pollination occurs in both wild and cultivated forms (Dostálová et al., 2005; Olowick et al., 2002; Loenning et al., 1984). Suso et al. (2016) demonstrated that most legumes, including pea, possess floral structures enabling outcrossing. A study by Saboor et al. (2016) in Pakistan identified seven Diptera species, two Hymenoptera species, two Lepidoptera species, and one Coleoptera species acting as pollinators of cultivated pea. Field experiments have shown that pea pollen can disperse over several hundred meters (Dostálová et al., 2005; Olowick et al., 2002). For comparison, outcrossing rates in wild cowpea (*Vigna unguiculata* (L.) Walp.) populations in West Africa range from 1% to 9.5% (Kouam et al., 2012), classifying cowpea as a predominantly self-pollinating species. In *Medicago truncatula*, the outcrossing rate is 3–5% (Bonnin et al., 2001). However, some outcrossing events may remain undetected in highly genetically homogeneous populations because offspring may not show visible heterozygosity. In this context, we plan further research to determine outcrossing levels under field conditions in pea.

Self-pollination in wild pea populations was not necessarily advantageous during domestication but is part of a mixed mating system. This trait remains valuable for breeders working under decreasing pollinator abundance. Insect-mediated outcrossing can exploit heterosis in crop species, while in the absence of pollinators, self-pollination ensures minimal but sufficient seed set. This provides reproductive assurance while allowing high outcrossing rates when pollinators are available (Suso et al., 2016).

Thus, we conclude that pollination efficiency in our crossing combinations was substantially higher when performed immediately after emasculation, although the influence of parental genotype cannot be excluded.

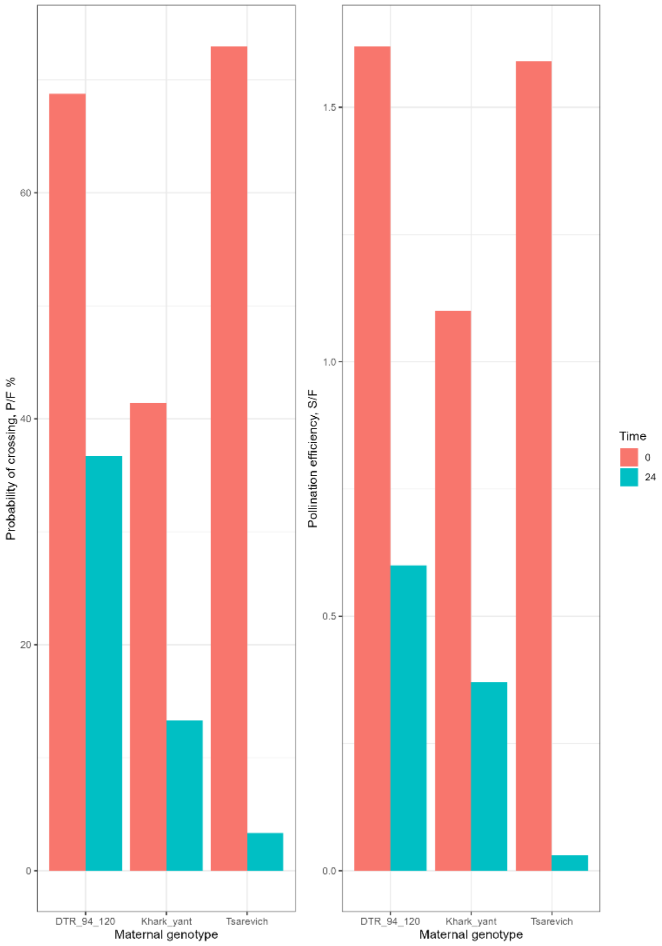

Fig. 2. Pea hybridisation efficiency, 2020 depending on time after emasculations: immediately after emasculation or the next day (24-hour delay). Paternal component – *Pisum fulvum*

Combining different options for combining the paternal and maternal components allowed us to assess the pollination efficiency and the possibility of including the wild component in the hybrid material (Table 3).

Table 3. Effect of year condition on hybridization success in *Pisum*, 2020-2021

Maternal component	Paternal component	Year	N of flowers	N of seeds	SFInd
DTR 94-120	*P. fulvum*	2020	62	70	1.13
DTR 94-120	*P. fulvum*	2021	20	0	0.00
P. fulvum	DTR 94-120	2020	30	18	0.60
P. fulvum	DTR 94-120	2021	13	6	0.46
P. elatius	Hayduk	2020	30	56	1.87
P. elatius	Hayduk	2021	35	55	1.57
P. elatius	DTR 94-120	2020	21	43	2.05
P. elatius	DTR 94-120	2021	35	1	0.03
P. elatius	Kharkivskyi yantarnyi	2020	20	25	1.25
P. elatius	Kharkivskyi yantarnyi	2021	15	21	1.40

Differences between years were assessed using bootstrap resampling of crossing combinations present in both years. Bootstrap analysis confirmed significant differences between years of experiment in seed production efficiency (Δ = 1.1, 95% CI: 0.06 to 1.16). A significant negative effect of *P. fulvum* on seed production efficiency was observed compared to *P. elatius*. Crosses involving *P. fulvum* showed significantly lower seed production efficiency compared to *P. elatius*. Bootstrap analysis confirmed a negative effect (Δ = −0.68, 95% CI: −1.24 to −0.12), which was consistent with the quasi-Poisson model (rate ratio = 0.57, p = 0.034). Bootstrap analysis revealed significant variation among *Pisum sativum* genotypes, indicating that genotype identity plays an important role in cross success (Δ = 0.92, 95% CI: 0.04–0.96). This result highlights the importance of selecting appropriate genotypes for interspecific hybridization.

The evaluation of reciprocal crosses proved a significant difference between pollination efficiency and direction of crossing and confirmed a strong dependence on the conditions of the year (Fig. 3). Thus, if for hybrid combinations involving *P. elatius* two combinations out of three had a significant advantage in pollination efficiency when the wild component was used as the maternal form (direct crossing), and only one – reverse. For combinations with *P. fulvum*, using the wild component as the parent was more effective and had a significant difference when crossing with Tsarevich and Kharkivskyy Yantarnyy. In the combination of *P. fulvum* – DTR 94-120, the difference between direct and reciprocal crossing was significant only in the conditions of one year, 2020.

The higher cross-breeding efficiency in combinations involving *P. elatius* and *P. sativum*, compared to combinations where *P. fulvum* acted as one of the parents, may be explained by the fact that *P. elatius* is a member of the species *Pisum sativum* (Yang et al., 2022). Although some authors have reported cases of nuclear – cytoplasmic incompatibility between *P. elatius* accessions and cultivated pea, most representatives of the genus *Pisum* — wild *P. fulvum*, *P. elatius*, *P. humile* and the cultivated taxon *P. abyssinicum* — can be crossed with *P. sativum*, particularly when cultivated pea is used as the female parent (Ben-Ze’ev and Zohary, 1973; Ochatt et al., 2004). Our results indicate that the number of hybrid seeds obtained in *P. sativum* × *P. elatius* combinations may vary depending on the genotype of the *P. sativum* parent.

In 2023, seeds obtained in 2021 were grown in the hybrid nursery. Based on the seed-coat coloration in the combinations Haiduk, Tsarevich and Oplot with *P. elatius*, true hybrids were obtained (Table 4).

For combinations where *P. elatius* served as the maternal parent, the next generation is required to observe segregation for anthocyanin pigmentation (flowers, leaf petioles, stem) or white corolla coloration. Thus, interspecific hybrids have already been obtained, and work will continue in subsequent years.

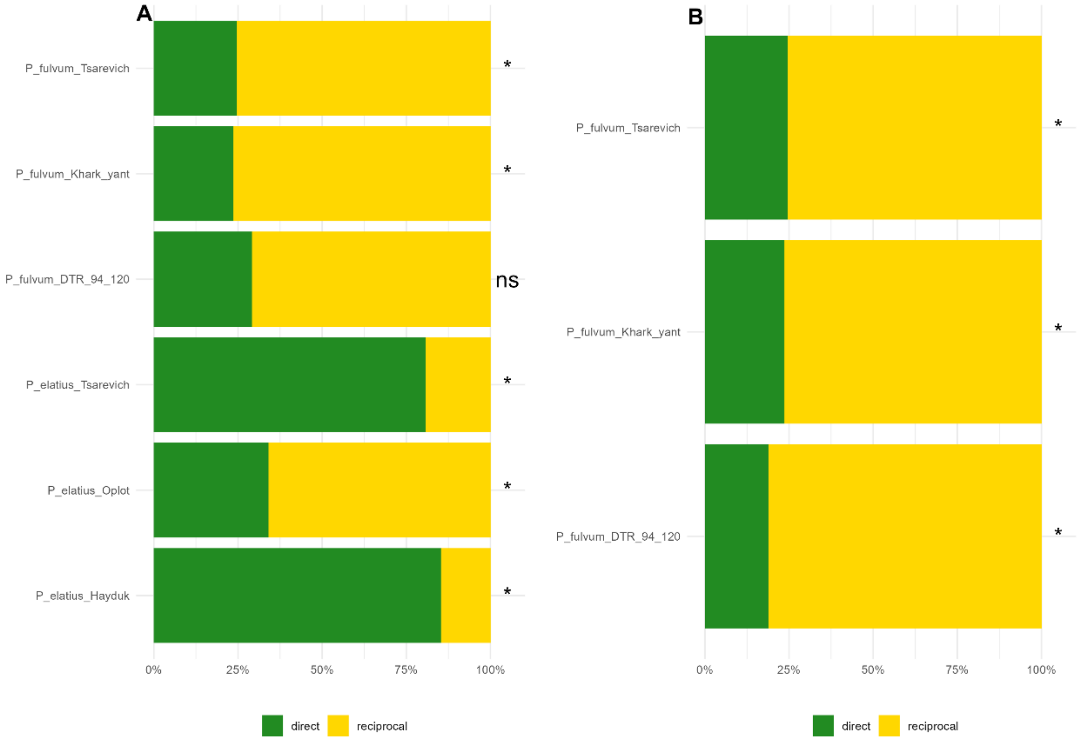

Fig. 3. Pollination efficiency of direct and reciprocal crosses. Asterisks indicate statistically supported differences between direct and reciprocal crosses based on 95% bootstrap confidence. A - all combinations, B - for *P. fulvum* 2020 only.

Table 4. Color of hybrid seeds F2

Accessions		Seeds sown (2021 harvest, pcs.)	Plants obtained (2023)	Seed color, F2
♀	♀			
P. elatius	Haiduk	55	27	brown-green
Haiduk	*P. elatius*	26	24	brown-green
Tsarevich	*P. elatius*	40	37	brown-green
Oplot	*P. elatius*	78	76	brown-green
P. elatius	DTR 94-120	1	1	brown-green
P. elatius	Kharkivskyi yantarnyi	21	19	brown-green

Conclusions

Considering the obtained results, we can state that in field conditions, to increase the efficiency of crossing for Pisum L. and successfully obtain hybrid seeds, both for interspecific and intraspecific crossing combinations, it is necessary to carry out pollination with the parental component immediately after emasculation. While delayed pollination for 24 hours leads to a decrease in the number of hybrid seeds.

Our results demonstrate the possibility of obtaining true hybrids between representatives of *P. sativum* x *P. fulvum* precisely in field conditions. It has been confirmed that both in field conditions (as well as in controlled conditions) for combinations of *P. sativum x P. fulvum* the probability of obtaining hybrid seeds increases significantly when *P. fulvum* is used as a pollinator. When obtaining hybrid offspring between *P. sativum* and *P. elatius*, a tendency for the influence of the parental component was noted.

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Ефективність штучного запилення у представників роду *Pisum* L.

А.О. Василенко, Н.О. Вус, Л.М. Шевченко, О.М. Безугла,
А.В. Глянцев, В.О. Скидан, Ю.О. Зимогляд

Дикі родичі культурного гороху (*Pisum sativum* L.) є важливим резервуаром алелів, пов'язаних зі стійкістю до біотичних та абіотичних стресорів; однак їх використання в селекційних програмах обмежене інформацією про їхню різноманітність та труднощами в отриманні гібридів у польових умовах. У цьому дослідженні було оцінено ефективність міжвидової гібридизації між *P. sativum, P. elatius* та *P. fulvum* у польових умовах, зосереджуючись на впливі часу запилення після кастрації квіток, батьківського генотипу та напряму схрещування. Морфологічні характеристики батьківських зразків використовувалися для полегшення ідентифікації гібридів на основі забарвлення насіннєвої оболонки при прямому та реципрокному схрещуванні. Порівнювали два підходи до запилення: негайне запилення після кастрації квіток та відкладене запилення, проведене через 24 години після неї. У всіх комбінаціях за участю *P. sativum* / *P. fulvum* набір гібридного насіння (індекс S/F) був стабільно вищим, коли запилення проводилося одразу після кастрації. Затримка запилення призвела до помітного зниження ефективності гібридизації, що, ймовірно, відображає як фізіологічні обмеження, так і високий температурний стрес під час цвітіння. Реципрокні схрещування виявили низьку та відносно рівномірну ефективність гібридизації в комбінаціях з *P. fulvum*, тоді як схрещування за участю *P. elatius* дали значно вищий набір насіння, що узгоджується з його близькою генетичною спорідненістю з *P. sativum*. Стабільність ефективності гібридизації протягом років (2020 – 2021) була підтверджена для кількох комбінацій схрещування. Оцінка партій насіння F_1 та F_2 продемонструвала успішне отримання справжніх міжвидових гібридів, при цьому діагностична сегрегація спостерігалася в комбінаціях, де дикий вид служив материнською лінією.

Ці результати підтверджують, що життєздатні гібриди серед представників роду *Pisum* можна надійно отримати в польових умовах, і підкреслюють, що негайне запилення після кастрації є найефективнішою стратегією для максимізації виробництва гібридного насіння. Отримані результати підтверджують ширше використання зародкової плазми дикорослих представників *Pisum* у передселекційних та генетичних дослідженнях і надають практичні рекомендації щодо покращення успіху міжвидової гібридизації в селекційних програмах.

Ключові слова: *Pisum sativum L., Pisum elatius, Pisum fulvum, гібридизація*

Цитування: *Vasylenko A.O., Vus N.O., Shevchenko L.M., Bezuhla O.M., Hlyantsev A.V., Skydan V.O., Zimohlyad Yu.O. The effectiveness of artificial pollination in various representatives of the genus Pisum L. Вісник Харківського національного університету імені В.Н. Каразіна. Серія «Біологія», 2026, 46, с. 82–92. https://doi.org/10.26565/2075-5457-2026-46-7*

Про авторів:
Василенко А.О. – Інститут рослинництва імені Юр'єва НААН, проспект Героїв Харкова, 142, Харків, Україна, 61060, avase2015@gmail.com, https://orcid.org/0000-0003-0081-5894
Вус Н.О. – Agroecologie, INRAE, Institute Agro, University Bourgogne Franche-Comté, 17 Sully st, Dijon, France, Інститут рослинництва імені Юр'єва Національної академії аграрних наук (НААН), проспект Героїв Харкова, 142, Харків, Україна, 61060vus.nadezhda@gmail.com, https://orcid.org/0000-0001-7098-9158
Шевченко Л.М.– Інститут рослинництва імені Юр'єва НААН, проспект Героїв Харкова, 142, Харків, Україна, 61060, larashevchenko667@gmail.com, https://orcid.org/0009-0000-5213-4527
Безугла О.М. – Інститут рослинництва імені Юр'єва НААН, 61060, Україна, Харків, проспект Героїв Харкова, 142, olgabezuglaya61@gmail.com, https://orcid.org/0000-0002-1458-1630
Глянцев А.В. – Інститут рослинництва імені Юр'єва НААН, проспект Героїв Харкова, 142, Харків, Україна, 61060, avglyancev@gmail.com, https://orcid.org/0009-0007-6331-1286
Скидан В.О. – Інститут рослинництва імені Юр'єва Національної академії аграрних наук (НААН), Героїв Харкова, 142, Харків, Україна, 61060, VScydan@gmail.com, https://orcid.org/0000-0001-5592-2107
Зимогляд Ю.О. – Інститут рослинництва імені Юр'єва НААН, проспект Героїв Харкова, 142, Харків, Україна, 61060, zimogladyulia25@gmail.com, https://orcid.org/0009-0003-1808-5727

Внесок авторів: Василенко А.О.: ідея, дизайн дослідження, обговорення результатів, написання тексту, редагування тексту; Вус Н.О.: обговорення результатів, аналіз та візуалізація результатів, підбір та аналіз літературних джерел, написання тексту; редагування тексту; Безугла О.М.: обговорення результатів, підбір та аналіз літературних джерел; Скидан В.О.: обговорення результатів, підбір та аналіз літературних джерел; Шевченко Л.М. виконання експериментальної роботи; Глянцев А.В. виконання експериментальної роботи; Зимогляд Ю.О. підбір та аналіз літературних джерел. **/ Authors Contribution:** Vasylenko A.O.: idea, research design, implementation of experimental work, participation in discussion of results, writing the text, editing the text; Vus N.O.: participation in discussion of results, analysis and visualization of results, selection and analysis of references, writing the text, editing the text; Bezuhla O.M.: participation in discussion of results, selection and analysis of references; Skydan V.O.: participation in discussion of results, selection and analysis of references; Shevchenko L.M.: Implementation of experimental work; Glyantsev A.V. Implementation of experimental work; Zymoglyad Yu.O.: selection and analysis of references.

Conflict of interest: The authors declare no conflict of interest. / **Конфлікт інтересів**: Автори заявляють про відсутність конфлікту інтересів

Use of Artificial Intelligence: The authors certify that no generative artificial intelligence tools were used in the conduct of the research or in the preparation of this manuscript. / **Використання штучного інтелекту:** Автори засвідчують, що під час проведення дослідження та підготовки цього рукопису генеративний штучний інтелект не використовувався.

Подано до редакції: 27.11.2025 / Прорецензовано: 02.01.2026 / Прийнято до друку: 09.05.2026 / Оприлюднено: 31.05.2026