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Animal pollination shapes fruits market features, seeds functional traits and modulates their chemistry

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In this study, we experimentally addressed the impact of different pollination treatments on the morphological, reproductive and chemical traits of fruits and seeds of two crop species, the wild strawberry (*Fragaria vesca* L.) and cowpea (*Vigna unguiculata* (L.) Walp.). Multiple flowers from each plant were exposed to different pollination treatments: (1) self pollination, (2) hand cross pollination and (3) open pollination. Both crops were positively affected by open pollination in terms of morpho-chemical parameters concerning the marketability (e.g., 35% decrease in sugar/acid ratio in open pollinated strawberries compared to the autogamous ones) and the seed germination rate as a proxy of reproduction efficiency (e.g., the almost complete absence of seed abortion in the open pollination treatment). Remarkably, the pollination treatment also strongly influenced the phytochemical composition. Open-pollinated strawberries exhibited a higher relative concentration of compounds endowed with nutraceutical properties such as anthocyanins, ellagic acid derivatives and flavonoids. At the same time, cowpea seeds displayed higher concentrations of anti-nutrients in the self pollination treatments, such as saponins, compared to the open and hand cross pollinated seeds. This study suggests the presence of a link between the pollination mechanism, market quality, plant reproduction and chemical properties of fruits and seeds, supporting the intricate interplay between pollinators, plants and human nutrition, highlighting the crucial importance of animal pollination in the ecological and dietary contexts.

Keywords High resolution mass spectrometry, Plant-pollinator interaction, Pollination ecosystem service, Post-harvest quality, Food security

The global decline of insects poses a significant risk to key ecosystem functions and services. Among these, plant pollination service ranks first[1], with most angiosperms mainly relying on pollinator insects to support sexual reproduction[2]. Recently, Tong et al.[3] estimated that 90% of flowering plant species are somewhat dependent on animal pollination. The major advantages of relying on insect-mediated pollination concern the pollinator ability to recognize flowers of the same species and their effectiveness in transporting adequate quantities of pollen to the floral stigmas compared to wind-driven mechanisms, thus favouring allogamy. The crucial role of animal pollination for plant reproduction emerges clearly by looking at agricultural production with about 35% of global crop production by volume depending on pollinators[4,5]. Usually, the relevance of pollinators for crops is measured with parameters related to plant yield and commercial quality, such as the number of seeds and fruits produced or the shelf-life of the harvest[6,7]. In the last few years, pioneer studies provided evidence that a link between pollinators and crop nutritional quality for humans could exist[8,9]. Indeed, it is estimated that crops dependent on animal pollination could contribute to the provisioning of many micronutrients, such as vitamin B9, vitamin A, and ascorbic acid, globally[10]. Most of these micronutrients are the base of common global nutritional deficiencies, especially in the case of vitamin and mineral components, such as niacin, iron and zinc[11]. A modelling analysis by Smith et al.[10] further stressed the importance of pollinators for the human diet

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by showing that the extinction of pollinators could lead to an annual increase of approximately 1.4 million deaths worldwide due to non-communicable and malnutrition-related diseases, as well as an additional 27 million disability-adjusted life-years (DALYs).

The growing awareness of the importance of pollinators for ensuring food security has stimulated scientific efforts towards replacing real pollinators with surrogates. In this context, biotechnologies have opened new frontiers such as plant genome editing to stimulate the production of secondary metabolites in the plant without pollinator interventions[12] or practices such as spraying hormones to artificially trigger the metabolic processes activated after a floral visit[9], as well as the use of pollinator robots consisting in wheeled machines and micro-scale drones designed to mimic in different ways the pollination functions[13,14]. This study aims to understand the implications on seeds and fruits development and metabolism of insect-mediated pollination compared with self pollination or hand cross pollination. We studied the direct effects of the pollination treatment on the functional parameters linked to seeds germination (plant fitness), marketability and the composition of phytochemicals relevant to the human diet. Considering the first evidence of a connection between pollination and human diet, we hypothesize that —compared to autogamy— animal pollination may specifically shape the morphological, functional and phytochemical phenotype of the derived fruits and seeds, hence influencing the quality of the food consumed, including its nutritional and nutraceutical features, with consequent impacts for human health.

The experimental plan involved the analysis of two different plant species: (1) the wild strawberry *Fragraria vesca* L., characterized by an actinomorphic flower visited by a wide range of pollinators from Coleoptera to Hymenoptera and fleshy false fruits eaten by humans and frugivores and (2) cowpea, *Vigna unguiculata* (L.) Walp., characterized by zygomorphic flowers usually pollinated by bees and producing legumes bearing seeds of food interest.

By investigating the response of these plants to the pollination treatment, we seek to identify common elements related to the pollination processes to derive shared morphofunctional and metabolic features that may highlight the relevance of pollinators beyond pollen transport.

Results and discussion

Analysis of the commercial quality

The pollination treatment significantly affected the morphological traits of both wild strawberries and cowpeas. Specifically, in both cases, the OP treatment showed better parameters in terms of dimensions, colours, weight and size compared to the SP and HP treatments, which conversely did not show any significant variation between each other (Fig. 1, Table S1). The overall improvement in the morphological parameters in the OP treatment compared with SP and HP are likely to be linked to a more effective pollen deposition. Interestingly, these processes are confirmed—based on the present data—irrespective of of the fruit and seed type. These findings complement those of previous studies[6,14–17], which have already provided some indications of the impact of pollinators on these aspects carrying significant implications for the food industry. Indeed, suboptimal food production resulting from inadequate pollination services poses a challenge for growers. Providing further

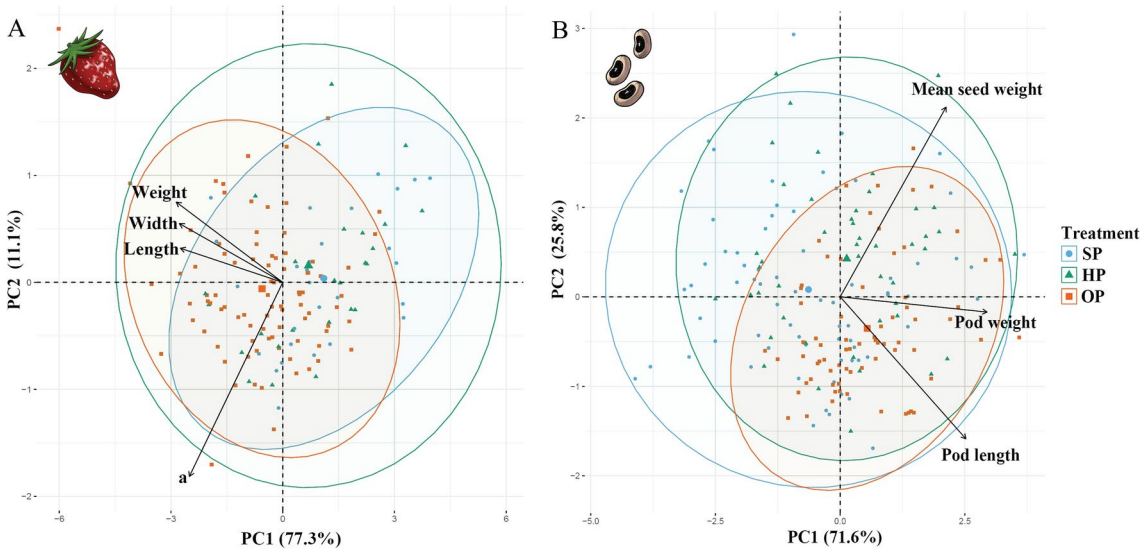

Fig. 1. Principal Component Analyses performed on the morphological traits of wild strawberry (**A**) and cowpea seeds (**B**). Each point represents a strawberry or a cowpea seed. The vectors indicate the position in the multivariate space where the specific variables tend to be more represented. In both species, the open pollination (OP) treatment was associated with larger fruits and seeds ($p = 0.002$) compared to SP (Self Pollination) and HP (hand cross pollination) which were found to be morphologically similar. For wild strawberries, the weight, width, length, and "*a*" value, which indicate the green/red composition of the fruit, were considered. For cowpea, mean seed weight and pod length and weight were considered.

Species	Quality parameter	SP	HP	OP
Fragaria vesca	TSS (%)	12.95 ± 0.24^{a}	11.07 ± 0.79^{b}	11.2 ± 0.94^{b}
Fragaria vesca	TA (%)	0.97 ± 0.25^{a}	0.86 ± 0.05^{a}	1.3 ± 0.35^{a}
Fragaria vesca	pH	3.55 ± 0.13^{a}	3.45 ± 0.06^{a}	3.47 ± 0.19^{a}
Fragaria vesca	TSS:TA	13.81 ± 2.55^{a}	12.72 ± 1.14^{a}	9.06 ± 1.79^{b}
Vigna unguiculata	TSC (%)	44.5 ± 3.24^{a}	45.28 ± 1.53^{a}	45.1 ± 0.6^{a}
Vigna unguiculata	TPC (%)	24.4 ± 0.18^{a}	24.49 ± 0.14^{a}	24.49 ± 0.12^{a}

Table 1. Different chemical quality parameters of the two study species. Data are reported as mean ± SEM. Different uppercase letters indicate differences significant at the statistical level ($p < 0.05$). *TSS* totable soluble solids, *TA* titratable acids, *TSC* total starch content, *TPC* total protein content, *SP* self pollination, *HP* hand cross pollination, *OP* open pollination.

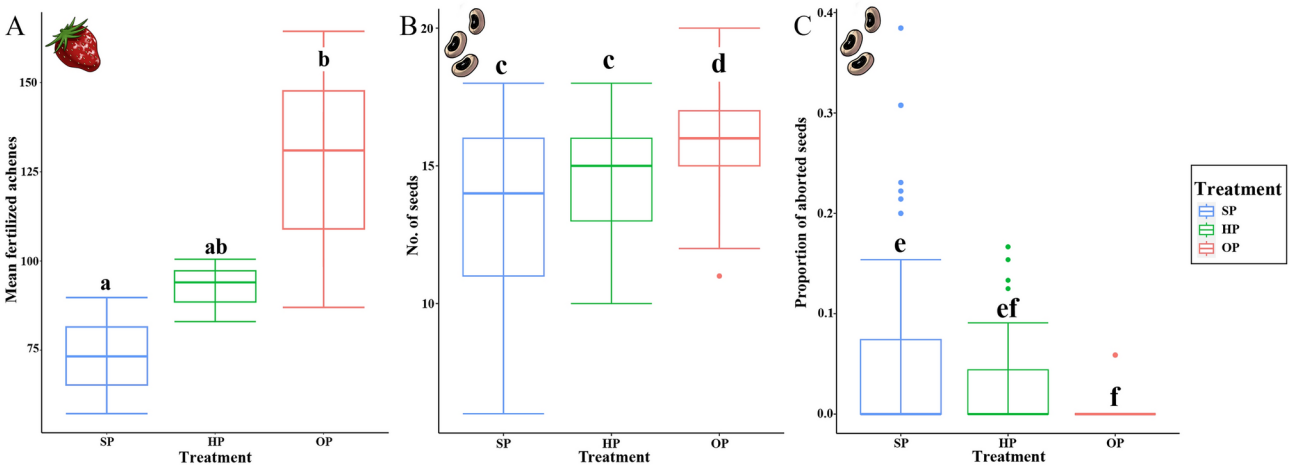

Fig. 2. Number of the mean fertilized achenes per strawberry (**A**), number of seeds per cowpea pod (**B**), and proportion of aborted on total seeds per pod in cowpea (**C**). *SP* self pollination, *HP* hand cross pollination, *OP* open pollination. Treatments identified by different letters are to be considered significantly different ($p < 0.05$).

evidence of the crucial role of animal pollination in enhancing economic yields can stimulate the adoption of mitigation strategies.

In the wild strawberry, the pollination treatment significantly affected both the TSS and the TSS:TA ratio, while TA and pH were found similar (Table 1). In cowpea seeds, no significant variations in the total starch and protein content were observed based on the pollination treatment (Table 1). Market quality in terms of seed and fruit size, and the sugar/organic acid ratio in wild strawberry displayed significant improvements in the OP treatment in both investigated species. A recent study by Umemura et al. [16] indicated that the development of achenes and receptacles in wild strawberry is a mainly hormone-driven process. Although there is no straight evidence about the ability of pollinators to alter the hormonal balance of the plant directly, there is clear agreement that greater fertilization success can stimulate the biosynthesis of hormones involved in the development of strawberry achenes and receptacles. Specifically, Wietzcke et al.[15] suggested that the biosynthesis of phytohormones, specifically auxin, depends on the proportion of fertilized achenes, which is in turn related to the visiting of pollinating insects. Therefore, the fertilization success induced by the OP treatment could stimulate the metabolism of endogenous auxins, besides other phytohormones, such as gibberellins, guiding the formation of high-quality seeds and fruits[18]. This hypothesis has been partially investigated in previous studies (e.g.,[19]) suggesting that insect-mediated pollination is more efficient than other pollination treatments. Furthermore, some studies suggest that the interaction with insects may trigger variations in plant metabolism at the level of different organs, such as leaves, fruits and flowers[16,20,21] mainly due to phenomena dealing with vibration patterns, such as the frequency produced by the buzzing[22]. What has still to be clarified is to define whether the effect of insect pollination is only indirect and linked to the ability to increase pollination success or whether the insect also acts directly on the hormonal balance of the plant.

Analysis of the seed functional traits

Wild strawberries produced through the OP treatment showed a higher number of fertilized achenes (Fig. 2A) and a similar pattern was observed in cowpea, which exhibited an increased seed count per pod in the OP treatment (Fig. 2B), along with a significant reduction in the proportion of aborted seeds in the OP treatment compared to SP (Fig. 2C). The HP treatment ranged between the other two conditions. Additionally, cowpea seeds originating from the OP treatment showed significantly higher germination rate and success compared to

the other experimental conditions (Fig. S1). Cowpea is a pulse that shows a high rate of self pollination, generally estimated to range between 90 and 99%[17], while wild strawberry produces fleshy fruit and its yield is dependent on cross pollination for more than 20% of the fertilization events[23]. Furthermore, these two species exhibit very different fruit morphologies. Both the species belong to the Euroside I clade; however, they experienced very different evolutionary paths despite maintaining relationship processes with pollinators resulting in a higher seed set, an increased number of viable seeds and a higher germination rate when animal pollination occurred compared to self pollinating flowers, as shown by the results of the present study. Specifically, we observed that the OP treatment improved the seed set irrespective of the high auto-fertility rate, known especially in the domesticated varieties of cowpea[17,23]. Moreover, both species exhibited a lower yield in the hand cross pollination treatment compared to the insect-mediated one. This pattern suggests that plant fitness does not depend only on the exchange of an adequate amount of pollen among different individuals but is probably linked to the biophysical mechanism of pollination promoted by the interaction between plants and pollinators.

Untargeted metabolomic investigation and identification of discriminant phytochemicals

The pollination treatment influenced the phytochemical profile of both species. In detail, in the wild strawberry, a net separation between OP and SP ($p=0.015$) and OP and HP ($p=0.033$) was found in negative ion current (Fig. 3A), while the positive ion current indicated significant separation only between SP and OP ($p=0.018$), as shown in Fig. 3B. These data correlate with a higher TPC and TFC in SP and HP compared to OP, while the total antioxidant activity (TEAC) of the samples was not affected by the pollination treatment (Table S3). The metabolomic investigations performed on cowpea seeds revealed a significant separation between SP and the other two pollination treatments (both HP, $p=0.006$, and OP, $p=0.003$) in negative ion current (Fig. 3C), while no clear clusterization emerged in the positive ionization mode (Fig. 3D).

In Table 2, the discriminant phytochemicals responsible for the separation of the pollination treatments and identified based on MSMS experiments are reported. In strawberries, a higher relative amount of many secondary metabolites was detected, such as flavonoids, ellagic acid derivatives, and anthocyanins in the OP treatment, while the SP treatment was characterized by a higher relative concentration of ellagitannins. Concerning cowpea seeds, the discriminant phytochemicals (mainly flavonoids, flavanols, and saponins) mainly occurred in the SP treatment.

Only two compounds in cowpea seeds showed higher relative concentration in the OP treatment compared to the other two experimental groups (i.e., m/z 465.12, identified as taxifolin-O-hexoside, and m/z 505.21, characterized as an acetyl-hexoside of quercetin). Furthermore, some discriminant ions were characterized by a bicharged pattern and were identified as unknown peptides. On the other hand, one of the most relevant data obtained in the present study deals with the increase in the relative concentration of many compounds endowed with putative nutraceutical properties in strawberries originating from the OP treatment, some of which are also involved in the colouring of the receptacles. This study agrees with previous experiments performed on tomatoes which found significantly higher lycopene (responsible for fruit redness) and soluble sugar content in bumblebees pollinated fruits compared to the self pollinated ones[9]. These observations are further supported by recent research which found that sound stimulation, such as insect buzzing, can trigger the biosynthesis of several secondary compounds, such as flavonoids[22].

From the ecological perspective, the observed variation in the metabolome of fruit and seeds offers new insights into ecological interactions. For instance, the receptacle colour is an honest signal for birds to assess the nutritional value of berries[29,30]. Therefore, the increased concentration of compounds belonging to the class of anthocyanins (such as pelargonidin and cyanidin derivatives) in OP derived wild strawberries correlates with their higher redness and makes them more attractive to frugivores, with implications for seed dispersal. Indeed, in a recent study by Lam et al.[31] about the analysis of the success of pollination and the dispersal ability of the derived fruits in *Hedera helix*, there was a notable signal that frugivores tend to feed primarily on insects and/or hand cross pollinated fruits compared to the self pollinated ones. The higher attractiveness may likely be related to patterns dealing with the colouring intensity, guided by the group of anthocyanins, as shown in the present study in wild strawberries. All these aspects pave the way for a more effective seed dispersal from the plant point of view, while from the animal point of view, the foodstuff gains added value[32].

Furthermore, a class of polymers, the ellagitannins, displayed a higher relative occurrence in SP and HP strawberries compared to OP treatment which could also account for the higher TPC and TFC of the extracts, as the reaction driving these colorimetric assays is primarily determined by the concentration of hydroxyl groups within a compound[33,34] and tannins are polymers rich in hydroxyl units. However, excessive concentration of molecules belonging to this class may jeopardize the palatability of the fruit, as they can confer an astringent taste to foods[35], therefore worsening their overall value.

The marked phytochemical differences observed in strawberries are not as evident in cowpea. Specifically, in cowpea seeds, we found that the starch and protein content were similar in all the investigated treatments. This lack of an effect may be explained by the fact that these nutritional classes are not affected by genetic and environmental factors, differently from phytochemicals[36,37].

Conversely to the pattern observed in wild strawberries, open pollinated cowpea seeds were generally found to exhibit lower relative concentrations of phytochemicals compared to HP and, particularly, SP. However, the boiling adopted for pulses, such as cowpea, tends to promote the loss of most of the phytochemicals[38], reducing the relevance of their occurrence in this matrix at the dietary level.

From a physio-ecological point of view, since the fruit of cowpea does not depend on animal dispersal, the occurrence of secondary compounds in its seeds is not due to the need to attract any interactors. Still, it is likely to depend more on the overall stress status of the seed and embryo[39]. Also, seed dormancy (identifiable by the lower germination rates of SP seeds) was found to positively correlate with the amount of phenolic compounds in the seed itself, which may behave as dormancy inducers[40].

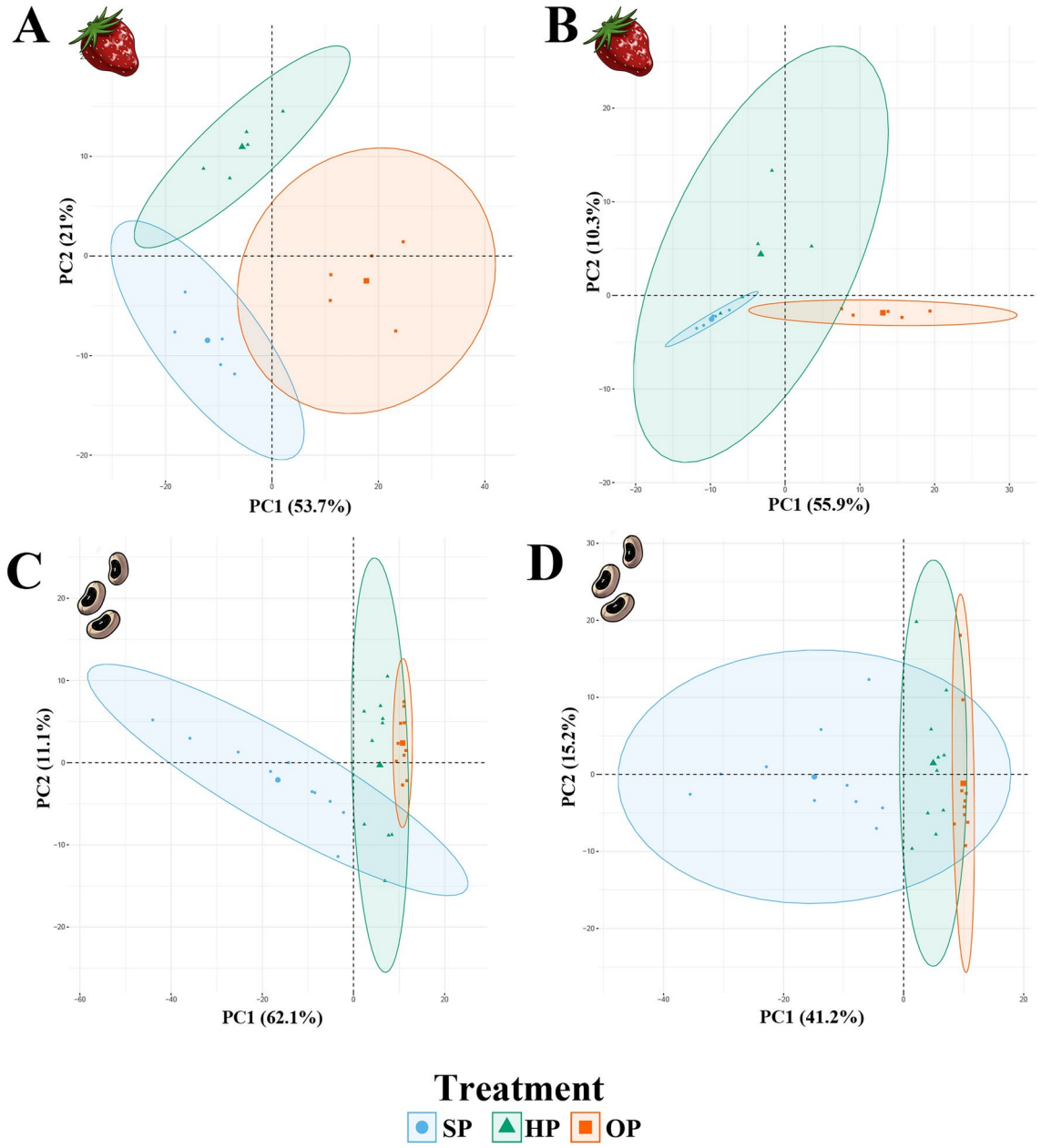

Fig. 3. Ordination analysis performed on the metabolic profiles of *F. vesca* phytochemical fractions in negative (**A**) and positive ion current (**B**) and on *V. unguiculata* seeds extracts in negative (**C**) and positive ion current (**D**). Each point represents a different extraction bulk collected during the productive season. *SP* self pollination, *HP* hand cross pollination, *OP* open pollination.

Arguably, in the SP treatment, the deposition of pollen on the floral stigmas may result uncompleted. An ineffective pollen deposition during the fertilization event could influence the seeds germination ability and their phytochemical composition, due to stress phenomena occurring during the development of seed embryos or inducing a higher dormancy rate, as supported by the reduced germination success of seeds as well as by the increased proportion of abortion on the total seed set (Figs. S1; 2C).

Conclusion

In the context of sustainable agricultural production, many studies are focusing on pollinators well-being, encouraging the adoption of good management practices for their safeguard. The present study provides compelling evidence that pollinators play a crucial role in enhancing the morphological features of fruits and seeds. The variations observed in fruits and seeds functional traits are indicators of an impact of pollination on ripening and embryo development process, with direct influence on crop yield and commercial quality. Furthermore, pollinator insects are shown also to play a relevant role in the definition of the chemical features of fruits and seeds, therefore for their overall health-promoting value for humans. A better understanding

Id.	Species	Rt	m/z	Adduct	Fragments	Ontology	Tentative ID	Family	Reference	*P*-value
1	*F. vesca*	6.12	203.08	[M-H]−	142, 130, 116	$C_{11}H_{12}N_2O_2$	Tryptophan	Amino acid	Public library (Compound Discoverer)	(SP = HP) < OP
2	*F. vesca*	6.93	947.05	[M-H]2−	901, 883, 301	$C_{41}H_{24}O_{27}$	Unknown ellagitannin	Ellagitannin	[24]	(SP = HP) > OP
3	*F. vesca*	8.58	447.15	[M-H]−	285, 285	$C_{21}H_{20}O_{11}$	Kampferol-*O*-hexoside	Flavonoid	[25]	(SP = HP) < OP
4	*F. vesca*	8.67	331.1	[M-H]−	127	$C_{14}H_{20}O_9$	Tetra-*O*-acetyl-dexoyhexoside	Sugar derivative	Public library (Compound Discoverer)	(SP = HP) < OP
5	*F. vesca*	8.97	465.1	[M-H]−	447, 285, 247	$C_{21}H_{22}O_{12}$	(2R,3R)-Taxifolin-3′-*O*-β-D-glucopyranoside	Flavonoid	Public library (Compound Discoverer)	(SP = HP) < OP
6	*F. vesca*	9.38	449.11	[M-H]−	355, 329, 287, 269, 193, 165, 137	$C_{21}H_{22}O_{11}$	Ferulic acid hexose derivative	Phenolic acid	[24]	(SP = HP) < OP
7	*F. vesca*	9.4	431.09	[M-2H]−	269, 268, 224, 147	$C_{21}H_{20}O_{10}$	Pelargonidin-3-*O*-glycoside	Anthocyanin	[24]	(SP = HP) < OP
8	*F. vesca*	10.29	371.1	[M-H]−	249, 121	$C_{16}H_{20}O_{10}$	3-Benzoyloxy-2-hydroxypropyl glucopyranosiduronic acid	Phenolic acid derivative	Public library (Compound Discoverer)	(SP = HP) > OP
9	*F. vesca*	11.14	447.05	[M-H]−	301, 300, 257, 229	$C_{20}H_{16}O_{12}$	Ellagic acid rhamnoside	Ellagic acid derivative	[25]	(SP = HP) < OP
10	*F. vesca*	11.36	567.21	[M-HCOO]−	521, 359	$C_{26}H_{34}O_{11}$	Methylated flavonoid hexoside	Flavonoid	[26]	(SP = HP) < OP
11	*F. vesca*	11.43	477.06	[M-H]−	301, 300	$C_{21}H_{18}O_{13}$	Quercetin glucuronide	Flavonoid	[27]	(SP = HP) < OP
12	*F. vesca*	11.86	463.09	[M-H]−	315, 300, 151	$C_{21}H_{20}O_{12}$	Myrcitrin	Flavonoid	Public library (Compound Discoverer)	(SP = HP) < OP
13	*F. vesca*	12.28	519.07	[M-H]−	315, 300	$C_{23}H_{20}O_{14}$	Methyl ellagic acid acetyl hexoside	Ellagic acid derivative	[24]	(SP = HP) < OP
14	*F. vesca*	12.58–13.13	461.0723	[M-H]−	315, 301, 275	$C_{21}H_{18}O_{12}$	3-*O*-Methylellagic acid-3′-*O*-α-L-rhamnopyranoside	Ellagic acid derivative	Public library (Compound Discoverer)	(SP = HP) < OP
15	*F. vesca*	12.86	315.01	[M-H]−	300	$C_{15}H_8O_8$	3-*O* Methyl ellagic acid	Ellagic acid derivative	Public library (Compound Discoverer)	(SP = HP) < OP
16	*F. vesca*	14.5	491.2	[M-HCOO]−	313, 161	$C_{21}H_{34}O_{10}$	(Z)-(1S,5R)-β-Pinen-10-il-β-vicinoside	Monoterpene	Public library (Compound Discoverer)	(SP = HP) < OP
17	*F. vesca*	14.94	549.16	[M-H]−	255	C26H30O13	Liquiritin apioside	Flavonoid	Public library (Compound Discoverer)	(SP = HP) < OP
18	*F. vesca*	16.87	255.07	[M-H] −	213, 185, 171, 151, 145, 107	$C_{15}H_{12}O_4$	Pinocembrin	Flavonoid	Public library (Compound Discoverer)	SP < HP < OP
19	*F. vesca*	6.05	205.1	[M-H]+	188, 170, 118	$C^{11}H_{12}N_2O_2$	Tryptophan	Aminoacid	Public library (Compound Discoverer)	(SP = HP) < OP
20	*F. vesca*	7.96	579.15	[M-H] +	409, 287, 127	$C_{30}H_{25}O_{12}$	B-type procyanidin	Procyanidin	Public library (Compound Discoverer)	SP < OP < HP
21	*F. vesca*	8.17	291.09	[M-H]+	161, 147, 139, 123	$C_{15}H_{14}O_6$	Quercetin glucuronide	Flavanol	Public library (Compound Discoverer)	HP > SP > OP
22	*F. vesca*	9.79	463.12	M+	301	$C_{22}H_{23}O_{11}$	Peonidin 3-*O*-glycoside	Anthocyanin	[25]	(SP = HP) < OP
23	*F. vesca*	10.01	433.11	M+	271	$C_{21}H_{21}O_{10}$	Pelargonidin-3-*O*-glycoside	Anthocyanin	[24]	(SP = HP) < OP
24	*F. vesca*	12.02	303.05	[M-H]+	285, 257	$C_{14}H_6O_8$	Ellagic acid	Ellagic acid	Public library (Compound Discoverer)	(SP = HP) < OP

Continued

Id.	Species	Rt	m/z	Adduct	Fragments	Ontology	Tentative ID	Family	Reference	*P*-value
25	*F. vesca*	16.9	257.07	[M-H]+	239, 215, 153, 131, 103, 77	$C_{15}H_{12}O_4$	Pinocembrin	Flavonoid	Public library (Compound Discoverer)	SP < HP < OP
26	*F. vesca*	17.2	246.24	[M-NH4]+	124, 57	$C_{14}H_{28}O_2$	Ethyl laurate	Fatty acid	Public library (Compound Discoverer)	SP < HP < OP
27	*V. unguiculata*	2.4–2.7	451.1	[M-H]−	289, 137, 109	$C_{21}H_{24}O_{11}$	Catechin-*O*-glucoside	Flavanol	[28]	SP > HP > OP
28	*V. unguiculata*	3.58	385.1	[M-HCOO]−	134, 85	$C_{15}H_{16}O_9$	Caffeic acid derivative	Phenolic acid derivative	Public library (Compound Discoverer)	SP > HP > OP
29	*V. unguiculata*	3.9	289.1	[M-H]−	123, 109	$C_{15}H_{14}O_6$	Catechin	Flavanol	Public library (Compound Discoverer)	SP > HP > OP
30	*V. unguiculata*	4.05	493.15	[M-H]−	289, 245, 203	$C_{23}H_{26}O_{12}$	Catechin-*O*-glucoside-*O*-acetoside	Flavanol	[28]	SP > HP > OP
31	*V. unguiculata*	4.97	465.12	[M-H]−	285, 151	$C_{21}H_{22}O_{12}$	Taxifolin-*O*-hexoside	Flavonoid	Public library (Compound Discoverer)	(SP = HP) < OP
32	*V. unguiculata*	5.16–5.3	625.16	[M-H]−	301, 300	$C_{27}H_{30}O_{17}$	Quercetin-di-*O*-hexoside isomers	Flavonoid	Public library (Compound Discoverer)	SP > HP > OP
33	*V. unguiculata*	5.53	567.23	[M-HCOO]−	521, 506, 359, 344, 217	$C_{26}H_{34}O_{11}$	Neolignan	Lignan	Public library (Compound Discoverer)	SP > HP > OP
34	*V. unguiculata*	6.28	505.11	[M-H]−	463, 301, 300	$C_{23}H_{22}O_{13}$	Quercetin-*O*-(acetyl-hexoside)	Flavonoid	Public library (Compound Discoverer)	(SP = HP) < OP
35	*V. unguiculata*	7.64	663.33	[M-H]2−	–	–	Uknown peptide	Peptide	See text	SP > HP > OP
36	*V. unguiculata*	7.81	677.32	[M-H]2−	–	–	Uknown peptide	Peptide	See text	SP > HP > OP
37	*V. unguiculata*	7.93	648.32	[M-H]2−	–	–	Uknown peptide	Peptide	See text	(SP = OP) > HP
38	*V. unguiculata*	8.01	640.32	[M-H]2−	–	–	Uknown peptide	Peptide	See text	SP > HP > OP
39	*V. unguiculata*	10.5–10.9	987.54	[M-HCOO]−	941, 733, 615, 457	$C_{48}H_{78}O_{18}$	Azukisaponin isomers	Triterpene saponins	Public library (Compound Discoverer)	SP > HP > OP
40	*V. unguiculata*	10.7	327.23	[M-H]−	211, 183, 125	$C_{18}H_{32}O_5$	Trihydroxy-octadecadienoic acid	Polyunsaturated fatty acid	Public library (Compound Discoverer)	SP > HP > OP
41	*V. unguiculata*	11.2	957.54	[M-H]−	615, 263, 221	$C_{48}H_{78}O_{19}$	3-Glucose-Galactoside-Glucurunate Soyasapogenol B	Triterpene saponins	Public library (Compound Discoverer)	SP > HP > OP
42	*V. unguiculata*	11.4	955.52	[M-H]−	613, 455	$C_{48}H_{76}O_{19}$	Soyasaponin	Triterpene saponins	Public library (Compound Discoverer)	SP > HP > OP
43	*V. unguiculata*	12.1	985.54	[M-HCOO]−	921, 613, 455	$C_{48}H_{76}O_{18}$	Dehydrosoyasaponin	Triterpene saponins	Public library (Compound Discoverer)	SP > HP > OP

Table 2. Identification of metabolites significantly contributing to treatment related differences is shown in Fig. 3. The symbols " > " and " < " indicate differences significant at the statistical level ($p < 0.05$), while " = " indicates non-significant differences. *Rt* retention time, *m/z* mass to charge ratio, *SP* self pollination, *HP* hand cross pollination, *OP* open pollination.

of this phenomenon is pivotal to identifying strategies aimed at enhancing the production of metabolites of nutraceutical interest in plant foods and arranging conservation guidelines for pollinators safeguard. Overall, the higher occurrence of compounds involved in antioxidant and anti-inflammatory properties and the reduction of antinutrient factors in animal-pollinated products highlighted in the context of the present study is a relevant sign of the role of pollinators in the enhancement of the nutritional quality of foods, in line with the One-Health concept, suggesting that both environmental and animal health strongly correlates with human well-being.

Methods

General experimental procedures

The chemical extractions were carried out using a rotary evaporator (Hei-VAP, Heidolph, Germany). The purification of the phytochemicals extracted was done by using a VisiPrep SPE Manifold (Merck, Germany). The titration assays were done by using a pH-meter (HANNA, USA) and the total soluble solids (TSS) were estimated by a hand-held refractometer (Krüss, Germany). The analysis of fruit redness was done by using a portable colorimeter (CR-410 Chromameter, The Netherlands). The colorimetric and enzymatic assays were performed through a Cary 60 UV–Vis spectrophotometer (Agilent, USA) while the metabolomic analyses were performed through a high-resolution mass spectrometer (Xevo-G2-qToF) coupled to an ESI source (Waters, USA).

Chemicals and reagents

Ultrapure H_2O (18 MΩ) was obtained using a Milli-Q purification system (Millipore, USA). Solvents and reagents for samples extraction and characterization (ethanol, methanol, gallic acid, Trolox, quercetin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium nitrite ($NaNO_2$), sodium hydroxide (NaOH), aluminium chloride ($AlCl_3$), sodium carbonate (Na_2CO_3), Folin–Ciocalteu Reagent) were purchased from Sigma Aldrich®, Germany. Mass-grade solvents (methanol, acetonitrile, and formic acid) were obtained from Romil®, Italy.

Plant material

Two plant species were considered, and simultaneous experiments were carried out to evaluate the effect of different pollination mechanisms on the morphological and phytochemical features of fruits and seeds as well as on seed germination success. Specifically, a total number of 120 seedlings of wild strawberries (*F. vesca*) were obtained from Valitutto s.r.l (Sicignano degli Alburni, Italy) and 100 seeds of cowpea (*V. unguiculata*, accession number TVU11733), from the International Institute of Tropical Agriculture, Nigeria.

Wild strawberries were cultivated in 9 L pots filled with a peat pumice substrate (Hochmoor–Terflor, Italy), within an experimental open greenhouse, divided into four distinct plots, each comprising 30 plants.

Cowpea plants were cultivated in an experimental field, arranged in a single plot consisting of two rows (12 m long), with 50 plants per row, as described in[38].

The cultivation of both species was conducted at the CREA Institute (Council for Agricultural Research and Agricultural Economics Analysis) of Sanremo, Italy, and lasted for two subsequent years (2021 and 2022).

Pollination treatments

The flowers of both species were subjected to three different pollination treatments. The treatments were carried out simultaneously on the same plants to minimize the effect on the individuals.

Specifically, the chosen floral units were treated with one of the following conditions: (1) the self pollination (SP) treatment was set up by covering the flowers using Osmolux bags (Pantek, France) to avoid pollination events due to insects or wind, (2) the hand cross pollination (HP) treatment, based on the application to the stigmas of selected flowers of the pollen obtained from a different individual by using a soft brush (the procedure was repeated 10 times to promote a complete pollen saturation), and (3) the open pollination treatment (OP) where the floral units were left free to be visited by pollinator insects. The pollen to enable the HP treatment was gathered by using the E-PoSa device described in[41]. The selection of the Osmolux bags was made based on previous studies that highlighted their ability to maintain unaltered micro-environmental conditions, such as light, temperature and humidity[15]. The bags were removed at the beginning of the fruiting (i.e., after the petal fall and at the initiation of fruit swelling). The wild strawberries were collected at complete ripening, while cowpea pods at the end of fruit development to minimize differences related to the ripening stage. Concerning wild strawberries, the term "fruit" was defined as the combination of both achenes and receptacles. The collected fruits were analysed for their morphological traits and subsequently stored at − 20 °C before metabolic analyses.

Analysis of morphological features and the reproductive success

The length, weight and horizontal diameter of treated strawberries were measured immediately after harvesting to avoid alterations in their properties. The fruit colour was evaluated by a portable colorimeter (CR-410 Chromameter, The Netherlands) at two opposite sides of the fruits in the L * a * b colour space, with the a-value indicating the green–red composition[15] on a total of 100 fruit per treatment.

Cowpea pods length and weight were measured post-harvest, the total number of seeds per pod and mean seed weight were recorded on a total of 50 fruit per treatment.

The reproductive success of the two species under different pollination treatments was determined by the number of fertilized achenes per fruit in wild strawberries on a total of 100 fruit per treatment, by evaluating the proportion of aborted out of the total seeds per pod and by conducting a germination assay cowpea. Fertilized achenes were manually counted after separating them from unfertilized ones based on their differential sedimentation abilities in water. For cowpeas, 100 seeds per pollination treatment were tested for germination rate. The seeds were set in five petri dishes ($N = 20$ for each trial) and the proportion of germinated seeds was estimated every 24 h for a total of 3 days.

Organoleptic features and macronutrient composition

Strawberries were analyzed for their total soluble solids (TSS), titratable acids (TA) and the pH values of the juice. After being homogenized by a tube mill (IKA, Germany), fruits were centrifuged twice at 7000 rpm for 15 min. The supernatant was then analyzed to determine its pH value, TSS, and TA. TSS estimation was conducted using a hand-held refractometer (Krüss, Germany) while pH value measurement was performed using a pH meter (HANNA, USA). TA was quantified following the protocol described in[15]. Finally, the sugar to acid ratio was obtained by dividing the TSS by the TA. For each treatment, 30 fruits were used by considering the whole seasonality of the experiment and therefore including fruits originating from the six different harvesting periods.

The total starch content and total protein content of cowpeas were analyzed to investigate potential variations in the macronutritional composition related to pollination treatment. Fifty seeds per experimental group were grinded by a laboratory mill (IK, Germany) and the powder obtained was freeze-dried to eliminate water residual content. Starch content was determined using the total starch kit (Megazyme, Ireland) according to the method reported in[42], while proteins were extracted and quantified following the protocol described in[43].

Phytochemical analysis by untargeted metabolomics

The metabolomic analysis of strawberries and cowpea seeds was conducted by analytical chemistry approaches. Before extracting the phytochemicals from strawberries, the fruits were freeze-dried to remove the water content and subsequently grinded into a fine powder. For each treatment, 100 fruits were used equally divided into experimental bulks, according to the seasonality of the experiment for a total of 6 different harvesting periods.

A hydro-alcoholic solvent composed of MeOH 70% v/v, pH = 3.5 was used for the extraction process. The drug-to-solvent ratio was equal to 1:20 w/v. The extraction process involved maceration on an orbital shaker (Asal 711, Italy) for two cycles of 15 min each, followed by centrifugation at 5000 g. The supernatant was collected, and total extracts were evaporated to dryness using a rotary evaporator (Heidolph, Germany) before resuspension in 10 mL of ultrapure milli-Q H_2O.

Due to the high concentration of sugars in the total extracts, a solid-phase mediated purification was conducted using Reverse Phase cartridges (Strata-X, Phenomenex, USA) to isolate the phytochemical fraction. After loading, the cartridge was washed with MeOH 5% and then eluted with MeOH+0.1% HCOOH v/v. Then, the eluted fractions were kept at − 80 °C before the metabolomic analyses.

The total phenol content (TPC), trolox equivalent antioxidant capacity (TEAC), and total flavonoid content (TFC) of these fractions were estimated as reported in[44].

Cowpea seeds were ground according to Par. 4.4.1. For each treatment, 100 seeds were considered for the phytochemical experiments.

The powder was extracted by a hydro-alcoholic solvent (EtOH 50% v/v) for three subsequent cycles lasting 10 min each in a drug/solvent ratio equal to 1:10 *w/v* using by using a bath sonicator (ArgoLab, Italy) at a frequency of 37 Hz at room temperature. The obtained extracts were evaporated to remove the organic solvent and then kept at − 80 °C before the subsequent analyses.

The metabolomic analysis of the obtained samples was carried out by using a Xevo-G2-qToF mass spectrometer (Waters, USA). The analysis was performed in reverse-phase chromatography by using a Zorbax SB-C_{18} column (2.1 × 100 mm, 3.5 µm). H_2O + 0.1% formic acid and acetonitrile + 0.1% formic acid were used as mobile phases A and B, respectively. The flow rate was set to 0.4 mL/min and the injection volume was 5 µL. Full scan analyses (MS^1) were aquired both in negative and positive mode, while the identification of phytochemicals affected by the pollination treatment was carried out by subsequent MS^2 analyses.

Statistical analysis

All the statistical analyses and figures, graphs and images were obtained on R (Version 4.3.1). To evaluate the effect of the pollination treatment on the morphological parameters, Principal Component Analyses (PCA) coupled with PERMANOVA analysis and post hoc Tukey test were employed. Packages used were *vegan*, *ggplot2*, and *RVAidememoire*. Data about the TSS, TA, pH, TSS:TA ratio of wild strawberries, the number of fertilized achenes, and the proportion of aborted seeds in cowpeas, together with the data about primary metabolism were analyzed by regression models accounting for the pollination treatment as a fixed effect. The germination success of cowpea seeds was analyzed considering as covariate the interaction between the duration time of the experiment and the pollination treatment. Packages used were *MASS*, *glmmTMB*, and *ggplot2*. Details concerning the statistical distribution assumed are reported in Table S2.

Finally, the data resulting from the untargeted metabolomic analysis were first processed on MS-Dial (version 4.9) for peak peaking, deconvolution, noise level setting, alignment on a quality control (QC) sample, and normalization. Then, the normalized data were analysed by PCA followed by PERMANOVA analysis followed by post-hoc Tukey tests to evaluate the effect of the pollination treatment on the metabolomic variables. If significant differences among the pollination treatments were detected, each ion composing the metabolic profiles was tested for significant differences by one-way ANOVA followed by a Tukey post-hoc test. The significant features were studied for their chemical characterization by interpreting their MS^2 spectra and matching them with both libraries of natural products (EXPV17 on MS-Dial and The Waters Traditional Medicine on UNIFI) besides bibliographic research.

Data availability

Data used in this article can be found online at https://doi.org/10.6084/m9.figshare.25343485.v1

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Author contributions

Emiliano Pioltelli: conceptualization, methodology, data curation, formal analysis, writing—original draft, visualization. Lorenzo Guzzetti: conceptualization, methodology, formal analysis, investigation, writing—review & editing. Paolo Biella: conceptualization, methodology, writing—review & editing. Davide Sala: investigation. Andrea Copetta: investigation, data curation. Paolo Mussano: investigation. Andrea Galimberti: resources, writing—review & editing. Massimo Labra: resources, writing—review & editing. E.P., L.G. and P.B. conceived the idea and designed the experiments. E.P., L.G., A.C. and P.M. performed the experiments. E.P.,L.G. and D.S processed the experimental data, performed the analyses, drafted the manuscript and designed the figures. A.G. and M.L supervised the project. All authors provided critical feedback on the results and contributed to the final version of the manuscript.

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Competing interests

The authors declare no competing interests.

Ethical statement

The experimental procedures complied with relevant institutional, national, and international guidelines and legislation.

Additional information

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