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Reproductive Biology and Field Performance of New Uruguayan Promising Mandarins

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Abstract

Three promising new mandarin hybrids from the Uruguayan Citrus Breeding Program offer the organoleptic and quality standards demanded by the premium fresh citrus market. This study aimed to characterize the reproductive biology, parthenocarpic capacity, and field performance of F4P7, F3P8, and F2P3 genotypes to assess their suitability for seedless fruit production. For this purpose, pollen tube growth was evaluated under self-pollination, and potential limiting factors of fruit set and their type of parthenocarpy were evaluated. In addition, sprouting, flowering, and yield under open-pollination were studied. All three genotypes exhibited gametophytic self-incompatibility. Besides, these genotypes exhibited facultative autonomous parthenocarpy and low parthenocarpic capacity. Specifically, seedless fruits were produced only when compatible pollination was absent; moreover, parthenocarpic fruit set was not stimulated by self-pollination and remained lower than that observed under open-pollination conditions. Our results revealed that fruit set limitations were associated with insufficient GA_1 and soluble carbohydrate levels in the ovary during early fruit development. Cross-pollination significantly enhanced GA_1 levels and carbohydrate accumulation in the ovary, thereby improving fruit set and increasing the number of seeded fruits. Field performance showed year-to-year yield variability, with flowering intensity correlating with yield in F4P7 and F3P8. Conversely, F2P3 displayed more stable production and greater variations in shoot-type distribution across different flowering levels. Our findings highlight that preventing cross-pollination via bee-proof netting or spatial isolation is essential for seedless fruit production. In addition, agronomic practices aimed at increasing bioactive gibberellin levels and soluble carbohydrates availability are required to ensure consistent high yield in these genotypes.

Keywords: soluble carbohydrates, flowering, fruit set, gibberellin, parthenocarpy, yield

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Biología reproductiva y comportamiento agronómico de nuevas mandarinas promisorias uruguayas

Resumen

Este trabajo tuvo como objetivo investigar la biología reproductiva, la capacidad partenocárpica y el comportamiento agronómico de tres destacados híbridos de mandarina (F4P7, F3P8 y F2P3) del Programa de Mejoramiento Genético de Cítricos del Uruguay. Se evaluó el crecimiento de los tubos polínicos en autopolinización, se analizaron los factores limitantes del cuajado y el tipo operante de partenocarpia. Además, se evaluó la brotación, la floración y el rendimiento en condiciones de polinización cruzada. Los tres genotipos exhibieron autoincompatibilidad gametofítica y partenocarpia facultativa de tipo autónoma, presentando baja capacidad partenocárpica en todos los casos. Los resultados revelaron que las limitaciones en el cuajado de frutos se asociaron con el nivel de GA_1 y carbohidratos solubles en el ovario durante el desarrollo inicial del fruto. La polinización cruzada aumentó significativamente el contenido de GA_1 y la acumulación de carbohidratos en el ovario, lo que favoreció un mayor cuajado de frutos con semillas. En dichas condiciones, el rendimiento de F4P7 y F3P8 mostró variabilidad interanual, correlacionando directamente con la intensidad de la floración. F2P3 mostró una producción más estable y cambios en la distribución del tipo de brote según el nivel de floración. Nuestros hallazgos indican la necesidad de prevenir la polinización cruzada para la producción de frutos sin semillas e implementar prácticas agronómicas para un alto rendimiento constante de estos híbridos de mandarina.

Palabras clave: carbohidratos solubles, floración, cuajado de frutos, giberelina, partenocarpia, rendimiento

Biologia reprodutiva e desempenho agronômico de novas tangerinas uruguaias promissoras

Resumo

Este estudo investiga a biologia reprodutiva, a capacidade partenocárpica e o desempenho agronômico de três promissores híbridos de tangerina (F4P7, F3P8 e F2P3) do Programa Uruguaio de Melhoramento de Citros. O crescimento do tubo polínico foi avaliado sob autopolinização; a porcentagem de fixação de frutos foi determinada sob isolamento com rede à prova de abelhas e condições de polinização aberta; e fatores limitantes da fixação de frutos foram analisados, bem como o tipo de partenocarpia sob polinização manual. Além disso, a brotação, a floração e a produtividade foram avaliadas sob condições de polinização aberta. Todos os três genótipos exibiram autoincompatibilidade gametofítica e partenocarpia autônoma facultativa com baixa capacidade. Nossos resultados revelaram que as limitações na fixação de frutos estavam associadas à incapacidade de manter níveis suficientes de giberelina ativa (GA_1) e carboidratos solúveis no ovário durante o desenvolvimento inicial do fruto. A polinização cruzada aumentou significativamente o teor de GA_1 e o acúmulo de carboidratos no ovário, resultando em maior fixação de frutos e produção de sementes. O desempenho em campo apresentou variabilidade anual na produção, com a intensidade da floração correlacionada com a produção em F4P7 e F3P8. F2P3 apresentou produção mais estável e uma mudança na distribuição dos tipos de brotos sob diferentes níveis de floração. Nossos resultados indicam a necessidade de evitar a polinização cruzada para a produção de frutos sem sementes e de implementar práticas agronômicas para uma alta produtividade consistente desses híbridos de tangerina.

Palavras-chave: carboidratos solúveis, floração, frutificação, giberelina, partenocarpia, rendimento

1. Introduction

Seedlessness is a key trait for the citrus fruit fresh market and has driven breeding efforts to develop high-quality seedless cultivars (Food and Agriculture Organization of the United Nations [FAO], n.d.; Caruso et al., 2020; Spreen et al., 2020). It results from gynosterility and obligatory parthenocarpy, or by self-incompatibility (SI) combined with facultative parthenocarpy (Vardi et al., 2008). SI, commonly gametophytic in Citrus, is the inability to set seeds following self-pollination in fertile cultivars (de Nettancourt, 2001; Zhang et al., 2018), while parthenocarpy refers to production of seedless fruits without fertilization. It can be classified as either stimulative, if

pollen stimulation is required to enhance fruit set, or autonomous, which occurs independently of such stimuli (Vardi et al., 2008).

At the physiological level, fruit set is known as the transition from the ovary to the developing fruit stage. During this period, a first wave of abscission occurs within three to four weeks after anthesis, and initial fruit set depends on hormonal factors mainly related to the ability to synthesize GA_1 in the ovary (Mesejo et al., 2013; Olimpieri et al., 2007; Talón et al., 1992). A second wave of abscission occurs two weeks later, triggered by carbohydrate depletion (Iglesias et al., 2003). In non-parthenocarpic cultivars, fertilized ovules promote ovary growth (Bermejo et al., 2015), whereas in parthenocarpic ones, fruit set is promoted by the constitutive synthesis of gibberellins (GAs), which reactivates cell division in the ovary (Mesejo et al., 2016).

In addition, fruit set regulation is a critical physiological process because it determines fruit number and, consequently, has a strong impact on fruit yield. Optimal yield across years needs a well-balanced flowering level and an appropriate self-regulated crop load. Otherwise, different performance patterns can be observed due to low yields associated with high flowering levels and low fruit set, or sharp year-to-year fluctuations between heavy and light crops, which may lead to alternate bearing (Agustí & Primo-Millo, 2020).

Against this background, three mandarin hybrids developed by the Uruguayan Citrus Breeding Program are of great interest to the Mediterranean basin and show potential for the worldwide mandarin market. These genotypes were selected for their excellent organoleptic characteristics, low seed number, and extended market window (early-season F4P7, mid-season F3P8, and late-season F2P3) (Rivas & Vignale, 2015). However, despite their promising commercial traits, information on their reproductive biology and parthenocarpy is still lacking, particularly for the early- and mid-season genotypes. Therefore, this study aimed to determine male and female gametophyte performance, self-incompatibility, parthenocarpic capacity, and field performance of these three genotypes, to assess their suitability for seedless fruit production and support their introduction into major citrus-growing regions.

2. Materials and Methods

2.1 Plant Material

Experiments were carried out at INIA Salto Grande Experimental Station in Uruguay (31° 16' SL, 57° 53' WL), from 2020 to 2023. Adult trees of mandarins, all hybrids of 'Ellendale' (*Citrus reticulata* Bl.) × 'Page' [*Citrus clementina* Hort. ex Tan. × (*Citrus paradisi* Macf. × *Citrus reticulata* Bl.)], coded as F4P7, F3P8, and F2P3, grafted onto 'Trifolia' (*Poncirus trifoliata* L. Raf.) were used. The trees were planted at a spacing of 3 × 6 m with a drip irrigation system and fertilizer application (700 g $plant^{-1}$ containing 18% N, 5% P_2O_5 and 22% K_2O). The soil was a Typic Argiudoll with sandy texture (Altamirano et al., 1976). Climate conditions correspond to a temperate-humid subtropical classification, without a marked dry season and warm summers (Cfa) (Köppen, 1918). The average maximum, mean, and minimum annual temperatures are 25 ºC, 19 ºC, and 13 ºC, respectively. Annual rainfall ranges between 1200 and 1600 mm without a rainfall season (Castaño et al., 2011). Two air temperature sensors (DS1923 Hygrochron, iButton, USA) were placed inside two tree canopies during the three years of evaluation.

2.2 Experimental Procedure

Experiment 1 was conducted to evaluate SI. To this end, 100 leafy-single-flowered shoots were selected from 20 trees of each genotype. A completely randomized design was used. Two treatments [self-pollination and pollination with 'Afourer' mandarin (*Citrus reticulata* Bl.)] and five sampling dates [0, 2, 4, 8, and 12 days after

pollination (DAP)] were included, using one flower as the experimental unit and 10 replicates per treatment. Treatments were applied to flowers at the hollow balloon stage, corresponding to BBCH stage 59 (0 DAP). In vivo pollen germination, self-incompatibility, pollen tube kinetics, and ovule senescence were evaluated by histological analysis (Mesejo et al., 2007).

After verifying the SI, Experiment 2 was carried out to determine parthenocarpic capacity by comparing fruit set of seeded fruit (open pollination) and seedless fruit (isolation) in 18 trees per genotype. In all genotypes, a completely randomized design with two factors (treatment and sampling date) was used. Two treatments (open pollination and netting) and three sampling dates [0, 7, and 14 days after anthesis (DAA) for GA_1 analysis and 30, 45, and 60 DAA for carbohydrate analysis] were used, with one tree as the experimental unit and three replicates per treatment. Anthesis was considered when flowers exhibited fully expanded petals and exposed stamens and stigma (0 DAA). The netting treatment consisted of complete tree isolation using a bee-proof net (50 g m^{-2}) to prevent cross-pollination, installed from the beginning of bud break until the end of petal fall. Two branches, with at least 100 nodes, were tagged on each tree to evaluate flowering intensity (flowers per 100 nodes), fruit set (%), seeded fruits (%), and seed number per fruit. Additionally, in each genotype, 450 leafy-single-flowered shoots per experimental unit were selected at BBCH stage 59 (Agustí et al., 1997) for gibberellin and carbohydrate analysis.

Experiment 3 was conducted to determine the type of parthenocarpy through controlled pollination. For this experiment, genotypes were considered as obligately parthenocarpic when fruits remained seedless even after compatible pollination, and as facultatively parthenocarpic when seedless fruits developed without compatible pollination, but seeded fruits developed under it. Autonomous parthenocarpy was defined when fruit set in the absence of pollination was comparable to that of self-pollinated flowers. Conversely, stimulative parthenocarpy was assigned when self-pollination significantly increased seedless fruit set relative to unpollinated controls. For each genotype, 300 leafy-single-flowered shoots at BBCH stage 59 (0 DAP) were selected from 10 trees. The experiment was arranged as a randomized complete block design, using one tree as the block, 10 blocks, and 10 leafy-single-flowered shoots as a subsample. Three treatments: 1) emasculation, 2) self-pollination, and 3) pollination with 'Afourer' mandarin were applied. Within each tree, treatments were randomly assigned to groups of 10 leafy-single-flowered shoots (subsamples), which constituted the experimental units. Fruit set (%) was calculated for each experimental unit as the proportion of flowers that set fruit relative to the total number of treated flowers (n = 10), and seed presence was determined at harvest. Fruit set was evaluated from anthesis until the end of physiological fruit drop. Additionally, in F3P8, a set of 225 single-flowered leafy shoots per treatment were tagged at BBCH stage 59 for gibberellin analysis.

Experiment 4 was carried out to evaluate the agronomic performance of all genotypes under open-pollination conditions. Five trees of each genotype were used during three consecutive years. A two-factor completely randomized design with three genotypes (F4P7, F3P8, and F2P3) and three years (2020, 2021, and 2022) was used, with one tree as the experimental unit and five replicates per treatment. Two branches per tree were selected to evaluate sprouting intensity (shoots per 100 nodes), flowering intensity (flowers per 100 nodes), and shoot type distribution (%). At harvest, fruit yield (kg $tree^{-1}$) was evaluated, and the alternate bearing index (ABI) was calculated with the following equation:

$$[\text{ABI} = \frac{1}{n-1} \times \left\{\frac{|a2-a1|}{a2+a1} + \frac{|a3-a2|}{a3+a2} + \frac{|an-an\text{-}1|}{an+an\text{-}1}\right\}$$

where n = number of years, and a1, a2, an–1, an = yields of the corresponding years (Pearce & Doberšek-Urbanc, 1967).

2.3 Pollination Procedure

Pollen was collected from flowers at BBCH stage 59, and anthers were removed and kept in silica gel until dehiscence. Pollen viability was assessed using the acetocarmine staining method (Singh, 2001). Samples were incubated in 1% acetocarmine for 30 min at room temperature, and pollen was considered viable when stained red. In vitro germination was assessed under light microscopy (E200, Nikon, Japan) using 1,000 pollen grains obtained from five flowers. Pollen was incubated in Brewbaker & Kwack's medium (Brewbaker & Kwack, 1963) at 23.6 ± 0.5 ºC and 80.9 ± 1.6% relative humidity. Pollen was considered germinated when the tube length doubled the grain diameter after 48 h (Mesejo et al., 2007). For pollination, leafy-single-flowered shoots at BBCH stage 59 were selected, emasculated, pollinated with a brush, and isolated from cross-pollination by covering the pistil with an aluminum cap.

2.4 Histological Analysis

Ten flowers per treatment were sampled at 0, 2, 4, 8, and 12 DAP, fixed in FAA solution (5% formaldehyde, 5% acetic acid, 90% ethanol at 70%) and stored at 4 ºC for histological analysis. Style length (mm) was measured using an electronic caliper (500-171 Absolute Digimatic, Mitutoyo, USA). In vivo pollen germination (200 grains per flower), pollen tube growth (expressed as the percentage of the style length recorded), number of pollen tubes reaching the middle of the style length and style-ovary junction, and chalazal callose deposition as an ovule senescence sign (15 ovules per flower) were evaluated using aniline blue staining and epifluorescence optical microscopy (14-6, Labotecgroup, China), following Mesejo et al. (2007). Pollen was considered germinated when the pollen tube doubled the grain diameter, as defined for in vitro germination; genotypes in which pollen tubes did not exceed 50% of the style length were considered self-incompatible, and ovule senescence was determined as the percentage of ovules with callose deposition in the chalazal zone (Mesejo et al., 2007).

2.5 Gibberellin Analysis

Three samples of 25 flowers per treatment were collected at 0, 7, and 14 DAA to evaluate ovary GA_1 concentration, as the first wave of abscission is primarily hormonally regulated and occurs shortly post-anthesis (Mesejo et al., 2013). Ovaries were frozen in liquid N_2, freeze-dried (Lyophilizer 01. JLG, Labotecgroup, China), and then finely ground. GAs analysis was performed by LC-MS/MS (Seo et al., 2011). In brief, extraction was made from 30 mg DW material with 80% methanol and 1% acetic acid containing internal standards and mixed by shaking for 1 h at 4 ºC. Extracts were purified by reverse phase column Oasis HLB (30 mg, Waters, USA) and a cationic exchange Oasis MCX column, eluted with methanol (MeOH). The final residue was dissolved in 5% acetonitrile, 1% acetic acid, and GA_1 was separated by UHPLC with a reverse Accucore C18 column (2.6 μm, 100 mm length; Thermo Fisher Scientific, USA) with a 5 to 50% acetonitrile gradient containing 0.05% acetic acid at 400 μl min^{-1} over 14 min. For the quantification, extracts were passed consecutively through HLB (reverse phase) and MCX (cationic exchange) columns, and were eluted with MeOH 80%, 1% acetic acid and 60% MeOH, 5 % NH_3, respectively, and analyzed by electrospray ionization (spray voltage 3.0 kV, heater temperature 150 ºC, sheath gas flow rate 40 μl min^{-1}, auxiliary gas flow rate 10 μl min^{-1}) in negative mode and targeted-tSIM (capillary temperature 300 ºC, S-lens RF level 70, resolution 70.000) in a Q-Exactive spectrometer (Orbitrap detector; Thermo Fisher Scientific, USA). GA_1 concentrations were determined using external calibration curves and the Xcalibur 4.0 and TraceFinder 4.1 SP1 programs, and deuterium-labeled hormone (OlChemim, Czech Republic) were used as an internal standard.

2.6 Carbohydrate Analysis

Three samples of 25 fruitlets per treatment were collected at 30, 45, and 60 DAA to determine fructose, glucose, and sucrose concentrations by HPLC, following the methodology reported by Rivas et al. (2006). This sampling period corresponded to the second wave of abscission, which occurs two weeks following the initial wave and

driven primarily by carbohydrate availability (Iglesias et al., 2003). Briefly, fruitlets were frozen in liquid N_2, freeze-dried (Lyophilizer 01. JLG, Labotecgroup, China), and finely ground. Carbohydrates were extracted from a sample of 100 mg of the freeze-dried material with 80% (v/v) ethanol at 80 ºC for 5 min, adding 100 μl of fucose (60 mg ml^{-1}) as an internal standard, and then centrifuged at 12,000 g for 10 min. The supernatant was evaporated to 0.5 ml (Speed-Vac, 40 ºC) and purified with a cationic column [AmberChrom™ 50WX8 50-100 mesh, Sigma-Aldrich, USA], an anionic column [AmberChrom™ 1X4 100-200 mesh, Sigma-Aldrich, USA], a membrane filter (0.45 μm membrane, Waters-Millipore, USA), and a C18 cartridge (Sep-Pak, Waters-Millipore, USA). The cationic column was washed with 2 M HCl in mQ water to adjust pH to 4.5, whereas the anionic column was washed with 1 M Na_2CO_3 in mQ water until reaching pH 7.5. The samples were evaporated to dryness (Speed-Vac, 40 ºC) and dissolved in 100 μl of mQ water. Identification and quantification were made from 20 μl of sample injected into an HPLC (1260 Infinity II, Agilent, USA) with guard column (4.6 × 12.5 mm, ZORBAX, USA), attached to pre-column (4.6 × 250 mm, ZORBAX, USA), G7162A refractometer and G7110B Iso pump, and acetonitrile: water (80:20, v/v) as solvent at a flow rate of 1 ml min^{-1} over a 20 min run. Fucose, fructose, glucose, and sucrose identification were carried out by retention time and corrected by a fucose recovery factor.

2.7 Statistical Analysis

Statistical analyses were performed using analysis of variance (ANOVA) followed by Tukey's test ($p < 0.05$). Model assumptions were verified using Q-Q plots, the Shapiro-Wilks test, plots of predicted versus residuals values, and Levene's test. Percentage data were analyzed using generalized linear mixed models (GLMMs), with treatment and sampling date as fixed effects and trees (replicates) as random effects, assuming a binomial distribution with a logit link. When the fixed effect was significant, differences between treatments were assessed by multiple comparisons of adjusted means using the DGC test ($p < 0.05$) as implemented in InfoStat. Relations between sprouting or flowering intensity and yield were analyzed with Pearson's correlation coefficient. Analyses were carried out using InfoStat statistical software version 2020 interfaced with R version 3.6.3.

3. Results

3.1 Self-Incompatibility, Pollen Tube Kinetics, and Ovule Senescence

Pollen used for pollination exhibited more than 85% of viability on average, without difference among genotypes (Table S1 in Supplementary material). The in vitro pollen germination of 'Afourer' (53 ± 2%), F4P7 (51 ± 6%), and F3P8 (54 ± 1%) was significantly higher than F2P3 (21 ± 2%). In all genotypes, the in vivo pollen germination was higher when pollinated with 'Afourer' than in self-pollination (Table 1). Overall, pollen viability and germination were high across all evaluated genotypes.

Table 1. Percentage of in vivo pollen germination of F4P7, F3P8, and F2P3 evaluated at 2 days after self-pollination and pollination with 'Afourer'

Seed parent	*Pollen parent*	*In vivo germination (%)*
F4P7	F4P7	48.2 ± 10.2 b
	'Afourer'	73.7 ± 9.5 a
F3P8	F3P8	59.5 ± 11.6 b
	'Afourer'	64.6 ± 11.9 a
F2P3	F2P3	48.9 ± 10.2 b
	'Afourer'	67.6 ± 8.9 a

Different letters indicate significant differences in the same seed parent genotype ($p < 0.05$, Tukey's test). Data are the means of 10 biological replicates ± standard deviation (SD).

In all genotypes, pollen tube growth and ovule senescence showed significant differences between treatments, showing interaction between treatment and sampling date (Figure 1). The 'Afourer' pollen tube reached the style-ovary junction of F4P7 and F3P8 at 12 DAP, whereas in F2P3 this occurred earlier, at 8 DAP, consistent with its higher growth rate (0.73 mm d^{-1}) than in F4P7 (0.61 mm d^{-1}) and in F3P8 (0.57 mm d^{-1}) ($p < 0.0001$). Furthermore, on these dates, 15 ± 4, 5 ± 1, and 10 ± 2 'Afourer' pollen tubes reached the style-ovary junction in F4P7, F3P8, and F2P3, respectively (Figure S1 in Supplementary material). In contrast, pollen tube growth of self-pollinated flowers did not exceed 50% of the style length in any genotype; consequently, no pollen tubes were reached at the style-ovary junction (Figure S1 in Supplementary material). On the other hand, a sharp increase in senescent ovules was found in self-pollinated flowers of F4P7 and F3P8 at 8 DAP, whereas in 'Afourer'-pollinated flowers it occurred at 12 DAP. In F2P3, this behavior was evident earlier, at 4 DAP, then gradually increased, regardless of the pollen genotype. On the whole, although all three genotypes exhibited high pollen viability and germination, their own pollen tubes were unable to reach the style-ovary junction, and the earliest ovule senescence was found to occur in F2P3.

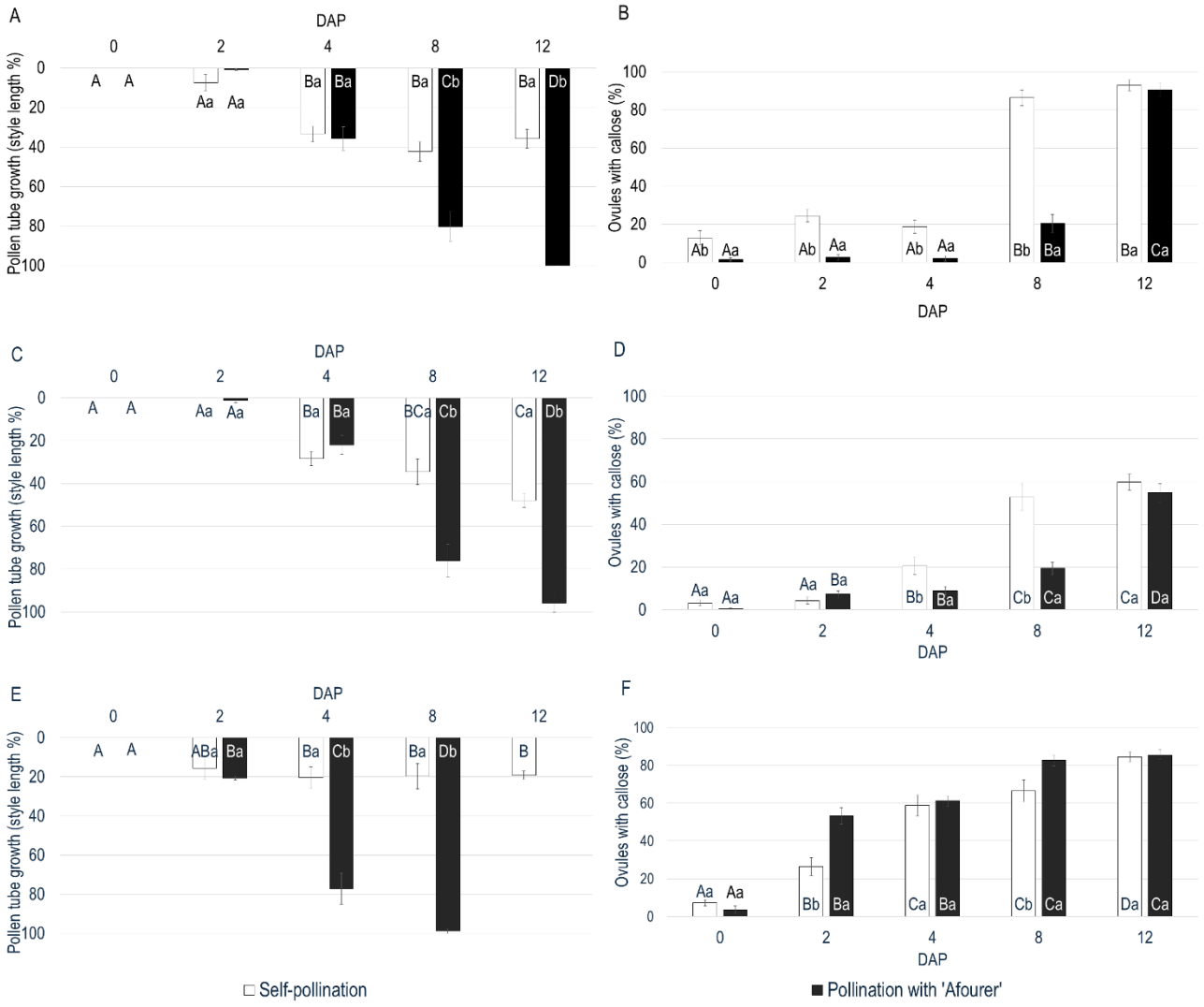

Figure 1. Pollen tube growth and ovule senescence at 0, 2, 4, 8, and 12 days after pollination (DAP) in self-pollinated (white columns) and 'Afourer' pollinated (black columns) flowers of F4P7 (A, B), F3P8 (C, D), and F2P3 (E, F) genotypes. In C, at 12 DAP, the style of all 'Afourer' pollinated ovaries had abscised

Different capital letters indicate significant differences between dates within each treatment, and different lowercase letters indicate significant differences between treatments within each date ($p < 0.05$, Tukey's test). Data are the means of ten biological replicates, and bars indicate the standard error.

3.2 Parthenocarpy Capacity

In all genotypes, a low fruit set was determined during the early abscission period when avoiding cross-pollination (Figure 2 and Figure S2 in Supplementary material). When isolated from cross-pollination, a lower GA_1 ovary concentration was observed in F2P3 at 0 DAA (Figure 3 C), and from 7 DAA onward in F4P7 and F3P8 (Figure 3 A, B).

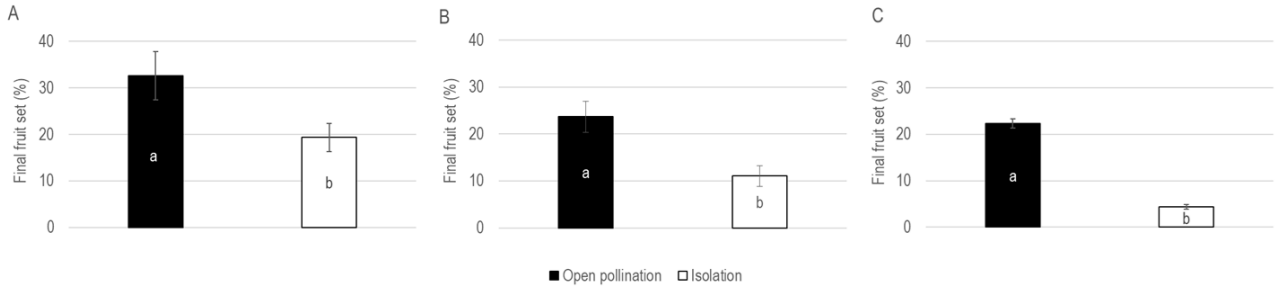

Figure 2. Fruit set (%) under open pollination (black columns) and isolation (white columns) at 40 days after anthesis in F4P7 (A), F3P8 (B), and F2P3 (C) genotypes, grafted onto 'Trifolia'

Different letters indicate significant differences in the same seed parent genotype ($p < 0.05$, DGC's test). Data are the means of 10 biological replicates, and bars indicate the standard error.

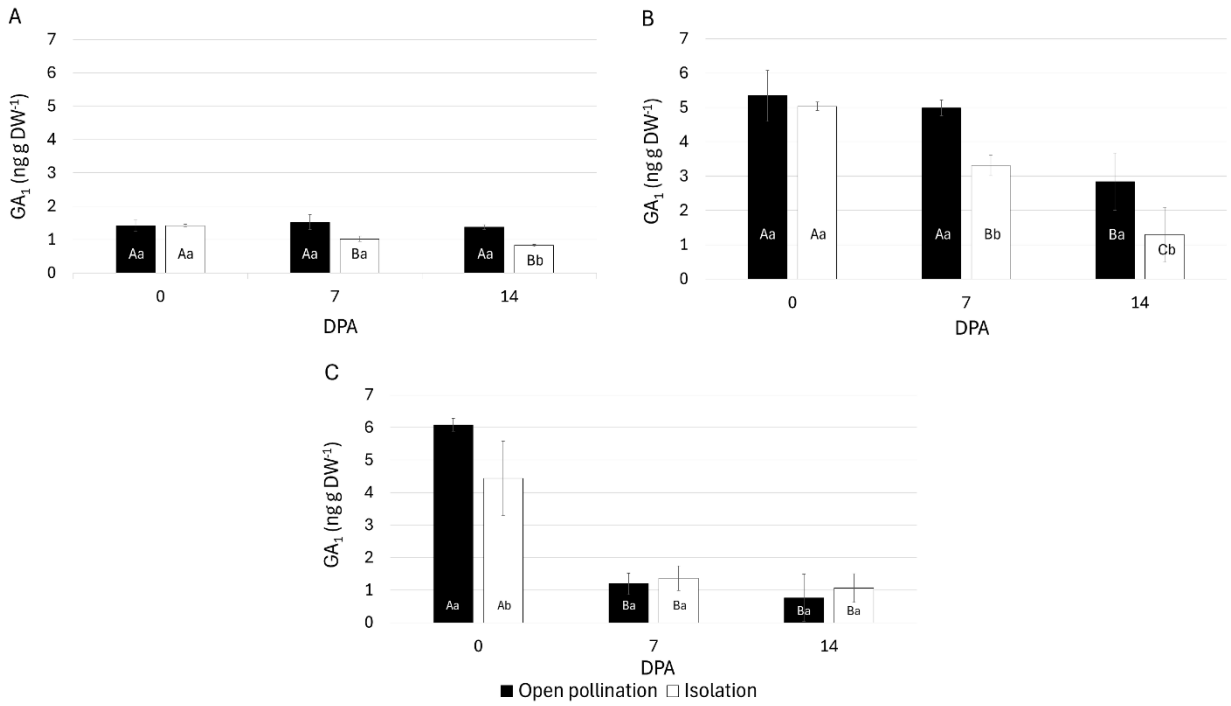

Figure 3. Evolution of ovary GA_1 concentration under open pollination (black columns) and isolation (white columns) treatments at 0, 7, and 14 days after anthesis (DAA), in F4P7 (A), F3P8 (B), and F2P3 (C) genotypes, grafted onto 'Trifolia'

Different capital letters indicate significant differences between dates within each treatment, and different lowercase letters indicate significant differences between treatments within each date ($p < 0.05$, Tukey's test). Data are the means of three biological replicates, and bars indicate the standard error.

Total soluble carbohydrate and sucrose concentrations in the ovaries increased during fruitlet development from 30 to 60 DAA in all genotypes under both, open-pollination (OP) and isolation (I) conditions; however, the magnitude of this increment differed among genotypes (Figure 4). In F4P7 and F2P3, total carbohydrate accumulation was consistently higher under OP than under I, with a sharp increase at 60 DAA, driven mainly by sucrose (Figure 4 A, C). A similar pattern was observed in F3P8, although overall concentrations were lower (Figure 4 B). Collectively, the results indicate that sucrose was the predominant carbohydrate across all genotypes. Moreover, OP consistently resulted in higher carbohydrate accumulation compared to I conditions, particularly at 60 DAA.

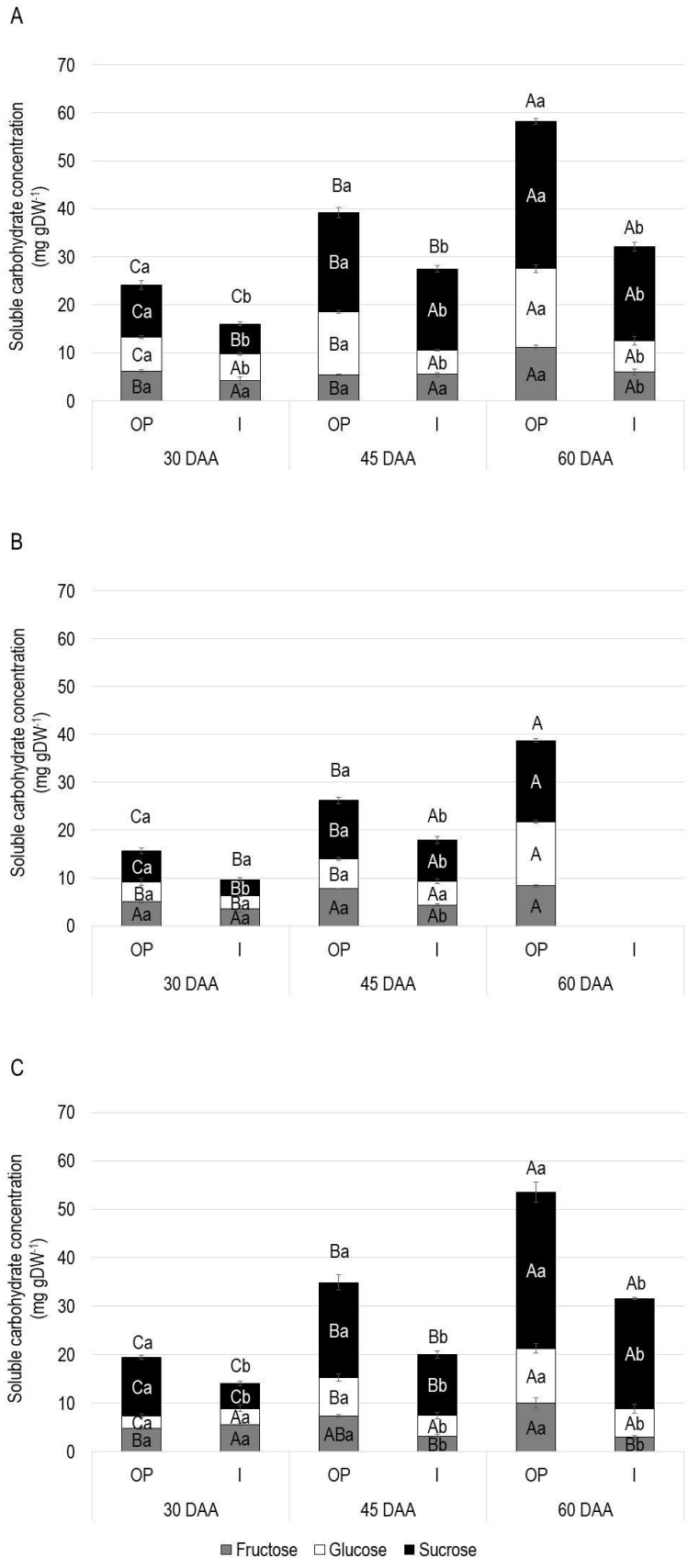

Figure 4. Concentration of soluble carbohydrate in the ovary under isolation (I) and open pollination (OP) conditions at 30, 45, and 60 days after anthesis (DAA), in F4P7 (A), F3P8 (B), and F2P3 (C) genotypes, grafted onto 'Trifolia'. In B, all flowers under isolation had abscised at 60 DAA

For fructose, glucose, sucrose, and their sum, different capital letters indicate significant differences between dates within each treatment, and lowercase letters indicate significant differences between treatments within each date ($p < 0.05$, Tukey's test). Data are the means of three biological replicates, and bars indicate the standard error.

Seeded fruits were null under isolation (0 ± 0%), while reaching 96 ± 5%, 89 ± 2%, and 96 ± 6% in fruits from open-pollination treatment of F4P7, F3P8, and F2P3 genotypes, respectively. Seed number per fruit achieved 3.7 ± 0.6, 3.3 ± 0.8, and 4.4 ± 1.1 under open pollination in F4P7, F3P8 and F2P3, respectively.

3.3 Type of Parthenocarpy

In all three genotypes, the fruit set of emasculated and self-pollinated flowers was significantly lower than that of the flowers pollinated with 'Afourer' throughout the fruit set period (Figure S3 in Supplementary material). In F4P7, the fruit set of self-pollination and emasculation treatments was similar at the end of the physiological drop, reaching 1.1 ± 0.9% and 1.1 ± 1.0%, respectively; however, both were significantly lower than in flowers pollinated with 'Afourer' (73.3 ± 3.9%). Similarly, in F3P8 and F2P3, no significant differences were detected between self-pollination and emasculation treatments at the end of the physiological drop (1.0 ± 1.0% in both genotypes and treatments), and 'Afourer'-pollination treatment resulted in a significantly higher final fruit set (43.0 ± 7.5% in F3P8 and 44.3 ± 4.6% in F2P3). On the other hand, pollination with 'Afourer' set seeds in all fruits, while fruits from the emasculation and self-pollination treatments were seedless (Table S2 in Supplementary material). Overall, across the three genotypes, flowers pollinated with 'Afourer' showed higher fruit set and produced seeded fruits, and conversely, self-pollination and emasculation resulted in lower fruit set and seedless fruits.

Pollination treatments affected the evolution of GA_1 concentration in the ovaries (Figure 5). In emasculated and self-pollinated flowers, GA_1 concentration was significantly higher at 0 DAA and then declined, with no differences at 7 and 14 DAA. However, in the ovaries of flowers pollinated with 'Afourer', GA_1 concentration remained high until 7 DAA and declined thereafter.

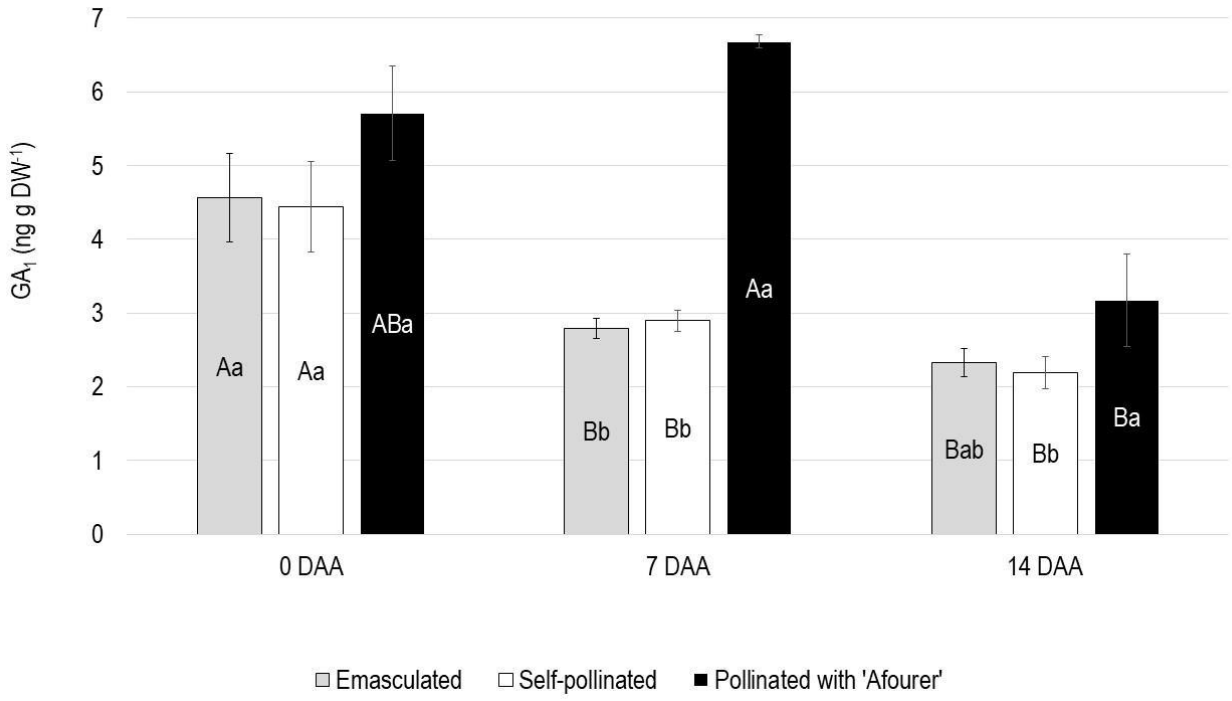

Figure 5. Concentration of GA_1 in ovaries of emasculated (grey columns), self-pollinated (white columns), and pollinated with 'Afourer' pollen (black columns) flowers at 0, 7, and 14 days after anthesis (DAA), in F3P8 grafted onto 'Trifolia'

Different capital letters indicate significant differences between dates within each treatment, and different lowercase letters indicate significant differences between treatments within each date ($p < 0.05$, Tukey's test). Data are the means of 10 biological replicates, and bars indicate the standard error.

3.4 Field Performance under Open Pollination

Under open-pollination conditions, with 89-90% seeded fruits, sprouting, flowering, and yield varied across genotypes and years (Figure 6). All genotypes presented high yields in 2020, followed by declines in sprouting and flowering intensity and a subsequent reduced yield in 2021. This was followed by increases in both sprouting variables, resulting in higher yields again in 2022 (Figure 6). Sprouting and flowering intensity were correlated with the subsequent yield in F4P7 (r: 0.89 and 0.74) and F3P8 (r: 0.79 and 0.71), whereas no significant correlations were observed in F2P3 (r: 0.42 and 0.45). Despite similar temporal patterns among genotypes, differences in ABI were evident: F3P8 showed the highest value (0.40), followed by F4P7 (0.34) and F2P3 (0.21). Overall, although sprouting, flowering, and yield varied according to genotype and year, under open-pollination conditions, all genotypes exhibited a low ABI.

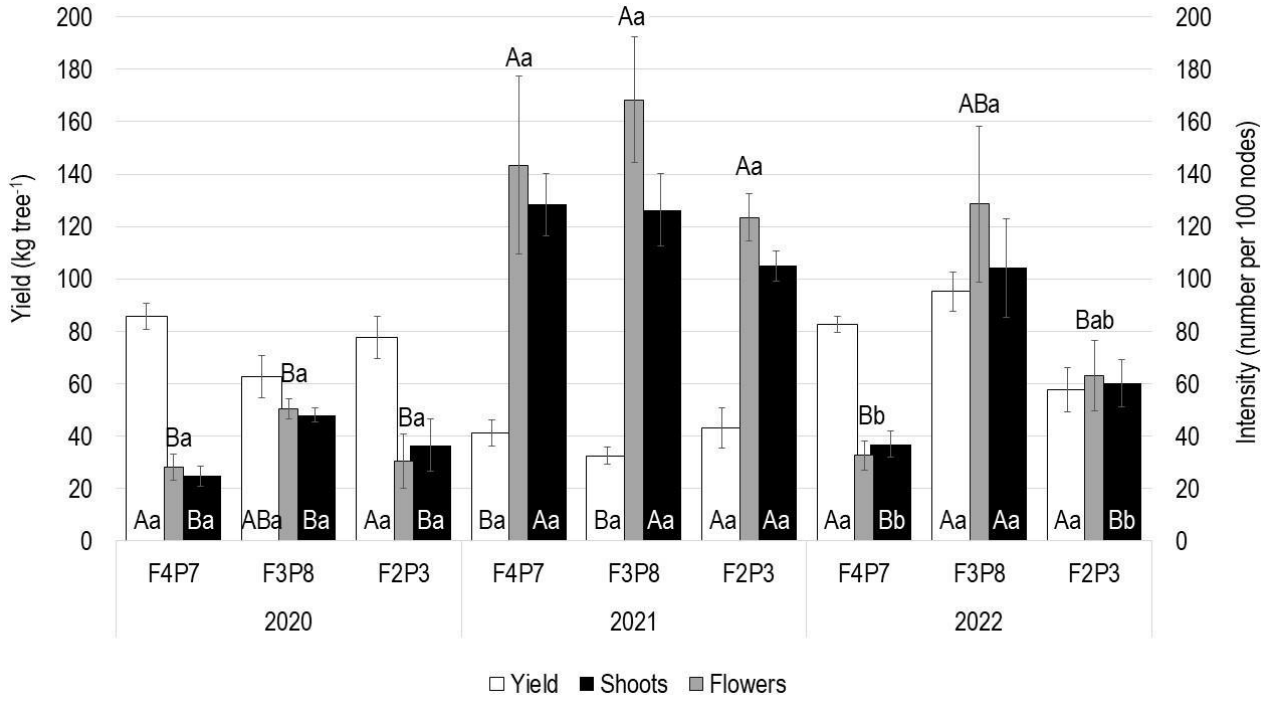

Figure 6. Yield (kg tree^{-1}) (white columns), flowering intensity (flowers per 100 nodes) (gray columns), and sprouting intensity (shoots per 100 nodes) (black columns) in the F4P7, F3P8, and F2P3 genotypes grafted onto 'Trifolia', in three consecutive years

For each variable, different capital letters indicate significant differences between years within each genotype, and different lowercase letters indicate significant differences between genotypes within each year ($p < 0.05$, Tukey's test). Data are the means of five biological replicates, and bars indicate the standard error.

Single-flowered leafless shoots were the most abundant across genotypes and flowering intensities, except for F2P3 in 2020, which exhibited a higher frequency of vegetative shoots (41%) and leafy reproductive shoots (46%) (Figure 7). It is interesting to note that F2P3's shoot-type distribution, especially single-flowered leafless shoots, changed according to the flowering intensity, more than in the other genotypes. Indeed, in F2P3, 11 ± 2% of single-flowered leafless shoots were recorded, with 31 ± 23 flowers per 100 nodes and 82 ± 2% of this shoot type, with 123 ± 20 flowers per 100 nodes (Figure 6 and Figure 7). As shown, single-flowered leafless shoots predominated across genotypes irrespective of flowering intensity, except for F2P3, which exhibited a shoot-type distribution that markedly varied with flowering intensity.

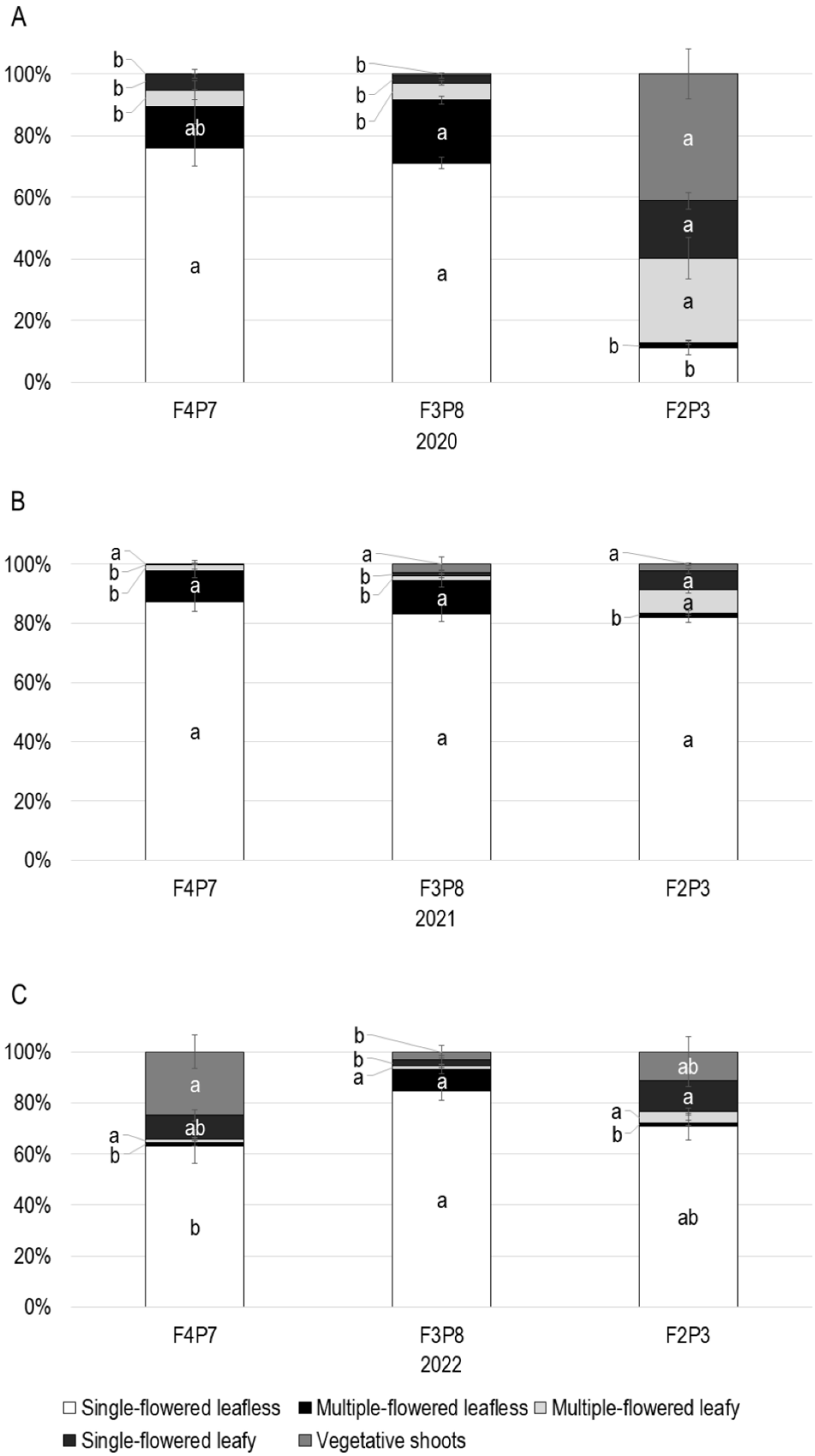

Figure 7. Shoot-type distribution (%): vegetative shoots, single-flowered leafy shoots, multiple-flowered leafy shoots, single-flowered leafless shoots, and multiple-flowered leafless shoots, in F4P7, F3P8, and F2P3 genotypes grafted onto 'Trifolia', evaluated in 2020 (A), 2021 (B), and 2022 (C)

Different letters indicate significant differences between genotypes within each shoot type ($p < 0.05$, Tukey's test). Data are the means of five biological replicates, and bars indicate the standard error.

4. Discussion

Citrus is the most extensively cultivated fruit crop worldwide, exhibiting a consistently increasing trend over recent decades (FAO, n.d.). Additionally, there is an increasing demand for mandarins due to their fresh, convenient, easy-to-peel, and their health benefits features (Saini et al., 2022). The presence of high-quality seedless fruit is a significantly important trait in the global citrus fresh market. Consequently, the absence of seeds is a pivotal characteristic in the context of Citrus Breeding Programs. In recent years, three new mandarin hybrids, coded as F4P7, F3P8, and F2P3, have been developed in Uruguay, and released worldwide due to their excellent organoleptic characteristics and great potential for the global mandarin exportation. However, several aspects of their reproductive biology have not yet been studied, indicating a knowledge gap that must be addressed to overcome productivity limitations.

SI is common in most woody crops, including the genus *Citrus* (Liang et al., 2020; Zhang et al., 2018). In our work, despite their high gamete viability, seedless F4P7, F3P8, and F2P3 mandarins were obtained under self-pollination and isolation treatments. Furthermore, self-pollen tubes did not exceed 50% of the style length (Figure 1 A, C, E), indicating gametophytic self-incompatibility in these three genotypes, as reported for other *Citrus* cultivars (Gambetta et al., 2013; Kahn & DeMason, 1988; Zhang et al., 2018). Additionally, in F2P3, the incompatibility reaction occurred in a previous section of the gynoecium compared to F4P7 and F3P8, suggesting that the kinetics of pollen tube growth in self-pollinated treatments depended on the genotype, as demonstrated in other *Citrus* (de Nettancourt, 1997; Soost, 1965). On the other hand, it has been demonstrated that incompatibility reaction is affected by temperature, reporting its inhibition in conditions of 10-15 ºC (Aloisi et al., 2020; Montalt et al., 2022). However, in our experiment, the growth arrest of incompatible pollen tubes occurred under field conditions with a daily mean temperature greater than 15 ºC and a daily minimum close to 9 ºC (Figure S4 in Supplementary material).

Regarding seed formation, our results indicated that the newly studied genotypes presented high gamete viability (Table 1, Figure 1) and thus the ability to set seeds when compatible genotypes were growing nearby. To prevent seeded fruit production, the distance to compatible varieties should be considered, since bees can carry citrus pollen up to 960 m (Chao et al., 2005). On the other hand, the effects of pollen-pistil interactions on pollen tube growth, fertilization success, and seed set have been reported in many *Citrus* (Distefano et al., 2012; Montalt et al., 2019). In F4P7 and F3P8, 'Afourer' pollen tubes required more time to reach the style-ovary junction than in F2P3, possibly due to higher air temperature during pollen tube growth in F2P3 (Figure S4 in Supplementary material). High temperatures increase the rate of pollen tube growth, reducing the time to reach the ovules (Irenaeus & Mitra, 2014; Montalt et al., 2019). Moreover, the pollinated flowers of F4P7 and F3P8 have a low proportion of ovules with callose, an early indicator of ovule senescence (Mesejo et al., 2007), until 8 DAP (Figure 1 B, D, F). This suggests that these genotypes lack the brief ovule viability period required to minimize seed content, a mechanism previously described for *C. unshiu* (Mesejo et al., 2007). However, in all genotypes, 'Afourer' pollen tubes reached the style-ovary junction when the ovule senescence was high (Figure 1 A, C, E), which allows us to explain the lower number of seeds obtained in these genotypes (3.3 to 4.4 seeds per fruit), considering that the mandarin ovary has more than 32 ovules (Tadeo et al., 2003). Furthermore, these results agree with the previous report (Rivas & Vignale, 2015), who highlighted the low seed number determined in these genotypes under open pollination, despite the high proportion of seeded fruit found in this work.

In terms of the type of parthenocarpy, F4P7, F3P8, and F2P3 genotypes presented facultative parthenocarpy, since they produced seedless fruit under isolation conditions, but they set seeded fruits under open-pollination conditions, according to the definition proposed by Vardi et al. (2008). On the other hand, all genotypes exhibited autonomous parthenocarpy, as self-pollination treatment did not stimulate seedless fruit set compared with emasculation treatment (non-pollinated flowers), supporting the presence of autonomous parthenocarpy in these

three genotypes, as reported in other *Citrus* cultivars (Gambetta et al., 2013; Mesejo et al., 2013). Nevertheless, fruit set was drastically reduced when flowers were emasculated, self-pollinated, or isolated from cross-pollination (Figure 2), conditions that prevented fertilization. These results demonstrate that these genotypes have low parthenocarpic capacity, consistent with reports for their parental lines 'Ellendale' and 'Page' (Gravina et al., 1997), and in agreement with previous studies indicating that parthenocarpy is genetically determined (Sykes, 2008).

The fruit set process is hormonally and nutritionally regulated, with active GAs associated with the regulation of the first wave of abscission (Agustí & Primo-Millo, 2020). According to our findings, these low parthenocarpic capacity genotypes could not maintain GA_1 ovary concentrations during the first week after anthesis (Figure 3), as we have previously found (Gambetta et al., 2026). The lack of autonomous GAs biosynthesis at anthesis limits cell division and, thus, the ovary-to-fruit transition process (Mesejo et al., 2016). Furthermore, autonomous parthenocarpy is uncoupled from pollination, and self-pollination does not trigger this process (Mesejo et al., 2013). Our results indicated that self-pollination did not enhance GA_1 concentration nor fruit set in these self-incompatible genotypes. In contrast, fertilized ovules (open pollination and 'Afourer' pollination treatments) maintained the GA_1 levels during the first week after anthesis, and reached higher fruit set than unfertilized ovules (isolation, emasculation, and self-pollination treatments) (Figure 5), as observed in non-parthenocarpic genotypes where GAs synthesis and ovary growth depend on fertilization (Ben-Cheikh et al., 1997; Bermejo et al., 2016).

Regarding ovary carbohydrate availability during the second wave of abscission period, in our experiment fruitlets from open pollination (seeded fruits) had higher total soluble carbohydrates and sucrose levels than parthenocarpic fruitlets (isolation conditions), associated with higher fruit set (Figure 4). The carbohydrate role in citrus fruit set is supported by a wide body of evidence (Goldschmidt, 1999; Iglesias et al., 2003; Mahouachi et al., 2009). In our work, preventing cross-pollination for seedless fruit production reduced fruit set due to a decline in GA_1 during the early stage of phase I of fruit development, and a limited increase in soluble carbohydrates later in this phase, as found in other citrus cultivars (Ben-Cheikh et al., 1997; Bermejo et al., 2015). Therefore, agronomical practices to stimulate initial fruit set and reduce physiological fruit drop should be considered to reach high yields of seedless fruits when cross-pollination is avoided (Agustí & Primo-Millo, 2020).

In the open-pollination field performance study, other yield-limiting factors were identified in these new genotypes. All of them exhibited medium to high flowering intensities, which varied according to the year (Figure 6), and the yield ranged from low (less than 45 kg tree^{-1}) to high (more than 75 kg tree^{-1}). In F4P7 and F3P8, although a positive correlation between flowering and subsequent yield suggests light alternate bearing, both genotypes showed low ABI values (less than 0.5). ABI value greater than 0.5 corresponds to an alternate bearing, while an index below 0.5 indicates a regular bearing (Bassal, 2009). In F2P3, the very low ABI value (less than 0.25) and the lack of correlation between flowering intensity and yield indicate stable performance. This finding suggests the need for specific horticultural management to regulate flowering intensity and yield even under open-pollination conditions. Moreover, our results indicate that in F2P3, generative shoots were abundant only under high flowering intensity, while in F4P7 and F3P8 the generative shoots were the most abundant, irrespective of the flowering level, which could be indicative of a varietal characteristic (Agustí et al., 2022). This feature corresponds with 'Ellendale', one of their parents, which presents very high flowering intensities with a predominance of leafless inflorescences (Gravina et al., 1997). The results on shoot-type abundance may suggest a genotype-specific response to agronomic management practices, as their effectiveness depends on the level of flowering (Gravina et al., 1997, 1998).

5. Conclusions

The newly promising mandarin hybrids coded as F4P7 (early season), F3P8 (middle season), and F2P3 (late season) presented gametophytic self-incompatibility and facultative, autonomous, low-parthenocarpic capacity. All genotypes presented viable pollen and ovules, indicating their ability to both act as pollen donors and undergo successful fertilization. In the absence of cross-pollination, the limiting fruit set capacity of the three genotypes is likely explained by their inability to maintain GA_1 concentration in the ovary and the reduced capacity to increase soluble carbohydrates in fruitlets. Under open pollination conditions, a high proportion of seeded fruits were found, although the number of seeds per fruit was low. In this condition, cross-pollination must be prevented to produce seedless fruits, and appropriate practices to stimulate fruit set should be implemented. Regarding implications for the breeding program, the irradiation method could be used to obtain materials with obligate parthenocarpy, or these genotypes could be used as male parents. However, their degree of polyembryony should be evaluated before their use as female parents. Despite the excellent characteristics of these genotypes, this work provides evidence of their reproductive biology and physiological limitations, bringing forward that agronomic management is needed to produce high and constant yields. Lower gibberellin and soluble carbohydrate levels in the ovary may indicate that these genotypes could respond to spring applications of gibberellic acid and girdling, thereby representing relevant avenues for future study.

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Transparency of Data

Data not available: The data set that supports the results of this study is not publicly available, but it is available from the corresponding author upon request.

Author Contribution Statement

	N Guimaraes	A Mautone	A Gravina	F Rivas	G Gambetta
Conceptualization	■		■	■	■
Data curation	■				
Formal analysis	■				
Funding acquisition	■		■	■	■
Investigation	■	■			
Methodology	■		■	■	■
Supervision				■	■
Visualization	■				
Writing – original draft	■			■	■
Writing – review and editing	■	■	■	■	■

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Supplementary Material

Table S1. Viability of pollen from 'Afourer', F4P7, F3P8, and F2P3, evaluated with 1% acetocarmine, kept 30 min at 20.9 ± 0.6 ºC

Genotype	Pollen viability (%)
'Afourer'	87.0 ± 1.8 a
F4P7	87.9 ± 1.4 a
F3P8	86.2 ± 1.4 a
F2P3	85.9 ± 1.8 a

Different letters indicate significant differences ($p < 0.05$, Tukey's test). Data are the means of 10 biological replicates ± standard deviation (SD).

Table S2. Seeds' presence in fruits from emasculated, self-pollinated, or 'Afourer' pollinated flowers of F4P7, F3P8, and F2P3 genotypes, grafted onto 'Trifolia'

Treatment	*Genotype*					
	F4P7		F3P8		F2P3	
	Seeded fruit (%)	Seed number per fruit	Seeded fruit (%)	Seed number per fruit	Seeded fruit (%)	Seed number per fruit
Emasculation	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Self-pollination	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Pollination with 'Afourer'	100 ± 0	16 ± 4	100 ± 0	15 ± 4	100 ± 0	20 ± 6

Data are the means of 10 biological replicates ± standard deviation (SD).

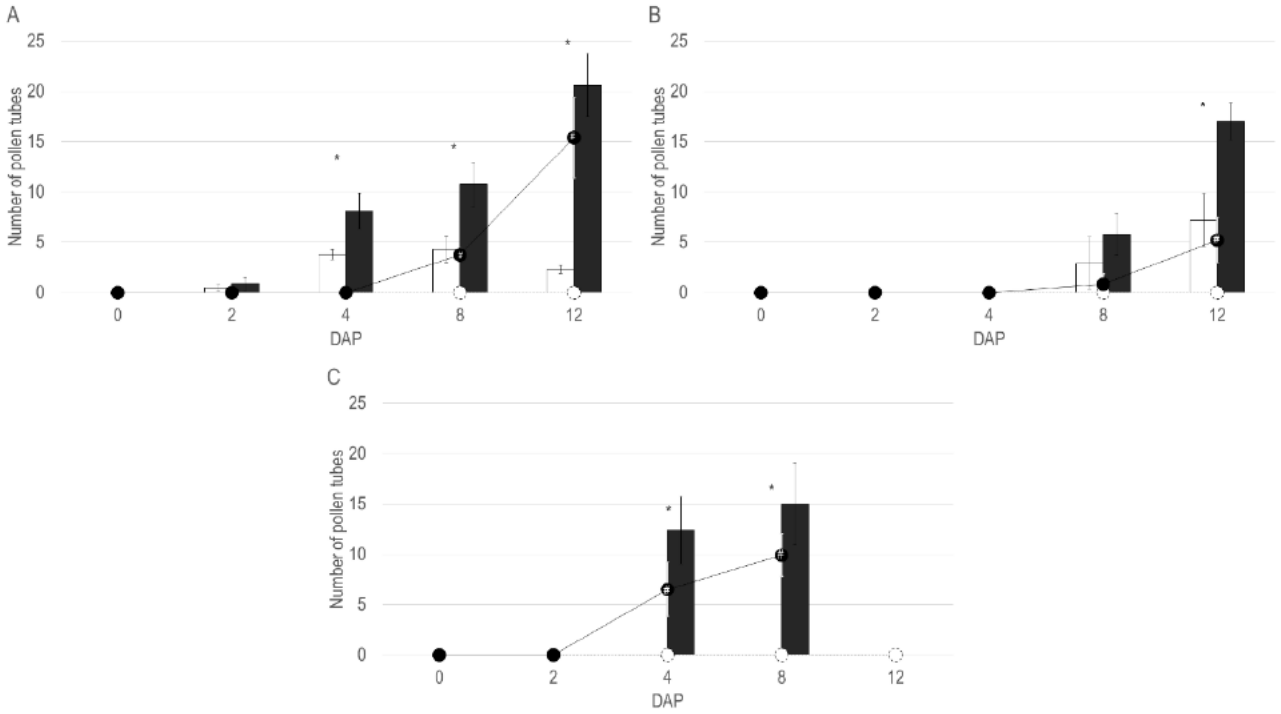

Figure S1. Number of pollen tubes that reached the middle (columns) and style-ovary junction (circles) at 0, 2, 4, 8, and 12 days after pollination (DAP) in self-pollinated flowers (white) and pollinated with 'Afourer' (black), in F4P7 (A), F3P8 (B), and F2P3 (C) genotypes

* Indicate significant differences between treatments in the number of pollen tubes that reached the middle of the style ($p < 0.05$, Tukey's test). # Indicate significant differences between treatments in the number of pollen tubes that reached the style-ovary junction ($p < 0.05$, Tukey's test). Data are the means of 10 biological replicates, and bars indicate the standard error.

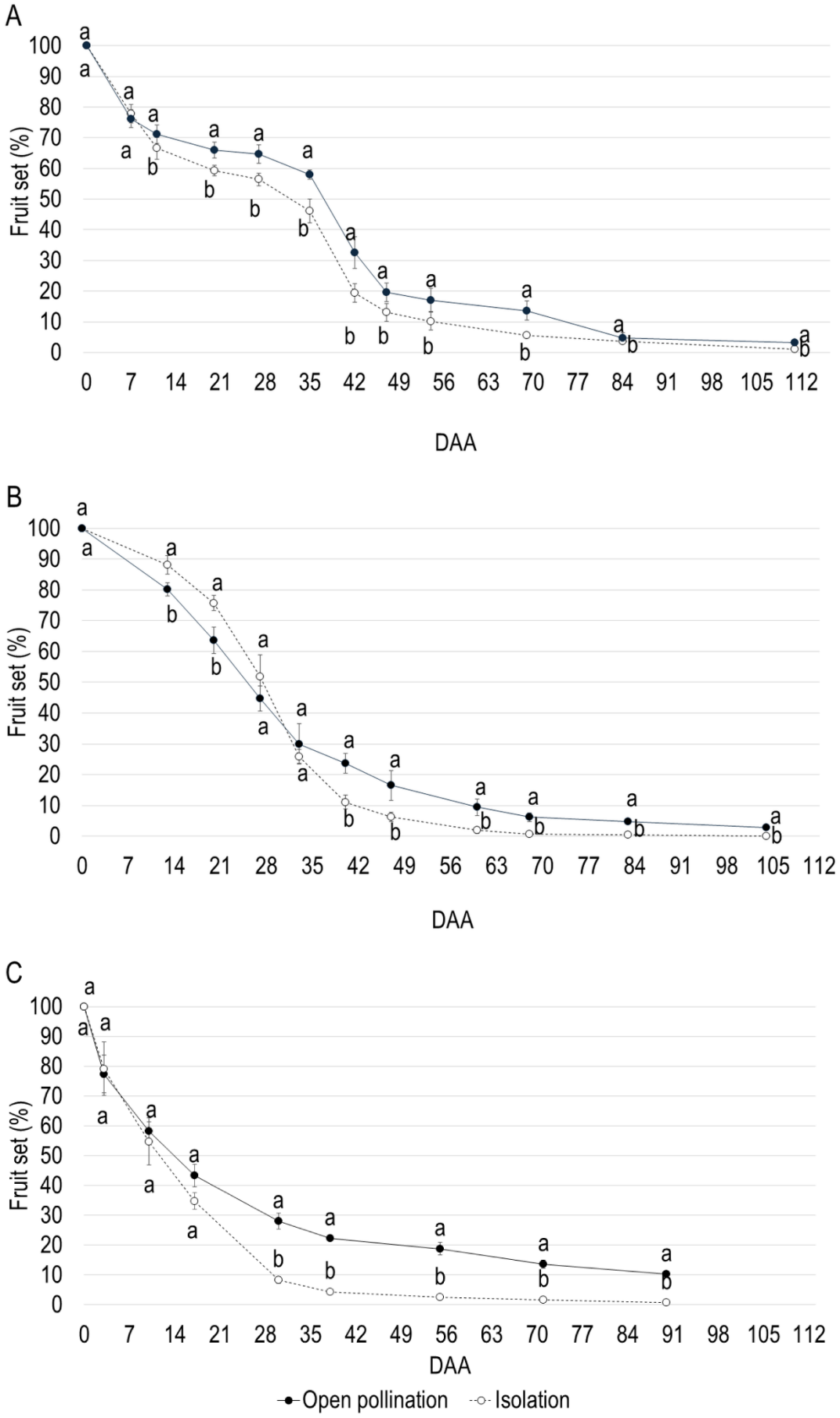

Figure S2. Fruit set (%) in isolation (dashed line) and open pollination (solid line) treatments during the physiological drop (days after anthesis, DAA) in F4P7 (A), F3P8 (B), and F2P3 (C) genotypes, grafted onto 'Trifolia'

Different letters indicate significant differences between treatments ($p < 0.05$, DGC's test). Data are the means of 10 biological replicates, and bars indicate the standard error.

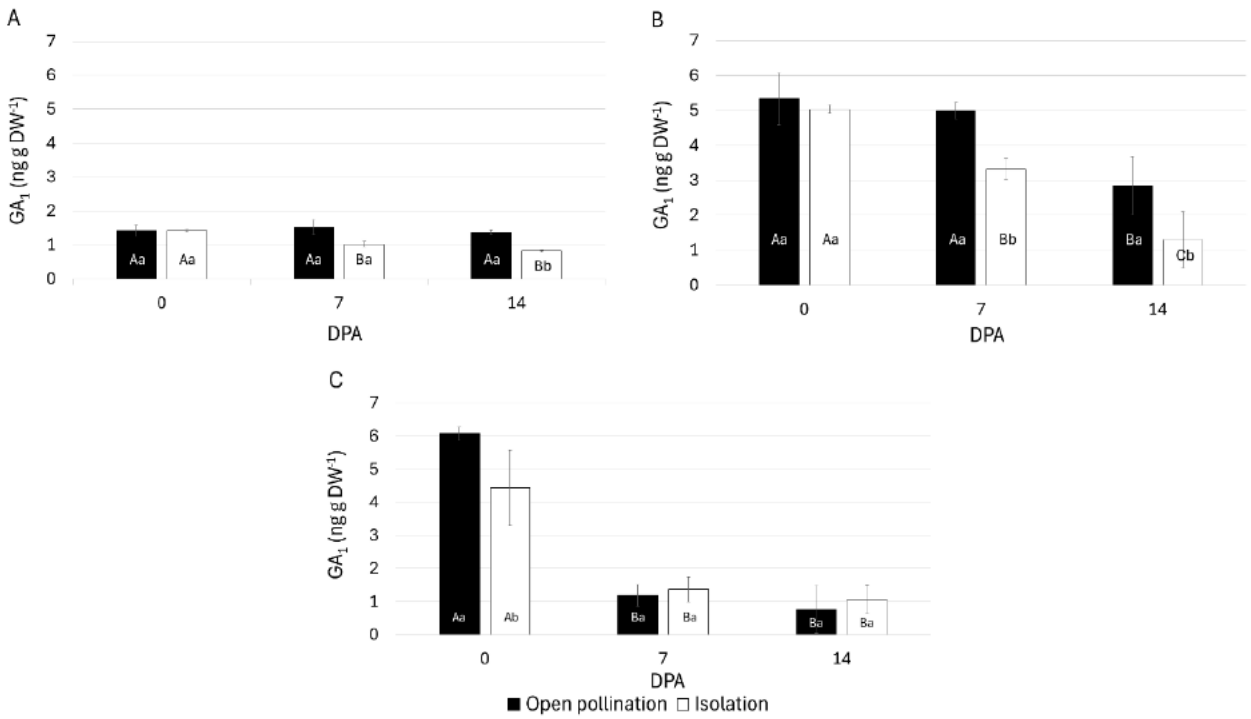

Figure S3. Evolution of the fruit set percentage of emasculation (dashed line), self-pollination (dotted line), and pollination with 'Afourer' (solid line) treatments during the physiological drop (days after anthesis, DAA) in F4P7 (A), F3P8 (B), and F2P3 (C) genotypes grafted onto 'Trifolia'

Different letters indicate significant differences between treatments ($p < 0.05$, DGC's test). Data are the means of 10 biological replicates, and bars indicate the standard error.

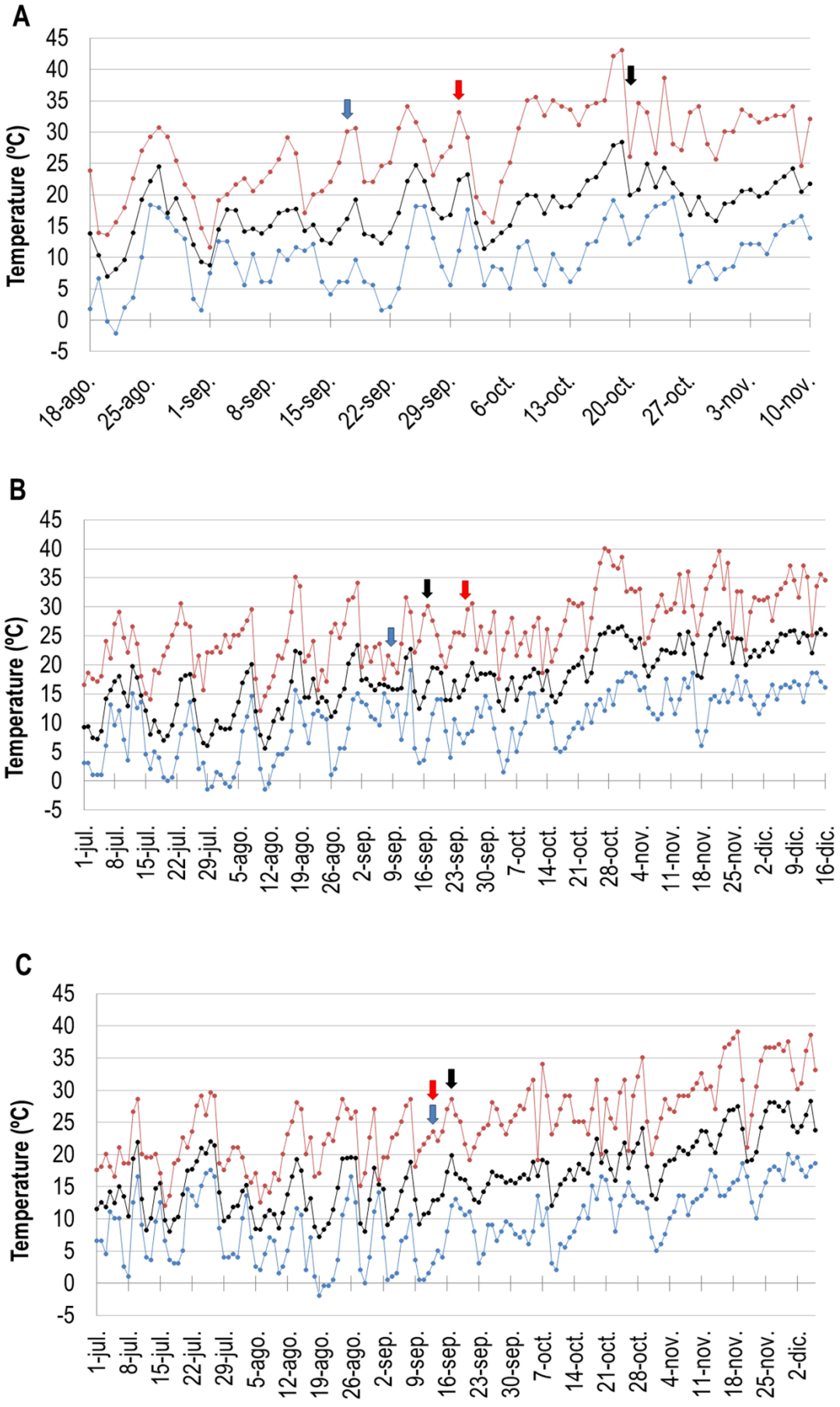

Figure S4. Minimum (blue), mean (black), and maximum (red) daily air temperature (°C) during the evaluation period, recorded in 2020 (A), 2021 (B), and 2022 (C)

Arrows indicate the full boom date in F4P7 (blue arrows), F3P8 (red arrows), and F2P3 (black arrows) genotypes. Data are daily means calculated from hourly air temperatures (24 readings per day).