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HYBRID SEED SUCCESS OF *COFFEA CANEPHORA* X *C. ARABICA* INTERSPECIFIC HETEROPLOID CROSSING DIRECTION

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SUMMARY

Coffea canephora × *C. arabica*-crossing direction has shown to have a complete post-zygotic barrier. The study sought to unravel the degree of seed failure of paternal excess interspecific hybridization of *C. canephora* × *C. arabica*. The present research was conducted at the Pakuwon Experimental Station, Indonesian Industrial and Beverage Crops Research Institute (IBCRI), from August 2019 until March 2022. The *C. canephora* "Sidodadi" hand pollination used freshly collected pollen from *C. arabica* "Mangening" and *C. arabica* "AGK," as well as from *C. canephora* "Kriting" and *C. liberica*. The fruit set was observed three months after anthesis (MAP) and six MAP. Cherry fruit and seed morphometric traits were measured at harvest time, with the number of seeds with collapsed endosperm (empty seed) also recorded. The surviving healthy seeds subsequently were sown to observe germination percentage. The number of leaf pairs on developing seedlings was recorded at a 2-month interval. Results showed that paternal excess had generated larger fruit and seeds but mostly contained collapsed endosperm. Few healthy developed seeds could germinate and subsequently develop into seedlings that are more vigorous. Interestingly, the interspecific homoploid crossing of *C. canephora* × *C. liberica* also exhibited a robust post-zygotic barrier.

Keywords: *Coffea,* interploidy, parent-of-origin, paternal excess, reproductive isolation

Key findings: These findings could become preliminary information related to the early-acting post-zygotic reproductive barrier between diploid maternal of *C. canephora* and tetraploid paternal of *C. arabica*. The low frequency of hybrid seeds succeeded to germinate and develop into normal seedlings. These novel F1 hybrids could potentially be integrated in future coffee breeding programs.

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INTRODUCTION

Populations of *Arabica* coffee species (*C. arabica*) often have mixed with (sympatry) or adjacent to (parapatry) populations of its diploid relatives, especially Robusta and Liberica coffee (*C. canephora* and *C. liberica*). Such conditions have become potential zones for the emergence of interspecific hybrids (Gomez *et al.*, 2016). Several putative spontaneous hybrids of these three coffee species have been reported elsewhere, including Hybrido de Timor (HdT), the most popular and utilized genotype in Arabica coffee breeding programs (Setotaw *et al.*, 2020).

A single plant of first-generation HdT was among the population of Arabica coffee in Timor Island, Indonesia. The first generation of HdT identification as allotriploid cytotype (2*n*=3*x*=33) resulted from karyological and cytometric analysis. However, some accessions generated from this allotriploid HdT have a higher 2C value and are more similar to *C. arabica* (Clarindo *et al*., 2013). The Indonesian Government introduced some lines derived from HdT and subsequently developed by local farmers with the local name of Arabusta Tim-Tim (Hulupi *et al*., 2013). These HdT-based cultivars have become highly resistant to leaf rust disease (*Hemileia vastatrix*) and nematode (*Meloidogyne exigua*) (Bertrand *et al*., 2003).

Interestingly, Indonesia produces more Robusta coffee instead of Arabica. Consequently, the total area of Robusta coffee production was significantly larger. In some regions, few Arabica coffee plants remained to grow among Robusta coffee populations (personal observation). This interspecific contact zone might be a potential source of novel interspecific hybrid between *C. canephora* and *C. arabica* (Gomez *et al*., 2016). However, no reports of recovered interspecific hybrids from *C. canephora* populations exist.

Even though the interspecific hybridization between *C. arabica* and its diploid relatives can produce fertile hybrids, accounting for the direction of the cross is necessary in artificial hybridization. Successful production of triploid hybrids resulted when positioning the tetraploid *C. arabica* as the female parent (4*x* × 2*x*), whereas reciprocal crosses (2*x* × 4*x*) so far, have never been successful (Herrera *et al.*, 2002). However, in several other plant species, the paternal excess crosses (2*x* × 4*x*) had been able to produce several F1 hybrid seeds that survived and developed into regular plants. Cao *et al.* (2002) reported that tetraploid pears could produce pollen with 1*x* and 2*x* genomes. The progeny resulting from the paternal excess crosses are mostly diploid. Meanwhile, triploid offspring production occurs at a minimum frequency. However, research on the influence of male parents with higher ploidy (paternal excess) on the success of interspecies hybridization between *C. canephora* and *C. arabica* is still insufficient.

In many cases, it is a fact that *paternal excess* can harm the endosperm cellularization process and, ultimately, failure of seed formation (Lafon-Placette and Köhler, 2016). However, in the model plant of *Arabidopsis thaliana*, this paternal effect had conclusive variation among maternal × paternal genotype combinations (House *et al*., 2010). Consequently, there is still the possibility to gain success of paternal excess cross combinations. This research aims to determine the success rate of seed formation due to interspecific hybridization with *paternal excess* between the diploid *C. canephora* and tetraploid *C. arabica* species. The hypothesis to consider is that there is a strong but not complete post-zygotic barrier to the success of the *C. canephora* × *C. arabica* crossing direction.

MATERIALS AND METHODS

Plant material

Three species of *Coffea*, namely, *C. arabica*, *C. canephora,* and *C. liberica*, began their parapatric growing from 2012 to 2014 at the Pakuwon Experimental Station (6°50’00.8”S 106°44’31.5”E). *C. arabica* species comprises two distinct cultivars– ‘Mangening’ and ’AGK.’ The Mangening cultivar has a dark purple leaf and young fruit, where its epicarp color would turn to dark red at the mature-ripe stage.

"AGK" cultivar has a dark green leaf and young fruits. The berries will turn yellow before harvest.

Interspecific hybridizations

Hand pollination of some *C. canephora* 'Sidodadi' (C1) clonal trees used freshly collected pollen from *C. arabica* 'Mangening' (A1) and *C. arabica* 'AGK' (A2), respectively, to study the paternal excess effect on fruit and seed size of heteroploid interspecific crossing between diploid *C. canephora* and tetraploid *C. arabica*. Cross combinations of homoploid-intraspecific *C. canephora* Sidodadi (C1) × *C. canephora* 'Kriting' (C2) and homoploid-interspecific *C. canephora* Sidodadi (C1) × *C. liberica* 'CLvL07' (L1) were used for comparison. Simplified recording had the cross combinations designated as C1 × A1, C1 × A2, C1 × C2, and C1 × L1. Controlled pollination commenced during the co-anthesis of those three *Coffea* species in August 2020 following the first rain in late July. Intra- and interspecific co-anthesis had made it possible to transfer immediately freshly prepared paternal pollen into the stigma of the maternal parent.

The emasculation of flowering branches of sample plants and their bagging one day before the anthesis (during the candle stage) helped minimize potential pollen contamination. Hand pollination using fresh pollen from A1 and A2 started early on the day of anthesis, from 08:00 am until 10:00 am, on seven and 15 flowering branches C1, respectively. Moreover, each of the 10 C1 flowering branches bore hand-pollination using fresh pollen from C2 and L1. Pollinated flowers' immediate bagging used fine nylon mesh, and their tagging followed the respective cross combinations. Two weeks after pollination proceeded to the bags' removal. Counting each cross combination fruit setting started three months after pollination (MAP) and six MAP, respectively.

Morphometric measurements

Mature cherry fruit handpicking occurred at seven MAP when the epicarp color began to turn red or yellow (for *C. arabica* AGK). Morphometric measurement of cherry fruit continued immediately after harvesting. The measurement of the polar diameter, equatorial diameter, and thickness of cherry fruits used a digital caliper.

The immediate processing of harvested cherries had seeds separated from the fruit epicarp. Afterward, the seeds soaked in tap water ran for 24 hours, and then washing in flowing tap water freed the seed's mucilage. Finally, the seeds underwent wind drying overnight. After counting the number of surviving seeds, their calculation of the previously mentioned morphometric variables for survived seeds transpired.

Endosperm failure proportion

Coffee seeds consist of endocarp or parchment as the outer layer, covering the seed endosperm and embryo, wrapped by a thin layer of silver skin (Eira *et al.*, 2006). Therefore, to study the endosperm failure requires removing the parchment. Abnormal seed endosperm visual differentiation can be distinct from the rest of the normal endosperm.

Table 1. Interspecific heteroploid, intraspecific heteroploid, and interspecific homoploid cross combinations between *Coffea canephora*, *C. arabica*, and *C. liberica*.

Code	Cross combinations	Ploidy Combinations
C1×A1	*C. canephora* "Sidodadi" × *C. arabica* "Mangening"	2*x* × 4*x*
C1×A2	*C. canephora* "Sidodadi" × *C. arabica* "AGK"	2*x* × 4*x*
C1×C2	*C. canephora* "Sidodadi" × *C. canephora* "R-Mut"	2*x* × 2*x*
C1×L1	*C. canephora* "Sidodadi" × *C. liberica* var. *liberica*	2*x* × 2*x*

Table 2. Cross success of interspecific heteroploid (C1 × A1 and C1 × A2), intraspecific homoploid (C1 × C2), and interspecific homoploid (C1 × L1) hybridizations.

Cross combination	Pollinated flowers	Recovered seeds	Endosperm failure	Normal seed	Cross success (%)
C1 × A1	568	76	73	3	0.26
C1 × A2	1,238	215	204	11	0.44
C1 × C2	846	1,095	84	1,011	59.75
C1 × L1	821	115	108	7	0.43

Note: C1 and C2 are diploid *Coffea canephora* (2*n*=2*x*=22), A1 and A2 are tetraploid *C. arabica* (2*n*=4*x*=44), and L1 is *C. liberica* var. *liberica.*

Seed and seedling performance

Finally, the remaining healthy developed seeds underwent further germination. The number of regular seeds is in Table 2. Seed sowing occurred on fine sand. The germinated seeds were counted and then transferred into a polybag. The number of leaf pair was observed at two-month intervals.

Data analysis

Data of fruit set, seed germination, and seedling performance attained presentation by a bar or line chart using MS-Excel Version 2309. The analysis of fruits and seeds morphometric data distribution through a boxplot and the principal component analysis (PCA) biplot visualizations used the R Studio version 2023.06.1 with R packages version 4.3.1. Those datasets underwent a non-parametric statistical analysis of the Mann-Whitney U Test using SPSS version 26. Pairwise comparisons were shown for each interspecific cross combination to standard intraspecific cross combinations (C1 × C2).

RESULTS

High fruit and seed set failure

The fruit set three MAP of both interspecific heteroploid crosses (C1 × A1 and C1 × A2) was comparable to the intraspecific standard cross (C1 × C2). A significantly lower fruit set at three MAP ($P < 0.01$) appeared for the interspecific homoploid cross (*C. canephora* × *C. liberica*). However, fruit loss was high between three and six MAP for all interspecific crosses, leaving only a few normally developed fruits attached to hand-pollinated flowering branches (Figure 1). A drop of reddish-immature cherries during this period was the primary cause of fruit loss. The high shedding of young fruit can be due to an imbalance between maternal and paternal genomes in interspecific crossing, thereby triggering failure in ovary development (He *et al*., 2022).

Paternal excess produced larger fruits and seeds

Interspecific heteroploid crosses (*C. canephora* × *C. arabica*, 2*x* × 4*x*) generated hybrid cherry fruits and seeds with a larger polar diameter than intra- and interspecific homoploid crosses. However, no statistical differences emerged in the cherry fruits and seeds' equatorial diameter and thickness. It means that the interspecific heteroploid crossing promoted only the elongation growth of fruits and seeds. In contrast, interspecific homoploid hybridization (*C. canephora* × *C. liberica*, 2*x* × 2*x*) had produced comparatively reduced cherry fruits and seeds. Only the seed thickness variable showed no difference statistically among cross combinations (Figure 2).

Based on the PCA analysis of fruit and seed morphometrics, the cherry's polar diameter, equatorial diameter, thickness, and seed equatorial diameter were variables that chiefly contributed to PC1. Meanwhile, only the seed thickness variable contributed mainly to PC2. C1 × A1 and C1 × A2 cross combinations had unclear separation from C1 × C2. Instead, the C1 × L1 cross combination has had a

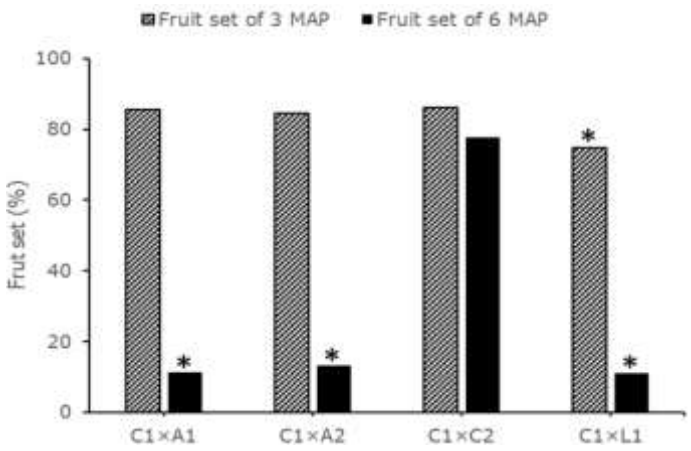

Figure 1. Initial and final fruit set of paternal excess (2x × 4x, C1 × A1, and C1 × A2) and homoploid (2x × 2x, C1 × L1) interspecific crossing compared with homoploid intraspecific crossing as a standard (2x × 2x, C1 × C2). Note: C1 and C2 are diploid *Coffea canephora* (2n=2x=22), A1 and A2 are tetraploid *C. arabica* (2n=4x=44), and L1 is *C. liberica var. liberica*. MAP is an abbreviation for months after pollination. Sign * above bars is significantly different based on the Mann-Whitney U test.

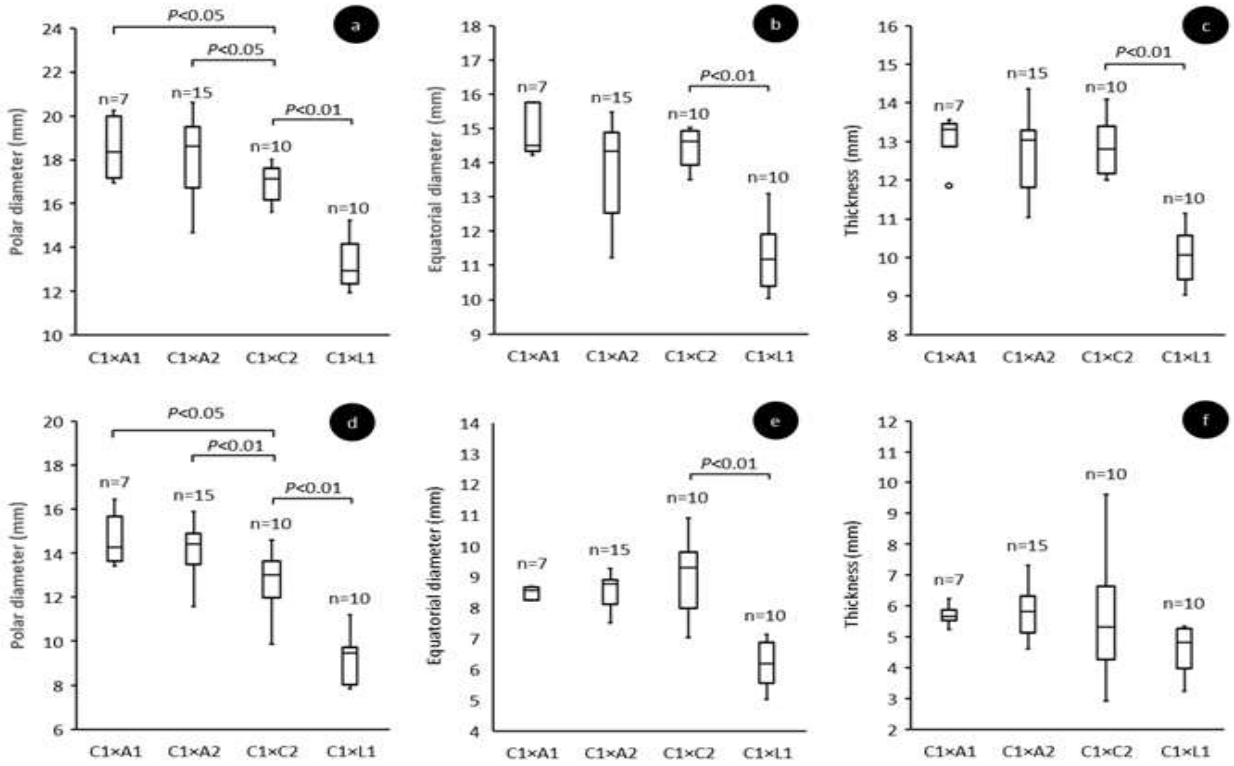

Figure 2. Morphometric size of the cherry fruit (a-c) and seed (d-f) of paternal excess (2*x* × 4*x,* C1 × A1, and C1 × A2) and balanced (2*x* × 2*x*, C1 × L1) interspecific crossing compared with the balanced homoploid intraspecific crossing as a standard (2*x* × 2*x*, C1 × C2). C1 and C2 are diploid *Coffea canephora* (2*n*=2*x*=22), A1 and A2 are tetraploid *C. arabica* (2*n*=4*x*=44), and L1 is *C*. *liberica* var. *liberica*. The bracket is a sign of the significant difference between pairwise medians based on the Mann-Whitney U test.

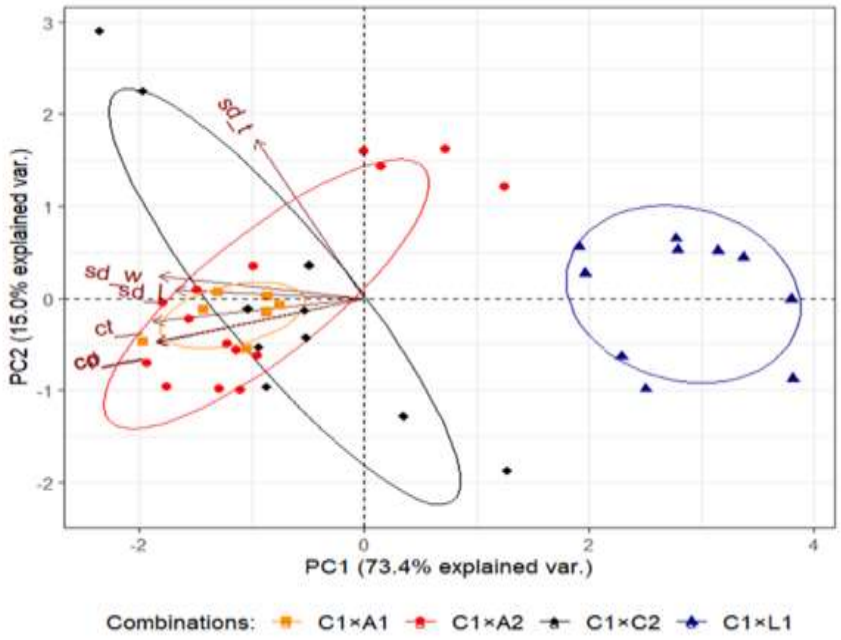

Figure 3. Principal component analysis (PCA) biplot of the coffee cherry fruit and seed morphometric of two interspecific heteroploid (2*x* × 4*x,* C1 × A1, and C1 × A2) and interspecific homoploid (2*x* × 2*x*, C1 × L1) crossings compared with the balanced intraspecific homoploid crossing as a standard (2*x* × 2*x*, C1 × C2). C1 and C2 are diploid *Coffea canephora* (2*n*=2*x*=22), A1 and A2 are tetraploid *C. arabica* (2*n*=4*x*=44), and L1 is *C*. *liberica* var. *liberica*.

distinct position (Figure 3). This result followed the PCA biplot analysis for flower morphometrics of these three coffee species. The flower morphometric of *C. liberica* had a grouping that distantly separated from *C. arabica* and *C. canephora* (Dani *et al*., 2023).

High frequency of endosperm failure

Despite larger seed size, endosperm development of C1 × A1 and C1 × A2 incurred high disruptions. Only a minimal proportion of survived seeds contained regularly developed endosperm. Interestingly, a similar result also appeared for C1 × L1. The cross success of C1 × A1, C1 × A2, and C1 × L1 was below 0.5%, which means that only less than five normal hybrid seed individuals recovered from 1,000 hand-pollinated flowers (Table 1). Most surviving seeds only contained shriveled blackish undeveloped endosperm covered by naturally developed parchment (Figure 4).

Better seedling performance of paternal excess

Most surviving seeds with normal endosperm of C1 × A2 (11 seeds) had successfully germinated, while all three C1 × A1 surviving seeds (3 seeds) had failed to germinate. On the other hand, a slight proportion of survived seeds of C1 × L1 (seven seeds) germinated.

Germinated seeds, transferred into a polybag for further development, had a two-month interval observation. The results showed that the seedling growth performance of C1 × A2 was superior based on the number of leaf pairs formed. Meanwhile, the seedling growth of C1 × L1 was the poorest (Figure 5).

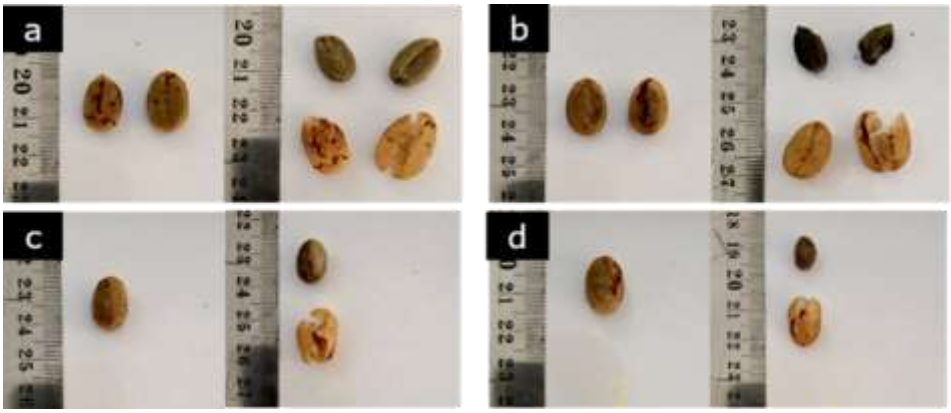

Figure 4. Fully developed seeds harvested from diploid *C. canephora* "Sidodadi" pollinated by tetraploid *C. arabica* "AGK." Few seeds, both double flat or single-rounded seeds, contained a fully developed endosperm (a and c), while others showed endosperm failure with normal parchment layer (b and d).

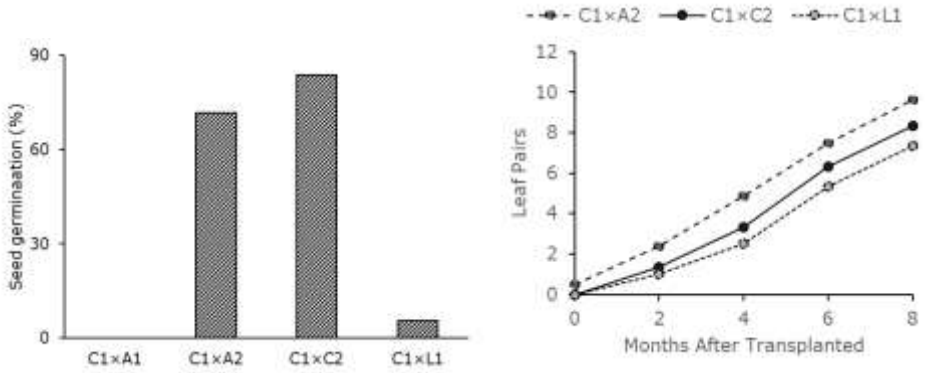

Figure 5. Seed germination and seedling growth performance of two interspecific heteroploid (2*x* × 4*x,* C1 × A1, and C1 × A2) and interspecific homoploid (2*x* × 2*x*, C1 × L1) crossings compared to the balanced intraspecific homoploid crossing as a standard (2*x* × 2*x*, C1 × C2). C1 and C2 are diploid *Coffea canephora* (2*n*=2*x*=22), A1 and A2 are tetraploid *C. arabica* (2*n*=4*x*=44), and L1 is *C. liberica* var. *liberica*.

DISCUSSION

Hybrid seed failure is the chief factor of post-zygotic barriers and plant speciation. Endosperm-based hybridization barriers were then widely proposed as the current concept (Lafon-Placette and Köhler, 2016; Lafon-Placette *et al.*, 2017; Städler *et al.*, 2021). This concept's proposal has long existed but has experienced a revival in the last five years (Städler *et al.*, 2019).

The endosperm is the nourishing tissue for developing embryos in many taxa of flowering plants (Baroux *et al.*, 2002), including coffee plants (Eira *et al.*, 2006). However, in other specific plant species, such as the water lily *Nymphaea thermarum,* the perisperm tissue, instead of the endosperm, has acted as the primary nourishing tissue for developing embryos in the seed. It has become a strategy to reduce parental conflict and to recover the maternal control over resource

allocation to their offspring (Povilus *et al.*, 2018).

The seed's embryo and endosperm are the products of double fertilization, which means that both consist of maternal and paternal genomes (Johnston *et al.*, 1980). However, an imbalanced genome between parental species is a foremost factor of interspecific and interploidy hybrid seed failure (HSF), which has a role as an early-acting post-zygotic reproductive barrier (Roth *et al.*, 2019). Nonetheless, according to the concept of endosperm balance number (EBN), the role of genes is more reasonable than the effect of the ploidy level on endosperm development (Johnston *et al.*, 1980). Imprinted genes have prevailed to function significantly during endosperm development (Scott *et al.*, 1998). The results of this research followed those hypotheses that observing a robust inhibition for endosperm development was not merely on interspecific interploid crossing (C1 × A1 and C1 × A2) but also interspecific homoploid crossing (C1 × L1).

The larger size of cherry fruits and seeds of paternal excess has become an indicator of the endosperm cell's precocious growth. Endosperm growth and proliferation of paternal excess are presumably extended compared to typical intraspecific crossings of *C. canephora* (Köhler *et al.*, 2021). However, this faster maternally plus paternally controlled tissue growth failed to cellularize. In turn, developing endosperm of paternal excess could become a shriveled collapsed structure (Zumajo-Cardona *et al.*, 2023). However, despite endosperm failure, surrounding maternal tissues, such as an endocarp and silver skin, continued normal development until the cherry fruit reached its maximum size and ripened (Figure 4). This result could indicate the complete control of those maternal tissues by specific maternal gene(s) (Johnston *et al.*, 1980).

Interestingly, a minimum frequency of usual hybrid seeds had their recovery from C1 × A2 and C1 × L1 combinations. Several hybrid seeds with regularly developed endosperm germinated and developed into healthy seedlings, except for the C1 × A1 cross combination. Indeed, this result aligned with the hypothesis of an incomplete post-zygotic barrier among *Coffea* species (Gomez *et al*., 2016). However, a larger seed size of paternal excess (C1 × A2) showed a more vigorous seedling growth. On the contrary, reduced hybrid seed sizes of interspecific homoploid crossing (C1 × L1) result in a less vigorous seedling performance. A positive correlation between size and seedling performance has also occurred in other studies (Souza and Fagundes, 2014; Makinde *et al.*, 2020).

Better seedling survival of interspecific hybridization with paternal excess (C1 × A2) compared with the interspecific homoploid (C1 × L1) could be due to high genetic similarity between *C. canephora* and *C. arabica*. Based on the genomic and archeological analysis, it has been a fact that *C. canephora* is one of the progenitors for allotetraploid *C. arabica*. However, the higher genetic diversity of *C. canephora* could act as an essential source of valuable genes for *C. arabica* breeding programs (Merot-L'anthoene *et al*., 2019).

The performance of F1 hybrid individuals requires further observation to determine the effect of paternal excess on their growth and development. Cytotype identification and genomic analysis of each hybrid individual are also necessary to ensure successful genome introgression between species. The introgressed valuable genes need utilization in future breeding programs for the next generation of coffee hybrid cultivars.

CONCLUSIONS

This study has revealed a robust but incomplete early-acting post-zygotic barrier of crosses between coffee species with paternal excess (*C. canephora* × *C. arabica*). However, similar results with sturdier intensity also appeared in interspecies crosses without paternal excess (*C. canephora* × *C. liberica*). The success of crosses between species, with and without paternal excess, is minimal, only at < 0.5%. These results indicated the existence of any factors other than a ploidy level on the success of crosses between coffee species. Although achieving crosses between coffee species with paternal excess is sparse,

an opportunity to form heteroploidy in the F1 generation, i.e., from diploid, triploid, to tetraploid, is promising. Ploidy variations in the F1 generation have never occurred in crosses between coffee species in the opposite direction (maternal excess).

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