Alborea: A New Mid-late Mandarin Triploid Hybrid [(*Citrus clementina* × *C. tangerina*) × (*C. nobilis* × *C. deliciosa*)]

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Citrus is one of the most important fruit crops worldwide, with more than 130 million tons produced in 2017. Mandarins represent 25% of this production (Food and Agriculture Organization, 2018) and are mainly destined for fresh consumption. The Mediterranean area is the most important region for mandarin exports, with 60% of the total volume, and Spain is the leading country. The fresh market demands high-quality, seedless fruit that can be harvested throughout the marketing season. Therefore, mandarin breeding programs worldwide are mainly aimed at obtaining new, seedless, easy-peeling varieties with an attractive fruit color and flavor, and with high organoleptic characteristics (Grosser et al., 2010; Navarro et al., 2015; Rapisarda et al., 2008).

The mandarin varietal structure in Spain has several problems. It includes satsumas [*Citrus unshiu* (Mak.) Marc.], clementines (*C. clementina* Hort. ex Tan.), and mandarin hybrids. Satsumas are harvested from the end of August to mid November. Their pollen and ovules are not viable, and thus they are seedless. Clementine varieties are the most important group of mandarins in Spain and can be harvested from mid September until the second half of January. They are self-incompatible, but their pollen and ovules are viable and, consequently, they are able to pollinate and be pollinated with other compatible cultivars. 'Hernandina' clementine, our latest maturing clementine, is characterized by low fruit quality when grafted onto 'Carrizo' citrange [*C. sinensis* (L.) Osbeck × *Poncirus trifoliata* (L.) Raf.], which is by far the predominant rootstock in our country. Fruit peel deteriorates quickly after mid January, and in practice no clementine fruit is available in the second half of January. Mandarin hybrids, such as 'Nova' [*C. clementina* × (*C. paradisi* Macf. × *C. tangerina* Hort. Ex Tan.)], 'Fortune' (*C. clementina* × ?), 'Murcott' (*C. reticulata* Blanco × *C. sinensis*), and 'Ortanique' (natural hybrid between mandarin and *C. sinensis*), were introduced to our citriculture to cover the demand of late-maturing mandarins by international markets. These cultivars are self-incompatible, but their pollen and ovules are viable and they cross-pollinate with clementines, producing seedy fruit in both groups of mandarins. In addition, some late mandarin hybrids, such as 'Fortune', 'Nova', and 'Murcott', are susceptible to *Alternaria alternata* fungus, which reduces production and depreciates the fruit commercially for the fresh-fruit market. This fungus has forced the top-grafting of trees of these susceptible cultivars, particularly 'Fortune', and during the past few decades there has been a decrease in late-mandarin plantations and an increase in clementine plantings, mainly 'Clemenules'. This has created an imbalance between production and market demand that has caused a drastic reduction in prices for the growers such that, in many cases, they cannot even sell their products. As a consequence of excessive clementine production, a lot of growers decided to cultivate other late mandarin cultivars, especially 'Nadorcott' [(*C. reticulata* × *C. sinensis*) × ?], 'Tango' (irradiated variety from 'Nadorcott' mandarin), and 'Orri' (irradiated variety from 'Orah' mandarin) and, more recently, 'Spring Sunshine' (irradiated variety from 'Murcott' mandarin), which is susceptible to *Alternaria*. These varieties cover the period from February to the end of April and are managed by different private companies that limit the number of plants or the cultivated area, and also impose high royalties that, in many cases, are difficult for growers to pay. Under this scenario, the recovery of a new, high-quality seedless mandarin cultivar that matures from mid December until the end of January, when there are no other high-quality mandarins in the market, and that is resistant to *Alternaria* is a very important objective for our citriculture.

In 1995, a triploid breeding program was started at the Instituto Valenciano de Investigaciones Agrarias (IVIA) with the objective of producing new mid- and late-maturing triploid cultivars resistant to *A. alternata* fungus through sexual hybridization, embryo rescue, and ploidy analysis by flow cytometry (Navarro et al., 2015). Triploid plants generally produce aneuploid gametes, thus leading to very low fertility of their pollen and ovules (Otto and Whitton, 2000). For this reason, citrus triploid hybrids can be considered sterile, producing seedless fruit, and do not induce seed formation in other varieties by cross-pollination, even in the presence of bees (Navarro et al., 2015). A routine strategy exploited for triploid citrus breeding is spontaneous female unreduced gamete formation in diploid × diploid crosses (Aleza et al., 2012; Cuenca et al., 2011, 2015), where triploid hybrids arise usually from the union of an unreduced megagametophyte formed through a second-division restitution mechanism with a haploid pollen (Cameron and Frost 1968; Cuenca et al., 2011; 2015; Esen and Soost 1971, 1973; Geraci et al., 1975; Luro et al., 2004). Triploid embryos are predominantly found in small seeds, which generally do not germinate in greenhouse conditions. Thus, embryo rescue from these small seeds is required to reach high germination rates (Aleza et al., 2010b). In addition, ploidy level determination by flow cytometry is also required in extensive triploid citrus breeding programs (Aleza et al., 2012). From this program, the mid- to late-maturing triploid varieties 'Garbı' [(*C. clementina* × *C. tangerine*) × (*C. reticulata* × *C. sinensis*)] (Aleza et al., 2010a) and 'Safor' [(*C. clementina* × *C. tangerina*) × (*C. unshiu* × *C. nobilis* Lour.)] (Cuenca et al., 2010) were released, with more than 600,000 plants commercialized until 2018.

We describe a new triploid hybrid named 'Alborea' mandarin [(*C. clementina* × *C. tangerina*) × (*C. nobilis* × *C. deliciosa* Ten.)] that is resistant to *Alternaria alternata* and characterized by the production of high-quality, seedless fruit that can be harvested from mid-December until the end of January.

Origin

'Alborea' mandarin was originally obtained from an open pollination in Spring 1995 of a diploid 'Fortune' mandarin tree grown at the IVIA plots. Subsequently, the male parent has been investigated through molecular marker profiles and was identified as 'Wilking' mandarin (*C. nobilis* × *C. deliciosa*). Procedures for performing this analysis are explained later.

In Jan. 1996, 120 'Fortune' mandarin fruit that contained 219 small seeds were collected. Embryos were isolated from these

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seeds and cultured in vitro in the culture medium described by Murashige and Skoog (1962), supplemented with 50 g·L^{-1} sucrose, 500 mg·L^{-1} malt extract, 100 mg·L^{-1} i-inositol, 1 mg·L^{-1} pyridoxine hydrochloride, 1 mg·L^{-1} nicotinic acid, 0.2 mg·L^{-1} thiamine hydrochloride, 4 mg·L^{-1} glycine, and 8 g·L^{-1} Bacto agar [Murashige and Skoog (MS) culture media]. After germination, 117 plantlets were subcultured in an elongation medium that consisted of the MS culture media without malt extract. Cultures were maintained at 24 ± 1 °C, 60% humidity, and 16 h of daily exposure to 40 µE·m^{-2}·s^{-1} illumination. The ploidy level of all plants was analyzed by flow cytometry in the flow cytometer Ploidy Analyzer (Partec GmbH, Münster, Germany). Small pieces of leaves, measuring ≈0.5 cm^2, were taken from the in vitro growing plants and chopped together with a piece of leaf from a control diploid plant, placed in a Partec CyStain ultraviolet Precise P nuclei extraction buffer, stained with DAPI (4′,6-diamidino-2-phenylindole), and analyzed in the cytometer. All 117 plantlets were found to be triploid and were transplanted to the greenhouse. All plants were grafted onto 'Carrizo' citrange rootstock for field evaluation at IVIA plots in Oct. 1996.

'Alborea' mandarin flowered for the first time in Spring 1999. After 3 years of regular production of high-quality fruit, it was selected for further evaluation and propagated in several additional test plots.

Investigating the Male Parent of 'Alborea' Mandarin

The open-pollinated 'Fortune' mandarin tree was located at the IVIA Germplasm Bank in Moncada, Valencia, Spain. Cultivars grown at the same and in the nearest orchards were considered as candidate male parents of 'Alborea' mandarin (Table 1). Forty-three citrus genotypes, including predominantly mandarin hybrids, one clementine, one satsuma, one sweet orange, and one pummelo, were analyzed. Fifteen simple sequence repeat (SSR) markers were used to investigate the pollen donor among the candidate genotypes (Supplemental Material 1). In addition, 'Alborea' mandarin together with its female parent, 'Fortune' mandarin, and the candidate male parent ('Wilking' mandarin) were analyzed with an additional 12 SSR markers (Supplemental Material 1). In total, we used 27 SSR markers covering the nine citrus linkage groups of the reference clementine genetic map (Ollitrault et al., 2012b). Polymerase chain reaction (PCR) amplification was performed using a Thermocycler EP gradient S (Eppendorf, Germany) in 10 µL final reaction volumes containing Taq DNA polymerase (Fermentas, Germany) (0.8 U), citrus template DNA (2 ng·µL^{-1}), wellRED (Sigma, Germany) dye-labeled forward primer (0.2 mM), unlabeled reverse primer (0.2 mM), deoxynucleotide triphosphate (0.2 mM each), 10× PCR buffer, and magnesium chloride (1.5 mM). The PCR protocol was as follows: denaturation at 94 °C for 5 min followed by 40 cycles of 30 s at 94 °C, 1 min at 50 or 55 °C (depending on the primer annealing temperature), 45 s at 72 °C, and a final elongation step of 4 min at 72 °C. Capillary electrophoresis was performed using a CEQ8000 Genetic Analysis System (Beckman Coulter Inc., Brea, CA). PCR products were initially denatured at 90 °C for 2 min, injected at 2 kV for 30 s, and subsequently separated at 6 kV for 35 min. Alleles were sized using a 400-bp DNA size standard. GenomeLab GeXP v.10.0 genetic analysis software was used for data collection. Alleles were converted to binary unit characters (1.0 = present, 0 = absent) using a Microsoft Excel (2007) template, and the similarity index was calculated for each male parent as the average of unit characters. Among the male candidates, only 'Wilking' mandarin shared one allele with 'Alborea' mandarin for all 15

Table 1. Candidate male parents of 'Alborea' mandarin, and similarity index calculated from genetic analysis with simple sequence repeat markers

Genotype	IVIA germplasm accession no.	Group	Similarity (%)
Wilking	28	Hybrid mandarin	100
Kara	218	Hybrid mandarin	80
Kinnow	33	Hybrid mandarin	80
Daisy	362	Hybrid mandarin	67
Moncada	421	Hybrid mandarin	67
Encore	155	Hybrid mandarin	67
Gold Nugget	—	Hybrid mandarin	67
N-27	423	Hybrid mandarin	67
Murcott	196	Tangor	67
Oronules	132	Clementine	60
Avasa 16	—	Hybrid mandarin	60
Avasa 17	—	Hybrid mandarin	60
Nova	74	Hybrid mandarin	60
Pixie	210	Hybrid mandarin	60
Simeto	413	Hybrid mandarin	60
Temple	81	Mandarin	53
A'-12	424	Hybrid mandarin	53
D-19	447	Hybrid mandarin	53
Page	79	Hybrid mandarin	53
Sunburst	200	Hybrid mandarin	53
Wallent	404	Hybrid mandarin	53
Seminole	348	Tangelo	53
Ortanique	276	Tangor	53
Valencia Late Delta	363	Sweet orange	47
Parson's Special	168	Mandarin	47
Okitsu	195	Satsuma	47
Avasa 15	—	Hybrid mandarin	47
C-54-4-4	—	Hybrid mandarin	47
Fairchild	83	Hybrid mandarin	47
Fallglo	466	Hybrid mandarin	47
Freemont	82	Hybrid mandarin	47
Osceola	573	Hybrid mandarin	47
Palazzelli	188	Hybrid mandarin	47
Mapo	190	Tangelo	47
Minneola	84	Tangelo	47
Ellendale	194	Tangor	47
Umatilla	100	Tangor	47
Primosole	414	Hybrid mandarin	40
Orlando	101	Tangelo	40
Avasa 9	—	Tangelo	40
Dancy	434	Mandarin	27
Chandler	207	Pummelo	20
Dweet	165	Tangor	20

IVIA = Instituto Valenciano de Investigaciones Agrarias.

Table 2. Summary of fruit quality characteristics of 'Alborea' mandarin grafted onto 'Carrizo' citrange rootstock.

Trait	Second half of December[z]	First half of January	Second half of January	First half of February	Mean[y]
Diameter (mm)	55 ± 4	58 ± 5	55 ± 5	55 ± 7	56 ± 5
Height (mm)	44 ± 3	45 ± 4	43 ± 5	42 ± 6	44 ± 5
CCI[x]	24 ± 2	23 ± 3	23 ± 3	22 ± 2	23 ± 3
Soluble solids (%)	14 ± 1	14 ± 1	14 ± 1	14 ± 1	14 ± 1
Acids (%)	1.7 ± 0.4	1.4 ± 0.3	1.4 ± 0.3	1.2 ± 0.1	1.4 ± 0.3
Juice content (%)	47 ± 6	44 ± 7	44 ± 6	43 ± 4	44 ± 6

[z]Averaged between 5 to 9 years of the three plots under evaluation. Each sample comprised 10 fruit.
[y]Average of four harvesting periods.
[x]Citrus color index according methodology described by Jiménez-Cuesta et al. (1981) and Pannitteri et al. (2017).

markers analyzed, and therefore 'Wilking' mandarin was revealed as the pollen donor (Table 1). In addition, the genetic analysis with the extra 12 SSR markers confirmed 'Wilking' mandarin as the male parent (Supplemental Material 1). Furthermore, the presence of two alleles from 'Fortune' mandarin and one allele from 'Wilking' mandarin using the SSR loci mCrCIR02D09, mCrCIR03G05, mCrCIR06A12, mCrCIR07F11, Ci02E08, and CIBE0733 indicated that 'Alborea' mandarin originated from the fusion of an unreduced gamete of 'Fortune' mandarin and a haploid (normal) gamete from 'Wilking' mandarin. Next highest similarity (80%) was found with 'Kara' and 'Kinnow' mandarins, which can be explained because 'Kara' mandarin is a hybrid with *C. nobilis*, and 'Kinnow' mandarin has the same pedigree (*C. nobilis* × *C. deliciosa*) as 'Wilking' mandarin. The results obtained with all the SSR markers used allow distinguishing 'Alborea' mandarin from other commercial hybrids. Its genetic profile will help protect 'Alborea' mandarin rights and control traceability of nursery-propagated plants.

Description of 'Alborea' Mandarin

The data presented here are based on three different plots, located in Moncada (Valencia, Spain; 17 trees), Carcaixent (Valencia, Spain; three trees) and Villarreal (Castellón, Spain; three trees), all of them grafted onto 'Carrizo' and 'Troyer' citrange. Data were collected following the Guidelines for the Conduct of Tests for Distinctness, Uniformity and Stability, *Citrus* L. Group 1 Mandarins, from the International Union for the Protection of New Varieties of Plants (2009). No significant differences were found in any parameter among tress growing in the different plots, and the variety was stable and uniform. Data of 'Alborea' mandarin were compared with 'Nova' and 'Fortune' mandarins because the first one matures more or less at the same harvesting period, although it displays completely different fruit organoleptic qualities than clementines, and the last one is the known female parent.

The 'Alborea' mandarin tree is vigorous, obloid in shape, and exhibits an open growth. The main branches in the first propagations had thorns of 24 mm in length in most buds. The new branches in further propagations presented small thorns of 8 mm in 50% of the buds.

The leaves of 'Alborea' mandarin are evergreen and simple, lanceolate, with an average length of 4.8 cm and an average width of 2.3 cm. The margin of the leaves is slightly crenate with an acute apex. The petiole is medium in length without wings.

Flowers of 'Alborea' mandarin are hermaphrodite, with white petals and around 20 stamens. Blooming occurs in mid April at Valencia conditions, with mostly single terminal flowers. 'Alborea' mandarin is seedless in an open-pollinated environment in the presence of many cultivars with fertile pollen. Seed counts of more than 1000 fruit of 'Alborea' mandarin during 18 seasons showed an average of 0.06 seed/fruit, compared with 3.12 and 7.26 seeds/fruit found in 'Clemenules' and 'Fortune' mandarin, respectively, under the same conditions. In addition, the pollen of 'Alborea' mandarin has a very low fertility in vitro, with only 0.1% of pollen grains germinating, whereas 90% of 'Fortune' mandarin pollen grains germinated. These results confirm the seedlessness of the new triploid cultivar 'Alborea' mandarin and the inability of this variety to cause seed formation in other varieties by cross-pollination.

Fruit characteristics are presented in Table 2. The optimum maturity period is in the beginning of January, with a solid/acid ratio of 10, with a percentage of soluble solids and acids content of 14 ± 1 and 1.4 ± 0.3, respectively, although they can be harvested from the second half of December until the end of January. During this time, 'Alborea' mandarin has always displayed an intermediate solid/acid ratio between 'Fortune' and 'Nova' mandarins (Fig. 1). These differences are mainly a result of the acid contents, because the sugar content is very similar in the three cultivars. The average juice content varies between 38% and 50%, measured with an automatic press juice machine (Zumonat, C-40 model) with a constant pressure of 14 $kg \cdot cm^{-2}$. The fruit is obloid, with a diameter of 56 ± 5 mm and a height of 44 ± 5 mm, and the broadest part is located in the equatorial line, with a circular shape in the transverse section without neck (Fig. 2). The fruit does not present persistent style, navel, or areola at the distal end. Peel color was measured with a Minolta CR-400 chroma-meter (Minolta Corp., Osaka, Japan) according to the methodology described by Jiménez-Cuesta et al. (1981), Pannitteri et al. (2017) and Continella et al. (2018). The results are expressed as a citrus color index (CCI), calculated with the formula CCI = $(1000 \times a)/(L \times b)$, where a indicates chromaticity on a green (–) to red (+) axis, b indicates chromaticity on a blue (–) to yellow (+) axis, and L measures the light reflected by the sample in the spectrum's green zone. The CCI is used widely and officially in the citrus industry in Spain as a parameter for color measurements and minimum quality conditions (DOGV, 2006). The fruit of 'Alborea' mandarin develop a deep orange–red rind at whole maturity (average CCI = 23 ± 3) with white albedo and orange flesh, and an intermediate number of well-developed segments (9–11 segments/fruit) (Fig. 2). The fruit of 'Alborea' mandarin are

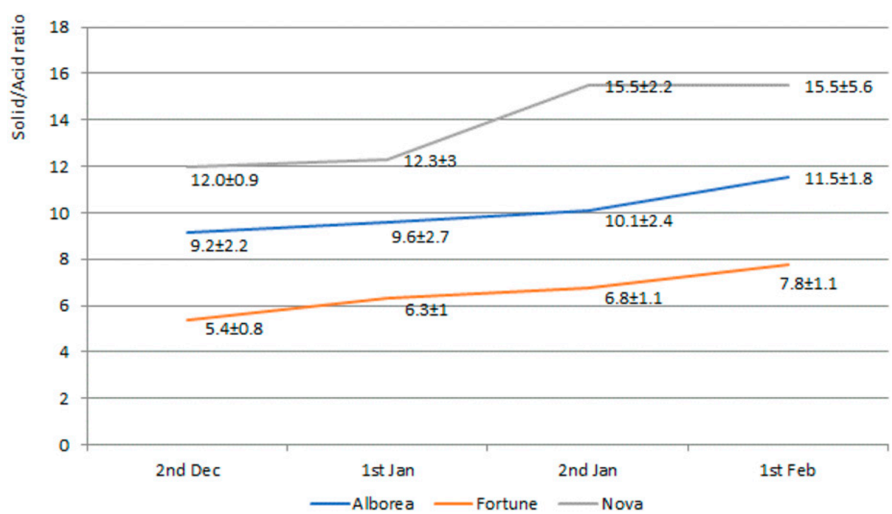

Fig. 1. Comparison of the solid/acid ratio evolution among 'Alborea', 'Nova', and 'Fortune' mandarins. Data are the average of more than five seasons.

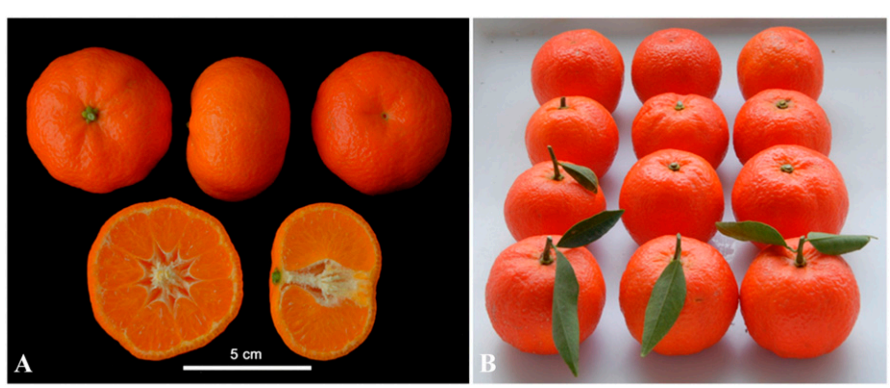

Fig. 2. (**A**) Typical fruit of 'Alborea' mandarin. (**B**) Fruit of triploid 'Alborea' mandarin harvested with and without leaves, resembling clementines with improved color.

easy to peel, like clementines. The flavor of 'Alborea' mandarin fruit is intense as a result of the high solid and acid contents, with an easy-to-eat texture, and a pleasant aroma somehow resembling that of the 'Willow leaf' mandarin, which is a direct parent of 'Wilking' mandarin.

'Alborea' Is a Resistant Cultivar to *Alternaria alternata* Fungus

'Alborea' mandarin is a direct hybrid from 'Fortune' mandarin, which is highly susceptible to *A. alternata* fungal infection. However, 'Alborea' is an *A. alternata*-resistant cultivar, as assessed by four different tests. In the first test, the trees of 'Alborea' mandarin and several mandarin cultivars were planted in experimental plots at the IVIA that have a high level of *A. alternata* inoculum. Under these conditions, and during a 15-year observation period, 'Fortune' and 'Nova' mandarins displayed symptoms in leaves and fruit, with 54% and 11% of the fruit affected by the fungus, and an average of 6.5 and 3 spots/fruit, respectively, whereas no symptoms were ever observed in the leaves or fruit of resistant 'Clemenules' clementine and 'Alborea' mandarin (Fig. 3A).

In the second test, buds from 'Alborea' and 'Fortune' mandarins were grafted onto 'Carrizo' citrange rootstock and cultured in pots along with ungrafted Alemow (*C. macrophylla* Wester) rootstock as an *A. alternata*-resistant control. New shoots with small leaves were sprayed with a high concentration of *A. alternata* spores and were covered with plastic bags to ensure high humidity, following the methodology described by Bassimba et al. (2017). The pots were then placed in a culture chamber at 25 °C to favor *A. alternata* infection. After 2 d, leaves of 'Alborea' mandarin and Alemow showed no symptoms of *A. alternata*, whereas 'Fortune' mandarin leaves were highly affected (Fig. 3B).

In the third text, young leaves (about 50% developed) from 'Alborea', 'Fortune', and 'Clemenules' were collected in spring, sprayed with a high concentration of *A. alternata* spores, and placed in a culture chamber at 25 °C inside petri dishes, as reported previously in Cuenca et al. (2013). After 3 d, leaves of 'Alborea' and 'Clemenules' showed no symptoms of *A. alternata*, whereas 'Fortune' leaves were highly affected (Fig. 3C).

In the fourth and final test, 'Alborea' mandarin, along with 20 susceptible and 20 resistant cultivars, were analyzed with the SNP08 molecular marker, tightly linked to the *A. alternata* resistance locus (Cuenca et al., 2016). *A. alternata* resistance is recessive—meaning, only one susceptibility allele is needed for a cultivar to be susceptible (Cuenca et al., 2013). 'Alborea' mandarin and all resistant cultivars showed homozygosity for the resistance allele, whereas all susceptible cultivars showed at least one susceptibility allele (Fig. 4).

Availability

Protection of Plant Breeders' Rights of 'Alborea' mandarin has been obtained in the

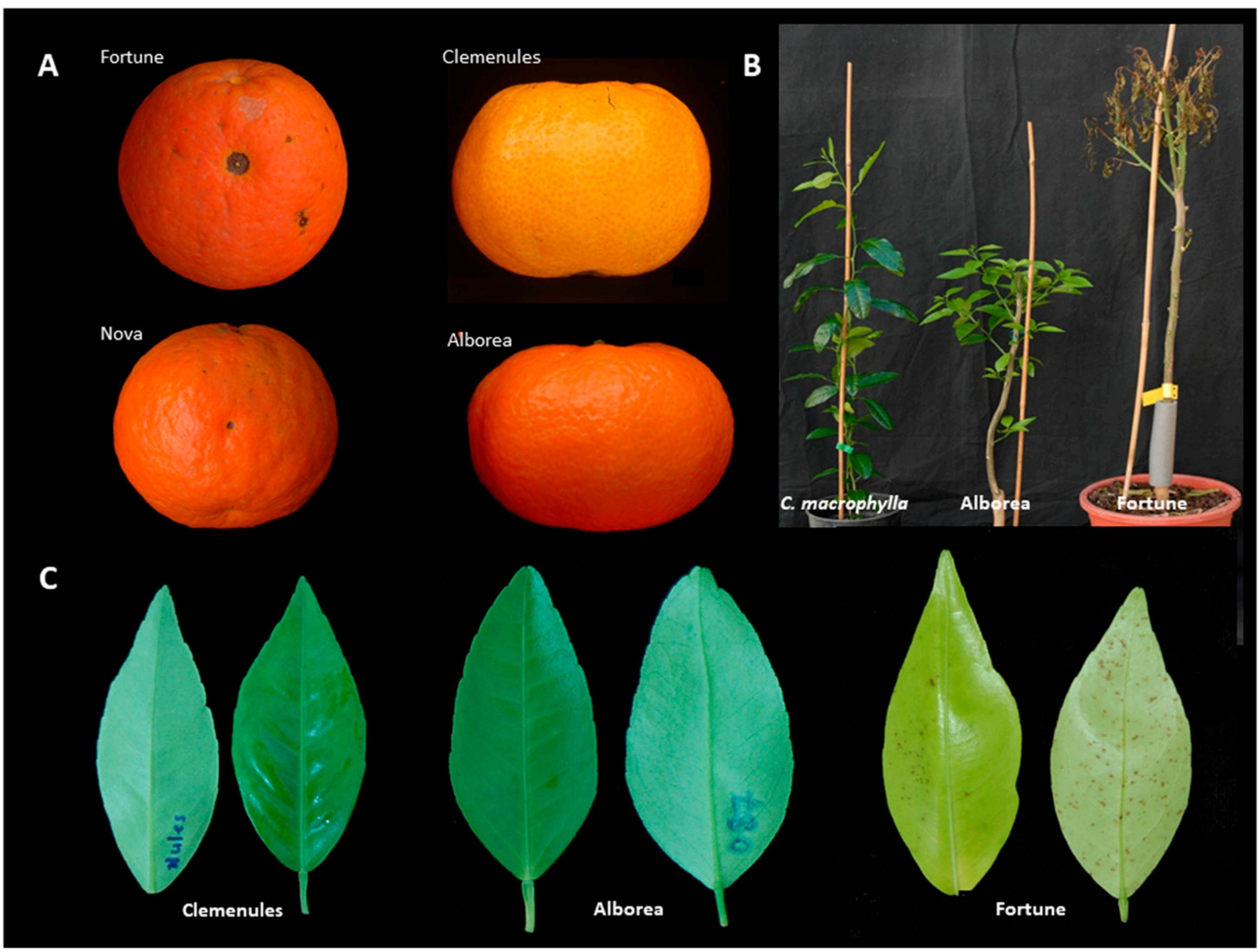

Fig. 3. (**A**) *Alternaria alternata* brown spots in fruit of susceptible cultivars and 'Alborea' mandarin collected from Instituto Valenciano de Investigaciones Agrarias experimental plots with a high level of *A. alternata* inoculum. (**B**) Plants of resistant cultivars *C. macrophylla* and 'Alborea' mandarin, and susceptible 'Fortune' mandarin showing *A. alternata* symptoms after inoculation with a suspension of fungus conidia. (**C**) Leaves of resistant cultivars 'Clemenules' clementine and 'Alborea' mandarin, and susceptible 'Fortune' mandarin showing *A. alternata* symptoms after inoculation with a suspension of fungus conidia.

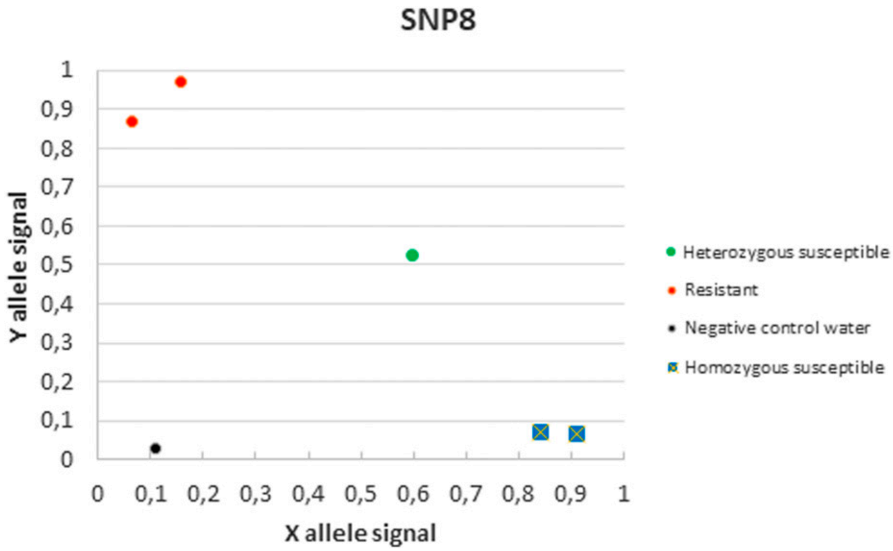

Fig. 4. SNP8 genotyping results for 'Alborea' mandarin, its parents, and other *A. alternata*-resistant and -susceptible genotypes selected for this study. Resistant cultivars are represented by red dots (TT); heterozygous (GT) and homozygous (GG) susceptible cultivars are represented by green and blue dots respectively. The black dot corresponds to water used as negative control. Resistant cultivars: 'Alborea' and 'Wilking' mandarins. Susceptible cultivar in heterozygosis: 'Fortune' mandarin. Susceptible cultivars in homozygosity: 'Dancy' mandarin (*C. reticulata*) and 'Minneola' tangelo (*C. reticulata* × *C. paradisi*). Single nucleotide polymorphism genotyping was performed by Kbioscience services, using the KASPar technique. A detailed explanation of specific conditions and reactives can be found in Cuppen (2007).

European Union until 2049 (Community Plant Variety Office Decision no. EU53392). Commercial release is expected to be licensed under royalty agreements through an external organization at moderate prices, with the objective that small growers can access this new cultivar. Pathogen-free bud source plants of 'Alborea' mandarin have been obtained by shoot-tip grafting in vitro according to the methodology described by Navarro et al. (1975).

Final Remarks

'Alborea' mandarin is a triploid hybrid recovered from the fusion of an unreduced female gamete of 'Fortune' mandarin and a haploid pollen gamete from 'Wilking' mandarin. Fruit reach optimum maturation in January with a deep orange color and pleasant aroma, and are very easy to peel, like clementines. In addition, 'Alborea' fruit are resistant to *Alternaria alternata* fungus. The fruit of 'Alborea' mandarin are seedless and have high and well-balanced sugar and acid contents. Altogether, 'Alborea' mandarin is a very interesting strategy to keep marketing late 'Clementine-like' high-quality fruit when there are no other clementine fruit with adequate organoleptic qualities.

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