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ORIGINAL PAPER

Cytogenetic and molecular characterization of almond trees treated with plant biostimulants or boron-based fertilizers

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Abstract
Almond is highly produced in the NE of Portugal, where late frosts during flowering, low precipitation, and high temperature in summer affect productivity and quality. Despite the use of late-flowering cultivars, plant biostimulants (PBs) and boron-based fertilizers can also be used to improve cell division, vegetative growth, photoassimilates rate, and nutritional status. PBs are widely used in some food crops, but the evaluation of their effects is still scarce. We treated three-year-old almond trees of cv. 'Vairo,' growing in a rainfed orchard in the NE of Portugal, with four individual treatments: two PBs (based on seaweed extract and free amino acids) and two boron-based fertilizers (applied on soil and leaves). Three monthly applications of seaweed extract (AN), free amino acids (AA), and boron ethanolamine (BE) were made. A single application of boron on the soil (BS) was made. Leaf samples were collected in treated and untreated (control) trees in cytogenetic and molecular analyses through the summer of 2019. The mitotic cell cycle analysis was performed to prepare mesophyll dividing cells stained with silver nitrate, and the molecular stability was assessed with five marker systems. This work aimed to extrapolate which individual treatment conferred higher mitotic cell cycle regularity and molecular stability. The cytogenetic and molecular data achieved in the cv. 'Vairo,' under the edaphoclimatic conditions studied, revealed that the individual treatments AA, BE, or BS induced higher leaf mitotic indexes, regular mitosis, and molecular stability, which might increase the photosynthetic area and production.

Keywords Chromosomal anomalies · Fertilizers · Leaf mitotic cell cycle · Molecular markers · Plant biostimulants · *Prunus dulcis*

Introduction

Almond (*Prunus dulcis* Miller D.A. Webb) has various benefits to human health, and its consumption has been increasing in the last years, requiring an increased production. *P. dulcis* is a Mediterranean crop with high adaptability

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to regions with hot summers, such as the Algarve and the NE of Portugal, where it has been widely cultivated (Queirós 2020; Pascoalino et al. 2021). However, the agricultural practices and edaphoclimatic conditions such as low fertility soils or late frosts compromise the *P. dulcis* productivity and its biochemical profile (Queirós 2020; Pascoalino et al. 2021).

Given the consensus on rising crop productivity without the intensive use of synthetic fertilizers and pesticides that negatively impact the environment, various plant biostimulants (PBs) of microbial and non-microbial origins have been used (Rouphael and Colla 2020; Colla et al. 2020; García-Santiago et al. 2021; Gupta et al. 2022). Recently, the European Union (EU) reached a consensual definition for PBs (EU Regulation 2019/1009), as follows: '*A plant biostimulant shall be an EU fertilizing product the function of which is to stimulate plant nutrition processes independently of the product's nutrient content with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere: nutrient use efficiency, tolerance to abiotic stress, quality traits, or availability of confined nutrients in the soil or rhizosphere'* (EU 2019).

The treatment of *P. dulcis* (foliage and rootstocks) with PBs or boron-based fertilizers can improve growth, stress tolerance, yield, and nutrient uptake (Saa et al. 2015; Torres et al. 2015; Mondragón-Valero et al. 2019, 2020; Pascoalino et al. 2021).

Leaves are the organs more exposed to temperature, radiation, and pollutants. These abiotic stresses impair leaf cell division and differentiation, plant growth, photosynthetic rate, and efficiency, negatively impacting production and quality. Although most plant cytogenetic studies use meristematic root cells, leaves are more accessible in woody species for evaluating the mitotic cell cycle, as demonstrated previously in *Vitis vinifera* L. under heat stress (Carvalho et al. 2018). Therefore, we tested this technique in *P. dulcis* leaves for the first time in this work.

Abiotic stress induces oxidative damage to the DNA (Santos et al. 2015), causing irregularities in the cell cycle and chromosomes Carvalho et al. 2018; Castro et al. 2021, among others) and molecular instability. The latter can be assessed by DNA markers, such as inter-simple sequence repeat (ISSR); random amplified polymorphic DNA (RAPD); inter-retrotransposon amplified polymorphism (IRAP), and retrotransposon-microsatellite amplified polymorphism (REMAP) (Bozari and Aksakal 2013; Correia et al. 2014; Silprasit et al. 2016; Yigider et al. 2016, 2020; Al-Ashkar et al. 2020). The LTR-retrotransposons (RTNs) are responsible for the dynamics of plant genomes and are involved in various somatic mutations, some of which with agricultural value (Foster and Aranzana 2018). Recently, 38.21% of transposable elements (TE), including long terminal repeat (LTR) retrotransposons, were annotated in the genome of *P. dulcis* cv. 'Texas' (Alioto et al. 2020). In this line of thought, we are confident that the inter-priming binding site (iPBS) markers could constitute an additional RTN-based marker system able to trace molecular instability in *P. dulcis*. To evaluate the molecular instability or polymorphism induced by any stressful factor, usually, the inverse is determined, namely the genomic template stability (GTS). GTS is the percentage of monomorphic bands (or markers) among the control plants and those under stress. The sequence instability induced by stress generates DNA breakage or mutations that impair primer binding during the PCR. Therefore, the disappearance or appearance of new bands in the 'group of plants under stress' comparatively to the control bands (the ones amplified in control plants) are considered polymorphisms.

PBs have been widely used in some food crops, namely in organic farming, but their effects on the plant and environment should be more exploited (Calvo et al. 2014; Carrasco-Gil et al. 2018; Klein et al. 2021). Therefore, rapid analyses to screen for the PBs effectiveness in enhancing plant growth and tolerance to abiotic stress would be desirable (Klein et al. 2021).

We hypothesized that a PB or boron-based fertilizer suitable for *P. dulcis* trees of the cv 'Vairo' growing under stressful edaphoclimatic conditions would increase the leaf mitotic index, mitosis's regularity, and molecular stability compared to the control (untreated) trees. Such effects will probably allow the improvement of leaf growth, photosynthetic area, and yield and will mitigate the negative impacts of abiotic stress. In this line of thought, this work focused on the cytogenetic and molecular characterization of *P. dulcis* trees of cv. 'Vairo', grown in one rainfed orchard in the NE of Portugal and treated with two PBs and two boron-based fertilizers, aiming to select the individual treatment(s) that induced the highest values of leaf mitotic index, regular mitosis, and higher genomic template stability (GTS).

Materials and methods

Experiment site and plant material

The field experiment was conducted in the 'Mogadouro' region (41º 21' 5''N 6º 42'43''W) in the NE of Portugal, 746 m above sea level, through the spring and summer of 2019, in a rainfed orchard with three-year-old almond (*P. dulcis* cv. 'Vairo') trees. The orchard has a planting area of 0.33 ha and a plant density of 92 trees per ha. Its soil is classified as Leptosol due to its reduced depth and low clay content, decreasing water holding capacity. The Spanish cv. 'Vairo' (syn. 'Vayro,' breeder's reference IRTAMB-A21-323) was

developed at the Institute of Agrifood Research and Technology (IRTA) (Vargas et al. 2008, 2011). The cv. 'Vairo' had its origin in seedlings that resulted from crosses made in 1991 between the '4-665' and 'Lauranne' cultivars. [https://www.irta.cat/en/servei/varietats-ametller-vairo/] (Vargas et al. 2008, 2011). 'Vairo' is a late-blooming, self-fertile, highly productive cultivar with high plant vigour, consistent yield, and high-quality nuts (Vargas et al. 2008, 2011).

Plant biostimulant and boron-based fertilizer treatments

Commercial PBs or boron-based fertilizers were individually applied to 48 trees (12 trees × 4 rows). Each row received a particular treatment. One row received no treatment (12 control trees). The treatments were applied according to the manufacturer instructions as follows: (i) foliar application (3.0 L ha^{-1}) of an aqueous solution of seaweed extract 100% based on *Ascophyllum nodosum* (AN) at 16.5% (*w/v;* on the dry matter); (ii) foliar application (1.5 L ha^{-1}) of an aqueous solution of a liquid fertilizer carrier of 15.4% (*w/v*) of boron (B) in the form of boron ethanolamine (BE); (iii) foliar application (1.5 L ha^{-1}) of an aqueous solution of free amino acids (AA) of animal origin at 28.8% (*w/v*); and (iv) soil application (1 kg B ha^{-1}) of a solid fertilizer carrier of 11.0% of boron (BS), at the basis of each tree. The treatments will be referred to as AN, BE, AA, and BS through the text. The AN, BE, and AA treatments started in May 2019 and were repeated monthly until August. The soil application of boron (BS) was made once in the last week of March.

Sampling of leaves

Five young leaves were sampled in six treated and control trees one month after applying each foliar treatment, namely in June, July, and August. The leaf samples for the cytogenetic analysis were immediately fixed in absolute ethanol and glacial acetic acid in the proportion of 3:1 (*v/v*), transported to the lab and stored at -20 ºC until the preparation of suspensions of mesophyll dividing cells. The leaves for the molecular analyses were immediately frozen in liquid nitrogen, transported to the lab, and kept at -80 ºC till the isolation of genomic DNA.

Cytogenetic analyses

A small leaf fragment with an area of 0.5 cm^2 was cut and used to prepare a suspension of mesophyll dividing cells following Carvalho et al. (2018), except for the enzymatic digestion, whose duration was four hours. Per treatment, we used three fixed leaves from different *P. dulcis* trees to achieve individual mitotic preparations constituting three biological repetitions. Due to the small size of the *P. dulcis* nuclei and chromosomes, we stained the preparations with silver nitrate or DAPI to analyse the leaf mitotic cell cycle as described in Carvalho et al. (2018). The interphase and/or mitotic cells of 50 observation fields per mitotic preparation were scored. The different phases of mitosis and the various types of anomalies observed per field in interphase and mitotic cells were identified. Per preparation, we determined the mitotic index (MI) as follows: [MI (%) = number of dividing cells/ number of counted cells × 100], where the counted cells constitute the sum of the interphase and mitotic cells. We also calculated the percentage of dividing cells with anomalies (%DCA) as follows: [%DCA = number of dividing cells with anomalies/ number of dividing cells × 100].

Molecular characterization

The frozen leaves were grounded with liquid nitrogen, and the genomic DNA was isolated using the CTAB-based protocol of Doyle and Doyle (1987). The genomic DNA samples were diluted to 40 ng μL^{-1}. After testing individual primers and combinations (Online Resource 1), ISSR, RAPD, IRAP, REMAP and iPBS markers were amplified.

The ISSRs amplification and visualization followed the conditions described by Carvalho et al. (2005). For the RAPDs amplification, we used: 80 ng of genomic DNA, 10× PCR buffer with $(NH4)_2SO_4$, 17 µM primer, 50 mM $MgCl_2$, 7.5 mM dNTPs mixture, and 5 units of *Taq* DNA polymerase (Thermo Scientific, ThermoFisher Scientific), and the amplification conditions reported by Lima-Brito et al. (2006). For the amplification of iPBS, IRAP, and REMAP markers, we used: 100 ng of genomic DNA, 10× PCR buffer with $(NH4)_2SO_4$, 5 µM primer(s), 50 mM $MgCl_2$, 5 mM dNTPs mixture and 5 units of *Taq* DNA polymerase (Thermo Scientific, ThermoFisher Scientific). The amplification conditions were the same for iPBS, IRAP, and REMAP markers, namely: an initial denaturation at 94 ºC for 3 min followed by 34 cycles of 94 ºC for 30 s, 53 ºC for 1 min, and 72 ºC for 1 min, and a final extension at 72 ºC during 6 min. Each PCR reaction was repeated twice. The amplified products were visualized after electrophoresis on 1.5% agarose gels stained with SYBR Safe DNA Gel Stain (APExBIO), upon UV exposure. The amplified products were analysed for the presence (1) or absence (0) of bands among treated and untreated (control) plants for each treatment and sampling date (June, July, and August of

2019). Since SSR and LTR primers, when used alone, can produce ISSRs and IRAPs, respectively, to ensure the valuable analysis of REMAPs, bands with equal molecular size among these three matrices were removed from the REMAP matrix. The appearance of a new band or the disappearance of bands in the molecular patterns of the treated plants relative to the ones of the control plants was considered polymorphism. Based on these binary data, the percentage of genomic template stability (%GTS) was determined as follows: $\mathrm{GTS}\,(\%) = [(1 - \frac{a+b}{n}) \times 100]$, where 'a' is the number of newly appeared bands in the patterns of treated plants, 'b' is the number of disappeared bands in the profiles of treated plants relative to the control and 'n' is the total number of bands amplified in the control plants.

Statistical analyses

The results were statistically analysed by two-way analyses of variance (ANOVA), the post hoc Fisher's protected least significant difference (PLSD) test, and the equality of variances F test using StatView 5.0.1 (SAS Institute Inc. Copyright) to study the effects of treatment (T), sampling date (S), and the T × S interaction. The *p*-value significance due to the individual effects and interaction was established for probabilities lower than 5% ($p < 0.05$).

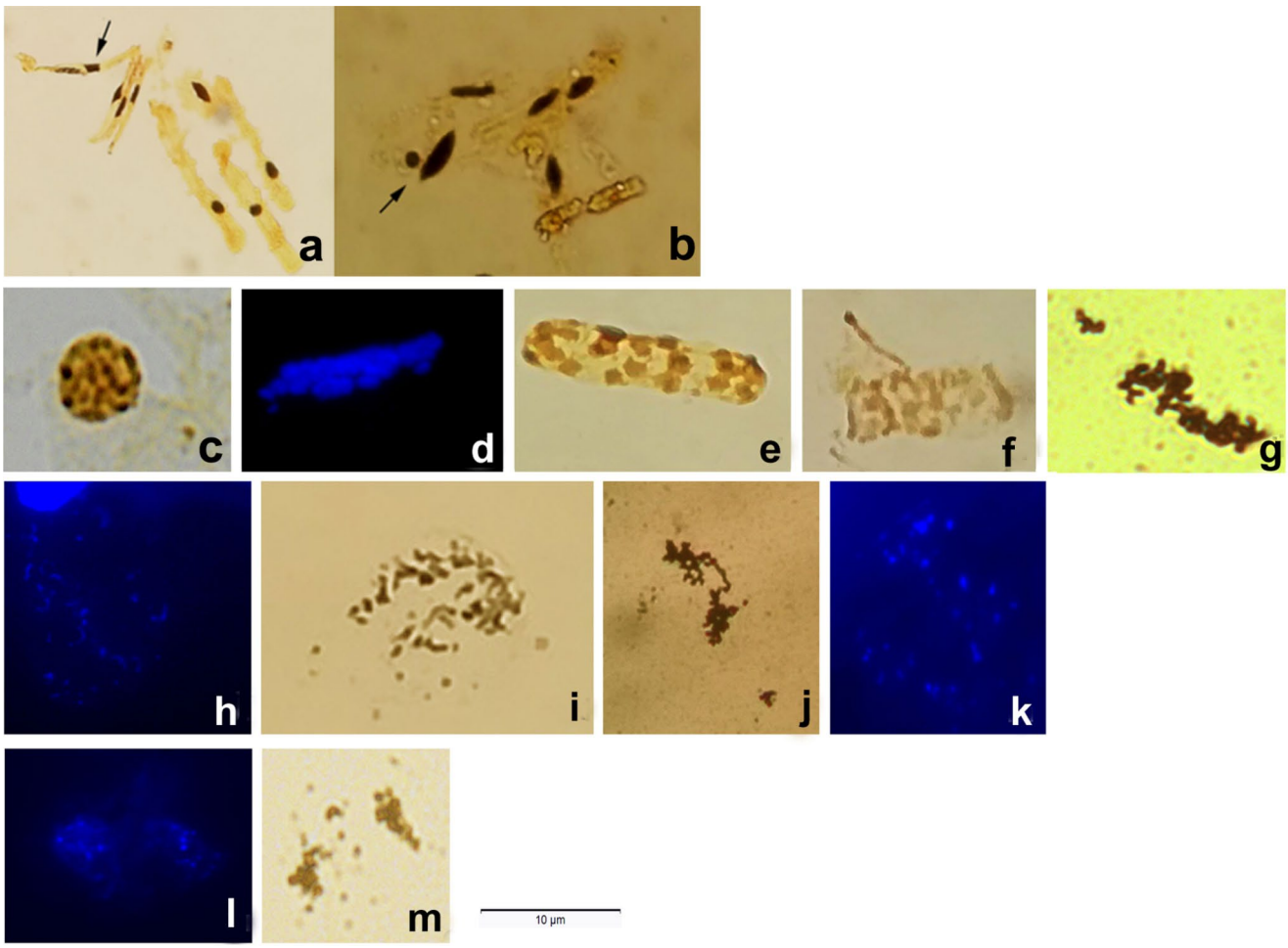

Fig. 1 Normal and irregular interphase (a, b) and mitotic (c - m) cells of *P. dulcis* stained with silver nitrate (a, b, c, e, f, g, i, j, m) or DAPI (d, h, k, l), showing: (a) one nucleolus with irregular shape (arrow); (b) one micronucleolus (arrow); (c) normal prophase; (d) normal metaphase; (e) C-mitosis; (f) sticky metaphase with one laggard chromosome; (g) metaphase with disturbed chromosomal orientation and laggard chromosomes; (h) late metaphase-early anaphase; (i) anaphase with disturbed chromosomal orientation and laggard chromosomes; (j) anaphase with disturbed chromosomal orientation and one chromatin bridge; (k) multipolar anaphase; (l) normal telophase; and (m) sticky telophase with laggard chromosomes

Results

Analysis of the leaf mitotic cell cycle

Normal and irregular interphase and dividing cells were observed in the preparations of each treatment and sampling date (Fig. 1). Among the 7,556 interphase cells scored, 19.1% were irregular and showed mostly nucleoli with irregular shape but also the presence of one or more micronucleoli (Fig. 1a, b; Online Resource 2). Among the scored 5,569 mitotic cells, 54.1% showed various types of anomalies (Fig. 1e-k, m).

The mean number of normal and irregular interphase cells showed statistically significant differences ($p < 0.05$) among treatments (T), sampling dates (S), and their interaction (T × S) (Online Resource 2). For each treatment and sampling date, the mean number of normal interphase cells was higher than that of the irregular ones (Online Resource 2). The highest mean number of normal interphase cells was detected in the AA treatment, June, and the AA × June interaction (Online Resource 2). The mean number of interphases with irregularly shaped nucleoli was lower in AA × June and AA × August than in control × June and control × August interactions (Online Resource 2). Also, the mean number of interphases with micronucleoli was lower in the interactions: AA × June, BE × July, and BS × August than in the respective control × sampling date interaction (Online Resource 2).

Concerning the average MI values, the leaves treated with BS and AA in June showed a significantly higher value than the control trees (Fig. 2; Online Resource 3).

In July, only the AA treatment increased significantly the average MI relative to the control (Fig. 2; Online Resource 3). In August, after three applications of AN, AA, and BE, the treated trees showed average values of MI that were significantly higher than those found in the control trees (Fig. 2; Online Resource 3). For the analysis of the %DCA, we looked at each treatment and verified a gradual increase from June to August in trees treated with AN (Fig. 2; Online Resource 3). The control trees showed the best results in July and August: the highest MI and the lowest %DCA values (Fig. 2; Online Resource 3). Apart from the control, the trees that showed the lowest %DCA average values were treated with BE in July and AA in August (Fig. 2; Online Resource 3). The highest average value of %DCA (68.74%) was registered in the AA × July interaction, but it diminished drastically and significantly ($p < 0.05$) in the AA × August interaction (30.58%).

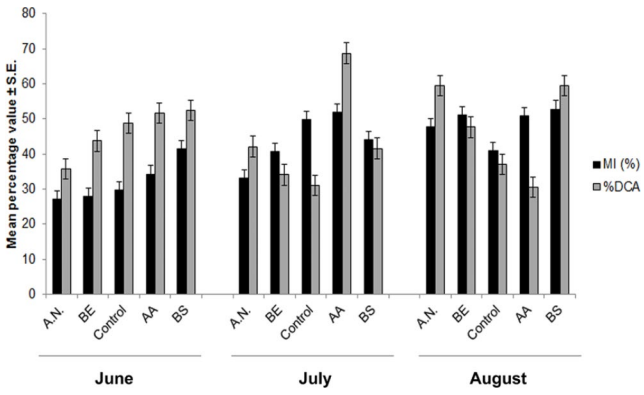

Fig. 2 Mean percentage values (± standard error, S.E.) of mitotic index (MI) and dividing cells with anomalies (DCA) determined for the treatment × sampling date (T × S) interaction

Most normal and irregular dividing cells were in prophase (Online resource 4). Nonetheless, the mean numbers of normal and/or irregular prophase cells found in the BE × August, and AN × August interactions were lower than those observed in control × August interaction (Online Resource 4). The highest average numbers of normal telophase cells were verified in July in BS and BE treatments and in the interactions control × July and AA × July (Online Resource 4). The mean number of irregular prophase, metaphase, anaphase, and/or telophase cells showed statistically significant ($p < 0.05$) differences among treatments, sampling dates, and their interactions (Online Resource 4). The highest average numbers of irregular telophase cells were detected in control, AA, and BS treatments, in July and August and the AA × July, and BS × August (Online Resource 4).

Independently of the mitotic phase, each irregular dividing cell showed one or more types of chromosomal and/or mitotic spindle anomalies (Fig. 1; Online Resource 5). Chromatin with stickiness was observed in all mitotic phases (Online Resource 5). We detected statistically significant differences ($p < 0.05$) among treatments, sampling dates, and their interaction for each type of anomaly.

Estimation of the genomic template stability (GTS)

A reduced polymorphism among individual trees that received the same treatment was observed (Online Resource 6). This result supported our decision to make DNA pools of six individuals per treatment. The individual and combined primers were selected based on their successful amplification and production of polymorphic patterns among the DNA pools of the control and treated plants that were sampled from June to August (Fig. 3).

The detection of polymorphisms (newly appeared and/or disappeared bands) in the molecular patterns of the treated plants, relative to the ones found in the control plants, was considered a decrease in the genomic template stability (GTS). The GTS (%) was determined for each treatment

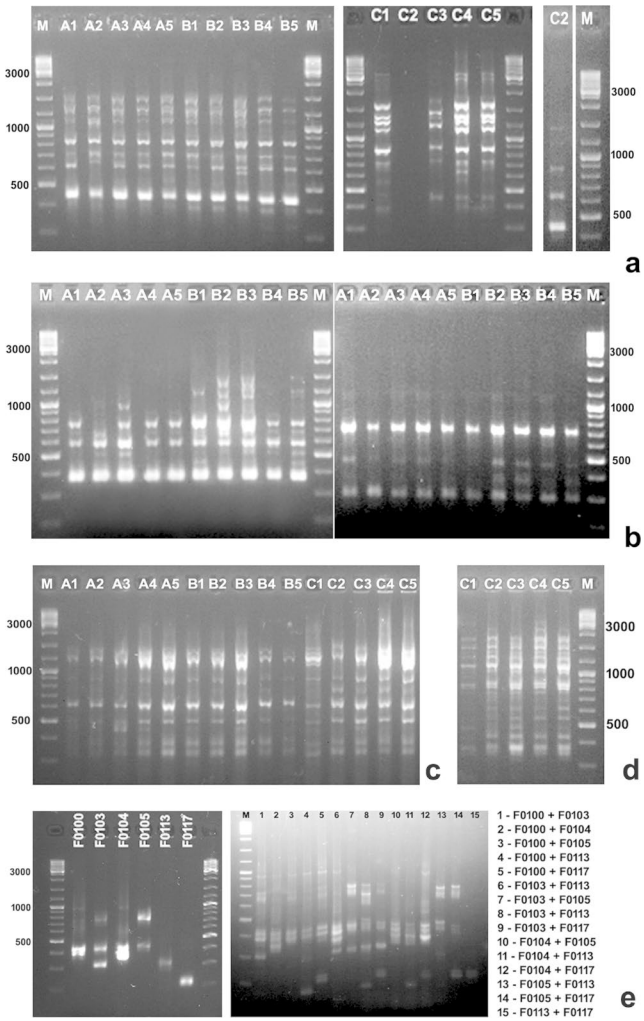

Fig. 3 Polymorphic molecular patterns achieved with (a) RAPD; (b) ISSR and (c - e) RTNs-based markers in the DNA pools of *P. dulcis* trees treated with AN (1), AA (2), BE (3) and BS (4), sampled in June (lanes A1, A2, A4, A5), July (lanes B1, B2, B4, B5) and August (lanes C1, C2, C4, C5), and detected after comparison with the patterns of the control DNA pools (lanes A3, B3 and C3). (a) RAPDs amplified with primer OPB-11; (b) ISSRs amplified with primers UBC857 and UBC888; (c) REMAPs amplified with primers *Sukkula* + *UBC857;* and (d) IRAPs amplified with *Sukkula*. (e) Testing of individual PBS primers (left) and their 15 possible combinations (right) in the same DNA pool to amplify iPBS markers. M – Molecular weight marker GeneRuler DNA Ladder Mix (Thermo Scientific, ThermoFisher Scientific)

and sampling date with the different marker systems (Online resource 7).

The ISSR and RAPD markers amplified more bands in control than the RTNs-based markers (Online resource 7). The higher GTS values were determined with: (i) ISSRs in the DNA pools of trees treated with BS on all sampling dates; (ii) RAPDs in the treatments AN and BE in July, and in the AA treatment in June; (iii) IRAPs in the treatment BS in August and July; (iv) REMAPs in the treatments BE and AA in June and August, respectively; and (v) iPBS in treatment AN in July, and the treatments BE and BS in August (Online resource 7). Excluding the BS treatment applied once, we did not verify an increase in the GTS values with the number of applications. Nevertheless, regarding the hottest months, July and August, the highest GTS values were reached in plants treated with boron (BE and/or BS) (Online resource 7).

Discussion

The wide use of PBs and fertilizers in organic and conventional agriculture requires in-depth knowledge of their impacts on target and non-target species and the environment (Calvo et al. 2014; Silva et al. 2020; Klein et al. 2021). Additionally, given the vast offer of commercial products, the development and application of rapid tests are needed to select the most suitable treatment for each species, variety, and/or environmental conditions where it is growing.

The proper functioning of plant biological processes depends on cell division and molecular stability (Thomas 2017). As far as we know, this work constitutes the first cytogenomic evaluation performed on *P. dulcis* trees treated with PBs and boron-based fertilizers. The molecular mechanisms underlying the cell cycle regulation in plants under stress are not entirely unravelled. The study of growing organs such as leaves has contributed to its understanding (Qi and Zhang 2020). In this work, we isolated mesophyll dividing cells from *P. dulcis* leaves using the same previously developed protocol for *V. vinifera* (Carvalho et al. 2018). This approach successfully evaluated the studied trees' nucleolar activity and mitotic cell cycle.

The interphase cells with irregularly shaped nucleoli or micronucleoli were more frequent in the AA treatment. Similar irregularities were found in other plant species in response to different abiotic stresses, such as heat, drought, salt, and exposure to agrochemicals or excess micronutrients (Berendsen et al. 2012; Nefic et al. 2013; Pekol et al. 2016; Carvalho et al. 2018, 2019). The nucleolus responds to abiotic stress with changes in its area, number, composition, and structure (Berendsen et al. 2012; Pekol et al. 2016; Carvalho et al. 2019). However, the interphase irregularities do not permanently impair nucleolar activity and protein synthesis (Carvalho et al. 2019).

When plants are exposed to abiotic stress, the checkpoints of the cell cycle are activated, leading to the arresting of cells in the G1/S phase and the up-regulation of inhibitors expression, making mitosis more prolonged (Qi and Zhang 2020). This assumption explains the highest average values of normal interphase cells detected in June when a single foliar treatment of PBs and BE was accomplished. The increase of interphase cells implies a reduced MI that

impairs the leaf and root growth (Glosh et al. 2016; Pekol et al. 2016; Qi and Zhang 2020; Klein et al. 2021). Additionally, most of the dividing cells were in prophase, indicating cell cycle arresting. Nonetheless, the highest mean number of normal prophases was detected in the control × July interaction. The studied *P. dulcis* trees were under summer stress (a combination of high temperature, water deficit and high irradiation), which can explain this result. Despite the high percentage of prophases, dividing cells into other mitotic phases were also seen (Online resource 4). The highest mean values of telophase cells were found in the two boron-based treatments, BE and BS, suggesting the progression and completion of the mitotic cell cycle. Besides, some of the anomalies detected in the control trees were absent in trees treated with BE and BS.

From June to August, with the monthly application of the PBs and BE, the average MI increased, leading to the significant reduction of interphase cells, probably due to the mitigation of the negative impacts of abiotic stress. Although the AA, BS, and/or BE treatments showed the highest average values of MI on all sampling dates, we gave particular attention to the %DCA values because the MI calculation encloses the number of irregular dividing cells. Hence, the lowest %DCA value was shown by AA-treated trees in August. Based on the global cytogenetic data, the treatments that enhanced the MI, the regularity of the mitotic cell cycle, and showed the lowest frequencies of anomalies, were AA and the two-boron-based fertilizers, BE and BS.

The molecular patterns with reduced polymorphism verified among trees of the same treatment, including the control, can be explained by the use of cuttings clonally propagated (Gray 2003). Therefore, we prepared DNA pools of six plants per treatment which allowed the test of a higher number of primers and marker systems. Per sampling date, the DNA polymorphisms observed in the molecular patterns of the treated trees in comparison with the control were considered a result of the treatment practiced.

The RAPD and ISSR markers were highly discriminative in this work since they amplified the highest number of polymorphic bands among the DNA pools.

The plant genome responds to stress by activating transposable elements (Kumar and Hirochika 2001). Hence, the changes detected in molecular patterns produced by RTNs-based markers can express molecular instability (Yigider et al. 2016, 2020). However, in this work, all the RTNs-based marker systems, including the iPBS performed in *P. dulcis* for the first time, revealed high GTS values, particularly in the DNA pools of trees treated with AA, BE and BS.

The molecular data indicated higher GTS values in *P. dulcis* trees treated with AA, BE, and BS treatments, corroborating the cytogenetic results.

Conclusions

As far as we know, this study's novelty relies on cytogenomic evaluation of *P. dulcis* trees treated with PBs and boron-based fertilizers using approaches applied for the first time in this species, namely, the analysis of the leaf mitotic cell cycle and amplification of iPBS markers.

The cytogenetic and molecular data revealed that the individual AA, BE, or BS treatments conferred higher mitotic index, regularity of cell division, and molecular stability to the *P. dulcis* trees of the cv. 'Vairo' growing in the NE of Portugal, probably due to an increase in the tolerance to the summer stress common in this region. The gathered information can be valuable for other almond orchards growing under similar edaphoclimatic conditions. The approaches used in this work constitute fast screening methods to evaluate the effectiveness of PBs and boron-based fertilizers in *P. dulcis* and other plant species.

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Authors' contributions Ana Esteves and Ana Carvalho realized the cytogenetic and molecular analyses. Ana Esteves, João Roque, Manuel A. Rodrigues and Carlos M. Correia made the fieldwork, samples collection and/or maceration. Ana Carvalho and José Lima-Brito were responsible for the conception of the study and analyses of the cytogenomic results. All authors were involved in the writing and revision of the manuscript and approved the submission of the final version.

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Data Availability All data generated or analysed during this study are included in this article and its supplementary information files.

Code Availability Not applicable.

Declarations

Conflict of interest/ Competing interests The authors declare that there are no conflicts of interest.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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