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Assessing the Effect of Plant Biostimulants and Nutrient-Rich Foliar Sprays on Walnut Nucleolar Activity and Protein Content (*Juglans regia* L.)

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Abstract: The cultivation of walnuts (*Juglans regia* L.) has become increasingly popular worldwide due to the nutritional value of the nuts. Plant biostimulants (PBs) and nutrient-rich products have been increasingly used in agriculture to improve yield, quality, and abiotic stress tolerance. However, farmers need fast laboratory studies to determine the most suitable treatment per crop or ecosystem to take full advantage of these products. Evaluating nucleolar activity and protein content can provide clues about the most appropriate treatment. This study aimed to determine how five commercial products, four PBs based on seaweed extract and/or free amino acids and one boron-enriched fertiliser used as foliar sprays, affect walnut cv's nucleolar activity and protein content. "Franquette" from an orchard located in NE Portugal was compared to untreated (control) plants. All treatments brought a low leaf mitotic index. The control showed the smallest nucleolar area, highest protein content, and highest frequency of nucleolar irregularities. Fitoalgas Green®, Sprint Plus®, and Tradebor® showed the highest nucleolar area and lowest frequencies of nucleolar irregularities. The recruitment of proteins/enzymes for response against abiotic stresses may explain the high protein content in the control. Hence, the enhanced abiotic stress tolerance of the treated trees explains their lower protein content and frequency of nucleolar anomalies. Globally, the Fitoalgas Green®, Sprint Plus®, and Tradebor® seem better suited for "Franquette" walnut trees under the edaphoclimatic conditions where trials were conducted.

Keywords: cytogenetics; fertilisers; nucleolus; plant biostimulants; protein content; walnut

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1. Introduction

Juglans regia L., commonly known as walnut, is one of the most cultivated nut-producing species in the world. Walnuts are healthy due to their high nutritional value [1–4]. The interest in cultivating walnut trees for fruit production in Portugal has risen due to recent market tendencies [5]. Walnut is the third most cultivated nut species in Portugal, occupying approximately 5% of the total area of cultivation of nut trees [5]. The production of walnuts in Portugal is roughly four thousand tons per year, with a growing tendency in recent years, and it is located essentially in the Northern and Alentejo regions [5].

The growing world population and climate change are causing the demand for higher crop productivity and food security to increase [6]. However, such pressure imposes challenges to agriculture in terms of reliability, sustainability, and maximisation of productivity

while reducing negative impacts on human health and the environment [7–9]. An innovative technology to tackle these issues is the foliar application of plant biostimulants (PBs), which, in general, can enhance or modify physiological processes to optimise plant growth, productivity and abiotic stress tolerance [6–13]. Despite producers' and enterprises' wide use of PBs, further knowledge about their effects on the plant and environment should be gathered to contribute to treatment efficacy and food security.

PBs are complex mixtures of raw substances with synergistic/antagonistic effects whose effectiveness depends on various factors, such as the plant genetic variability, environmental conditions, and mode of action, limiting the categorisation and commercialisation of new formulations [6,12,14–16]. There are few PBs for which a specific biochemical or molecular target and mode of action have been identified [12]. However, for a small subset of PB, their impacts on general biochemical and molecular pathways and physiological processes were designated as "mechanisms of action" by [12]. As PBs have not been characterised enough at specific levels to establish their modes of action, transcriptomics, proteomics, metabolomics, and high-throughput phenotyping have been contributing to unravelling their mechanisms of action [6]. These authors reviewed the mechanisms of action of PBs at the molecular and cellular levels (e.g., plant mineral nutrition, primary and specialised metabolism, photosynthetic processes, oxidative metabolism, signalling-related processes) and at the whole-plant level (germination, root and shoot growth and morphology, flowering, fructification, and fruit quality) [6].

The benefits of PBs may vary among crops, especially the open-field fruit-producing species where the application of such products aims to increase production [7].

Micronutrients, such as boron, are required by plants in reduced dosages, and their excessive application can lead to toxicity [17–21]. Micronutrient deficiencies are constraints that plants experience and can be exacerbated by genetic and edaphoclimatic factors. Boron deficiency in crops is among the most widespread, particularly in plants cultivated in poor soils such as NE Portugal [18,20,21]. An effective delivery technique that can correct boron deficiency is foliar spraying during spring. Depending on the plant species and supply mode of this micronutrient, boron can have a higher or lower mobility in the phloem upon being absorbed by roots or leaves. The foliar application of boron alone or combined with zinc was previously performed in walnuts. It contributed positively to flowering and fruit sets, considering their crucial roles in pollen germination and pollen tube growth for yield improvement [22]. Boron is required for higher plants' proper physiological and metabolic functioning. Still, its primary role has been the synthesis and maintenance of the structure and integrity of cell walls, which results in substantial barrier microorganisms, improving the plant tolerance to biotic stresses [19,23]. Boron also enhances cell division and elongation, which is also essential in leaves since it contributes to the enlargement of the photosynthetic area, improving productivity or promoting root growth, whose deepening is crucial in dry soils [19,22,23]. This micronutrient is also related to the functioning and structure of the plasma membrane, which is involved in enzymatic reactions and the transport of ions, metabolites, sugars, hormones, and other molecules [19,22,23]. In situations of boron deficiency, DNA synthesis is inhibited, impairing cell division in growing organs, cell differentiation, translation, and transcription [19].

PB- and boron-based fertilisers enhance the biomass of growing organs and the whole plant through the improvement of nutrient use efficiency, including micronutrients such as boron, which is highly involved in cell division and regulation, DNA synthesis, and stimulation of enzymatic activity and protein synthesis. For this work, we hypothesised that the cytogenetic evaluation of leaves sampled in walnut trees treated with these products would provide new insights about their effects.

Leaves are the plant organs that are most exposed to abiotic stresses. Moreover, leaf damage impairs the photosynthetic apparatus and process. Therefore, the cytogenetic analysis of mesophyll cells under environmental constraints or treatments with various products can reveal how external stimuli impact leaves on a cytological level. Examining the mesophyll cell cycle in interphase and dividing cells of grapevine and almond revealed

the effects of abiotic stress and/or the selection of the best leaf treatment [24–27]. Nevertheless, angiosperm species have chromosomes with a reduced size, which makes cell cycle analysis difficult. Therefore, taking into consideration the optimisation of protein synthesis as a consequence of PB- and boron-based fertilisers as well as the functions of the nucleolus in protein synthesis, cell cycle regulation, and sensing intracellular stress [28], we proposed an alternative cytogenetic study based on nucleolar activity. The staining of mitotic preparations with silver nitrate is required to study the transcriptionally active nucleoli. The staining of nucleoli and nucleolar organiser regions (NORs) with silver nitrate in interphase and metaphase cells, respectively, enables the analysis of RNA (rRNA) gene expression, which influences the ribosomal production and rate of protein synthesis [24–27]. However, the reduced chromosome size of angiosperm species impairs NOR visualisation. Nonetheless, the study of nucleolar activity in plants constitutes a fast approach to assess cytotoxicity caused by abiotic stresses in grapevine [25–27] or excess of micronutrients used in bread wheat seed biofortification [29], as well as to evaluate the suitability of foliar treatments [24,25], including biostimulants and boron-based fertilisers, performed in *Prunus dulcis* (almond) [24]. Nucleolar activity analysis has been used to verify how the induced abiotic stress impacts the nucleolar size, morphology, and number of different plant species [24–27,29–33]. With this in mind, for the present study, we hypothesised that the tested products (biostimulants and mineral-rich foliar sprays), certified for use in organic farming, can induce a stable nucleolar activity and improve the number of total soluble proteins of walnut trees, cv. "Franquette," grown in an NE Portugal organic orchard relative to the control (untreated leaves), for further selection of the best treatment.

2. Materials and Methods

2.1. Experimental Site and Nutritional Status of the Analysed Walnut Trees

The experiment was carried out in an organic walnut orchard composed of adult (20 years old) trees of the cv. "Franquette" with drip irrigation and a 7 × 7 m distance between trees. The orchard is located at Vale da Porca, Macedo de Cavaleiros, NE Portugal (41°54′34″ N, −6°88′91″ W, 583 m above sea level). Considering the Köppen–Geiger Climate Classification [34], the climate of the region is of the Csb type (warm-summer Mediterranean climate), characterised by hot summers and rainy winters [35]. The values of the climatological normal for monthly average temperature and accumulated precipitation, as well as the values recorded in 2019, are presented in Figure 1.

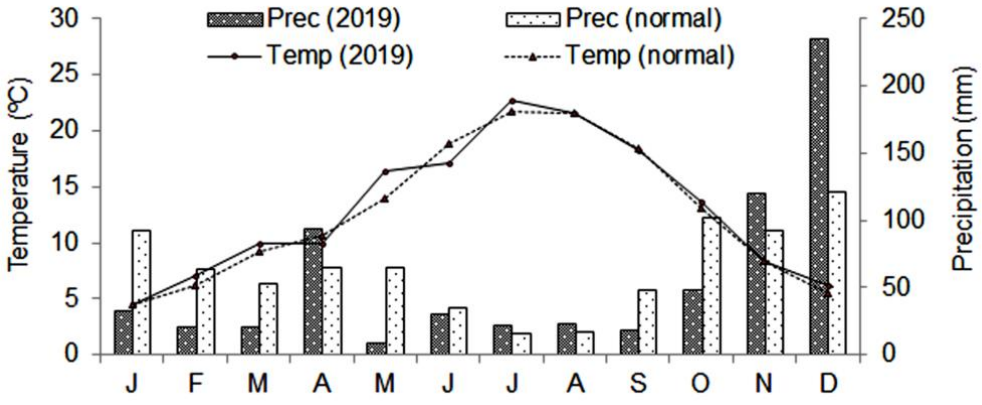

Figure 1. Climatological normal, monthly average temperature, and accumulated precipitation from a weather station near the experimental plot.

The soil in the plot where the experiment occurred is a eutric fluvisol with a sandy loam texture. Its organic carbon content is 24.5 g kg^{-1}, pH (H_2O) is slightly acidic (6.3), and phosphorus and potassium levels, determined by the Egnér–Riehm method, are medium (85.0 mg kg^{-1}, P_2O_5) and very high (406.0 mg kg^{-1}, K_2O), respectively. The cation exchange capacity is high (21.2 $cmol_+$ kg^{-1}).

In the previous growing season, the adult walnut trees of the plot where the experiment took place showed nitrogen and boron deficiencies and adequate concentrations of other nutrients. The nitrogen and boron concentrations in the leaves were 21.8 g kg^{-1} and 17.0 mg kg^{-1}, with sufficiency ranges established at 25–32.5 g kg^{-1} and 35–200 mg kg^{-1} [36], respectively.

2.2. Fertilising Materials and Treatments

This study featured the testing of five commercial products for foliar application, namely, Basfoliar® K premium (Compo Expert India Pvt. Ltd., Kharadi, Pune, India), Tradebor®, Sprint Plus®, Fitoalgas Green®, and Stimulus® (all from Tradecorp Nutri-Performance, Lisbon, Portugal). As a control treatment, foliar spraying was applied only with water. These products were chosen based on their widespread use among the walnut producers of the region due to the advantages advertised by their manufacturers and to test diversity in the type of product to enrich the experimental design.

The composition of the commercial products was the following: (i) Basfoliar® K premium (25% *w*/*v* K_2O, 2% *w*/*v* P_2O_5, 1% boron, 18% *w*/*v* soluble organic carbon, and 3% seaweed extract of *Ecklonia maxima*) at a dosage of 8 L/ha, (ii) Tradebor® (15.4% *w*/*v* B) at a dosage of 2L/ha, (iii) Sprint Plus® (28.8% *w*/*v* of free amino acids, 10.8% *w*/*v* of nitrogen, 44.4% of organic matter, and 28.8% organic carbon) at a dosage of 4 L/ha, (iv) Fitoalgas Green® (16.5 *w*/*v* seaweed extract of *Ascophyllum nodosum*) at a dosage of 3 L/ha, and (v) Stimulus® (6.6% *w*/*v* seaweed extract of *Ascophyllum nodosum*, 4% *w*/*v* of nitrogen, and 10.6% *w*/*v* free amino acids) at a dosage of 3 L/ha. The manuscript will refer to these treatments as SWE_{EM}, BE, AA, SWE_{AN}, and SWE-AA, respectively, and the control as C. The experiment was arranged as a completely randomised design, where each product (Basfoliar® K premium, Tradebor®, Sprint Plus®, Fitoalgas Green®, and Stimulus®) was applied according to the manufacturer-recommended dosage on three replicates, constituted by two adult trees with homogenous canopy dimensions. All spray applications were carried out according to standard procedures suitable for agricultural experiments to ensure good efficacy. The experiment was designed to avoid the over-spraying of non-target trees during the foliar spraying of the treated trees. Also, the foliar treatments were carried out on non-windy days. The foliar applications were made on the 21 June and 9 and 26 July of the 2019 growing season.

2.3. Leaf and Walnut Sampling

Six young leaves were sampled per treated and untreated (control) walnut tree on two sampling dates, namely, 25 July and 27 August. The leaves were collected and immediately fixed in acetic ethanol (3:1, *v*/*v*). Upon transport to UTAD, the fixed leaves were maintained at −20 °C until the preparation of chromosome spreads using the procedure described by [24].

Walnuts were harvested in treated and untreated trees on 24 October.

2.4. Nucleolar Activity Evaluation

The mitotic spreads were prepared with a small fragment (0.5 cm^2) of fixed leaf using a squashing method [37].

The slides were silver stained following the protocol described by [33] and observed with an optical microscope. The normal and irregular interphase cells identified in 50 observation fields per mitotic slide and treatment were scored. Three mitotic slides were performed per treatment, corresponding to three biological replicates. The anomaly types of irregular interphase cells were identified. The cytogenetic data were used to determine the percentage of irregular interphase cells with anomalies (%ICA) using Equation (1).

$$\mathrm{ICA}(\%) = \left(\frac{\text{no.irregular interphase cells}}{\text{total no.of interphase cells}}\right) \times 100 \quad (1)$$

Image capturing was performed on an Olympus BX41 microscope (Olympus America, New York, NY, USA) using a CCD digital camera XC10 (Olympus America, New York, NY, USA), a magnification of 1000×, and the cell Sens Entry 1.7 software (Olympus Soft Imaging Solutions GmbH, Münster, Germany). The number of nucleoli per cell was scored, and the nucleolar area (μm^2) was measured in interphases with one to two nucleoli with a regular shape using the Digimizer Image Analysis software (MedCal, Ostend, Belgium) and the scale bar (50 μm).

2.5. Concentration of Total Soluble Protein

Due to its speed and sensitivity, Bradford's method was used to determine the total soluble protein concentration [38]. The samples consisted of three to four milled walnut kernels sampled in different trees per treatment. Eight aqueous bovine serum albumin (BSA) solutions were prepared with 0 to 200 μg mL^{-1} concentrations. One volume of 1.4 mL of extraction buffer, constituted by 50 mM phosphate buffer (KH_2PO_4), pH 7.5, EDTA 0.1 mM, PMSF ($C_7H_7PO_2S$), and 2% insoluble Polivinyl pyridine, was added to 20 mg of sample. This mixture was centrifuged at 12,000 rpm for 30 min at 4 °C. Per sample, 20 μL of the supernatant were placed on a 96-well microplate and three technical replicates were performed. The same volume of extraction buffer and procedure were used for the BSA dilutions. Per well, 200 μL of Bradford's reagent were added. The samples and BSA dilutions were maintained at room temperature for 15 min in the dark. The absorbance (A) of each sample and BSA dilution was read at 595 nm using the Power Wave XS2 microplate spectrophotometer (BioTek® Instruments, Agilent Technologies, Inc., Santa Clara, CA, USA), the Gen5™ v.1.10 Microplate Reader and the Imager Software (BioTek®Instruments, Agilent, Technologies Inc., Santa Clara, CA, USA). The absorbance (A) values of the BSA dilutions were used to construct a standard curve, whose line equation ($y = ax + b$) enabled the determination of the concentration of total soluble proteins (x values) by replacing the y with the A values.

2.6. Statistical Analyses of the Data

The effects of treatment (T), sampling date (S), and (T × S) interaction on the nucleolar activity (mean nucleoli number per interphase cell and nucleolar area) and protein content were statistically analysed. The software IBM SPSS Statistics for Windows (Version 23.0. Armonk, NY, USA: IBM Corp) was used for these purposes.

The data satisfied the homogeneity of variances and normality assumptions required for the one-way ANOVA analysis. The post hoc Tukey test also analysed the cytogenetic and biochemical data. For all statistical tests, a significance level of 95% was considered. Therefore, significant differences were assigned to probabilities lower than 0.05 (alpha = 0.05).

3. Results

3.1. Nucleolar Activity

Each interphase nucleus of *J. regia* cv. "Franquette" presented one to three nucleoli (Figure 2). Among the 21,212 scored interphase cells, 20,444 (96.38%) were normal and 768 (3.62%) were irregular. Normal and irregular interphase cells were observed in all treatments and sampling dates (Table 1).

The number of normal interphases was high in all treatments and both sampling dates and there were no statistically significant differences ($p > 0.05$) among treatments or sampling dates; however, significant differences ($p < 0.05$) between each pair of T × S interactions per treatment were found (Table 1).

The irregular interphase cells presented two types of anomalies: (i) nucleoli with an irregular shape and (ii) nucleoli with the presence of micronucleoli, and the former was more frequent (Figure 2; Table 1). The mean number of interphase cells with each type of anomaly did not show statistical significance ($p > 0.05$) among treatments, sampling date, or their interaction (Table 1).

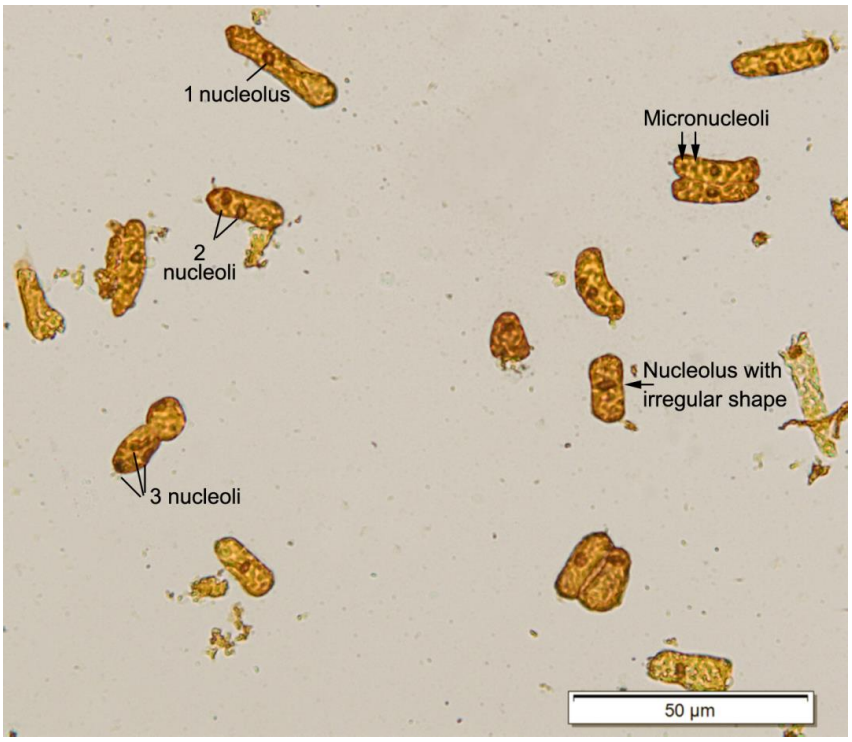

Figure 2. Normal and irregular *J. regia* interphase cells stained with silver nitrate showing a variable number of nucleoli (one to three) and two types of anomalies (presence of micronucleoli and nucleolus with an irregular shape). Bar scale: 50 µm.

Table 1. Total number of normal interphase cells, mean number (±S.E.) of normal interphase cells, and mean number (±S.E.) of irregular interphase cells with two types of anomalies, determined per treatment (T) and sampling date (S) and in the T × S interaction. Different lowercase letters among treatments (T) and T × S interactions represent statistically significant differences ($p < 0.05$).

		Normal Interphase Cells	**Normal Interphase Cells (Mean ± S.E.)**	**Irregular Interphase Cells with:**		**% ICA (Mean ± S.E.)**
				Irregularly Shaped Nucleoli (Mean ± S.E.)	**Micronucleoli (Mean ± S.E.)**	
T	Control	3163	451.86 ± 37.58	29.57 ± 10.00	0.43 ± 0.297	0.082 ± 0.027 [b]
	SWE_{EM}	3618	603.00 ± 64.42	3.67 ± 1.12	0.00 ± 0.000	0.009 ± 0.003 [a]
	SWE_{AN}	2928	488.00 ± 27.09	14.83 ± 5.04	0.83 ± 0.401	0.048 ± 0.019 [ab]
	SWE-AA	3612	516.00 ± 42.57	17.57 ± 6.51	1.43 ± 0.972	0.043 ± 0.013 [ab]
	AA	3344	557.33 ± 38.00	14.33 ± 5.81	0.83 ± 0.401	0.033 ± 0.013 [ab]
	BE	3779	539.86 ± 48.99	7.86 ± 1.81	0.14 ± 0.143	0.019 ± 0.003 [ab]
S	July 2019	11,148	530.86 ± 24.69	19.67 ± 4.52	0.76 ± 0.37	0.052 ± 0.012
	August 2019	9296	516.44 ± 29.06	9.39 ± 1.81	0.44 ± 0.17	0.025 ± 0.004
T × S	Control × July	1591	397.75 ± 24.00 [a]	40.50 ± 15.55	0.25 ± 0.25	0.118 ± 0.039
	Control × August	1572	524.00 ± 64.66 [b]	15.00 ± 5.51	0.67 ± 0.67	0.034 ± 0.011
	SWE_{EM} × July	1554	518.00 ± 50.00 [a]	4.33 ± 2.33	0.00 ± 0.000	0.011 ± 0.006
	SWE_{EM} × August	2064	688.00 ± 105.00 [b]	3.00 ± 0.58	0.00 ± 0.000	0.006 ± 0.002
	SWE_{AN} × July	1460	486.67 ± 30.12 [a]	21.00 ± 8.51	1.33 ± 0.88	0.068 ± 0.034
	SWE_{AN} × August	1468	489.33 ± 52.53 [a]	8.67 ± 4.06	0.33 ± 0.33	0.028 ± 0.012
	SWE-AA × July	2202	550.50 ± 71.35 [b]	25.50 ± 9.56	2.25 ± 1.65	0.059 ± 0.018
	SWE-AA × August	1410	470.00 ± 25.54 [a]	7.00 ± 4.16	0.33 ± 0.33	0.021 ± 0.011
	AA × July	1922	640.67 ± 15.07 [b]	14.00 ± 11.50	0.67 ± 0.67	0.026 ± 0.020
	AA × August	1422	474.00 ± 7.00 [a]	14.67 ± 6.06	1 ± 0.58	0.039 ± 0.019
	BE × July	2419	604.75 ± 49.58 [b]	7.75 ± 2.32	0.00 ± 0.000	0.017 ± 0.004
	BE × August	1360	453.33 ± 73.02 [a]	8.00 ± 3.51	0.33 ± 0.33	0.023 ± 0.003
ANOVA *p*-value	T	-	n.s.	n.s	n.s.	<0.05
	S	-	n.s.	n.s.	n.s.	n.s.
	T × S	-	<0.05	n.s.	n.s.	n.s.

The treatment significantly affected ($p < 0.05$) the ICA percentage (Table 1). The lowest average ICA value (0.009) was found in the SWE_{EM} treatment. The SWE_{EM} treatment did not differ significantly ($p > 0.05$) from the remaining ones, except from the control, which presented the highest average value of ICA (Table 1). Therefore, the following analyses focused on understanding how the treatment affected the nucleolar number, area, and total protein content (Tables 2 and 3).

Table 2. Mean (±S.E.) values of nucleolar area (μm^2) determined for interphase cells with one to three regularly shaped nucleoli per nucleus (N), treatment (T), and N × T interaction. Different lowercase letters per column indicate statistically significant differences ($p < 0.001$) among nucleoli number per nucleus (N) and treatment (T) and among the 2 × T and 3 × T interactions.

		Mean Nucleolar Area (μm^2) ± S.E. (Number of Interphases Measured)
Number of nucleoli per nucleus (N)	1	7.08 ± 0.08 ^{c} (600)
	2	5.31 ± 0.18 ^{b} (136)
	3	4.27 ± 0.38 ^{a} (27)
Treatment (T)	Control	5.82 ± 0.23 ^{a}(150)
	SWE_{EM}	6.10 ± 0.15 ^{ab} (134)
	SWE_{AN}	6.71 ± 0.18 ^{bc} (126)
	SWE-AA	6.44 ± 0.19 ^{ab} (128)
	AA	6.68 ± 0.19 ^{bc} (111)
	BE	7.39 ± 0.19 ^{c} (114)
N × T	1 × Control	6.70 ± 0.28 ^{a}
	1 × SWE_{EM}	6.67 ± 0.14 ^{a}
	1 × SWE_{AN}	7.00 ± 0.20 ^{a}
	1 × SWE-AA	6.96 ± 0.20 ^{a}
	1 × AA	6.80 ± 0.20 ^{a}
	1 × BE	7.38 ± 0.21 ^{a}
	2 × Control	5.20 ± 0.36 ^{a}
	2 × SWE_{EM}	4.72 ± 0.32 ^{a}
	2 × SWE_{AN}	5.59 ± 0.35 ^{a}
	2 × SWE-AA	4.43 ± 0.27 ^{a}
	2 × AA	5.15 ± 0.76 ^{a}
	2 × BE	7.50 ± 0.48 ^{b}
	3 × Control	3.08 ± 0.29 ^{b}
	3 × SWE_{EM}	3.90 ± 0.46 ^{b}
	3 × SWE_{AN}	4.97 ± 1.16 ^{c}
	3 × SWE-AA	4.97 ± 1.16 ^{c}
	3 × AA	6.76 ± 0.28 ^{d}
	3 × BE	0.00 ± 0.00 ^{a}
ANOVA *p*-value	N	<0.001
	T	<0.001
	N × T	<0.001

Table 3. Mean concentration of total soluble protein (mg g^{-1}) (±S.E.) determined per treatment. Different lowercase letters indicate statistically significant differences ($p < 0.001$) among treatments.

Treatment	**Concentration of Total Soluble Protein (mg g^{-1})**
Control	47.13 ± 0.54 ^{d}
SWE_{EM}	30.11 ± 0.87^{b}
SWE_{AN}	20.38 ± 1.05 ^{a}
SWE-AA	26.92 ± 0.75 ^{b}
AA	37.29 ± 1.55 ^{c}
BE	36.47 ± 2.75 ^{c}
ANOVA *p*-value	<0.001

Most normal interphase cells had one or two integer nucleoli per nucleus and a regular shape (Table 2).

As revealed in Table 2, most of the interphase cells showed a single nucleolus. The highest average values of the nucleolar area were found in interphases with one nucleolus (Table 2). In contrast, the smallest nucleoli were observed in interphases with three nucleoli per nucleus (Table 2). Regardless of treatments, the mean nucleolar area decreased significantly ($p < 0.001$) with the increasing number (N) of nucleoli per nucleus (Table 2). In addition, the mean nucleolar area differed significantly ($p < 0.001$) among treatments (T) (Table 2). The BE treatment showed the highest average nucleolar area, whereas the lowest value was detected in the control (Table 2). For the N × T interactions, the highest average nucleolar area was found in interphase cells with two nucleoli × BE and the lowest was found in interphases with three nucleoli × control (Table 2). We did not find nuclei with three nucleoli in the BE treatment (Table 2). The average nucleolar area evidenced statistically significant differences ($p < 0.001$) among the two nucleoli × T interactions and the three nucleoli × T interactions (Table 2). Globally, the ANOVA indicated that the average nucleolar area was significantly influenced ($p < 0.001$) by the effects of nucleolar number (N), treatment (T), and N × T interaction (Table 2).

3.2. Biochemical Analysis

The highest mean concentration of total soluble proteins was detected in the milled kernels of the control treatment, which differed significantly ($p < 0.001$) from the remaining treatments (Table 3).

The lowest average concentration of total soluble proteins was registered in the SWE_{AN} treatment (Table 3). Overall, the ANOVA *p*-values indicated that the average concentration of total soluble proteins showed statistically significant differences ($p < 0.001$) among treatments (Table 3).

4. Discussion

Given the wide use and offer of commercial biostimulants or fertilisers to organic and conventional agriculture, it is necessary to carry out studies that deepen the knowledge of their impacts on the target crops and the environment [16,24,39,40]. Furthermore, the diverse sources of materials and the uncertain modes of action of biostimulants require the development of approaches that ensure the fast selection of the most appropriate product(s) for each species, variety, and/or environmental growing conditions [24,40]. Indeed, a biostimulant on the market should only be considered adequate under some conditions [40].

In the present work, we analysed how five individual foliar treatments, namely, four biostimulants and one boron-based fertiliser, would affect the nucleolar activity and protein content of adult *J. regia* trees from cv. "Franquette" in one organic farmed orchard in NE Portugal. The results will be used to select the most suitable treatment(s) based on cytogenetic and biochemical data. These walnut trees are cultivated in a region with stressful edaphoclimatic conditions characterised by eutric fluvisol with a soil with a sandy loam texture that is slightly acidic and experiencing extreme environmental conditions typical of a Csb climate type, namely, hot and dry summers and cold and rainy winters [35]. Moreover, the leaves of the walnut trees of this orchard, when analysed in the previous growing season, evidenced deficiency in nitrogen and boron; accordingly, the interpretation of the values which was based on [36].

Plants' biological processes depend on regular cell division and molecular stability [41], which can be perturbed under abiotic stress. Therefore, the cytogenetic monitoring of dividing mesophyll or root meristem cells, which are plant organs exposed to stressful conditions, constitutes a suitable and fast approach to selecting adequate foliar treatments [24,25] or the most stress-tolerant genotypes [26,27,33]. Moreover, such studies can also highlight cytotoxicity, which is common when biostimulants or fertilisers are used in dosages above the manufacturer's recommendation [35,37–44]. Despite the common assumption held by

producers of fertilisers and different authors that boron stimulates growing tissues [19,42] as a consequence of the enhancement of cell division, the leaf mitotic index was low despite collecting young leaves in all treatments. As [43] demonstrated, the undifferentiated cell (mitotic cell) region covers most of the leaf area in young leaves. This result may have arisen from the high temperatures of the sampling dates and preceding days (Figure 1), since the plant may have had enough time during the evening and night, when the temperature decreased, to recover from the continuous heat stress occurring for successive days, coupled with dry soil and intense light incidence on the leaves (summer stress), which is common in the northern region of Portugal [25]. However, Ref. [44] also reported a reduced mitotic index on root meristematic cells of walnuts. The small size of the *Juglans* spp. chromosomes (ranging from 2 to 3 µm) probably impairs the detection of mitotic cells. This feature can also justify the few cytogenetic studies available for the walnut species [45,46].

Given the reduced number of detectable dividing cells, we could not analyse the leaf mitotic cell cycle in the different treatments. Our cytogenetic results revealed that under abiotic stress and boron deficiency, cell division was impaired in leaves; most of the cells were in interphase, suggesting cell cycle arrest. Among the various cell cycle checkpoints, the major one is G2/M, which prevents the cell from entering mitosis and explains the reduced mitotic index. Furthermore, Ref. [42] reported that boron deficiency is first noticed in the meristems, suggesting the role of this micronutrient in signalling mechanisms during cell differentiation and organogenesis. However, these authors could not provide an in-depth correlation between aberrant development and meristem function under boron deficiency.

Cell division inhibition due to abiotic stress and/or boron deficiency compromises leaf growth, reducing the photosynthetic area and, ultimately, the tree's productivity.

Since one of the several roles of the nucleolus is its involvement in cell cycle regulation, we focused our research on the interphase cells and nucleolar activity and its implication in protein synthesis considering the fact that nucleolar parameters can serve as biomarkers to assess the intracellular stress that can be generated by incorrect or excessive dosages of biostimulants or fertilisers, as demonstrated previously by other species under abiotic stress [29,30,33,47,48], constituting the novelty of the present work.

The nucleolus responds to heat, drought, salt stress, and exposure to agrochemicals or excess micronutrients by changing its area, morphology, composition, or quantity [26,27,29–31,33,47,48]. Nonetheless, in bread wheat, we verified that the interphase anomalies did not impair the nucleolar activity or protein synthesis [29]. In this study, only the SWE_{EM} treatment significantly affected the percentage of interphase cells with anomalies (%ICA). Nonetheless, the reduced frequency of irregular cells presenting micronucleoli or irregularly shaped nucleoli did not differ among treatments or sampling dates. Most interphases were normal and showed one to three nucleoli per nucleus, except for the BE treatment, whose maximum nucleolar quantity was two. The previous and few karyotypes or ideograms of *Juglans* spp. did not refer to the number of rDNA loci or satellited chromosomes in walnuts [45,46]. Instead, it was mentioned that the satellite regions of the walnut chromosomes are hardly identifiable [45]. The detection of three nucleoli per nucleus might suggest the existence of two satellited chromosome pairs in *J. regia*, and the detection of a maximum of three nucleoli per nucleus can be due to the phenomenon of nucleolar fusion, which also results in irregularly shaped nucleoli being the most frequent anomaly. Nuclear fusion is common in plant species and frequently occurs through the interphase or telophase due to the joining of active smaller nucleoli constituting a more prominent single nucleolus [49,50]. Moreover, nucleolar fusion contributes to a larger nucleolar area. Such a parameter was significantly influenced ($p < 0.001$) by the number of nucleoli per nucleus, decreasing significantly with the increase in the nucleolar number. A similar result was reported in previous studies developed with other plant species [29,30,32]. Large nucleolar size often correlates with high rRNA transcription activity, interphase duration, and ribosomal production [51]. Changes in the nucleolar number and area in response to abiotic stress may cause alterations in the cell kinetics and

duration of the cell cycle, the rate of cell proliferation, transcription of the rRNA genes, ribosomal biogenesis, and protein synthesis [28,52].

Irregularly shaped nucleoli may also originate from the exudation of silver-stained material to the nucleoplasm, and during that process, the nucleolus assumes a different morphology [53]. Beyond the silver-stained particles with irregular shapes that are released from the nucleolus to the nucleoplasm as a consequence of nucleolar disruption [29], in plant interphase nuclei, small and spherical nuclear bodies that appear in the vicinity of the nucleolus are known as micronucleoli [54]. In this work, we detected the presence of micronucleoli in a minor frequency of interphase cells. Nuclear bodies are composed of ribonucleoproteins and RNA molecules and result from trafficking in plant nuclei whose signalling pathways, regulated by post-transcriptional modifications and abiotic stress, such as high temperature, have been the research target [55]. Unfavourable environmental conditions contribute to nucleolar morphological alterations, disruption, and DNA damage that disturb or inhibit rRNA synthesis and ribosomal assembly [29].

The average concentration of total soluble protein in the walnut kernels was determined for all treatments to understand how the nucleolar activity affected the protein synthesis. We detected the highest average concentration of total soluble protein in the control kernels, which showed the smallest nucleolar area. This biochemical result was not expected, since previous works reported increased protein content when applying biostimulants in *Phaseolus vulgaris* L. under favourable weather conditions [53]. Other authors stated that biostimulants are not always beneficial for protein content [56,57]. These contradictory assumptions, along with the results achieved in our work, demonstrate that any biostimulant treatment success is highly dependent on the edaphoclimatic conditions, plant species or variety (genotype), and their interaction. Moreover, as seen in the present work, the significant differences detected among treatments revealed that different biostimulants must be tested for a proper plant species, variety, and site. Although the highest mean values of protein content were found in the control, AA, and BE treatments, the former showed statistically significant differences relative to AA and BE, which did not differ significantly between each other. The lowest nucleolar area of the control did not compromise the protein synthesis. Similar results were found by [29] in bread wheat. However, the control treatment showed the highest frequencies of nucleolar irregularities. Concerning the role of the nucleolus as a sensor of intra- and extracellular stress and its regulatory function of the cell cycle, we attributed the highest protein content of the untreated walnut trees to the potential recruitment of antioxidant enzymes involved in the plant defence response to abiotic stresses and boron deficiency. This assumption also justifies the lower protein content evidenced by the biostimulant-treated walnut trees concerning the expected enhancement of stress tolerance and boron availability provided by these treatments. Additionally, ongoing biochemical analyses performed in walnut kernels of the same trees have revealed a higher antioxidant enzymatic activity and a lower concentration of secondary metabolites involved in the antioxidant response in the control kernels.

As reported by [23] and references therein, the deficiency in boron triggers the production and accumulation of reactive oxygen species. Although boron-deficient leaves can activate antioxidant enzymatic activity, they are not strong enough to protect against oxidative stress. Young growing tissues have an enhanced boron requirement since the starvation of this micronutrient impairs cell division and elongation, compromising the growth and development of roots, flowers, fruits, and the entire plant [23]. Oxidative stress induces DNA damage, which may decrease the origin mitotic index and various cell cycle and chromosomal anomalies [24]. Recent cytogenomic evaluation of the effects of biostimulants and boron-based fertilisers in almond cv. "Vairo" grew in similar conditions to the ones analysed here, and it was revealed that individual AA or BE treatments, as well as the application of solid boron on the soil, allowed an increase in leaf mitotic index, more regular mitosis, and molecular stability [24]. Based on the overall data, in this work, we also considered the BE, AA, and SWE_{AN} to be the most suitable for the cv. "Franquette" walnut trees.

5. Conclusions

As far as we know, this study's novelty relies not only on the cytogenetic evaluation of *J. regia* leaves but also on the applications of biostimulants performed on the sampled trees for the first time in this species. At least one previous work reported the effects of one boron-based fertiliser treatment in walnuts [22].

This approach can provide a reliable and fast analysis of the effects of biostimulant treatments, bringing new insights to the state of the art, and has the additional advantage of being suitable for studying woody species growing in natural field conditions, where leaf sampling is the most viable option. The cytogenetic results revealed that the treatment's concentrations in the field trial had no adverse effects. Nonetheless, they were not optimal for increasing the leaf mitotic index, which could have been low due to sampling dates corresponding with extreme summer stress. However, the nucleolar activity was quite regular, and the reduced frequencies of nucleolar anomalies did not hamper protein synthesis. Although the highest total protein content was found in the control treatment, it also presented the highest average value of %ICA. The higher nucleolar area and/or protein content values were detected in the BE, SWE_{AN}, and AA treatments, which also presented the lowest %ICA values. Based on the present results, the BE and AA treatments, and ultimately the SWE_{AN}, can be considered suitable for walnut trees of cv. "Franquette" for these specific edaphoclimatic growing conditions.

Author Contributions: J.R. carried out the cytogenetic and biochemical methodologies and analysed their results. J.R., M.Â.R. and C.M.C. did the fieldwork and sample collection. A.C. and J.L.-B. conceptualised the study and acquired resources for the cytogenetic analyses. M.Â.R. and C.M.C. also contributed to resource acquisition. All authors were involved in the writing and revision of the manuscript, and the submission of the final version was approved. All authors have read and agreed to the published version of the manuscript.

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