Phenolic Compounds Regulating the Susceptibility of Adult Pine Species to Bursaphelenchus xylophilus

Cândida Sofia Trindade 1,2,*, Sara Canas 3,4, Maria L. Inácio 1,5, Santiago Pereira-Lorenzo 2,*, Edmundo Sousa 1,5 and Pedro Naves 1,5

1 Instituto Nacional de Investigación Agraria e Veterinaria, INIA-Oeiras, Quinta do Marquês, 2780-157 Oeiras, Portugal; lurdess.inacio@iniaiav.pt (M.L.I.); edmundos.souza@iniaiav.pt (E.S.); pedro.naves@iniaiav.pt (P.N.)
2 USC—Department of Crop Production and Projects of Engineering, University of Santiago de Compostela, 15705 Santiago de Compostela, Spain; santiago.pereira.lorenzo@usc.es
3 Instituto Nacional de Investigação Agraria e Veterinária, INIA-Dois Portos, Quinta da Almoinha, 2565-191 Dois Portos, Portugal; sara.canas@iniaiav.pt
5 GREEN-IT Bioresources for Sustainability, ITQB NOVA, Av. da República, 2780-157 Oeiras, Portugal

* Correspondence: candida.trindade@iniaiav.pt

Abstract: Pine wilt disease (PWD), caused by the pinewood nematode (PWN) Bursaphelenchus xylophilus, is one of the most destructive diseases in trees of the genus Pinus and is responsible for significant environmental and economic losses in North America, Eastern Asia, and Western Europe. However, pine species are not equally affected, with some being tolerant/resistant while others are susceptible to nematode infection for reasons still unclear. The present study aims to investigate differential chemical responses of susceptible and tolerant/resistant pine species shortly after nematode infection by characterizing the phenolic profiles of adult Pinus sylvestris, Pinus pinaster, Pinus pinaster, and Pinus halepensis. HPLC and LC-MS were used to identify and quantify the pine’s phenolic compounds: gallic acid, ferulic acid, taxifolin, rutin, resveratrol, (+)-secoisolariciresinol, (-)-epicatechin, protocatechuic acid hexoside, gallic acid hexoside, ferulic acid glucoside, quercetin hexoside, and two unidentified compounds (#A and #B). Prior to infection, we could not differentiate between nematode-tolerant/resistant and susceptible adult pine species based on their constitutive phenolic compounds. In the presence of the PWN, the phenolic profile allowed for a noticeable separation of the PWN-tolerant/resistant P. halepensis from the susceptible P. sylvestris, contrasting with a more homogenous response from P. pinea and P. pinaster. Observations on P. halepensis suggest that taxifolin, resveratrol, and rutin may have an active role in protecting against B. xylophilus, possibly in conjugation with other biochemical and anatomical characters. We emphasize the importance of studying pine tolerant/resistance on adult trees, and not on excised branches, saplings, or seedlings to accurately simulate the nematode–pine host interactions occurring under natural conditions.

Keywords: pine wood nematode; pine wilt disease; Pinus spp.; pine resistance; secondary metabolites

1. Introduction

Pine wilt disease (PWD), caused by the pine wood nematode (PWN) Bursaphelenchus xylophilus (Steiner & Büher, 1934) Nickle, 1970, is one of the most devastating diseases affecting pine forests worldwide [1,2]. The PWN is dispersed by insect pine sawyers of the genus Monochamus (Coleoptera: Cerambycidae) [3,4], invading pine branches through wounds created by the feeding activity of the beetles. Once inside the susceptible host trees, the nematodes migrate through the resin canals, causing serious damage to the xylem tissues of susceptible pine hosts while feeding and breeding [5,6]. In favorable climatic conditions, the resin flow is suppressed, the pine needles initially turn yellow and afterward brown, and the host tree wilts and dies within a few weeks (e.g., [7,8]).
In Europe, the PWN was first detected in the Portugal mainland in 1999 [9], and subsequently in Madeira Island [10] and Spain [11]. In Europe, several pine species can be affected by PWD, although with different levels of susceptibility [12]. In the Iberian Peninsula, maritime pine, *Pinus pinaster* Aiton, is the most affected pine species, with *Pinus nigra* Arnold [13] and *Pinus radiata* Don [14] also being susceptible; inversely, Stone pine (*Pinus pinea* Linnaeus) and Aleppo pine (*Pinus halepensis* Miller) seem to be tolerant/resistant to wilt disease under field conditions [15,16].

Tolerance/resistance to PWD has been hypothesized to result from an interaction of anatomical and/or chemical defenses of the trees (e.g., [7,17–19]), although the mechanisms underlying the host’s response and defense strategies against the nematode remain largely undetermined. According to some authors, secondary metabolites such as phenolic compounds may be involved in the recognition of the nematode by the host [20,21], preventing its spread and reproduction [22,23]. These phenolic compounds can range from simple molecules of low-molecular-weight (phenolic acids, flavonoids, stilbenes) to highly polymerized substances (lignins, tannins) [24], whose concentrations can be constitutive or induced by the herbivore insect and pathogen attack [24–26]. Constitutive phenolics [27,28] can play a role in the resistance mechanisms [29]. Other authors have hypothesized that tree death results from an excessive production of compounds after the PWN entrance [30], resulting in a phenomena of host hypersensibility.

Understanding the mechanisms underlying the susceptibility/tolerance/resistance of pines to PWD is an essential component of programs of integrated management against the invasive *B. xylophilus* [31]. The objective of this study was to characterize the differential chemical responses of susceptible and resistant pine species to *B. xylophilus* infection by quantifying phenolic compounds by HPLC before and shortly after nematode inoculation. To achieve this, we conducted trials on tolerant/resistant and susceptible adult pine trees that were 12-years-old instead of young plants or seedlings, resulting in a more realistic approach to extrapolate to natural conditions in the pine forests as different anatomical and biochemical responses can be expected between the seedlings and the adult trees.

2. Materials and Methods

2.1. Plant Material

Experiments were conducted on potted pine trees, 12 years-old, of four species, *P. sylvestris*, *P. pinaster*, *P. pinea*, and *P. halepensis*, selected because of their contrasting status as susceptible (the first two) or tolerant/resistant (the last two) to PWD under field conditions. Trees were kept under natural environmental conditions at the INIAV campus in Oeiras, Portugal (38°41′ N and 9°19′ W; 39 m above sea level), and watered daily from May to July 2017.

2.2. Nematode Culture and Tree Inoculation

The nematode *B. xylophilus* was supplied by the INIAV’s Nematology Laboratory isolate Bx013.003 (GenBank database (NCBI) accession number MF611984.1), reproduced in Erlenmeyer flasks filled with 10 g of barley seeds, 10 mL distilled water (autoclaved at 120 °C for 20 min) and *Botrytis cinerea* Pers., and kept at 26 °C in the dark.

Inoculation trials took place in July 2017, corresponding to the seasonal period of *Monochamus* highest activity and nematode-transmission [4,32]. For each *Pinus* species, three trees were artificially inoculated with *B. xylophilus*, three others with deionized water, and three additional trees were not inoculated or wounded, totaling 36 replicates. On each inoculated tree, two superficial wounds of approximately 4 cm (simulating the feeding wounds made by the *Monochamus* vectors), about 20 cm apart, were made with a sterilized scalpel on the middle of a living branch randomly selected from the lower canopy. Three trees of each species were inoculated with c.a. 400 µL of an aqueous suspension with approximately 2500 nematodes per inoculation point, while three additional trees were inoculated with the same amount of deionized water. Wounds were covered with sterile cotton and sealed with Parafilm to prevent dehydration.
2.3. Tree Sampling

Samples were collected from non-inoculated trees at the day of nematode/water inoculation (T0). After inoculation (with water or nematodes), wood samples were collected at 24 h (T24) and 72 h (T72). Samples consisted of ≈30 cm long branch sections (including the segment where inoculation was performed) that were cut and immediately wrapped in aluminum foil, labeled, and quickly (within 30 s) emerged in liquid nitrogen to prevent oxidation and hydrolysis, in order to avoid the phenolic decomposition of the tissues.

In the laboratory, the branch segments were removed from the liquid nitrogen, and wood samples of about 50 g of tissue (sections of circa 0.5 cm) were collected below the inoculation wound. The tissues were ground in an ultra-centrifugal mill, wrapped in aluminum foil, again frozen in liquid nitrogen, and kept at −80 °C until analysis.

2.4. Extraction of Phenolic Compounds

For each sample, 2 g of *Pinus* tissues were extracted with 10 mL of hexane, ultrasonicated (15 min; 20 °C) and stored in the darkness for 24 h, following the method described by Canas et al. [33]. The extracts were centrifuged at 6315 × g for 20 min at 2 °C, and the liquid phase containing the resin and volatiles was discarded. The tissues were placed in a laboratory oven (10 min; 35 °C) for the evaporation of the remaining hexane and weighted. One gram of tissue was then extracted with 20 mL of methanol–water (70:30 v/v) under rotary agitation for 180 min at 25 °C in duplicate. The extracts were centrifuged at 6315 × g for 20 min at 2 °C, and an aliquot (9 mL) of the liquid phase, containing the phenolic compounds, was evaporated to dryness at 40 °C. The residue was dissolved in 3 mL methanol–water (70:30 v/v). Samples were filtered through a 0.45 μm syringe filter to be analyzed by HPLC and LC-ESI-FT-ICR-MS.

2.5. Chemicals

Gallic acid, ferulic acid, (−)-epicatechin, rutin, and quercetin were purchased from Fluka (Buchs, Switzerland), while (+)-secoisolariciresinol, taxifolin, and resveratrol were purchased from Sigma-Aldrich (Steinheim, Germany) to be used as standards (purity > 98%) without further purification. The standards were prepared fresh prior to use with methanol/water (70:30 v/v)—methanol gradient grade and ultrapure water. The solvents, used in the sample extraction and in the spectrophotometric analysis, were analytical grade. All solvents used in the chromatographic analysis were HPLC gradient grade purchased from Merck (Darmstadt, Germany), filtered through a 0.45 m membrane, and degasified in an ultrasonic bath.

2.6. Analysis of Phenolic Compounds by HPLC and LC-ESI-FT-ICR-MS

The phenolic compounds included gallic acid, ferulic acid, protocatechuic acid hexoside, gallic acid hexoside, ferulic acid glucoside (phenols classified as phenolic acids), resveratrol (stilbene), and (+)-secoisolariciresinol (lignan) and flavonoid compounds included taxifolin, rutin, (−)-epicatechin, and quercetin hexoside, with two additional unknown compounds #A and #B identified by HPLC and LC-ESI-FT-ICR-MS as described by Canas et al. [33]. Quantification of each compound by HPLC was performed through a calibration curve using the corresponding commercial standard [33]; the concentration of the unknown compounds was expressed as mg of internal standard/L.

2.7. Total Phenolic Index

Total phenolic index (TPI) of the *Pinus* tissues was determined by the absorbance measurement at 280 nm (Ribéreau-Gayon, 1970) using a Varian Cary 100 Bio spectrophotometer (Santa Clara, CA, USA) and a 10 mm quartz cell. The extracts were diluted with ethanol/water (70:30). The analysis was performed in duplicate. Total phenolic index was calculated as follows: measured absorbance x dilution factor (1:20).
2.8. Statistical Analysis

The Shapiro–Wilk test was used to analyze data for normal distribution. Analysis of variance (ANOVA) was performed to assess the effects of sampling time and pine species on the contents of individual compounds and TPI between treatments. The effect of treatment and sampling time (24 h and 72 h) was analyzed for TPI and each phenolic compound and Pinus species with the non-parametric Kruskal–Wallis test.

A multidimensional analysis (principal component and classification analysis) was performed to examine patterns of clustering of the pine individuals/species of the water and nematode-inoculation treatments regarding the discriminating phenolic compounds. Analyses were carried out using the Statistica software, 9th edition (Statsoft Inc., Tulsa, OK, USA).

3. Results

3.1. Total Phenolic Compounds

3.1.1. Non-Inoculated Trees

Without inoculation of water or nematodes, mean values of TPI were similar between pines, differing only for P. sylvestris, which presented significantly lower mean values (Kruskal–Wallis test: $\chi^2 = 23.20$, d.f = 3, $p = 0.001$; Figure 1). Indeed, higher levels were observed on P. halepensis and P. pinea (4.705 and 4.484, respectively) and also on P. pinaster (4.644), while P. sylvestris presented contrasting lower levels (3.228).

3.1.2. Water-Inoculated Trees

After inoculation with water, mean values of total phenolic index decreased for all pines after 24 h and subsequently increased at 72 h, except for P. pinea. However, these differences were not significant (Figure 1).

3.1.3. PWN-Inoculated Trees

In the four nematode-inoculated pine species, we observed a decrease in total phenolic compounds after 24 h, followed by a significant increment at 72 h (Kruskal–Wallis test: $\chi^2 = 3.87$, d.f = 1, $p = 0.04$). In the last sampling time (72 h), the highest accumulation of phenolic compounds was found in P. halepensis.
3.2. Individual Phenolic Compounds

A total of 13 peaks were quantified in the four pine species studied (Figure 2). Eleven compounds were identified, while two additional peaks corresponded to unknown compounds (Figure 2b).

![HPLC chromatograms of standards (a) and of a *Pinus* (in this case *P. halepensis*) extract (b) recorded at 280 nm: 1—gallic acid; 2–5—hydroxymethylfurfural; 3—(−)-epicatechin; 4—taxifolin; 5—ferulic acid; 6—rutin; 7—resveratrol; 8—solariciresinol; 9—protocatechuic acid hexoside; 10—gallic acid hexoside; 11—ferulic acid glucoside; 12—quercetin hexoside; 13—unk #A; 14—unk #B.](image)

3.2.1. Non-Inoculated Trees

In general, the highest constitutive levels of flavonoid compounds and stilbene compounds were detected in *P. halepensis* and *P. pinea*, with significant differences for the other pines (Kruskal–Wallis test: $\chi^2 = 1.74$, d.f = 1, $p = 0.03$; Table 1). *Pinus halepensis* presented the highest concentrations of resveratrol (stilbene), (−)-epicatechin, and rutin, while *P. sylvestris* showed the lowest concentrations. *Pinus pinea* and *P. pinaster* presented similar intermediate mean values for the phenolic compounds described above (Kruskal–Wallis test: $\chi^2 = 0.92$, d.f = 1, $p = 0.064$; Table 1). Phenolic acids were more abundant on *P. sylvestris* and *P. pinaster* (Table 1), and the lowest contents were found in the remaining two pine species.
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**Table 1.** Mean concentration of phenolic compounds according to sampling time and treatment in four pine species (mg/L).
3.2.2. Water-Inoculated Trees

As shown in Table 1 and Figure 3, there was a response of pines to wounding and inoculation with water. At 24 h, phenolic compounds differed between species (Table 1), and at 72 h, there was a significant (ANOVA; \( p_{2,18} < 0.01 \)) variation in the phenolic acids, taxifolin, resveratrol, and rutin, particularly, for *P. halepensis* and *P. sylvestris* (Figure 3).

![Figure 3](image-url)

**Figure 3.** Plot of means two-way interaction (species x sampling time, ANOVA test) for phenolic acids (a), taxifolin (b), rutin (c), and resveratrol (d) in water-inoculated trees. Significant differences are indicated by an asterisk (**\( p < 0.01 \)** and *ns*—without significant difference).

Given the interactions shown in Table 1, a global evaluation of the data was performed by a principal component analysis (PCA, Figure 4) at 72 h after inoculation with water. The results of the PCA (Figure 4a,b) demonstrate that compounds were differentially involved in the response of the pine species: *P. sylvestris* was mainly influenced by gallic acid hexoside, *P. pinea* by ferulic acid glucoside, *P. halepensis* by protocatechuic acid hexoside, gallic acid and (−)-epicatechin, and *P. pinaster* by (+)-secoisolariciresinol and ferulic acid (Figure 4b).

3.2.3. PWN-Inoculated Trees

There was a specific nematode-induced response of some compounds by the pines, with taxifolin, rutin, resveratrol, ferulic acid glucoside, (+)-secoisolariciresinol, protocatechuic acid hexoside, gallic acid, and ferulic acid presenting significantly (ANOVA; \( p_{1,24} < 0.01 \)) different concentrations on the nematode-infested pines in relation to water-inoculated branches (Table 1, Figure 5).

After nematode inoculation, we observed a diverse and contrasting array of responses of individual compounds among pines (Figure 6). In general, phenolic acids in *P. halepensis* increased in the first 24 h after inoculation with *B. xylophilus*, whereas in *P. sylvestris*, there was a sharp decrease followed by a strong increment at 72 h (Figure 6).
For rutin, there was a significant increase immediately after nematode inoculation for both *P. halepensis* and *P. sylvestris*, while (+)-secoisolariciresinol presented contrasting responses for these species (Table 1).

We observed a time-effect (24 h vs. 72 h) in the phenolic contents for all pines. Significant statistical differences between the two time series were noticed for taxifolin, resveratrol, ferulic acid glucoside, gallic acid hexoside, (+)-secoisolariciresinol, and unknown compound #A with a higher increase in *P. halepensis*, and for gallic acid, ferulic acid, and protocatechuic acid hexoside in *P. sylvestris*.

Nevertheless, this pattern was not common to all compounds, and for (+)-secoisolariciresinol, a significant decrease was observed in *P. pinaster* in the last sampling time.

The PCA (Figure 4c,d) demonstrated a splitting of the four *Pinus* species according to the treatment (first component PC1, Factor 1), with a clear separation between *P. halepensis* and *P. sylvestris*, while *P. pinea* and *P. pinaster* showed a more homogenous response (Figure 4d). The PC1 exhibits strong positive loading vectors mostly for flavonoids and stilbenes, and strong negative loading vectors for phenolic acids.

**Figure 4.** Principal component analysis (PCA, ANOVA test) of the coordinates of the treatment (water-inoculated (a,b) and pinewood nematode-inoculated trees (c,d)) and the pine species (*P. halepensis*, *P. pinea*, *P. sylvestris*, and *P. pinaster*) at 72 h, on a graph defined by two components.
Figure 5. Plot of means of two-way interaction (treatment × sampling time, ANOVA test) for taxifolin (a), rutin (b), resveratrol (c), fag—ferulic acid glucoside (d), and (+)-secoisolariciresinol (e) in P. halepensis; pca—protocatechuic acid hexoside (f), gallic acid (g), and ferulic acid (h) in P. sylvestris. Significant differences are indicated by an asterisk (*** p < 0.01).
walls [36], and therefore, its presence as a response to mechanical damage is plausible, even which was not obvious in our results because maritime pine (a susceptible species) also presented high levels of constitutive phenols. According to some authors, differences in biochemical responses in the first days after inoculated trees. Significant differences are indicated by an asterisk (** p < 0.01 and ns—without significant difference).

4. Discussion

The main objective of this study was to discriminate between PWN-tolerant/resistant and susceptible pine species based on their constitutive and induced phenolic profile.

We could not differentiate between species based on the total phenolic index, which was similar except for P. sylvestris, which presented lower mean values. The importance of constitutive phenolic compounds in tolerance/resistance to initial infection has been reported for several plant pathogens (e.g., [34]), and also for B. xylophilus [20,21], with high levels of constitutive total phenolic index related to greater resistance to PWN infection [35], which was not obvious in our results because maritime pine (a susceptible species) also presented high levels of constitutive phenols.

Considering individual compounds, we observed a response of pines to wounding in the absence of PWN, allowing us to detect differences between physiological responses caused by the mechanical damage and specific induced responses to B. xylophilus. Among the analyzed compounds, only rutin was negatively correlated with mechanical injury after 24 h, while ferulic acid was strongly correlated with injury. Ferulic acid is known to be involved in the biosynthesis of lignin, acting as a binding agent and stabilizer of cell walls [36], and therefore, its presence as a response to mechanical damage is plausible, even in the absence of B. xylophilus.

In general, we observed that high levels of taxifolin, rutin, and resveratrol seem to be associated with the intrinsic tolerance/resistance of pines to PWN, while phenolic acids (gallic acid, ferulic acid, and protocatechuic acid hexoside) were dominant in the susceptible P. sylvestris, although this tendency was not noticeable for all pine species. According to some authors, differences in biochemical responses in the first days after nematode inoculation are crucial in defining the pine’s susceptibility to wilt disease [37–39].

Some compounds were significantly induced by the presence of B. xylophilus including taxifolin, (+)-secoisolariciresinol, resveratrol, rutin, and unknown compound #A in P.
The flavononol taxifolin is characterized by a powerful antioxidant activity [40], particularly against fungi [26,41,42]. The lignan (+)-secoisolariciresinol may act as an antimicrobial and antioxidant with antifeedant properties [28], similar to resveratrol [43,44], which has been associated with the resistance of pines to PWN [27,29]. Suga et al. [45] suggested that the resistance of Pinus massoniana Lamb. and Pinus strobus L. to PWD was related to the presence of resveratrol due to its strong nematicidal activity. In our study, we observed a wide variation of resveratrol in *P. sylvestris* over time, however, the low concentration compared to *P. halepensis* may account for a lack of effectiveness in response to the invasive agent.

Rutin was significantly induced by *B. xylophilus* in *P. halepensis*, contrasting with the sharp decrease in water-inoculated trees. Previous studies with entomopathogenic nematodes suggest that high concentrations of rutin may affect their reproduction [46]. The unidentified compound #A (Figure 1) showed significant responses to the nematode’s presence in *P. halepensis*, and therefore should be investigated in detail to determine its identity and putative importance.

Scots pine (*P. sylvestris*), one of the species most susceptible to PWD, presented a significant variation of hydroxybenzoic acids (protocatechuic hexoside and gallic acids), which significantly increased 72 h after nematode-infection. Protocatechuic hexoside is renowned for its antibacterial and antioxidant activities [47], also presenting nematicidal properties [47,48]. Gallic acid also presents a strong antioxidant activity [49] and is reported to act as a prooxidant induced by pathogen attack, which could inhibit the antioxidant properties [26,50,51] and therefore cause an adverse hypersensitive reaction with negative impacts to the pine host, as suggested by some authors (e.g., [6,52]).

5. Conclusions

We observed that constitutive total phenolic index cannot be used to clearly separate between PWN-tolerant/resistant and susceptible adult pine species prior to nematode infection. Nevertheless, in the presence of *B. xylophilus*, this index allowed a noticeable separation of the PWN-tolerant/resistant *P. halepensis* and the susceptible *P. sylvestris*; in contrast, *P. pinea* and the susceptible *P. pinaster* presented a more homogenous and less obvious response, which is surprising considering that *P. pinaster* is heavily affected by the PWD in the Iberian Peninsula whilst *P. pinea* is not affected, even in regions with high frequency of the pathogen [16].

Considering individual compounds, observations on the tolerant/resistant *P. halepensis* suggest that taxifolin, resveratrol, and rutin may have an active role in protecting pine species against *B. xylophilus* infection, and/or in regulating associated microorganisms such as bacteria and fungi. Nevertheless, the same compounds did not display a comparable behavior on the similarly resistant *P. pinea*, which may suggest that resistance of pines to *B. xylophilus* probably results from a complex interaction of diverse biochemical and anatomical characteristics [20,33,53], and not from the presence or abundance of a single compound.

Several authors have studied the most important anatomical and biochemical characteristics that condition the susceptible/resistance of different pine species to *B. xylophilus* (e.g., [21,30,54,55]). Nevertheless, all these studies have been conducted on excised branches, saplings, or seedlings, and with artificial inoculations of nematodes into the xylem, which do not mimic with accuracy the interaction of the nematode and the adult pines in the field [54]. This is the first study comparing the reaction of adult pines (including susceptible and tolerant/resistant species) to PWN entering the phloem through a simulated feeding wound, and therefore may more rigorously characterize the responses of the trees to the presence of the parasitic nematode. Considering our observations, particularly on the resistant *P. halepensis*, future studies should focus primarily on characterizing the role of taxifolin, resveratrol, and rutin, individually and associated, on the resistance of pines against *B. xylophilus*. 
Author Contributions: Conceptualization, P.N., C.S.T. and E.S.; Methodology, C.S.T., S.C., M.L.L and P.N.; Formal analysis and investigation, C.S.T., S.C. and P.N.; Writing—original draft preparation, C.S.T., S.C., M.L.L, E.S. and P.N.; Writing—review and editing, C.S.T., S.C., M.L.L, S.P.-L, E.S. and P.N.; Funding acquisition, P.N.; Project Administration, P.N. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Foundation for Science and Technology (FCT) under the project CRM:0048124 “Fatores envolvidos na resistência e suscetibilidade de coníferas à doença da murchidão dos pinheiros” (FCT IF/00471/2013/CP1203/CT0001), and through the R&D Unit “GREEN-IT-Bioresources for Sustainability” (UIDB/04551/2020 and UIDP/04551/2020).

Data Availability Statement: Data is available through the following link: https://drive.google.com/drive/folders/1L5F1bsGjQ96kh0myDugDsL0RFlQzQF?usp=sharing, accessed on 7 March 2022.

Acknowledgments: The authors would like to thank INIA’s colleagues Marina Cardoso, Francisco Martins, Federico Preza, Teresa Valdiviesso, and Ana Margarida Fontes for their logistical and technical assistance during the experiments.

Conflicts of Interest: The authors declare that they have no conflict of interest.

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