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Exogenous Application of Salicylic Acid Modulates Oxidative Stress during the Seed Development of Rice (*Oryza sativa* L.) Grain

María Pinciroli [1,2], Raúl Domínguez-Perles [1,*], Sonia Medina [1], Camille Oger [3], Alexandre Guy [3], Thierry Durand [3], Mari Merce Cascant-Vilaplana [1,4], José Antonio Gabaldón-Hernández [5], Federico Ferreres [5] and Ángel Gil-Izquierdo [1,*]

1 Research Group on Quality, Safety and Bioactivity of Plant Foods, Department of Food Science and Technology, CEBAS-CSIC, University Campus of Espinardo-Edif. 25, 30100 Espinardo, Spain
2 Cátedra de Bioquímica y Fitoquímica, Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de la Plata, Calle 60 y 119, La Plata 1900, Buenos Aires, Argentina
3 Institut des Biomolécules Max Mousseron (IBMM), Pôle Chimie Balard Recherche, UMR 5247, CNRS, University of Montpellier, ENSCM, 34000 Montpellier, France
4 Neonatal Research Group, Health Research Institute La Fe, 46001 Valencia, Spain
5 Molecular Recognition and Encapsulation Research Group (REM), Health Sciences Department, Universidad Católica de Murcia (UCAM), Campus de los Jerónimos 135, 30107 Guadalupe, Spain
* Correspondence: rdperles@cebas.csic.es (R.D.-P.); angelgil@cebas.csic.es (Á.G.-I.); Tel.: +34-968396200 (ext. 445363) (R.D.-P.); +34-968396200 (ext. 445346) (Á.G.-I.)

Abstract: The present study aimed to discover the effects of exogenously foliar-applied salicylic acid (SA) in concentrations of 0, 1 and 15 mM (applied 7 and 21 days after heading) on oxidative stress. The effects were monitored through the concentrations of phytoprostanes (PhytoPs) and phytofurans (PhytoFs) in immature and mature grains of three genotypes of rice ('R52', 'R45' and 'Yerua'), and their influence on grains per panicle and chalkiness. Chromatographic separation of PhytoPs and PhytoFs was performed using a UHPLC coupled to triple quadrupole-MS/MS (Agilent Technologies, Germany). The concentrations of oxylipins showed differences in both harvest times (immature and mature) for each genotype. The advanced lines, 'R52' and 'R45', showed concentrations that were 24.0 and 79.0% lower than those of the immature grains, respectively. The PhytoFs concentration in "R45" was 46.0% lower in the mature grains. In unripe grains, SA reduced a single oxylipin of all those analyzed, while in mature grains, a significant decrease in six of the ten monitored biomarkers was observed. The SA produced an increase in grains per panicle, and a decrease in chalkiness. Therefore, salicylic acid-mediated antioxidant regulatory capacities due to oxylipin down-regulation could favor grain filling and, hence, rice production.

Keywords: rice; oxidative stress; phytofurans; phytoprostanes; salicylic acid; seed development

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1. Introduction

The ontogenesis of rice seeds involves diverse processes, including embryogenesis, reserve accumulation and maturation. During these phases, the plant's moisture and its metabolic activity, and consequently its level of reactive oxygen species (ROS) in the seeds, vary drastically [1,2]. In this aspect, the necessity of mitigating the generation of toxic amounts of ROS in plant cells at each stage of the life cycle to ensure their proper development has been previously reported [3]. From an agronomic and physiological point of view, it is known that abiotic stresses in rice, particularly high radiation or high temperatures, decreases the ability of cereals to scavenge ROS; this increases lipid peroxidation, and causes important metabolic changes related to secondary metabolism and lipid metabolism that lead to reduced grain filling [4,5].

In plant cells, the mitochondrial electron transport chain is one of the most important producers of ROS [3,6]; its level is proportional to the respiratory rate [7]. For this reason, higher plants have developed a compliant and dynamic regulatory system to remove ROS and maintain optimal CO_2 fixation rates in a wide range of light intensities [8]. In plant seeds, there are microbodies (glyoxysomes) that are characterized by their ability to produce and decompose hydrogen peroxide [9]. As a consequence of the oxidation of fatty acids present in cell membranes (such as linolenic acid) and catalyzed by ROS action, several classes of phytoprostanes containing prostaglandins D_1, E_1, F_1, A_1, B_1, or the deoxy J_1-ring system, as well as malondialdehyde, can be formed [6,10,11]. This pathway would be parallel and equivalent to the jasmonate pathway catalyzed by lipoxygenases, by which linolenic acid is converted to 12, 13-(S)-epoxy-octadecanoic acid (12, 13-EOT), which is the first intermediate in the biosynthesis of jasmonic acid [12]. In connection with this, Cuyamendous et al. [13] reported a new type of plant oxylipin that results from a similar lipid oxidation reaction. This process is triggered at oxygen pressures of 21% and higher, leading to an initial cyclization, and to the generation of compounds containing tetrahydrofuran cycles, the so-called phytofurans (PhytoFs) [13]. In this scenario, a relationship of PhytoPs and PhytoF with the regulation of the redox balance has already been proposed, not only in rice plants [14,15], but also in other crops [2,11,16]. It is presumed that this great diversity of oxylipins acts either as an elicitor/biomarker for the synthesis of hormones, or as activators of stress genes such as 12, 13-EOT that may serve as stored intermediates that allow the rapid synthesis of jasmonic acid during initial phases of stress signaling [17]. The difference would be that they were generated in a non-enzymatic way, which could be an archaic mechanism [17]; since the means is not enzymatic, they would be protected from oxidative damage caused by ROS [1].

Previous studies have reported the presence of the phytohormone salicylic acid (SA) in plant cells. Its major role is in the modulation of oxidative stress, photosynthesis and some plant hormones [3,18,19]. In this aspect, currently, there is controversial information regarding the actual influence of SA on the generation of ROS, while the origin of the ROS induced by SA could include a range of cellular compartments (chloroplasts, mitochondria and cell membrane) and enzymes (NADPH oxidase and polyamine oxidase) [8]. Moreover, it should be noted that the modulation of ROS levels by SA may depend to a large extent on diverse factors, including concentration, the duration and mode of application (when considering exogenous SA), the species and organ under study, and the environmental conditions [18]. Wang et al. [20] verified that SA played a critical role in rice cultivars by regulating antioxidant defense systems, reducing ROS formation, and preventing the degradation of internal cell organelles. It has also been observed that SA pre-treatment in rice seeds reduced the concentration of malonaldehyde [21], and also triggered detoxifying enzymes to eliminate ROS, such as aldehyde dehydrogenase (EC 1.2.1.3, ALDH), glutathione (GSH) and glycolate oxidase [22]. Additionally, SA application maintained the chlorophyll content in rice leaves by preventing chlorophyll breakdown, and simultaneously accelerating its de novo synthesis [22].

The seed set is an important agronomic trait that determines rice yield, and chalkiness is a significant concern in rice breeding because it is one of the critical factors determining quality and price [23]. In fact, rice quality is essentially determined by the chemical composition of stores of starch and protein that derive from carbon (C) and nitrogen (N) metabolism during grain filling. The early stage of grain filling is the critical point for chalkiness formation [24]. Changes in redox homeostasis during grain filling is likely responsible for altering grain chalkiness in rice [25]. Furthermore, the regulation of oxidative stress (ROS generation) during embryogenesis and maturation, including the transition phase, is not fully understood [26].

Based on these premises, it was suggested that the capacity of diverse genotypes to regulate the oxidative stress with the concourse of the hormonal system could influence the yield and quality of the harvested grain (rice seeds). As indicated above, abiotic stresses affect lipid metabolism; thus, the generation of linolenic acid, the precursor of

non-enzymatic oxylipins, was considered in this study. In addition, salicylic acid has the ability to decrease the activities of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), to enhance the concentrations of non-enzymatic antioxidants, and lower the concentrations of H(2)O(2) and malondialdehyde (MDA), indicating a lower generation of ROS under different stress conditions in rice [4,27]. Therefore, variations in linolenic acid metabolism, together with the antioxidant action of salicylic acid on oxygen species generation, could affect the generation of non-enzymatic oxylipins. However, this hypothesis has not yet been demonstrated. According to this, the present article aimed at conducting a preliminary study about the effect of exogenously applied SA on the oxidative stress of rice plants monitored through the assessment of rice seeds during the initial and final stages of grain formation in three rice genotypes, spp japonica ('R52', 'R45', and 'Yerua'), on the number of grains per panicle and chalkiness kernel (%) in mature grains, and their concentrations of PhytoPs and PhytoFs (plant oxylipins). The results of this preliminary study are intended to be a starting point for further in-depth trials considering targeted biochemical and molecular processes, grain filling, grain weight and ultimately yield per plot, to determine the level of interest for applying such a treatment in a production scheme.

2. Materials and Methods

2.1. Chemical and Reagents

Analytical standards of the PhytoPs 9-F_{1t}-PhytoP; *ent*-16-F_{1t}-PhytoP; *ent*-16-*epi*-16-F_{1t}-PhytoP; 9-*epi*-9-F_{1t}-PhytoP; 9-D_{1t}-PhytoP; 9-*epi*-9-D_{1t}-PhytoP; 16-B_1-PhytoP; and 9-L_1-PhytoP, as well as the PhytoFs, *ent*-16(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF; *ent*-9(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF; and *ent*-16(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF, were synthesized according to previously published procedures [13,28–33] provided by the Institut des Biomolécules Max Mousseron (IBMM) (Montpellier, France). Hexane was obtained from Panreac (Castellar del Valles, Barcelona, Spain); butylated hydroxyanisole (BHA), salicylic acid (SA) and bis–Tris (bis (2-hydroxyethyl) amino-tris (hydroxymethyl) methane) were purchased from Sigma-Aldrich (St. Louis, MO, USA); and all LC-MS grade solvents (methanol and acetonitrile) were supplied by J.T. Baker (Phillipsburg, NJ, USA). Water was treated in a milli-Q water purification system from Millipore (Bedford, MA, USA) to obtain the deionized water used in all extraction procedures, and in the chromatographic separation of the target oxylipins. The solid-phase extraction (SPE) cartridges used with the clean-up of sample purposes were Strata cartridge (Strata X-AW, 100 mg/3 mL), which were acquired from Phenomenex (Torrance, CA, USA).

2.2. Plant Samples and Crop Management

An assay was set up in the experimental station 'Julio Hirschhorn' in La Plata (34.982° S, 57.997° W, 9.8 m of altitude, Buenos Aires, Argentina), and included two advanced lines sourced from the *Rice Breeding Program* of the 'Facultad de Ciencias Agrarias y Forestales', 'R/03-5x desc/04-52-1-1' ('R52') and 'R/03-5x desc/04-45-1-1' ('R45'), as well as the genotype 'Yerua PA' ('Yerua'), broadly distributed in the Argentinean producing area. All three genotypes belong to the subspecies *japonica*, and the importance of two of the lines is that they improve the yields of the Yerua variety while maintaining its type of grain. Three types of genotypes were used, as they could influence the generation of plant oxylipins during the ontogenetic stage.

The experimental design consisted of randomized blocks with three replicates seeded in dry land manually at a rate of 350 seeds/m^2 in lines at 0.20 m, within plots of 5 m^2. The assay was conducted with flood irrigation 30 days after emergence, and weeds were controlled with commercial herbicides following the manufacturer's instructions. The treatments assayed in the present study included control rice plants untreated with SA (SA0), rice plants treated with 1 mM SA (SA1), and rice plants treated with 15 mM SA (SA15). The treatment with SA was applied 7 and 21 days after heading (DAH). The solutions were prepared in distilled water with the addition of Tween-20 surfactant (0.005%). The

plots were sprayed until runoff during late afternoon hours, when the wind speed was less than 10 km/h. Unripe and mature grains were harvested manually at 14 and 56 DAH, respectively. To understand the behavior of rice genotypes, the number of grains per panicle and the percentage of chalkiness kernel, in mature grain, were monitored.

2.3. Samples Preparation

The rice grain samples were harvested manually, and the kernels were dried in an oven at 40 °C. Dehulled grain samples (100 g) were ground into Cyclone Mill, sieved through a 0.4 mm mesh, and stored at 4 °C, protected from light, until the analytical determination of their contents of PhytoPs and PhytoFs.

2.4. Phytoprostane and Phytofuran Extracts

The PhytoPs and PhytoFs present in rice flours were extracted following the methodology described by Collado-González et al. [34] and Domínguez-Perles et al. [35], with minor modifications. Briefly, sample powders (4 g) were pestled with 10 mL of methanolic butylated hydroxyanisole (BHA) (99.9:0.1, v/w). The extracts were centrifuged at 2000× g for 10 min, and the supernatants were collected and cleaned up with SPE, using Strata X-AW cartridges (Phenomenex, Torrance, CA, USA) according to the procedure described [36].

2.5. UHPLC-ESI-QqQ-MS/MS Analysis

The chromatographic separation of PhytoPs and PhytoFs was performed using a UHPLC coupled to a 6460 triple quadrupole-MS/MS (Agilent Technologies, Waldbronn, Germany), using a BEH C_{18} analytical column (2.1 × 50 mm, 1.7 µm) (Waters, Milford, MA, USA), applying the chromatographic, ionization and fragmentation conditions described by Collado-González et al. [34] and Domínguez-Perles et al. [35]. Briefly, the injection volume and flow rate were 20 µL and 0.2 mL min^{-1}, respectively, through the following linear gradient (Time (min), %B): (0.00, 60.0%); (2.00, 62.0%); (4.00, 62.5%); (8.00, 65.0%); and (8.01, 60.0%). An additional post-run of 1.5 min was considered to equilibrate the column. Spectrometric analysis was performed in Multiple Reaction Monitoring (MRM), operated in negative mode, assigning quantification and confirmation MRM transition for each analyte [30,35]. The ionization and fragmentation conditions were as follows: gas temperature 325 °C, gas flow 8 L min^{-1}, nebulizer 30 psi, sheath gas temperature 350 °C, jetstream gas flow 12 L min^{-1}, capillary voltage 3000 V, and nozzle voltage 1750 V, according to the most abundant productions. Data acquisition and processing were performed using Mass Hunter software version B.08.00 (Agilent Technologies, Waldbronn, Germany). The concentrations of the PhytoPs and PhytoFs detected in the analyzed rice flours were quantified by calculating the area under the curve, taking as reference the standard curves of known concentrations prepared each day during the analyses.

2.6. Statistical Analysis

All of the assays were developed in triplicate (n = 3), and the values were provided as means ± the standard error of the mean (SEM). Maturation stage (2), SA treatment (3) and genotypes resource (3) were used as sources of variation to analyze the behavior of the oxylipins concentration. For the statistical analysis of the quality parameters (number of grains per panicle and chalkiness of mature grains expressed as a percentage), the factors were SA treatment (3) and genotypes resource (3). In this way, 2 × 3 × 3 and 3 × 2 multifactorial analyses of variance (ANOVA) were performed. The differences between means were compared via the multiple range test of Tukey, and the degree of significance was set at $p < 0.05$. Statistical analyses were carried out using Statgraphics Centurion XVI 16th version (StatPoint Technologies Inc., Warrenton, VA, USA).

3. Results and Discussion

Although the impact of agronomical and environmental growing conditions on the levels of PhytoPs and PhytoFs have previously been described in rice crops [14,15,37], there

is a gap in knowledge regarding the evolution of the PhytoP and PhytoF concentrations during grain development. In this regard, obtaining accurate information on the evolution of the quantitative profile of PhytoPs and PhytoFs during this stage would be informative, providing theoretical support for further improvements in agronomical management. The present research aimed to study changes in the concentrations of these oxylipins in rice grains during ontogenesis, as an approach that would contribute strongly to identify relationships between the concentrations of PhytoPs and PhytoFs, to hormonal regulation of oxidative stress, and their impact on final product quality.

3.1. Total Content of Phytoprostanes and Phytofurans in Unripe and Ripe Rice Grains

In the assessment of rice grains of three distinct genotypes at different ripening stages (unripe and mature grains) on their total content of PhytoPs and PhytoFs, it was noted that these plant oxylipins' concentrations varied significantly depending on the genotype considered and the ontogenetic stage.

With respect to total PhytoPs, their relative abundances in unripe and mature rice grains of the genetic resources under study presented the following decreasing order: 'R45' (2006.4 ng g^{-1} dw) > 'R52' (1898.7 ng g^{-1} dw) > 'Yerua' (1616.1 ng g^{-1} dw) for unripe rice grains, and 'Yerua' (1713.2 ng g^{-1} dw) > 'R52' (1445.5 ng g^{-1} dw) > 'R45' (420.4 ng g^{-1} dw) for mature grains. As demonstrated previously regarding other crops, namely almonds [38], melon leaves [39], rice [14], cocoa [40], olive oil [34,35,41–43], the diversity of the genetic background within a single species of higher plant can be responsible for specific profiles of fatty acids and resistance to biotic and abiotic stresses; this, in turn, can result in different quantitative profiles of plant oxylipins. The results retrieved in the current research reinforced this hypothesis, providing information that also extends this fact to oxylipin levels in rice grains during development.

In more detail, concerning the changes in the total PhytoPs concentration during ripening, the cv. 'Yerua' presented equivalent concentrations of total PhytoPs in unripe and mature grains. In contrast, in the advanced lines 'R52' and 'R45', the abundances of these plant oxylipins in mature grains decreased significantly relative to those in the immature stage (by 24 and 79%, respectively) (Figure 1A).

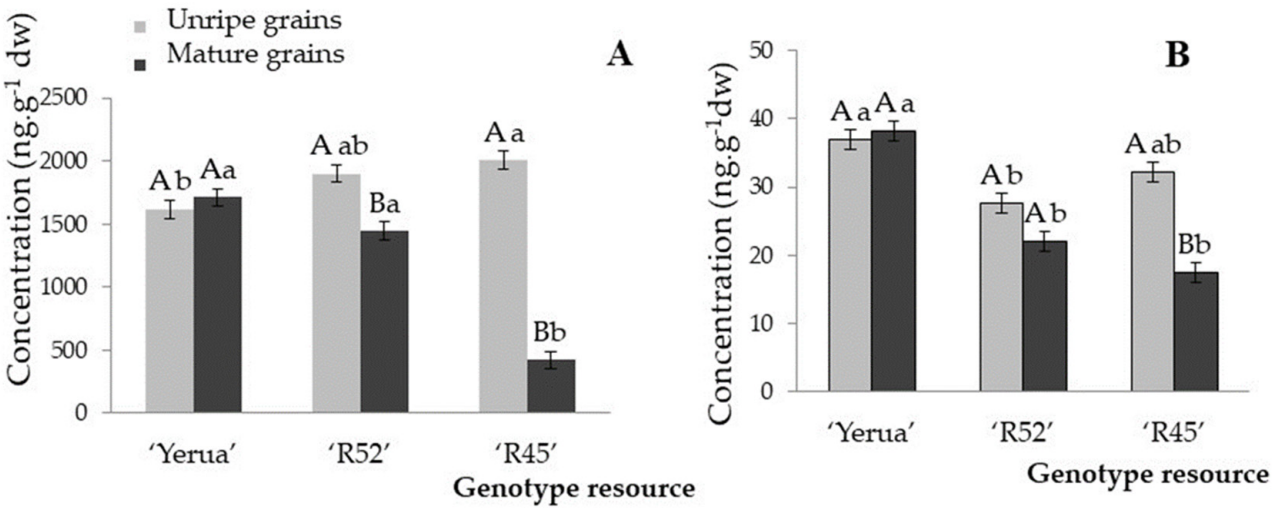

Figure 1. Concentrations of total phytoprostanes (**A**) and phytofurans (**B**) in unripe and mature grains, in 'Yerua PA' ('Yerua'), 'R/03-5x desc/04-52-1-1' ('R52') and 'R/03-5x desc/04-45-1-1' ('R45'). Different capital letters indicate significant differences between ripening stages at harvest for each (the same) genotype, and distinct lowercase letters indicate differences between genotypes at $p < 0.05$, according to a single-way analysis of variance (ANOVA) and a multiple range test of Tukey.

On the other hand, when comparing the total concentration of total PhytoPs in rice grains between genotypes at both maturation stages, it was found that, regarding unripe

samples harvested at 14 DAH, no significant differences were found between genotypes, which exhibited values ranging between 1616.1 and 2005.4 ng g^{-1} dw. Nonetheless, regarding mature grains, the highest significant concentration corresponded to the cv. 'Yerua' (1713.2 ng g^{-1} dw), which surpassed concentrations found in the advanced lines 'R52' and 'R45' by 18.5 and 307.5%, respectively (Figure 1A).

In addition to PhytoPs, according to the concentration of total PhytoFs generated in the rice grains, the genotypes evaluated evidenced the following decreasing trend: 'Yerua' (37.0 ng g^{-1} dw) > 'R52' (27.6 ng g^{-1} dw) > 'R45' (32.2 ng g^{-1} dw) for unripe grains, and 'Yerua' (38.1 ng g^{-1} dw) > 'R52' (22.0 ng g^{-1} dw) > 'R45' (17.4 ng g^{-1} dw) for mature grains. These plant oxylipins have also been studied in other plant seeds, namely pine nuts, walnuts, chia seeds, flax, rice, pea, almonds, pistachio and cocoa clones, which have been found in a concentration range of 0.3–28.0 ng g^{-1} [15,35,37,40,41,44–46]. The lower cellular content of PhytoFs observed could be due to the fact that the synthesis of this family of compounds is prioritized under a partial pressure of oxygen that is greater than that in plant cells during embryogenesis [13,47].

In addition to differences due to the genetic background, the ripening process also seems to have a critical influence on the final concentrations of PhytoFs in mature grains. In this respect, in 'Yerua', the ripening process did not affect the concentration of total PhytoFs (37.6 ng g^{-1} dw, on average, in both maturation stages) significantly, while in 'R52' and 'R45', which presented total concentrations of PhytoFs at the unripe stage of 29.9 and 19.7 ng g^{-1} dw, respectively, decreases of 20.0 and 46.0% were observed during maturation, respectively. However, this reduction was statistically significant only for 'R45' (Figure 1B).

The overall results regarding total PhytoPs and PhytoFs suggest a higher content of PhytoPs in the advanced lines ('R52' and 'R45') in the initial stages when grain filling occurs (unripe grains). This is in agreement with the fact that, in the embryogenesis stage, the intense mitochondrial respiration rate generates high amounts of ROS that are responsible for the oxidation of fatty acids which make up part of the cellular structures toward the synthesis of plant oxylipins [1]. In this aspect, totipotent protoplasts during the intense cell division occurring in embryogenesis have also been associated with the cellular machinery responsible for maintaining the redox balance [1]. On the hand, in later stages, and especially when the grain is dry and inactive, respiratory activity decreases, practically becoming null [40], thus minimizing ROS production and the oxidation of fatty acids toward the synthesis of PhytoPs and PhytoFs. This has been further demonstrated by several authors, who found an intense fall in the activity of protective enzymes responsible for the regulation of the redox balance (superoxide dismutase (SOD), catalase (CAT), peroxidase (POD)), by neutralizing the harmful effects of ROS, 14 or 21 DAH [48,49]. In agreement with this, this period could be required to achieve the physiological maturity of seeds, as well as their final size, which would entail a decrease in metabolic activity.

In this regard, the intense generation of ROS would be defining the high level of oxylipins, in this case, by auto oxidation. It should be taken into account that lipid metabolism, and more specifically, linolenic acid precursor of plant oxylipins as well as the generation of ROS in rice, depends on the genotype; therefore, this factor directly affects the generation of PhytoPs and PhytoFs [50]. This diversity of molecules could act as stress biomarkers. This mechanism would be equivalent to activating the enzymatic system that unfolds to neutralize ROS and restore cellular redox balance.

However, this fact seems to be closely dependent on the genetic resource considered and its specific physiological characteristics [14,36]. In this regard, after maturation, the grain of the cv. 'Yerua' did not show a reduction in the concentration of both families of stress biomarkers monitored in the present study (PhytoPs and PhytoFs). Moreover, the slow senescence that characterizes this genotype is associated with preserving active green leaf tissues during the advanced grain-filling period. Possibly, this intense metabolic activity is also present regarding the metabolism of the seeds, which constitutes a cornerstone for the presence of oxylipins related to limiting damage to the cell membranes, and the maintenance of the redox balance. On the other hand, in the advanced rice lines, the excess

energy may not dissipate efficiently, and ROS may be generated. The ROS accelerated the leaf senescence process, upon which the leaves turned yellow, showing severe damage to the photosynthetic apparatus [48]. Senescence is an oxidative process that involves the overproduction of ROS [49]. In the frame of this cellular process, ROS cause cell damage via lipid peroxidation, which is generally considered to be a major contributor to the senescence syndrome [51]. In this aspect, it has been reported that during the period of fast growth, as well as in plants with a significant biomass-producing capacity, the antioxidant enzymes' activities augment the prevention of ROS production [48].

3.2. Effect of Exogenous Salicylic Acid on the Quantitative Profile of Phytoprostanes and Phytofurans in Unripe and Mature Rice Grain

In addition to PhytoPs and PhytoFs in this study, the abundances of individual compounds belonging to both families of plant oxylipins were determined in the three rice genotypes, regardless of the harvest time and the supplementation with salicylic acid. These analyses allowed establishment of the following decreasing order of average concentration: co-eluting *ent*-16-F_{1t}-PhytoP and *ent*-16-*epi*-16-F_{1t}-PhytoP (625.9 ng g^{-1} dw) > 9-*epi*-9-F_{1t}-PhytoP (406.6 ng g^{-1} dw) > 9-F_{1t}-PhytoP (377.4 ng g^{-1} dw) > 9-D_{1t}-PhytoP (73.2 ng g^{-1} dw) > 16-B_1-PhytoP (13.9 ng g^{-1} dw) > 9-*epi*-9-D_{1t}-PhytoP (9.9 ng g^{-1} dw) > 9-L_1-PhytoP (9.8 ng g^{-1} dw). When comparing these results with the information available in the literature on rice and other plant-based foods and foodstuffs (dry melon leaves, aged wine, grape nuts, olive oil, almonds, and macroalgae), it was noticed that, in agreement with the outcomes described in this research, compounds belonging to the F_1-PhytoPs were the most abundant [35,38,39,52–55].

On the other hand, the concentrations of individual PhytoFs in the three genotypes analyzed were observed to be in the following decreasing order of average concentration: *ent*-16(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF (15.7 ng g^{-1} dw) > *ent*-16(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF (10.1 ng g^{-1} dw) > *ent*-9(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF (3.3 ng g^{-1} dw). This ranking matches with that observed in five indica-type rice genotypes [15], as well as with the most abundant PhytoF described in melon leaves by Yonny et al. [39] (*ent*-16(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF).

In addition to the basal concentration of individual PhytoPs and PhytoFs, the influence of 1 and 15 mM salicylic acid on the content of these oxylipins in unripe and mature rice grains was also evaluated in the 'Yerua' cv. and two advanced lines ('R52' and 'R45'). As a result, significantly different effects were observed in the concentrations of individual PhytoPs and PhytoFs (Figures 2 and 3). This is of particular relevance, since currently little is known about the oxidative state in the early stages of grain filling, even though the possible involvement of ROS in seed-filling processes has been well documented [1].

In this regard, developing embryos would potentially generate significant amounts of ROS, requiring strict control through antioxidant mechanisms [1]. In unripe grains, a single application of 1 or 15 mM SA demonstrated that the basal concentrations of individual PhytoPs and PhytoFs decreased inversely with the amount of SA applied. However, this response was only significant in respect to 9-*epi*-9-D_{1t}-PhytoP (23.3, 10.1, and 9.7 ng g^{-1} dw for 0, 1, and 15 mM SA, respectively) (Figure 2 and Table S1). The other plant oxylipins did not show a response with a simple application of SA in that short period (6 days after application), nor in the applied doses (1–15 mM SA) (Figure 2 and Table S1).

On the contrary, when analyzing the effect of the application of 1 and 15 mM SA on the quantitative profiles of PhytoPs and PhytoFs in mature grain, both 1 and 15 mM SA decreased the concentrations of 7 out of the 11 oxylipins monitored (*ent*-16-F_{1t}-PhytoP, *ent*-16-*epi*-16-F_{1t}-PhytoP,9-D_{1t}-PhytoP, 9-L_1-PhytoP, *ent*-16(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF, *ent*-9(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF and *ent*-16(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF). At the same time, 9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, 9-*epi*-9-D_{1t}-PhytoP and 16-B_1-PhytoPdid not respond significantly to the hormonal application (Figure 2 and Table S1).

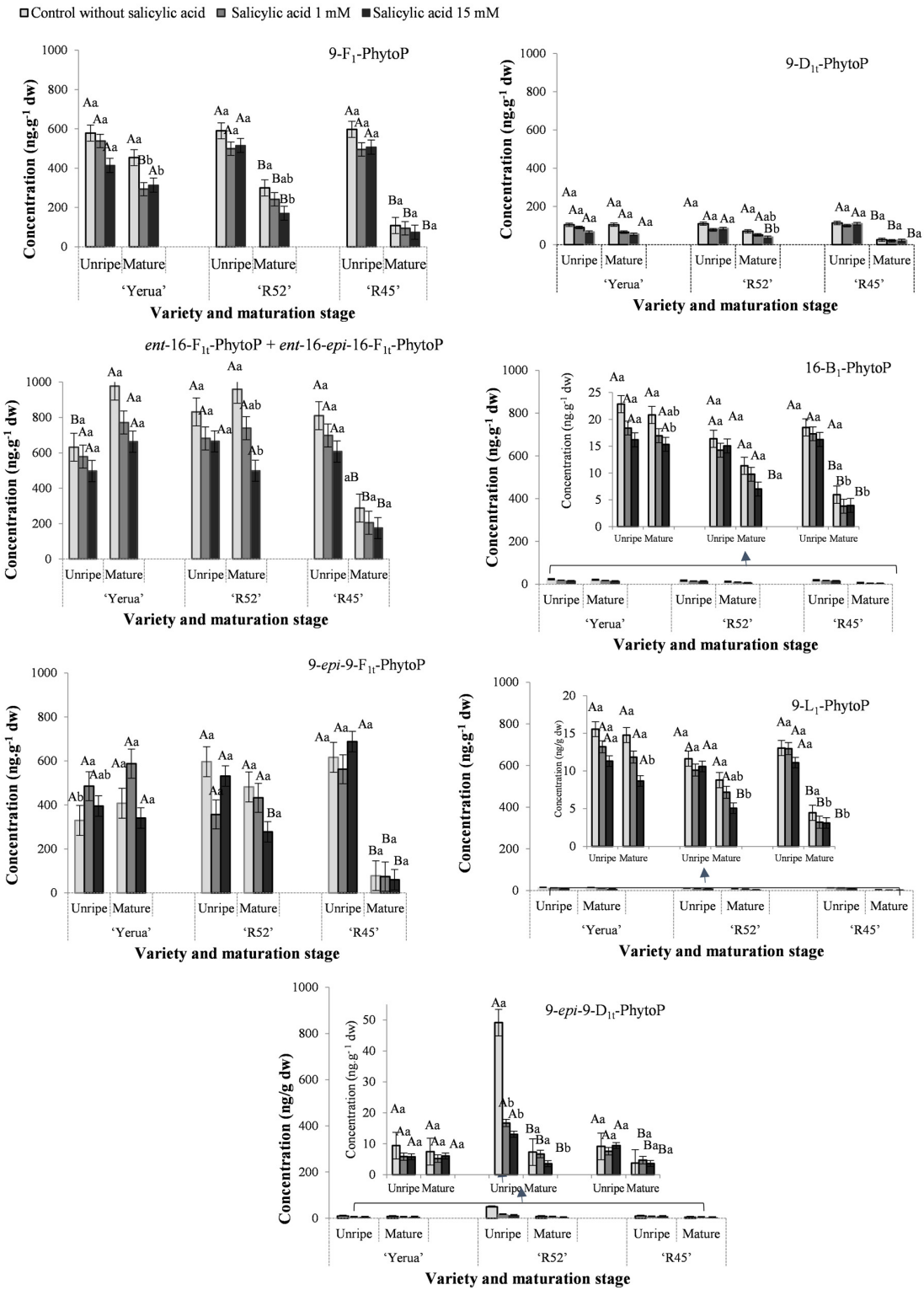

Figure 2. Comparison of concentrations of the individual phytoprostanes 9-F_{1t}-PhytoP, *ent*-16-F_{1t}-PhytoP, *ent*-16-*epi*-16-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, 9-D_{1t}-PhytoP, 9-*epi*-9-D_{1t}-PhytoP, 16-B_1-PhytoP and 9-L_1-PhytoP in unripe and mature rice grains at different salicylic acid supplementation levels (untreated, 1 mM and 15 mM) in each genotype ('Yerua', R52', and 'R45'). Distinct capital letters indicate significant differences between the maturation stage of rice grains at harvest for the same genotype, and different lowercase letters indicate significant differences between SA levels for the same genotype, both at $p < 0.05$, according to one-way analysis of variance (ANOVA) and multiple range test of Tukey.

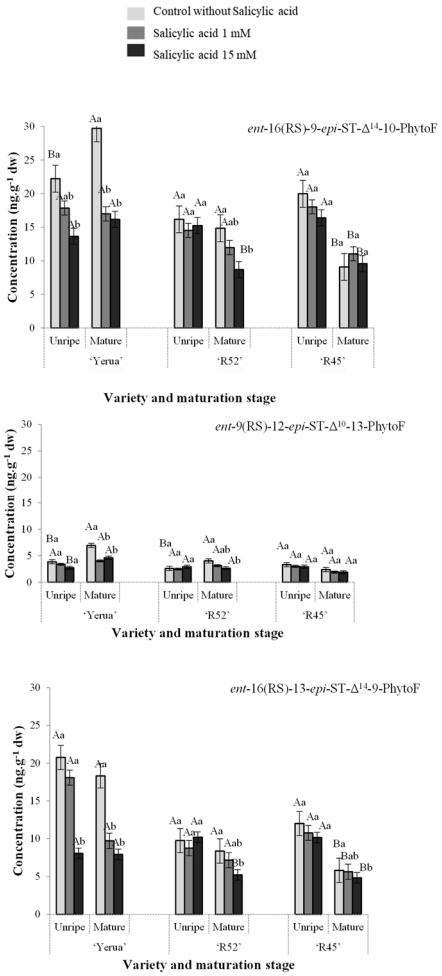

Figure 3. Comparison of concentrations of the individual phytofurans *ent*-16(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF, *ent*-9(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF and *ent*-16(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF in unripe and mature rice grains at different salicylic acid supplementation levels (untreated, 1 mM, and 15 mM) in each genotype ('Yerua', R52', and 'R45'). Distinct capital letters indicate significant differences between the maturation stage of rice grains at harvest for the same genotype, and different lowercase letters indicate significant differences between SA levels for the same genotype, both at $p < 0.05$, according to one-way analysis of variance (ANOVA) and multiple range test of Tukey.

Before the harvest of unripe rice grains, a single SA application was made, six days prior. Nonetheless, when harvesting the mature grain, rice plants had received two SA (1 or 15 mM) applications that raised the hormonal concentration of plants, causing a marked effect on the redox balance during filling and drying of the grain.

Concerning individual PhytoPs, the highest dose of SA assayed (15 mM) was the only one powerful enough to significantly increase their concentrations relative to untreated control. In the case of PhytoFs, and especially regarding *ent*-9(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF and *ent*-16(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF of mature grains, even the lower dose of SA (1 mM) significantly reduced the concentrations of these oxylipins, thus demonstrating their capacity to prevent oxidative stress during embryogenesis (Figure 3 and Table S2).

In light of antecedents regarding other higher plants such as tomato, the treatments with 1 mM SA seemed to enhance the ROS production and, consequently, the lipid peroxidation reactions in vitro in photosynthetic tomato leaves [3]. Indeed, the induction of ROS production by SA has been observed in treatments with different concentrations in an array of plant species and models, such as in suspension cultures of tobacco cells treated with 1 mM SA; in mitochondria isolated from tobacco leaves with 1 mM SA; in isolated tobacco cell mitochondria treated with 0.5 mM SA; in the appendix of *Sauromatumguttatum*

with SA 0.01 mM; in isolated soy cell mitochondria with 1 mM SA; in *Orobanche* seeds with 0.02 mM SA; in tobacco calluses with 0.02 mM SA; and in purified mitochondria of lupine yellow cotyledon treated with 1 mM SA [3].

Recently, in rice plants, Wang et al. [20] observed that the application of quinclorac damages the thylakoid membranes, and that the pre-treatment of SA 0.07 mM (10 mg L^{-1}) under quinclorac stress prevented the rupture of the thylakoid membrane by accelerating the production of chlorophyll and eliminating ROS; these findings indicate a key role in tolerance to SA-induced oxidative stress in rice plants. Furthermore, SA has the ability to decrease the activities of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), to enhance the concentrations of non-enzymatic antioxidants, and lower the concentrations of H(2)O(2) and malondialdehyde (MDA), indicating decreased generation of ROS under different stress conditions in rice [4,27]. Therefore, the variation in linolenic acid metabolism, together with the antioxidant action of salicylic acid on oxygen species generation, could affect the generation of these non-enzymatic oxylipins.

3.3. Oxidative Response of Unripe and Mature Grains after the Application of Exogenous Salicylic Acid Depending on Rice Cultivars

When analyzing the effect of the application of SA on the concentrations of the individual oxylipins (PhytoPs and PhytoFs) in unripe and mature grains, significant interactions were observed for all individual PhytoPs at harvest time (ANOVA, $p < 0.05$), considering the 'genotype' as the source of variation (Figures 2 and 3).

Regarding individual PhytoPs in unripe grains and their evolution due to exposure to different concentrations of SA, in general, the genotype 'Yerua' exhibited a decrease in PhytoP concentrations. In this aspect, the differences found were only statistically significant for 9-*epi*-9-F_{1t}-PhytoP (Figure 2, $p < 0.05$). On the other hand, for the mature grain of the 'Yerua' cv., changes in individual PhytoPs observed affected 9-F_{1t}-PhytoP, 16-B_1-PhytoP and 9-L_1-PhytoP. Hence, while the concentration of 9-F_{1t}-PhytoP reduced significantly with the application of 1 mM SA, the concentration of 16-B_1-PhytoP and 9-L_1-PhytoP only lowered significantly (by 53.1%, on average) when applying the highest dose (15 mM) (Figure 2). In respect to the advanced line 'R52' in unripe grains, only the concentration of 9-*epi*-9-F_{1t}-PhytoP modified significantly as a result of the application of SA, decreasing from 49.1 to 13.1 ng g^{-1} dw. However, this activity was observed when applying 1 mM SA. However, in mature grain, the concentrations of 9-F_{1t}-PhytoP, *ent*-16-F_{1t}-PhytoP + *ent*-16-*epi*-16-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, *ent*-9-D_{1t}-PhytoP and 9-L_1-PhytoP (299.6, 958.9, 7.3, 70.0 and 8.8 ng g^{-1} dw, respectively, under control conditions) decreased significantly after the application of 15 mM SA, by up to 103.0% (Figure 2). Finally, in respect to the advanced line 'R45', the application of 1 or 15 mM SA did not modify the concentrations of individual PhytoPs in unripe grains significantly; meanwhile, in mature grains of plants exposed to 1 mM SA, decreased concentrations were observed in 16-B_1-PhytoP and 9-L_1-PhytoP, from 5.96 to 3.82 ng g^{-1} dw, and from 4.45 to 3.20 ng g^{-1} dw, respectively (Figure 2).

The different responses observed in the three rice genotypes to exogenous SA agree with previous descriptions. Researchers who studied different rice genotypes described a significant difference between photosynthetic parameters and the antioxidant activities of specific enzymes [44,56]. This is in agreement with other studies that described different efficiencies in neutralizing ROS by distinct genotypes [57,58]. Indeed, in this regard, a differential modification of the quantitative profile of oxylipins when applying foliar fertilization in 5 rice genotypes has recently been specifically described [15], reporting significant changes of *ent*-16-F_{1t}-PhytoP, *ent*-16-*epi*-16-F_{1t}-PhytoP and 9-D_{1t}-PhytoP.

With respect to the different concentrations of PhytoPs between immature and mature rice grains in response to exogenous SA applied, the genotype 'Yerua' did not present significant differences. On the other hand, in the advanced line 'R52', the concentration was different in mature grain regarding 9-F_{1t}-PhytoP and 9-*epi*-9-D_{1t}-PhytoP for all SA doses, and in 9-*epi*-9-F_{1t}-PhytoP, 9-D_{1t}-PhytoP, 16-B_1-PhytoP and 9-L_1-PhytoP when applying the

highest amount of SA (15 mM). In contrast, in the advanced line 'R45', the concentrations of all individual PhytoPs in mature grain were significantly lower than those in unripe grain (Figure 2).

In addition, the analysis of the concentration of individual PhytoFs in unripe grains of the 'Yerua' cv. evidenced that the concentrations of *ent*-16(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF, *ent*-16(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF and 9-F_{1t}-PhytoP were significantly reduced by the highest concentration of SA (15 mM) (Figure 3). In mature grain, the lower concentration of SA (1 mM) was powerful enough to lower the concentration of all three PhytoFs monitored. Nonetheless, in unripe grains of both advanced lines under study, the application of SA did not produce any effect; meanwhile, for mature grains, in 'R52', a decrease in the concentrations of the three PhytoFs was observed as a result of the application of 15 mM SA. In the advanced line 'R45', the only PhytoF that evidenced a significant reduction in its concentration in plants exposed to 15 mM of SA was *ent*-16(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF, which after SA treatment achieved 4.82 ng g^{-1}.

When analyzing the quantitative profiles of the individual PhytoFs at harvest time, a decrease was observed in mature grains produced compared to immature grains in the three genotypes. However, in the genotype 'Yerua', this reduction was only significant for *ent*-16(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF after the application of 1 mM SA, while in 'R52' and 'R45', the significant differences observed were restricted to *ent*-16(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF and *ent*-16(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF in 'R52', as a result of exposure to 15 mM SA, and in 'R45' concerning both 1 and 15 mM SA (Figure 3).

The close relationship between the exogenous application of SA and the concentration of oxylipins observed in the results corroborates their link in the control of redox homeostasis. In this regard, a parallel can also be made between the reduction in oxylipins when applying SA and the decline in enzymatic antioxidant activity. Wang et al. [21] observed that SA pre-treatment further enhanced all enzymes' activities compared to a quinclorac herbicide treatment alone. Superoxide dismutase (SOD), ascorbate peroxidase (APX) and peroxidase (POD) activities showed the same increasing tendencies in the leaves of rice.

3.4. Effect of Exogenous Salicylic Acid on Yield and Quality of Rice Grain

To evaluate the effect of applying the phytohormone SA on the yield and quality of rice in the genotypes under evaluation, the number of grains per panicle and the percentage of chalkiness (often defined as the opaque parts in the endosperm [59] were assessed, since both parameters have significant economic repercussions, in addition to the influence of exogenous SA. The number of grains per panicle, together with the weight of the grain and the number of spikelets per panicle per growing area, define the productivity of the crop in kilograms per hectare [60]. Chalkiness is a defect that decreases the visual quality of rice, and generates a fragile structure in the grain, which increases breakage during milling, consequently reducing the percentage of whole grain, which is a fundamental parameter for the commercialization of this cereal [60].

The analysis of the number of spikelets per panicle evidenced an increase of between 2.0 and 19.0% when applying exogenous SA at the highest dose (15 mM) in the three genotypes (Table 1). This difference was only statistically significant ($p < 0.05$) in the advanced line 'R52' when applying the highest dose of SA, and resulted in values of the number of spikelets per panicle of 112.1, 110.3 and 132.2, on average, for the doses of SA of 0, 1 and 15 mM, respectively (Table 1). The number of spikelets per panicle did not present differences between genotypes, and its average value was 115.9 spikelets per panicle.

The application of SA also influenced the percentage of chalkiness. Although the physiological mechanisms involved in chalkiness are not fully understood, Zhang et al. [59] described certain hormonal regulation (ethylene and 1-aminocylopropane-1-carboxylic acid) in the grains, which can affect the arrangement of the starch granules in the cells; this can lead to an increase in dull parts in the endosperm [59]. In the case of damaged (central chalky) grains caused by high-temperature stress, abnormal- and round-shaped starch granules were loosely packed in the grain, and these were observed through irregular

reflections of the light. The mechanism for grain chalkiness under high-temperature stress is considerably complicated. The temperature at the grain-filling stage has been shown to influence the starch composition in rice grains [25].

Table 1. Mean values of numbers of spikelets per panicle and chalkiness in mature rice grain genotypes at different salicylic acid supplementation levels.

Parameter	Genotype [Z]	Treatment [Y]			Interaction SA by G
		Control	SA 1	SA 15	
Spikelets per panicle	'Yerua'	119.8 ± 1.7 a [X]	115.2 ± 6.3 a	120.4 ± 4.5 a	N.s.
	'R52'	112.1 ± 5.0 b	110.3 ± 1.3 b	132.2 ± 9.9 a	
	'R45'	106.2 ± 5.5 a	107.8 ± 1.1 a	119.5 ± 5.4 a	
Chalkiness (%)	'Yerua'	18.6 ± 1.9 a	17.8 ± 1.9 a	15.6 ± 0.7 a	N.s.
	'R52'	22.6 ± 2.1 a	15.3 ± 2.0 ab	13.8 ± 0.7 b	
	'R45'	15.4 ± 1.5 a	14.0 ± 1.7 a	11.7 ± 1.2 a	

[Z] Genotypes, 'Yerua', Yerua PA; 'R52', R/03-5x desc/04-52-1-1; 'R45', R/03-5x desc/04-45-1-1. [Y] Control, without exogenous application of salicylic acid; SA1, salicylic acid at 1 mM; SA15, salicylic acid at 15 mM. Data presented as mean ± SEM (n = 3). [X] Values in the same row followed by different letters are significantly different, at $p < 0.05$, according to one-way analysis of variance (ANOVA) and multiple range test of Tukey. N.s., not significant.

In the present study, the chalkiness was equivalent in all of the genotypes (Table 1). However, the application of SA induced a decrease in the average values for the three genotypes. Thus, the chalkiness values were 18.9, 15.7 and 13.7% for 0, 1 and 15 mM SA, respectively, with the application of 15 mM SA being the only dose responsible for differences observed relative to the control. When analyzing the effect of SA on each genotype, it was observed that, although all three genotypes improved in grain transparency, the chalkiness value was only statistically lower in 'R52' (Table 1). The observed differences between the lines may be due to the endogenous content of SA and different capacities to maintain redox homeostasis [58]. Although no trials have been reported regarding the influence of SA on chalkiness, it is known that ascorbic acid is a major plant antioxidant that is likely responsible for changing redox homeostasis in critical developmental stages associated with grain filling, and alters grain chalkiness in rice [23]. Furthermore, the chalking mechanism of a rice grain under HT stress has been discussed in terms of the grain starch glycome, transcriptome and proteome [25]. In this regard, and in relation to the oxylipins mentioned above, it has been described that stress conditions affect the concentration of fatty acids, including linolenic acid, a precursor of non-enzymatic oxylipins of the current study, leading to chalkiness (grain-filling defect). On the other hand, salicylic acid has the capacity to increase antioxidant enzymes and decrease the generation of ROS. Therefore, non-enzymatic oxylipins could indicate grain-filling defects.

3.5. Strengths and Limitations of the Study

Our study reveals that the exogenous application of salicylic acid regulates and alleviates oxidative stress linked to plant oxylipins (PhytoPs and PhytoFs), thus improving rice yield and quality traits. Limitations of the study include being the first of its kind to study the link between SA application during rice ontogenesis in the field, and changes in oxylipin varieties and content in the developing and mature seed; hence, an understanding of the biochemical and molecular processes involved has not been realized. It is also known that SA is a mediator of defence responses that can activate these responses in the panicle, and prevent further panicle diseases and environmental stresses. Changes in SA content in the seed after application could be monitored for comparison with natural endogenous levels. We are conducting a more in-depth study that considers the previous parameters evaluated, in addition to the number of spikelets, as the ontogenesis of the panicle is fully completed at the boot stage and grain-filling stage; we are also considering grain weight and ultimately yield per plot, in order to determine the interest of applying such a treatment

in a production scheme. In this way, the relationship of oxylipin changes as a consequence of salicylic acid application and selected agronomic traits could be better established.

4. Conclusions

In summary, the results obtained in this study corroborate the complexity of the regulatory system affecting the redox balance in plant cells. Despite this difficulty, it was demonstrated in this investigation that the application of SA improves the performance of whole and total grain, and contributes to explaining the positive effects of SA in the regulation of plant cell metabolism. In connection with this, since variations in plant oxylipin concentrations occur in parallel to changes in rice grain yield and quality, the major outcomes of this research, relative to the quantitative profiles of PhytoPs and PhytoFs in diverse rice genotypes at different maturation stages, allows prediction of the quality of the grains at harvest time with the exogenous application of SA. In this regard, the influence of these parameters (concentrations of PhytoPs and PhytoFs) on the economic results of rice crops becomes an important issue that should be further addressed in the future. Furthermore, the confirmation of the effectiveness of SA treatment on rice grain chalkiness will have economic importance, given the close relationship of this parameter with whole grain percentage. Although it would be interesting to analyze the relationship of oxylipins with characteristic enzymes of oxidative stress (i.e., CAT, SOD) in future research, this is the first time that the influence of the exogenous application of SA on the embryogenesis and its effect on the quality parameters of rice has been studied. Hence, the findings described in the present research provide new insights into grain embryogenesis regulation by SA, and offers a scientific foundation for rice cultivation practices. Moreover, this preliminary study serves as a starting point for further in-depth trials that consider targeted biochemical and molecular processes, grain filling, grain weight and ultimately yield per plot, in order to determine the level of interest for applying such a treatment in a production scheme.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13030636/s1, Table S1, Concentration of each oxylipin (PhytoPs) quantified according to salicylic acid dose, and harvest time; Table S2, Concentration of each oxylipin (PhytoFs) quantified according to salicylic acid dose, harvest time.

Author Contributions: M.P. developed the investigation, formal analysis and writing of the original draft. R.D.-P. provided conceptualization, investigation, validation, writing, review and editing of the current study. S.M. carried out the formal analysis. C.O. and A.G. carried out the validation and visualization. T.D. and F.F. contributed with the investigation, review, editing, visualization and supervision. M.M.C.-V. and J.A.G.-H. wrote and reviewed the manuscript. Á.G.-I. carried out the review, editing, supervision, conceptualization, methodology, investigation, project administration and funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All data presented in the study are available on request from the corresponding authors (angelgil@cebas.csic.es; rdperles@cebas.csic.es).

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