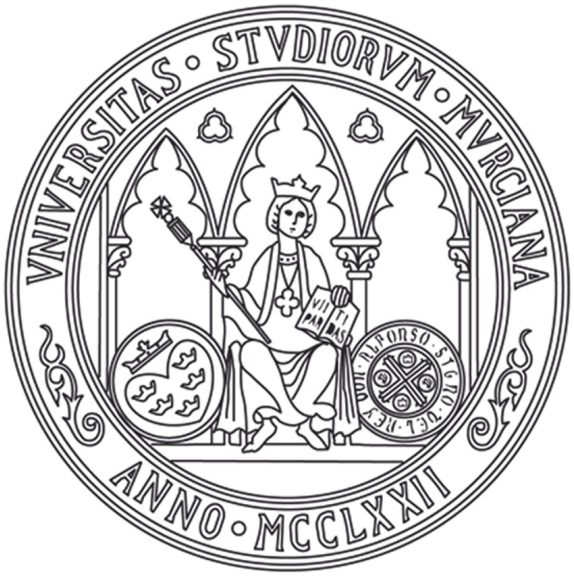

UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

Role of Phytoprostanes and Phytofurans in the Protection and Defense Mechanisms of Rice (*Oryza sativa* L.) Against Oxidative Stress Caused by Abiotic Agents and Different Technological Practices

Rol de los Fitoprostanos y Fitofuranos en los Mecanismos de Protección y de Defensa de Plantas de Arroz (*Oryza sativa* L.) Frente al Estrés Oxidativo Causado por Agentes Abióticos y Prácticas Tecnológicas

Dña. María Pinciroli

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Departamento de Ciencia y Tecnología de los Alimentos

TESIS DOCTORAL

Role of phytoprostanes and phytofurans for the protection of rice (O*ryza sativa* L.) against oxidative stress caused by abiotic agents and technological practices

Rol de los fitoprostanos y fitofuranos en los mecanismos de defensa en plantas de arroz (*Oryza sativa* L.) frente al estrés oxidativo causado por agentes abióticos y prácticas tecnológicas

Memoria realizada para optar el Grado de Doctor

presentada por

María Pinciroli

Bajo la dirección de los Doctores:

Dr. Ángel Gil-Izquierdo
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Murcia, 2018

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El Dr. Ángel Gil Izquierdo, Científico Titular del Consejo Superior de Investigaciones Científicas (CSIC), en el Centro de Edafología y Biología Aplicada del Segura de Murcia (CEBAS), autoriza:

La presentación de la Tesis Doctoral titulada '**Role of phytoprostanes and phytofurans for the protection of rice (O*ryza sativa* L.) against oxidative stress caused by abiotic agents and technological practices**' realizada por Dª. María Pinciroli, bajo mi dirección y supervisión, en el Departamento de Ciencia y Tecnología de los Alimentos CEBAS-CSIC, para la obtención del Grado de Doctor por la Universidad de Murcia.

En Murcia, a 14 de mayo de 2018

Dr. Ángel Gil Izquierdo

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El Dr. Raúl Domínguez-Perles, Investigador Postdoctoral contratado por el Consejo Superior de Investigaciones Científicas (CSIC), en el Centro de Edafología y Biología Aplicada del Segura de Murcia (CEBAS), a cargo del programa Juan de la Cierva de Incorporación del Ministerio de Economía, Industria y Competitividad, autoriza:

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La Dra. Mariana Garbi, Docente de la Facultad de Ciencias Agrarias y Forestales de la Universidad Nacional de La Plata (Argentina), autoriza:

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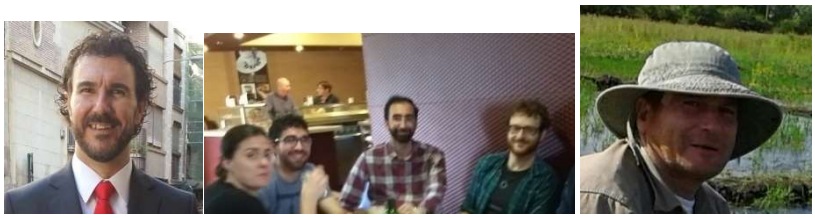
.



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Pinciroli, M.; Domínguez-Perles, R.; Abellán, A.; Guy, A.; Durand, T.; Galano, J. M.; Gil-Izquierdo. Comparative study of the phytoprostanes and phytofurans content of *indica* and *japonica* rice's (*Oryza sativa* L.) flours. *Journal of Agricultural and Food Chemistry*, **2017,** *65*, 8938-8947. http://pubs.acs.org/doi/ipdf/10.1021/acs.jafc.7b03482.

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RESUMEN

El cultivo del arroz (*Oryza sativa* L.) constituye una actividad agro-alimentaria fundamental para asegurar el suministro alimentario de más de la mitad de la población mundial. Se estima que, en 2050, la población mundial alcanzará los 9 mil millones, lo que obligará a aumentar la producción agrícola en 1,7 veces para garantizar el abasto de alimentos para toda la población. En la actualidad, al menos el 60% de los suelos cultivados en la producción de cultivos extensivos en todo el mundo se caracterizan por problemas de limitación de crecimiento. La mayor parte de estas pérdidas de rendimiento son debidas a diferentes factores de estrés abiótico como la escasez de agua, la salinidad, temperaturas extremas, acidez y aporte nutricional inapropiado para las plantas, es decir, deficiencias y toxicidades. Así, en relación con la temperatura, por ejemplo, a pesar de ser el arroz un cultivo tropical o subtropical, las altas temperaturas constituyen un factor limitante para el llenado de grano, lo que lleva a la pérdida de rendimiento debido a la disminución en el tamaño, número y calidad del grano. La temperatura óptima del aire para la maduración de variedades de subespecie japónica oscila entre 20 y 25 °C, mientras que la temperatura del aire por encima de 30°C puede causar efectos perjudiciales en el rendimiento de los cultivos de arroz y la calidad nutricional de la producción final.

Las plantas y los animales mantienen ácidos grasos poliinsaturados (PUFAs) en sus membranas, que son corresponsables de la modulación de la fluidez de las mismas, que pueden liberarse y metabolizarse enzimáticamente hacia ácidos grasos oxidados, algunos de los cuales actúan como moléculas de señalización en rutas de defensa en plantas superiores, *in vivo*. Además de la oxidación enzimática, las membranas vegetales (especialmente ricas en linoleato y linolenato) son propensas a la oxidación no enzimática que da lugar, en primer término, a fitoprostanos (FPs), en tanto en niveles de oxígeno molecular superiores, después de la ciclación inicial, conduce a la generación de estructuras furánicas llamadas fitofuranos (FFs). Actualmente, se sabe que estos compuestos bioactivos actúan como mediadores endógenos capaces de proteger las células de daños en diversas condiciones relacionadas con el estrés oxidativo en plantas superiores, en tanto que, en relación con su actividad biológica en mamíferos, se ha descrito su capacidad para la regulación de la función inmune (*in vitro*).

En relación con la inducción de estrés en cultivos de arroz, el ácido salicílico es una hormona vegetal que participa en varios procesos celulares y tiene la capacidad de modular diferentes mecanismos relacionados con la respuesta a situaciones de estrés. Por otro lado, en el cultivo de arroz, la fertilización con nitrógeno es una práctica tradicionalmente aplicada, sin embargo, la disponibilidad efectiva de nutrientes y eficiencia de su absorción por las plantas son

muy bajas, debido especialmente al manejo del agua de riego y al desconocimiento actual del momento oportuno de aplicación. Como alternativa, recientemente se ha propuesto la pulverización foliar, que permite una mayor disponibilidad de nutrientes en las fases de desarrollo específicas de la planta en que los requiere, al reducir el tiempo de latencia entre la aplicación y su absorción. En esta investigación se propone estudiar perfil y contenido en FPs y FFs en arroz blanco, arroz integral y salvado de arroz, en diferentes genotipos, así como su rol en los mecanismos de protección y defensa contra el estrés oxidativo causado por agentes abióticos y diferentes prácticas tecnológicas (suplementación con fertilizante foliar, ácido salicílico y cubierta plástica).

Con el fin de obtener información que permita una mayor comprensión de los niveles de estrés oxidativo inducido por las prácticas agronómicas mencionadas, en diferentes genotipos de arroz tradicionalmente cultivadas, se establecieron 3 ensayos durante las campañas 2016-2017 y 2017-2018, en un campo experimental de la provincia de Buenos Aires, en el centro-este de Argentina, perteneciente a la Facultad de Ciencias Agrarias y Forestales de la Universidad Nacional de La Plata. El diseño experimental fue, en todos los casos, bloques al azar con 3 repeticiones. Las siembras se realizaron en secano, en forma manual utilizando 350 semillas m^{-2} en líneas a 0,20 m, en parcelas de 5 m^2. Los ensayos se condujeron con riego por inundación a partir de los 30 días de la emergencia y se controlaron las malezas con bispiribac sodio.

En el primer ensayo se analizaron las harinas de grano pulido integral y salvado de 14 variedades de arroz, 8 de la subespecie *indica* (‘Cambá’, ‘Don Ignacio FCAyF’, ‘Don Justo FCAyF’, ‘El Paso 144’, ‘Guri INTA’, ‘IRGA 424’, ‘Nutriar FCAyF’, and ‘Puitá’) y 6 de la subespecie *japónica* (‘Amaroo’, ‘Arborio’, ‘Itapé’, ‘Quebracho FA’, ‘Yamani’, and ‘Yerua FA’) provenientes de cultivos de la Facultad de Ciencias Agrarias y Forestales de la Universidad Nacional de La Plata.

En el segundo ensayo, se sembraron cinco genotipos de arroz del Programa de mejoramiento de arroz: 1 variedad de normal contenido proteico en grano, ampliamente utilizada en el área de producción (‘Guri INTA’), 1 variedad (‘Nutriar FCAyF’) y 3 líneas avanzadas de elevado contenido de proteína en grano (‘H458-41-1-1-1’, ‘H475-3-1-1-2’ y ‘H484-9-1’). Los tratamientos fueron control (T0) y 0.3 Lha^{-1} de fertilizante foliar en iniciación de panícula (T3). El fertilizante foliar utilizado consistió en una solución de micronutrientes y se monitorizó el efecto de su aplicación en el rendimiento y calidad de grano del cultivo y los niveles de FPs y FFs como indicadores de estrés nutricional.

Finalmente, en el tercer ensayo, se sembraron 7 genotipos de arroz de tipo largo-ancho, subespecie japónica (‘Yerua PA’, ‘R/03-5x desc/04-52-1-1’, ‘R/03-5x desc/04-45-1-1’, ‘Amaroo x desc/08-1-1-1-2’, ‘R/03-5xdesc/04-27-3-1’, R/03-5xdesc/14-1-1-1’ y ‘H489-5-1-2’). Las

condiciones ensayadas fueron dos ambientes: a campo abierto y cubierta de plástico de 100 micrones de espesor y 3 dosis de pulverización con ácido salicílico de 0 (control), 1 y 15 mM. A través de este ensayo se estudió el efecto de dichas prácticas agronómicas sobre los niveles de los marcadores de estrés FPs y FFs.

Este trabajo permitió describir por primera vez, a nivel mundial, el efecto del procesado de arroz para la obtención de harina sobre los niveles de FPs y FFs, sintetizados como se mencionó previamente a través de reacciones de oxidación no enzimáticas en los tres ensayos realizados.

La cosecha y la trilla se realizaron en forma manual, los granos se secaron en estufa a 40 ± 1°C. A partir del grano con cáscara, se obtuvo grano pulido, integral y salvado utilizando un molino experimental. Los granos pulido e integral se molieron y tamizaron a través de mallas de 0,4 mm. Las harinas obtenidas y el salvado se almacenaron a 4°C, en oscuridad, hasta su evaluación analítica. La extracción y el análisis de los FPs 9-F_{1t}-PhytoP, *ent*-16-F_{1t}-PhytoP+*ent*-16-*epi*-16-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, *ent*-9-*epi*-9-D_{1t}-PhytoP, *ent*-9-D_{1t}-PhytoP, 16-B_1-PhytoP y 9-L_1-PhytoP y los FFs *ent*-16-*(RS)*-9-*epi*-ST-Δ^{14}-10-PhytoF, e*nt*-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF y *ent*-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF. La extracción y adecuación de las muestras para estos compuestos se realizó mediante extracción en fase sólida y su posterior análisis cualitativo y cuantitativo mediante UHPLC-ESI-QqQ-MS/MS. Las diferencias estadísticas entre genotipos y condiciones de cultivo/procesado se obtuvieron efectuando análisis de varianza multifactorial o de una vía (ANOVA) y las medias se compararon utilizando el test de rangos múltiples de Tukey.

La implementación de los diseños experimentales descriptos y la metodología indicada permitieron obtener los siguientes resultados generales:

En relación con el impacto del proceso tecnológico de la obtención de harinas de arroz sobre los niveles de FPs y FFs, se observó que, para los FPs, las concentraciones promedio fueron más altas en el salvado de arroz (<0,1-9,4 ng g^{-1}) que en las harinas de grano pulido e integral (<0,1-1,2ng g^{-1}). El estudio de FFs en harinas de arroz presentó valores promedio de 1,8; 4,2 y 10,3 ng g^{-1} en salvado de arroz, harina de grano integral y pulido, respectivamente. Se observó una correlación significativa entre la concentración de compuestos totales e individuales. Las concentraciones recuperadas sugieren que el salvado de arroz es una valiosa fuente dietética de FPs y FFs que debería considerarse en estudios adicionales sobre biodisponibilidad y bioactividad *in vitro* e *in vivo*.

Al analizar el contenido de FPs y FFs totales en granos de arroz de 5 genotipos como indicadores del impacto de la fertilización foliar sobre la concentración de moléculas diana totales, se encontraron efectos significativos. De hecho, la abundancia relativa de los diferentes FPs individuales cuantificados, promedio de los tratamientos y los genotipos oscilaron entre

213,3 y 3,1 ng g^{-1}, detectándose el siguiente orden decreciente de abundancia: *ent*-16-F_{1t}-PhytoP+ *ent*-16-*epi*-16-F_{1t}-PhytoP > 9-F_{1t}-PhytoP > *ent*-9-D_{1t}-PhytoP > 16-B_1-PhytoP > 9-L_1-PhytoP > *ent*-9-*epi*-9-D_{1t}-PhytoP > 9-*epi*-9-F_{1t}-PhytoP. Por otro lado, las concentraciones de los FFs individuales variaron entre 19,2 y 3,5 ng g^{-1} describiéndose el siguiente orden decreciente de concentración en el material vegetal analizado: *ent*-16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF > *ent*-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF > *ent*-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF. En términos generales, y considerando el promedio de los 5 genotipos, contenido total de de FPs y FFs disminuyó un 31,9% (de 442,9 a 335,9 ng g^{-1}) y en un 20,0% (de 36,4 a 29,8 ng g^{-1}), respectivamente, como consecuencia de la fertilización foliar aplicada. Los resultados sugieren que la fertilización generó una disminución del estrés nutricional, en tanto que los diversos genotipos incluidos en el estudio respondieron en función de su tolerancia. La realización del análisis estadístico de los resultados reveló que la fertilización foliar produce efectos significativos y variados dependiendo del genotipo considerado y del marcador objeto de estudio, lo que dará lugar a nuevas investigaciones y la selección de la mejor alternativa para la aplicación práctica de la tecnología evaluada en la presente investigación.

Finalmente, al analizar la concentración de FPs y FFs totales de granos de arroz de siete genotipos cultivados bajo diferentes ambientes y con la aplicación exógena de ácido salicílico, se encontraron respuestas significativamente diferentes. En términos generales, el incremento en 1°C de la temperatura del aire, debido a la cubierta de polietileno, produjo una reducción de la concentración de la mayoría de los biomarcadores de estrés analizados (FPs y FFs), tendencia que resultó significativa en los compuestos 9-F_{1t}-PhytoP y *ent*-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF. Este resultado podría indicar que las condiciones del ambiente cubierto, cálido y húmedo resultan beneficiosas, menos estresantes, para la mayoría de los genotipos, considerando que el arroz es una especie tropical o subtropical y las condiciones de localización del ensayo son subóptimas desde el punto de vista térmico. Los genotipos se comportaron en forma diferencial en los ambientes para las concentraciones de los compuestos 9-F_{1t}-PhytoP, *ent*-9-*epi*-9-D_{1t}-PhytoP, 16-B_1-PhytoP, *ent*-16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF y *ent*-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF. Estos resultados coinciden con diferentes autores que sostienen que existen diferencias varietales genéticas en respuesta a altas temperaturas, críticas según su capacidad de regular la homeostasis redox en las células. En términos generales, se observó una tendencia a la reducción de la concentración de los biomarcadores con la aplicación exógena de ácido salicílico 1 y 15 mM, con respecto al control no expuesto a esta hormona vegetal. Es importante destacar que las oscilaciones observadas fueron diferentes y dependientes de los compuestos (marcadores) analizados y genotipos considerados. La suma total de biomarcadores en cada genotipo resultó entre 1751,3 y 192,4 ng g^{-1} ordenándose de mayor a menor: 'Yerua'>'R/03-5x

desc/04-52-1-1'>'H489-5-1-2'>'R/03-5x desc/04-45-1-1'>'R/03-5x desc/04-27-3-1'>'R/03-5x desc/14-1-1-1'>'Amaroo x desc /08-1-1-1-2'. Como resultados de esta investigación, en la que se describe por primera vez la presencia de FPs y FFs en arroz, se pudo concluir que la localización de estos metabolitos, es preferentemente, en capas externas o pericarpio del grano donde se concentran los lípidos. Su concentración cualitativa y cuantitativa es fuertemente dependiente del genotipo. La suplementación con fertilización foliar de nutrientes y la hormona vegetal, ácido salicílico, genera una importante disminución en la concentración de FPs y FFs. En este sentido, estas prácticas tecnológicas pueden ejercer un efecto de mitigación del daño oxidativo causado como consecuencia del incremento de especies reactivas de oxígeno en situaciones de estrés abiótico. Los FPs y FFs son excelentes biomarcadores de estrés nutricional en plantas de arroz, dado que resultaron más sensibles que otros parámetros como rendimiento y calidad de grano. La concentración de los compuestos responde a la aplicación exógena de la hormona ácido salicílico, lo cual confirma su rol en el metabolismo de tolerancia al estrés. Se constató una respuesta diferencial de los materiales genéticos a las prácticas tecnológicas realizadas.

Esta Tesis, recoge tres trabajos de investigación multidisciplinar que combinan la Agronomía y la Tecnología de los Alimentos, lo que ha permitido describir, por primera, vez la presencia de FPs y FFs en arroz y harinas de arroz en relación con su perfil y concentración cuantitativa. Es posible que la profundización del conocimiento del metabolismo de los nutrientes minerales y su efecto sobre el estrés oxidativo permita obtener variedades de arroz más tolerantes al estrés nutricional o con mayor eficiencia en la respuesta a prácticas agrícolas como la fertilización foliar. La suplementación con ácido salicílico podría ser una práctica de gestión prometedora en los años más cálidos, que serán cada vez más frecuente en las próximas décadas debido al cambio climático, mitigando los efectos nocivos del estrés por calor. En este sentido, la información obtenida ayudará a comprender las herramientas fisiológicas de esta especie para protegerse contra las agresiones externas, lo cual es esencial para obtener, mediante enfoques de bioingeniería, materiales genéticos más tolerantes a deficiencias nutricionales o condiciones ambientales adversas o más eficientes en la respuesta a la fertilización foliar.

ABREVIATURAS

ALA, α-linolenic acid

APX, ascorbateperoxidase

BHA, butylatedhydroxyanisole

CAT, catalase

DHA, dehydroascorbate reductase

GPX, glutathione peroxidase

GR, glutathione reductase

GST, glutathione S-transferase

IRRI, International Rice Research Institute

IsoPs, isoprostanes

LOD, limit of detection

LOQ, limit of quantification

MDHAR, mono de hydroascorbate reductase

MRM, Multiple Reaction Monitoring

MS, mass spectrometry

N.s., no signification

N, nitrogen

PAR, photosynthetically active radiation

PhytoFs, phytofurans

PhytoPs, phytoprostanes

PLs, phospholipids

POX, peroxides

PR, Pathogenesis-related

PUFAs, Polyunsaturated fatty acids

ROS, reactive oxygen species

SA, Salicylic acid

SAR, systemic acquired resistance

SOD, superoxide dismutase

SPE, solid phase extraction

UHPLC-ESI-QqQ-MS/MS, ultra-high performance liquid chromatography coupled to electrospray ionization and triple quadrupole mass spectrometry.

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CHAPTER I. GENERAL INTRODUCTION

1.1. RICE (*Oryza sativa* L.)

1.1.1. General issues related to rice production

Rice (*Oryza sativa* L.) is the staple food of more than three billion people, more than half of the world's population (Yang *et al*., 2014). Of the 840 million people who suffer from chronic hunger, more than 50% lives in areas dependent on rice production (Redoña, 2004). It is the basis of food in Asian countries, where the population has an annual growth rate of 1.8% (Katsube-Tanaka *et al*., 2004).

Rice is grown in a wide range of locations and under a variety of climatic conditions, from the wettest areas of the world to the driest deserts. Hence, occurs along the Arakan coast of Myanmar, where the growing season records an average of more than 5,100 mm of rain, and in the Al Hasa oasis in Saudi Arabia, where the annual rainfall is less than 100 mm. World rice production is distributed in at least 114 countries (FAO, 2013), and is the main source of income and employment for more than 144 million farms worldwide, more than any other crop. Around 80.0 % of the world's rice is produced on small farms (FAO, 2004).

1.1.1.1. Origin, varieties, global production and world distribution

Rice cultivation began almost 10,000 years ago, in many humid regions of tropical and subtropical Asia (Arguisain, 2006). It is estimated that the geographical origin is the state of Oryza in northeastern India, on the slopes of the Himalaya. This hypothesis is supported by the presence and conservation of the genetic variation existing in the area, the spread of crosses and favored by the isolation of the environmental conditions of the place. The antiquity of the crop, its great diffusion, and the primitive conditions of work, as well as the biological characteristics of great adaptability of the plant, have made difficult its study and classification overs the time. The varieties of rice cultivated belong to the genus *Oryza* which in turn, belongs to the Oriceas tribe (subfamily Orizoideas de las Gramíneas), a small group of aquatic plant genera (or at least hygrophilous). The genus *Oryza* groups medium-high plants, annual or perennial graminiforms. To date, 24 species are included in the genus *Oryza*. According to several studies, there would be two evolutionary patterns of origin and domestication of cultivated rice, one in Asia, for the *O. sativa* species and other in Africa (Niger central delta) for

O. glaberrima. Besides the phylogenetic differences, these species present morphological differences (Acevedo *et al.,* 2006).

Global production. Rice is the second most important cereal crop in the world after corn. The world rice production of the last harvest (2016/2017) was 483.1 Mt (USDA, 2017). Since the beginning of the Green Revolution, in 1968, world rice production has increased sharply by almost 140%. In this regard 1968 to 2010, the field sown with rice increased from 129.0 Mha to approximately 159.4 Mha (**Figure 1**), and the average yield almost doubled, from of 2.23 to 4.32 t / ha, on average.

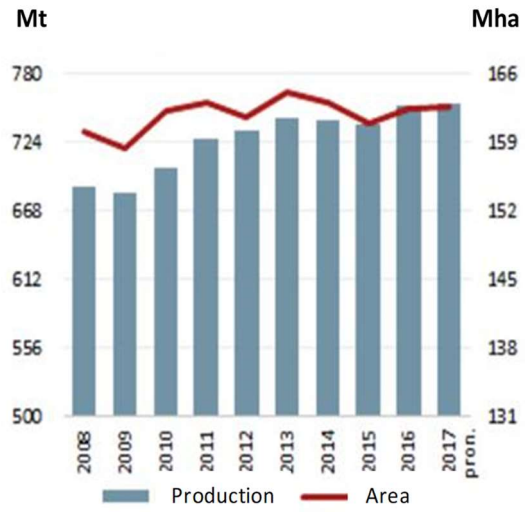


Figure 1. Global production and cultivated rice area in the world. Source: FAO, 2017

The largest rice producers in the world are by far China and Indiaresponsible 53.0% of the world production (FAOSTAT, 2013). Although its area harvested is lower than India's, China's rice production is greater because of higher yields and because nearly all of China's rice area is irrigated, whereas less than half of India's rice area is irrigated. After China and India, the next largest rice producers are Indonesia, Bangladesh, Vietnam, Myanmar, and Thailand (**Figure 2**). In Latin America, Brazil is by far the largest producer and has represented almost half (45.0% between 2008 and 2010) of rice production in the region. After Brazil, the largest producers are Peru and Colombia, followed by Ecuador. The most important production centers are located in the United States (California and the southern states near the Mississippi River). The main European producers are Italy, Spain, and Russia. Australia used to be an important producer, but its production has decreased substantially in recent years due to the recurrent drought (Mohanty *et al.,* 2013) (**Figure 2**).

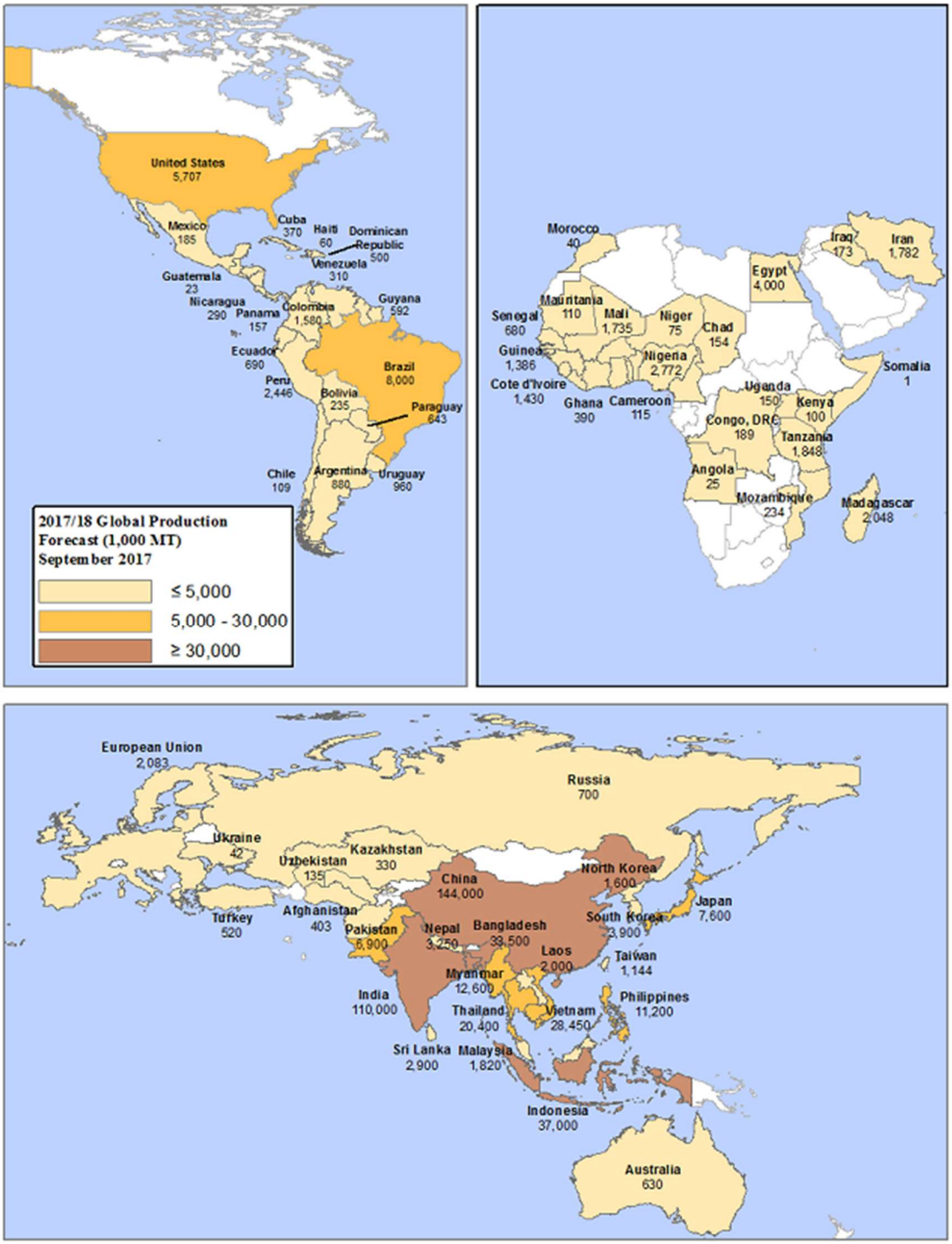


Figure 2. Production forecast for 2017/18, September 2017. Source**:** U.S. Dept. of Agriculture, Foreign Agricultural Service, Production, Supply and Distribution data base, 2017. Source: https://apps.fas.usda.gov/psdonline/app/index.html#/app/advQuery

Another important issue regarding rice a basic food for humans is marketing and consumption. In this respect, less than 7.0% of world rice production is traded internationally

and with this small marketable surplus; although prices fluctuate widely with droughts, floods and typhoons. Rice productivity and quality are seriously compromised by pests, diseases and physiological and environmental factors (Redoña, 2004). The crop engages the largest agrochemical market in the world, and consumes around EUR 3,000 million a year, in agrochemical costs, while crop losses associated to rice production are associated to rice production are almost tens of billions of dollars per year (DFID, 2004).

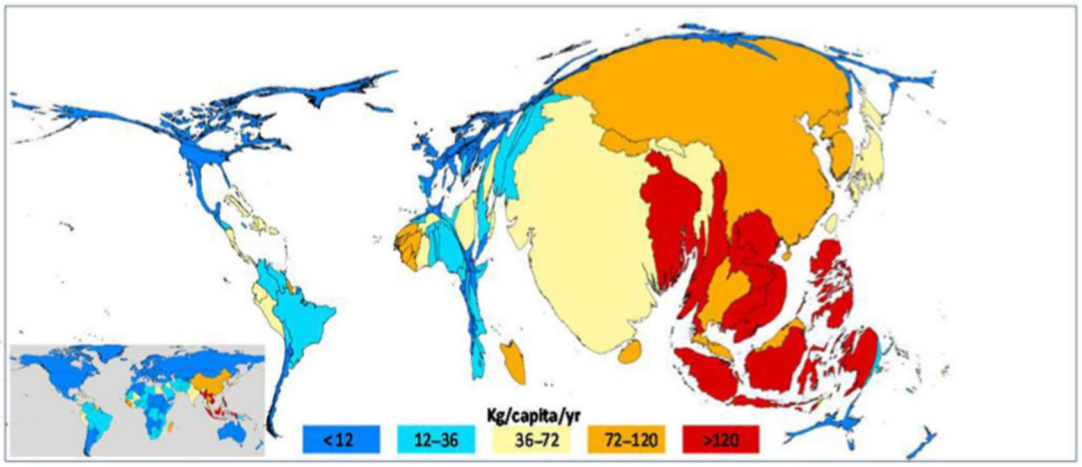


Figure 3. Consumption of rice per capita worldwide, Territory size represents the proportion of milled rice world vides that is consumed in that territory. Color shows the per capita consumption of milled rice. Source: Mohanty *et al*., 2013.

The global consumption of rice per capita was approximately 54.24 kilograms per capita in year (USDA, 2017), being very variable according to local customs. The expanded map in **Figure 3** shows an unusual view of the world, where each territory has been distorted based on the proportion of rice consumed (Mohanty *et al*., 2013). Regardless of this, Asia has a large part of the world population and has high consumption rates; therefore, only China and India account for more than 50% of world rice consumption. Higher rates are found in much of southern and southeastern Asia, western Africa, Madagascar, and Guyana. Several of these countries featured by per capita consumption rates that exceed 100 kg per year. Brunei tops the list with more than 20 kg per capita and month, compared to rates in Europe of less than 0.5 kg per month (Mohanty *et al*., 2013).

Four major types of rice are produced worldwide: i) *Indica* grown mostly in tropical and subtropical regions that accounts for more than 75 percent of global trade; ii) *Japonica* rice, typically grown in regions with cooler climates, accounts for around 8 percent of global rice trade; iii) *Aromatic* rice, includes jasmine from Thailand and basmati from India and Pakistan. This accounts for around 15 percent of global trade and typically sold at a premium category in

world markets; iv) *Glutinous* rice, grown mostly in southeast Asia and used in desserts, ceremonial dishes, and several other specialty rice account for most of the remainder (USDA, 2017).

1.1.1.2. Relevance of the genetics issues for rice cultures

Cultivated rice varieties have become differentiated from wild ones, as in other crops, by the intervention of man in selecting desirable forms. In the germplasm of International Rice Research Institute (IRRI) bank there are around 100,000 accessions, most belonging to the *O. sativa* species. This material is feature by adaptation capacity to different moisture conditions, growth habit, plant height, size and color of the stem, and of the flag leaf, as well as the characteristics of panicle and seeds; however, as well as its reaction to pests and diseases also constitutes a feature for years. All these properties are usable during breeding programs of the crop towards improved quality varieties (Acevedo *et al*., 2006). Genetic diversity motives that *O. sativa* species presents greater genetic variability, finding dos sub-species (*indica*, and *japonica)*, based on their ecology and morphology, this while was not observed in *O. glaberrima*.

Indica rice is mainly cultivated in tropical and subtropical environments, at lower latitudes or altitudes, whereas *japonica* rice is grown mainly in more temperate environments at higher latitudes or altitudes. During the long history of rice domestication, the *indica* and *japonica* rice varieties have clearly diverged in morphological features, agronomic traits, and physiological and biochemical features, as well as in yield, quality, and stress resistance. The genes and proteins responsible for these differences, and their roles in these two rice varieties, remain poorly characterized (Yang *et al*., 2014). Recently with the rapid development of molecular biology, a variety of molecular markers, including random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), microsatellite markers (SSR) and DNA insertion and deletion (InDel) have been widely used to identify *japonica* and *indica* rice varieties at the molecular level.In addition, concerning the proteomic field of study, Yang *et al*. (2014) has indented candidate proteins and important genes involved in *japonica* differentiation. Some authors do not recognize these subspecies. They do admit the emergence through the evolution of the cultivation of ecogeographic forms. So, that the morphological differences actually correspond to reactions of the plant adapted by man to areas of culture with extreme conditions. So if this hypothesis is true, this allows us to infer that the differentiation between *indica* and Japan also has a similar origin, with the environment being the main differentiating factor (Acevedo *et al*., 2006).

Genetic studies conducted on the *O. sativa* species revealed that it genome is composed of 430 million base pairs and approximately 50.0% is constituted by of repeated sequences (Acevedo *et al*., 2006). The narrow genetic base found in the varieties of rice released in the world in recent years, as a result of the uniformity of the nucleus and the cytoplasm, has brought as consequence a ceiling on productivity and vulnerability to biotic factors. As a result, numerous researchers from different countries have carried out genetic improvement with the help of biomolecular techniques to increase genetic diversity in the *O. sativa* species, using exotic germplasm of the genus *Oryza*, mainly the *O. glaberrima* species. For this purpose, *O. sativa* spontaneous, *O. rufipogon*, *O. officinalis*, belonging to the *O. sativa* and *O. officinalis* complexes were used as donors of valuable features (Acevedo *et al*., 2006). Hence, Martínez *et al*. (2004) reported that new alleles to increase the genetic variability of grain yield in commercial rice in Latin America are in the use of the 20 wild species of the genus *Oryza*. In the same work, they found that *O. glaberrima* exerts tolerance to the fungus *Polymyza graminis* transmitter of the rice grated necrosis virus (RSNV). The literature indicates that some *O. glaberrima* accessions are feature by tolerance to severe drought stress, upon a range of tolerance mechanisms such as: osmotic arrangement, stomata arrangement and deep root system (Acevedo *etal*., 2006) as well as to heat stress, presenting the early morning flowering, which escapes from the high temperature at midday (IRRI, 2017). Besides, the International Rice Research Institute (IRRI) is developing rice varieties that can with stand the conditions that are predicted to be more frequent and intense in the frame of the current climate change. This includes drought, floods, heat, cold, and soil problems, such as salinity and iron toxicity (IRRI, 2017).

1.1.1.3. Sowing and growing conditions

Rice is an annual plant of subtropical origin adapted to aquatic habitat and relatively high temperature and humidity. Rice roots receive the supply of oxygen from the aerial parts of the plant (stem and leaves). Growth and development of rice plants can be divided into three stages: 1) vegetative stage, 2) reproductive stage, and 3) stage of grain filling and maturation (**Figure 4**).

Vegetative stage includes the period from germination of seeds to the beginning of the differentiation of the floral primordium. This stage is featured by active tillering, gradual increase of the height, and emergence of the leaves. The reproductive stage covers from the end of the vegetative stage until the flowers pollination. In this stage the emergence of the flag leaf, the thickening of the stem, the emergence of the panicle, and flowering occurs. Maturity, third and last stage, goes from the filling of the grain until it is ripe (Gamarra, 1996). It implies the increase

of the size and weight of the grains, after the location of the starch and sugars from the tiller, flag leaf, and shoots to the grain. Throughout these stages the three components of yield are determined: the number of panicles per hectare, the number of grains per panicle, and the weight of one thousand grains (Gamarra, 1996). In **Figure 4** the different phenological phases of the crop and the moment of definition of the performance parameters can be observed. Each of them has specific requirements to develop their maximum potential.

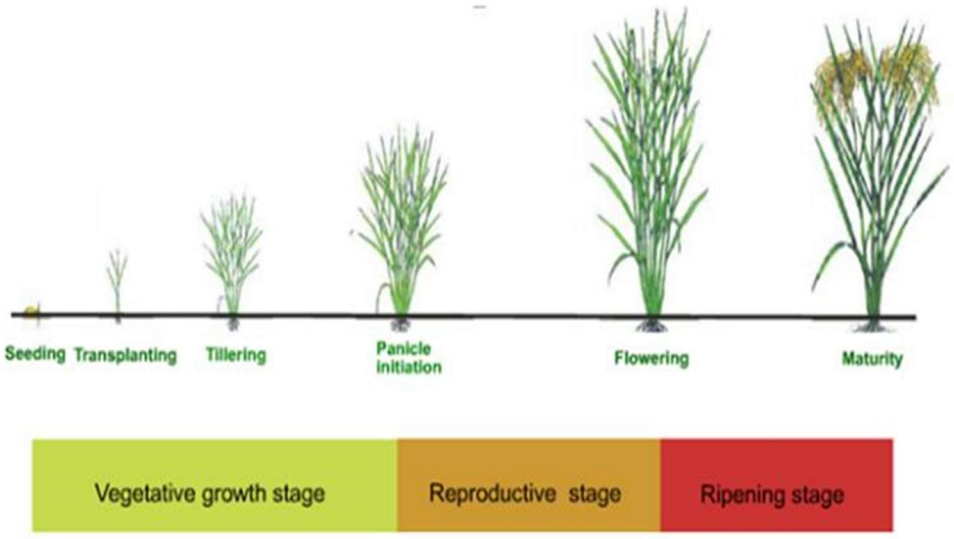


Figure 4. The growth stagefrom germinated seed to a mature plant. Source: http://knowledge.agriculturemachinerybusiness.com/CropAndPlantatin_212_11677_1.html

Shortly, it can be stressed the following characteristics for the separate stages considered:

a) Vegetative stage and number of panicles per square meter

a.1) *Germination*

When the required conditions of temperature and humidity are met, seeds swell and germinate. In the embryo, two structures, the radicle and the coleoptile, grow and lengthen. During this stage, the oxygen content in the soil determines the type of germination. Thus, high oxygen concentrations as in the case of dry sowing, cause that the radicle first emerges, while if it is low (sowing in water), the coleoptile first emerges, since this is less oxygen demanding than the radicle. In early plantings, cold and excess moisture can greatly delay the emergence while in late sowings; the lack of humidity can delay germination and emergence.

a.2) *Seedling*

Until the appearance of the third leaf, rice plants use the reserves of the endosperm and, generate seminal roots that are soon replaced by adventitious ones, which are born from the

underground node of the stems. At this time, temperature constitutes a critical factor. Regarding this, with values between 18-25 °C, the growth is normal, while low temperatures (less than 10-12 °C) reduce the probability of survival of the seedling.

a.3) *Tillering*

From the fourth leaf emerges the first tiller; the tillers arise from the node of the crown, located inside the leaf sheath. The first and perhaps second tiller ripens almost at the same time as the mother plant. Other tillers, if they are produced, mature later and have lower grain quality. The ability to emit tillers allows the rice crop to respond favorably to an array of diverse sowing densities. In general, both nitrogen and phosphorus are important for tillering. Early flooding and a high water sheet decrease tillering. For this, it is convenient to start watering at this stage, maintaining a low level of water (5-6 cm), such that it does not affect the tillering and allows the application of N in coverage. From this moment, the rice must remain flooded until ripe. The adaptation of rice to the flood derives from the presence of aerenchymae in the stem and root. The aerenchymae is formed as a consequence of the orderly and programmed death of the cells (apoptosis), producing large intercellular spaces that allow the conduction of oxygen from the air to the rhizosphere through the line of node and internodes (Gamarra, 1996).

a.4) *Formation of internodes*

The formation of knots and internodes above the crown is what gives rise to the stem and determines the length of it. The number of internodes is 5-6 per stem and is fairly constant for all varieties. In this stage, the upper internodes are not lengthened yet. The growth of the roots reaches its maximum when the formation of internodes begins. At this moment, the formation of tillers is stopped and some of the already formed tillers die (sterile tillers), since normally more than those that reach maturity are formed (Gamarra, 1996). At the end of the vegetative stage, the first component of yield is defined: potential number of panicles per hectare. Good management at this stage is essential to obtain high yields (Gamarra, 1996).

b) <u>Reproductive stage and number of grains by panicles</u>

b.1) *Panicle initiation*

From the differentiation of the primordium of the panicle, the internodes begin to elongate rapidly, the plant abandons the grass aspect and begins to grow at very high rates. It is possible to distinguish the floral primordium inside the stem; it presents the appearance of a small white cottony flame that can be recognized by making a longitudinal cut at the base of the

stem. The superior internode lengthens just before flowering and pushes the panicle through the leaf sheaths. In this stage, climatic conditions (especially solar radiation and temperature), and management practices (irrigation) are concise to define the rice yield (Gamarra, 1996).

b.2) *Booting stage*

At this time, the panicle is inside the sheath of the flag leaf, producing a visible thickening. In this stage, the plant is very vulnerable to adverse weather conditions, especially at low temperatures (Gamarra, 1996).

b.3) *Heading stage*

The panicle emerges through the sheath of the flag leaf and becomes visible. Pollination occurs when 15.0% of the panicles become fully visible. The actual flowering is the period in which flowers open, are fertilized, and close; the anthers appear white or yellow in the open flowers. It lasts 3 to 5 days after the emergence of the panicle and occurs from top to bottom. Strong winds or low temperatures can seriously hinder pollination. Rice is a species featured by self-pollinates. There is only a small percentage (0.0 to 4.0%) of cross-fertilization. At this stage of plant development, the second performance component is being formed: number of grains per panicle (Gamarra, 1996).

c) Ripening stage and weight of grains

Ripening begins when 50.0% of panicles are in bloom and ends when the average humidity of the grain is around 20.0%. The loss of humidity is gradual and, after 35-40 days after fertilization, it is considered that the grain has reached maturity. The carbohydrates are practically pumped from the leaves and the stem towards the grain, accumulating, fundamentally, as granules of starch in the endosperm. This stage is gradual, at first the grain has a milky appearance, it is the stage of milky maturity, and then, it loses moisture and becomes pasty: milky, dough, and maturity stages. As it loses moisture, the grain hardens and finally there is no more carbohydrate intake, a horny maturity stage (the water content is less than 20%). The endosperm is compressed between lemma and palea that give the final shape and size to the grain. The degree of filling and the climatic conditions determine the industrial and culinary quality of the grain. In this stage, the last component of yield is determined: the weight of a thousand grains (Gamarra, 1996).

1.1.1.4. Physiological issues

To obtain optimal harvests, rice requires mild temperatures (higher than 12 and 13 °C), that result in the physiological zero or base temperature for the subspecies *japónica* and *indica,* respectively (Ideas, 2007). Optimal temperatures for rice cultivation are defined according to the phenological development of rice plant (**Table 1**).

Table 1. Critical temperatures in the different stages of rice development (Source: De Datta, 1996).

Rice Development Stages	Critical temperatures (°C)		
	Low	High	Optimal
Germination	10	45	20-35
Emergency and establishment of seedlings	12-13	35	25-30
Elongation of leaves	7-12	45	31
Tillering	9-16	33	25-31
Panicle initiation	15-20	38	-
Heading	22	35	30-33
Maturation	12-18	30	20-22

During the maturation period, higher temperatures are necessary (more than 20 °C for 25 to 40 days) (Ideas, 2007). Therefore, for the expression of its productive potential, rice cultivation requires temperatures between 24 and 30 °C, as well as high solar radiation, sufficient availability of water and nutrients (Gamarra, 1996).

Besides, temperature requirements, in general, rice is considered a short-day plant, that is, blooms when the length of the night is longer. It has a critical photoperiod of 12-14 hours of light; however, the sensitivity of rice plants to the length of the day differs between varieties (Arguisain, 2006).

In respect to water requirements, rice is a semi-aquatic plant that develops in flooded soils. Flooding is a requirement for high yields. In general, the flooding period includes goes from tillering (about 30 days after emergence) to two weeks before harvest. This implies about 90-100 days during which the rice remains flooded (Gamarra, 1996). Numerous studies reveal the advantages and drawbasics of the different alternatives for water management. The most appropriated time-point depends on an array of factors, namely the variety under considers and have a significant influence on the levels of nutrition, efficiency in the use of the fertilizer, and

the development of diseases and pests. The selection of the cultivar and the texture of the soil are important factors to make decisions on the best water management option (Dou *et al.*, 2016).

Concerning nutritional requirements, the ideal soils for rice cultivation are those with clay-like texture, sandy clay or silty clay, with pH between 5.5 and 6.5. Sandy soils are not advisable, as they have low capacity to retain water, causing loss of nutrients by washing. The most important nutrients, as limiting factors for rice, are, by order, nitrogen (N), phosphorus (P), potassium (K), and zinc (Zn) (Cakmak, 2000). In greater proportion than in other crops, rice productivity depends on the availability and efficiency of N absorption, both for its direct contribution and for allowing the absorption of other nutrients. Although the extraction of N, P, and K is constant throughout the cycle, the phase of tillering and panicle initiation is distinguished by a higher intensity of absorption (Gamarra, 1996). The amount of nutrients removed from the soil in a rice crop depends on the cultivar, the biomass production, the soil, the climate and the management, but they are an important indicator of the nutritional needs of the plant.

1.1.2. Nutritional and phytochemical composition of rice

The grain of rice, commonly called seed, when newly harvested is formed by the fruit or cariopse and by the hull being; the latter composed of the glumella (palea and lema). The cariopseis formed by the embryo (preformed plant), the endosperm, aleurone layer (tissue rich in proteins), tegument (seminal cover, development of the ovule walls), and the pericarp (cover of the fruit, development of ovarian walls). The embryo is extremely small, located in the central area of the cariopse. The endosperm consists of parenchyma cells that elongate radially and is composed of starch granules and some protein bodies (Juliano, 1985). The aleurone layers vary from 1 to 5 cell layers, thicker in the dorsal part than in the ventral part, and thicker in the short grain rice than in the long grain. The aleurone and embryo cells are rich in proteins, which include phytates, and fatty compounds (Juliano, 1994). Five different products (or types of rice) can be produced from rough rice: hulls, bran, brown rice, whole-kernel milled rice, and broken-kernel milled rice (**Figure 5**). The first stage of milling removes the hull, producing brown rice that can be cooked and consumed. The next stage of milling removes the bran layer, leaving milled white rice. On average, every 100 pounds of rough rice yield around 55 pounds of whole-kernel milled rice, around 15 pounds of broken, about 10 pounds of bran, and 20 pounds of hull (Shih, 2003).

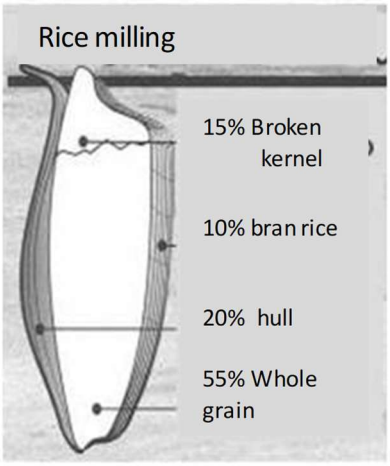


Figure 5. Products of rice milling.
Source: Shih, 2003.

1.1.2.1 Nutritional value and dietary burden of rice

Rice is the predominant staple food for 17 countries in Asia and the Pacific, nine countries in North and South America and eight countries in Africa. This cereal provides 19.0% of the world's food energy supply, as provides wheat, while corn supplies 5.0% (Ray *et al.*, 2013). The most widespread use of this cereal is as a polished grain, although currently the consumption of brown rice is being promoted due to the growing awareness on the attributes of this food matrix for health (Hanis *et al*., 2012). Whole grains are rich in fiber, B vitamins, minerals, tocopherols, and phytonutrients. Epidemiological studies have shown an inverse relationship between daily intakes of whole grains and the risk of developing chronic noncommunicable diseases, such as cardiovascular disease, Type 2 diabetes, and cancer (Nelina and Ruíz, 2005). Intake of whole grains can contribute to increase the body's antioxidant capacity, which is possibly one of the mechanisms through which whole grains might contribute to prevent such diseases. Among the antioxidants found in grains it has been described phenolic acids, flavonoids, tocopherol, tocotrienols, selenium, zinc, soluble fiber, and phytic acid (Nelina and Ruíz, 2005).

1.1.2.2. Phytochemical composition of rice

In **Table 2**, the chemical composition of rice can be observed comparatively with respect to that of other cereals grains, being noticed that has high digestible energy and low lipid and protein content, but high protein net utilization. The rice grains have good digestibility and although this content is low, the protein is of high quality due to its aminoacid composition. The amount of protein differs remarkably within the same cereal from one crop to another. This is

due to the strong interaction between the genotype and the environmental conditions that prevail during the development and maturation of the grain. The protein compounds of the grain are located in all its tissues, but the germ and aleurone layer concentrate the greatest amount of nitrogen compounds.

Table 2. Composition and energy balance of the mean cereals. Data calculated at 14.0% moisture. Source: Juliano, 1985.

Compound	Brown rice	Wheat	Corn	Barley	Sorghum	Rye	Oats
Protein (Nx6.25) (%)	7.3	10.6	9.8	11.0	8.3	8.7	9.3
Lipids (%)	2.2	1.9	4.9	3.4	3.9	1.5	5.9
Available carbohydrates (%)	64.3	69.7	63.6	55.8	58.0	71.8	62.9
Crude fiber (%)	0.8	1.0	2.0	3.7	4.1	2.2	5.6
Ash (%)	1.4	1.4	1.4	1.9	2.6	1.8	2.3
True digestibility (%)	99.7	96.0	95.0	88.0	84.8	77.0	84.1
Protein net utilization (%)	73.8	53.0	58.0	62.0	50.0	59.0	59.1
Digestible energy ($kJ.100^{-1}$)	1550.0	1360.0	1450.0	1320.0	1290.0	1330.0	1160.0

Table 3 shows the chemical composition of the rice grain according to the fraction of mill that is considered. Among the milling fractions of rice, the bran has revealed the highest energy andprotein content, while the hull is featured by the lowest.

The proteins, fats, vitamins, and minerals are concentrated in the germ and outer layer of the starchy endosperm. Only the brown ricefraction is edible. Abrasive or friction milling to remove the pericarp, seed coat, testa, aleurone layer and embryo, to yield milled rice results in loss of fat, protein, crude fiber, and ash. Available carbohydrates, mainly starch, are higher in milled rice than in brown rice. It has also been reported that the nutritional value and head rice recovery are reduced with the higher degree of milling (Roy *et al*., 2011).

Table 3. Proximate composition of paddy rice and its processing fractions at 14.0% moisture. Source: Juliano, 1994.

Rice fractions	Crude protein (g)	Crude fat (g)	Crude fiber (g)	Crude ash (g)	Available Carbohydrate (g)	Energy (Kcal)
Rough rice	5.8-7.7	1.5-2.3	7.2-10.4	2.9-5.2	64.0-73.0	378
Brown rice	7.1-8.3	1.6-2.8	0.6-1.0	1.0-1.5	73.0-87.0	363-385
Milled rice	6.3-7.1	0.3-0.5	0.2-0.5	0.3-0.8	77.0-89.0	349-373
Rice bran *	11.3-14.9	15.0-19.7	7.0-11.4	6.6-9.9	34.0-62.0	399-476
Rice hull**	2.0-2.8	0.3-0.8	34.5-45.9	13.2-21.0	22.0-34.0	265-332

*Rice bran refers to the layers of pericarp, tegument, and aleurone; ** Rice husk includes the outer layers of grain (palea and lemma).

Rice is not the only dietary source energy, but it is also a good source of thiamine, riboflavin, and niacin.

The B vitamins are concentrated in the bran layers as well as α-tocopherol (Vit. E) and phosphorus. In respect to minerals, rice is a rich source of phosphorus, most of which is retained in the bran. With respect to calcium, the largest amount remains in rice hull and milled rice is the least amount has (**Table 4**) (Juliano, 1994).

Table 4. Vitamin and mineral content of paddy rice and its processing fractions at 14.0% moisture. Source: Juliano, 1994.

Rice fractions	Thiamin (mg)	Riboflavin (mg)	Niacin (mg)	α-Tocopherol (mg)	Calcium (mg)	Iron (mg)	Zinc (mg)	Phosphorus (g)
Rough rice	0.26-0.33	0.06-1.11	2.9-5.6	0.9-2.0	10.0-80.0	1.4-6.0	1.7-3.1	0.17-0.39
Brown rice	0.29-0.61	0.04-0.14	3.5-5.3	0.9-2.5	10.0-50.0	0.2-5.2	0.6-2.8	0.17-1.43
Milled rice	0.02-0.11	0.02-0.06	1.3-2.4	0.0-0.3	10.0-30.0	0.2-2.8	0.6-2.3	0.08-0.15
Rice bran	1.20-2.40	0.18-0.43	26.7-49.9	2.6-13.3	30.0-120.0	8.6-43.0	4.3-25.8	1.10-2.50
Rice hull	0.09-0.21	0.05-0.07	1.6-4.2	0.0	60.0-130.0	3.9-9.5	0.9-4.0	0.03-0.07

In **Table 5**, the amino acid content can be observed, where it can be clearly seen that all the rice fractions have a high content of leucine, phenylalanine + tyrosine, and valine. Amino acid analysis showed lysine to be the first limiting essential amino acid in cereal proteins, but lysine content was highest in oats and rice among cereal proteins (Eggum, 1979).

Table 5. Amino acid content of paddy rice and its processing fractions at 14.0% moisture. **Source**: Juliano, 1994.

Rice fractions	His*	Iso	Leu	Lys	Met+Cys	Phe +Tyr	Thr	Trp	Val
Rough rice	1.5-2.8	3.0-4.8	6.9-8.8	3.2-4.7	4.5-6.2	9.3-10.8	3.0-4.5	3.0-4.5	4.6-7.0
Brown rice	2.3-2.5	3.4-4.4	7.9-8.5	3.7-4.1	4.4-4.6	8.6-9.3	3.7-3.8	3.7-3.8	4.8-6.3
Milled rice	2.2-2.6	3.5-4.6	8.0-8.2	3.2-4.0	4.3-5.0	9.3-10.4	3.5-3.7	3.5-3.7	4.7-6.5
Rice bran	2.7-3.3	2.7-4.1	6.9-7.6	4.8-5.4	4.2-4.8	7.7-8.0	3.8-4.2	3.8-4.2	4.9-6.0
Rice hull	1.6-2.0	3.2-4.0	8.0-8.2	3.8-5.4	3.5-3.7	6.6-7.3	4.2-5.0	4.2-5.0	5.5-7.5

*His, histidine; Iso, isoleucine; Leu, leucine; Lys, lysine; Met+Cys, methionine+cysteine; Phe+Tyr, phenilalanine + tyrosine; Thr, threonine; Trp, tryptophan; and Val, valine.

1.1.3. Impact of the agronomical management on rice productivity and composition

1.1.3.1. Relationship between biotic and abiotic stress and productivity of rice

Plants have to deal with diverse and complex interactions that involve numerous environmental factors. In the course of the evolution, they have developed specific mechanisms that allow them to adapt and survive stressful events. The exposure of plants to biotic and abiotic stress induces an interruption of the plant metabolism that implies physiological costs and therefore, leads to a reduction in the aptitude and, finally, limits the productivity (Rejeb *et al*., 2014). Among the relevant abiotic factors affecting the productivity of rice are noticed: air temperature, solar radiation, water and nutritional deficit, fertilizer management and pesticide use, while the biotic factors it has to be mentioned weeds, diseases, and pests.

In the coming years, global air temperature is expected to increase 0.2 °C - 0.4 °C per decade, which will lead to temperatures from 1.8 to 4.3 °C higher in 2100. It is likely that episodes of heat stress occur more frequently and exacerbate the current vulnerability of rice crops due to the global warming (Matsui *et al.,* 2000).

Abiotic factors

The appropriate climate conditions allow the maximum expression of the yield potential. Hence, the yield of the rice crops depends largely on specific climatic conditions and is greatly affected by climatic variations (Pandey and Shukla, 2015). In this regard, when analyzing the abiotic factors, it is revealed that.

Concerning solar radiation, the totals amount of photosynthetically active radiation (PAR 400-700 µm) intercepted by a crop is the most important determinant of the total dry matter produced (Ahmad *et al*., 2008). In connection whit this, achieving higher yields depends on the increase of the total biomass of a particular crop. Radiation in the reproductive stage affects the formation of reproductive structures and, fundamentally, filling and maturation of grain (Peng *et al*., 2004).

Figure 6. Thermal stress: non-o-uniform maturation.

In respect temperature, the optimum daily temperature for rice photosynthesis is between 30-35 °C (Zamorano Montañez, 2018). Temperature higher than 35 °C increases the transpiration rates in rice plants and, as a consequence, photosynthesis is reduced, as they produce stomatal closure, and reduce gas exchange. Temperatures higher than 40 °C, during ripening, result in reduced spikelet fertility and grain weight of the panicles (Maruyama *et al*., 2015) and cause yield losses of to 25% (Mohanty *et al*., 2013). High daily temperatures have been noticed to delay ripening, while those higher than 33 °C inhibited phenological development (Maruyama *et al*., 2015). Extensive studies have shown that the optimal daily temperature for rice flowering generally varies from 24 °C to 29 °C (Matsui *et al*., 2000) while ambient temperature above 32 °C at flowering initiation and throughout the development of this phase can alter the dehiscence of the anthers, and induce the sterility of the spikelets (**Figure 7**), begin responsible to loss of yield (Zhao *et al*., 2018). Optimal night temperatures are between 21 and 25 °C. Lower temperatures during the vegetative stage produce yellowing and death of seedlings; lower temperatures during the reproductive stage produce degeneration of spikelets and grain discoloration. In the other hand, night temperatures higher than 22 °C increase night respiration rates by reducing leaf area (by senescence) and therefore photosynthesis. According to Peng *et al.* (2004) grain yield decreases by 10% for each 1 °C increase in night mean

temperature of the growing season, while the effect of daily temperature on crop yield was negligible.

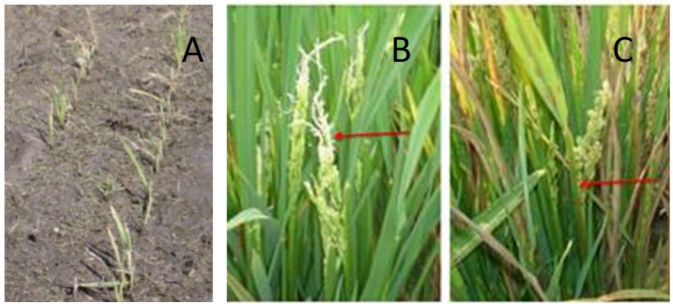


Figure 7. Cold damage in rice plant: A: chlorosis and death of seedlings, B: Spikelet degeneration, C: incomplet of panicle exsertion. Source: Zamorano Montañez, 2018 (A), Hasanuzzaman *et al*., 2013 (BC).

In comparison with other crops relatively to the central role of water per this crop, rice requires a greater amount of water throughout the development of plants. Indeed, water is a major constituent of plant tissues, working as a basic media for chemical reactions, as well as for translocation of metabolites and minerals. Besides, water is an essential component required for cell enlargement due to its valuable contribution to turgor pressure (Pandey *et al*., 2014). Actually, lack of proper water management is probably one of the most serious constraints to greater rice yield (Ahmad *et al*., 2008), given that stress related to water, causes a serious threat to production. In this regard, the magnitude of grain yield loss depends on the duration of the drought, the growth phase of the crop, and the severity of drought stress. When water stress occurs, plants react by slowing or stopping their growth (Pandey and Shukla, 2015). The plants' growth and development are reduced as a consequence of deficient root structure, which entails lower leaf surface features (shape, composition of the wax, cuticle, leaf pubescence and leaf color). This affects the radiation load on the foliage of the leaf. Besides, appropriate lack of water supply reduces photosynthesis, transpiration, stomatal conductance, relative water content, chlorophyll content, photosystem II (PSII) activity, membrane stability, carbon isotope discrimination and abscisic acid content, the accumulation of osmoprotectores like proline, sugars, polyamines and antioxidants and therefore affects the grain yield. According to Jin *et al*. (2013), during flowering, male fertility was dramatically affected due to the aberrant development of the anther under water stress. Drought also has a critical effect on decreasing grain yield, by shortening the grain filling period (Pandey and Shukla, 2015). In this concern, previous studies have shown that grain yield increases proportionally to water supply and is particularly high under flooded irrigation (Ahmad *et al*., 2008). In aromatic rice (cv. Basmati),

diminishing soil moisture at the time of grain filling is reported to flavor the aroma formation (Pandey and Shukla, 2015).

An addition relevant issue enclosed to rice production is referred to the fertilization management. About 60% of the cultivated soils around the world are featured by constrained true limit the growth of plants, due to deficiencies of mineral nutrients and toxicities. Therefore, the improvement of the mineral nutritional status of plants in marginal environmental conditions is of great importance for the maintenance of crop productivity (Cakmak, 2002). The management of fertility in the crop plays an important role in the defense against biotic factors. Some examples of this fact are represented by P which improves the tolerance to pests, silica (Si) that increases resistance to bacterial diseases, and leaf insects, and K fertilization that contributes to decrease the effect of pests, because it promotes the thickness of cell walls, reduces the content of free sugars and amino acids and increases the intake of Si. However, the administration of Zn has been recently related to the reduction of leaf-eating insects damage (Zamorano Montañez, 2018). On the other hand, some drawdarks have been notified regarding minerals fertilization. In this concern the nitrogen fertilization, although contribute to diminish populations of Trips; it makes the plants more succulent, facilitating the penetration of organs sucking of insects. In addition, it attracts and increases the rate of fecundity and reproduction of insects and favors the appearance of *Pyricularia oryzae* (the most important fungic disease in rice). Hence, nitrogen fertilization could increase the presence of weeds. It has been proven that weeds such as *Echinochloa* sp., and the perennial grasses (*Paspalum distichum, P. hydrophillum, Luziola* sp.) are more efficient in the use of applied fertilizer than rice (Gamarra, 1996).

Concerning salinity, salt stress limits the production of rice in large areas around the world, and the problem is increasing due to irrational human actions, which cause secondary salinization, as well as due to global warming, with the consequent increase in sea level and the increase in the occurrence of storms, particularly in coastal areas. Stomatal closure is often a rapid initial response to salt stress. Rice is particularly sensitive to salt stress during the seedling stage, with the consequent poor establishment of the crop, as well as during reproduction, where salinity can severely alter grain formation and yield (Mohanty *et al*., 2013).

The frequent acidity of tropical soils determines the high availability of some metals, such as Al, Fe, and Mn, in the soil. Traditional cultivation practices (fertilizers, herbicides, and pesticides), as well as industrialization and other human activities can incorporate metals into the soil (Olivares *et al*., 2002). Metals constitute a potential source of oxidative stress because they favor the production of reactive oxygen species ROS and radicals. A mechanism to protect the potential damage produced by these free radicals constituted by systems that trap these

compounds, as is the case of polyphenols and flavonoids. Indeed, it has been proposed that phenols, mainly hydrolysable tannins and polyphenols derived from gallic acid and tannic, are involved in the detoxification of metals in some aquatic plants (Olivares *et al*., 2002).

Finaly and concerning herbicides, whichare methods of weed control, chemical control stands out. However, even selective herbicides can trigger the production of reactive oxygen species and cause oxidative stress (Langaro *et al*., 2017). Herbicides cause phytotoxicity, reduce height, and alter the metabolism of plants, by generating ROS. This latter effect activates enzymatic and non-enzymatic defense systems and results in the degradation of photosynthetic pigments. Another consequence of stress caused by herbicides is the reduction of the total protein content. Recently, Langaro *et al.* (2017) evaluated the produced changes in rice plants by the application of preemergent herbicides (oxadiazon, pendimethalin, and oxyfluorfen). There three herbicides, although in different extent, reduced the photosynthetic rate and the effectiveness of carboxylation, and increased the activity of superoxide dismutase and ascorbate peroxidase, enzymes that are generated in situations of stress.

Biotic factors

Biotic factor-tolerant crops have revolutionized modern agriculture and have become a major tool of integrated management programs, leading to reduction in insecticide use while protecting the environment and human health. Biotic stresses include fungus, insect pests, bacteria, viruses, and herbicide toxicity (Ansari *et al.,* 2015). A virulent pathogen causes an accumulation of ROS that acts as direct reactive substrates to kill pathogens and to strengthen the cell walls of plants by crosslinking glycoproteins to obstruct further spread of the pathogen. ROS commonly triggers and precedes programmed cell death and also functions as signal molecules for protein production related to pathogenesis (PR). ROS induction has been implicated in rice against bacterial blight, blast, and gall midge resistance (Rawat, 2016).

The genetic improvement of rice for resistance to fungi is needed nowadays, because of the vulnerability of this crop to various pathogens. In this regard, rice blast disease, caused by the filamentous ascomycete fungus *Magnaporthe oryzae*, is the most important rice disease due to its severity and wide distribution (**Figure 8**).

Figure 8. Symptoms produced by *Magnaporthe oryzae* in rice. Source: Royal society publishing, 2016.

M. oryzae causes severe crop losses of up to 85%, depending on the agro-climatic conditions of the growing area. Resistant cultivars and pesticides have traditionally been used to control this disease; however, the fungus *M. oryzae* overcomes host resistance rapidly, and resistant cultivars become ineffective after few years (Bundó and Coca 2016). In addition to structural barriers and preformed antimicrobial compounds, plants have developed inducible immune responses against pathogens. The first defense reaction includes changes in ion fluxes through the membranes, an increase in intracellular calcium concentration, the activation of protein kinases or the synthesis of ROS.

The direct reactions consist of transcriptional reprogramming, alterations in the hormonal state, and reinforcement of the cellular wall, through depositions and lignifications of callose and in some cases, even by cell death at the site of infection (Liu *et al*., 2014). Locally activated defense responses in primary plant tissues infected with pathogens often extend to uninfected distal tissues, conferring acquired systemic resistance (SAR) (Bundó and Coca, 2016). Acquired systemic resistance is associated with the signal molecule salicylic acid (SA), and the accumulation of proteins related to pathogenesis (PR) that is believed to contribute to such resistances (Bundó and Coca, 2016). Transgenic rice containing Rir1b has been reported to have an enhanced resistance to rice Blast (Ansari *et al*., 2015). Several proteins have also been identified as good candidates for conferring resistance against various fungal species in rice plants, which are involved in tolerance to pathogen attack. Examples include lipid transfer protein, selenium binding protein homologue, genes that take part in flavonoid pathways, defensins, phytoalexins, protease inhibitor protein genes, and *Aspergillus flavus* antifungal protein, among others (Ansari *et al.,* 2015).

There are numerous reports indicating that rice genetically modified with the Bt (*Basillus thurigiensis*) gene can minimize losses due to lepidopteran pests and transgenic rice transformed with a synthetic cry1Ab gene has resistance to eight lepidopteran insects (Ansari *et al*., 2015). Biotic stress responses are preferentially mediated by phytohormones. The SA pathway is mainly connected with responses to biotrophic pathogens, while the JA and ethylene pathways generally act synergistically and are linked to plants defense against necrotrophic pathogens and insects (Rawat, 2016). Bacterial blight-resistant transgenic rice lines have been developed by transforming an endogenous gene, Xa21. In this regard, it has been found that this gene is the best candidate to induce resistance against bacterial blight (Ansari *et al*., 2015). The use of insecticides to control insect vectors is a feasible way to control rice crops, but the high costs of the insecticide and the risk to the environment are the main limiting factors.

In respect to genetic resistance against rice viruses or their insect vectors is also one of the most effective methods to protect rice plants from virus infection. Rice plants resistant to RDV and RSV have been developed and evaluated (Ansari *et al*., 2015). The use of pesticides is expensive and unfriendly to the environment. Since rice is a primary source of human food, new strategies should be developed to provide long-term protection against blast disease. The study of the defense responses of plants offers an array of possibilities to improve resistance to diseases in rice.

1.1.3.2. Effect of abiotic and biotic stress factors on the physical properties proximate composition of rice products and coproducts

Grain quality in rice is very difficult to be defining accurate by preferences for quality varies from country to country. For instance, the miller's basis of quality is dependent upon total recovery and the proportion of head and broken rice on milling. Consumers base their concept of quality on the grain appearance, size and shape of the grain, behavior upon cooking, taste, tenderness, and flavor (Pandey *et al*., 2014).

When considering the proximate compositions of rice and co-product it is noticed that starch is the major constituent of milled rice, constitution almost 90.0% of the dry matter. The lipid or fat content of rice is mainly in the bran fraction, specifically as lipid bodies or spherosomes in the aleurone layer and bran. The major fatty acids of these lipids are linoleic, oleic, and palmitic acids (Juliano, 1994).

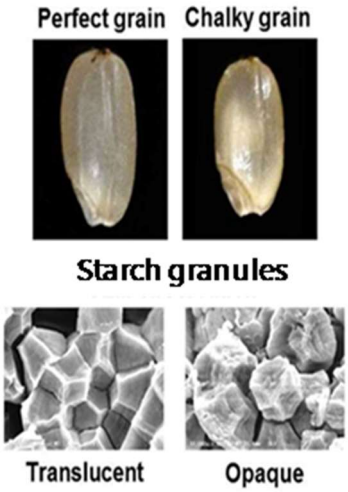


Figure 9. Perfect and chalky grains of rice. Source: Mitsui *et al*., 2013.

The proportion of amylose and amylopectin in the starch of rice grains is the factor that influences in a higher extent in the processing and quality of cooked rice. In this regard, air temperature during grain-filling period affects the amylose content of the grains, which may increase or decrease as lower as or higher than normal temperatures are recorded (Jenning *et al*., 1981). Besides, the amylose content generally becomes lower in water stress condition (Pandey and Shukla, 2015). The amylose content influences properties of cooked rice such as gelatinization temperature. The temperature of gelatinization has a reasonably high heritability, although this can vary as much as 10 °C in the same cultivar in exceptional cases according to the environmental conditions (Jenning *et al*., 1981).

Chalkiness is associated with the development of numerous air spaces between loosely packed starch granules and the resulting change in light reflection (Tashiro and Wardlaw, 1991). Chalkiness is a quality indicator that determines the market value of rice; grains tend to be weaker and more prone to break during grinding (Lisle et al., 2000). Stress at high temperatures during grain filling facilitates the formation of opaque grains through the loose packaging of amyloplasts (**Figure 9**) (Wakamatsu *et al.,* 2009). In rice, rice *japonica* subspecies is very sensitive to heat stress during the reproductive stages. Basmati rice grown under poor water supply conditions during the grain filling time shows excessive abdominal whiteness in the grains, while these factors negatively affect the cooking qualities (Pandey and Shukla, 2015). Excessive doses of nitrogen depress the whole grain and increase the chalk (Gamarra, 1996).

Proteins from rice are extensively use in the production of childs food because of their high caloric value and *in vitro* digestibility, as well as their hypoallergenic and anticancer properties that turn bran proteins in highly valuable relatively to those from the rest of cereals (Friedman, 2013). Concerning the effect of abiotic and biotic factors in the protein content,

which is also associated (remotely) with grain quality traits, such as head rice ratio, milled grain dimensions, milled grain appearance, and viscosity parameters; to date, it has been demonstrated that, in general, enriched nitrogen fertilization elevates protein content (Midorikawa *et al*., 2014). Like wheat, climate and nutrition give rise to a wide range of protein contents of rice grains, which is closely related to the nutritional quality and physical properties of the final products. Besides, surface hardness has been also directly associated to the protein content, being much higher with high protein. Although prolamines, globulins, and albumins are produced, the main storage protein in rice is oryzenin. Oryzenin is composed of subunits that are linked by intra- and intermolecular disulfide bridges. During storage of rice, the molecular weight of oryzenin increases significantly, which correlates with an augment in disulfide binding. It is believed that the decrease in solubility explains this decrease in adherence, observed in stored rice (Martin and Fitzgerald, 2002).

1.1.3.3. Effect of abiotic and biotic stresses on phytochemical composition of rice products

Non-enzyme compounds, such as tocopherols, anthocyanins, flavonoids, carotenoids, constitute a highly efficient antioxidant defense system, present in plant cells for the detoxification of ROS (Basu *et al*., 2010). The main groups of phytochemicals found in whole grains of rice, can be classified as phenolic compounds (ferulic acid, p-coumaric acid, catechin, vanillic acid and caffeic acid), carotenoids, vitamin E, lignans, β-glucan, and inulin (Huang and Ng, 2012).

Ferulic acid is the most abundant phenolic compounds in all parts of the grain. The content of phenols is higher in rice bran, lower in polished rice, and intermediate in the whole grain. The total polyphenolic content of *japonica* rice varieties has been noticed to be higher than that of *indica* rice varieties, while its content in pigmented rice varieties has been found in higher levels relatively to non-pigmented rice varieties (Huang and Ng, 2012). Phenolic compounds are products of the secondary metabolism in plants and exert beneficial effects on human health. Insoluble phenolic acids are typically involved in the structure of the cell wall (Huang and Ng, 2012) and are considered natural antioxidants, which act as radicals scavenging molecules that contribute to decrease the incidence of oxidative stress induced damage to large biological molecules, such as lipids, proteins, and DNA (Ghasemzadeh *et al*., 2018). Zhou *et al*., (2004) have observed that free phenolic acids provide grain resistance to external factors. In this regard, for instance, the content of phenols in the germ of corn grain is associated with tolerance to *Fusarium* sp.; while those of the pericarp are related to tolerance to storage pests (Cabrera-Soto

et al., 2009). In respect to abiotic stress, drought-tolerant rice varieties generally respond with significant increases in antioxidants during water deficit. There are substantial differences with respect to antioxidant responses in rice varieties to drought stress. Drought-tolerant species accumulate smaller amounts of peroxide and free radicals (Basú *et al*., 2010). High air temperatures produce an increase in most phenolic compounds (Nakano *et al.,* 2013).

Rice bran oil is not popular around the world but its demand increases due to its health benefits. It comprises 15-20% oil, depending on the variety, the degree of grinding, and other agroclimatic factors (Sohail *et al*., 2010). In Japan and in some Western countries, rice bran oil is more commonly recognized as 'heart oil' and has reached the status of 'healthy food' (Codex Alimentarius Commission (CAC), 2003). The lipid content and the fatty acid composition in non-glutinous brown rice are affected by daily mean temperature during the ripening stages, which has been described as significantly and positively correlated with the contents of myristic, palmitoleic, stearic, oleic, and arachidic fatty acids, but negatively correlated with the contents of linoleic and linolenic acids. Elevated temperatures during cultivation have been shown to change the profile of some phytosterols: increasing α-tocotrienol and/or α-tocopherol, while decreasing γ-tocopherol and γ-tocotrienol (Britz *et al*., 2007). Furthermore, oleic acid content was highly and negatively correlated with linolenic acid content (Taira *et al*., 1979). Brown rice also provides lipid-soluble antioxidants, including ferulated such as γ-oryzanol, tocopherols, and tocotrienols.

Recent published results suggest that products of non-enzymatic lipid peroxidation pathways, such as the isoprostanes / PhytoPs in animals and plants, might have an evolutionarily ancient function in host defense (Muller, 2004). Plants and animals maintain a certain level of polyunsaturated fatty acids (PUFAs) in their membranes. These fatty acids are important for modulating the fluidity of the membrane but can also be released from the membranes and used for the enzymatic synthesis of oxidized fatty acids (oxylipins), some of which function as defense signals (e.g. jasmonates and 2-hydroperoxy fatty acids) or antimicrobial lipids. However, before the development of enzymatic signaling pathways of oxylipins, another reaction sequence was already present, which gives rise to a large variety of oxylipins in all aerobic organisms containing PUFAs, namely catalyzed non-enzymatic lipid peroxidation by free radicals (Muller 2004). Phytoprostanes are apparently representatives of a much larger matrix of biologically active oxidized lipids that are generated during oxidative stress and could have an evolutionarily ancient function in the defense of the host (Muller, 2004). In a relatively short time, the structural variety, properties, and applications of the cyclic PUFA derivatives formed in an auto-oxidative manner have been discovered (Jhan *et al*., 2008). The concentration of

PhytoPs has been measured in tissues of several plants, for example, in tomato leaves, mint leaves, linden flowers, birch pollen, birch leaves, and valerian root, while other studies have also detected them in cell cultures of different types of plants, such as *Nicotiana tabacum*, *Glycine max, Rauvolfia serpentina, Agrostis tenuis, Salix alba, Arabidopsis thaliana, Tilia cordata*, and *Betula pendula* (Thoma, 2004). From the agronomic point of view in relation to the field of research on Food Science and Technology, further efforts are required to understand how the production of PhytoPs is affected by different types of abiotic stress. In addition, and nutritionally, additional studies would be necessary to know the physiological effects of PhytoPs in humans, since they show structures very similar to those of IsoP and PG, bioactive compounds relevant at a physiological level (Collado–González, 2015d).

1.1.3.4. Agronomic advantages and constraints of fertilization of rice crops

Against the backdrop of decreasing land, labor, and water that can be devoted to rice production due to increasing competition from non-farming sectors, the challenge to increase rice productivity is indeed enormous (Redoña, 2004). To achieve the required massive increase in food production, to meet the demand of the growing human population, a significant enhancement of the application of fertilizers and improvement of soils fertility are critical. Presently, in many developing countries, poor soil fertility, low levels of available mineral nutrients in soil, improper nutrient management, along with the lack of plant genotypes having high tolerance to nutrient deficiencies or toxicities are major related to food safety and, malnutrition (*i.e.*, micronutrient deficiencies) as well as to ecosystems degradation (Cakmak, 2002).

Flooding causes changes in soil properties due to physical reactions between soil and water. The most important change is the conversion of on root area of the rice plant from an aerobic environment to an anaerobic or near-anaerobic environment (De Datta, 1996). In flooded soil conditions there is a decrease in the redox potential, the pH tends to neutrality, and the dynamics of nutrients are modified realtively to aerobic conditions. The most important mineral changes can be summarized indication that most of mineral nitrogen is found into the ammonium form, the concentration of phosphorus, potassium, iron, manganese, and silica in the soil solution increases, and the concentration of zinc decreases (De Battista, 2006).

The supply of nitrogen to rice crops has its origin in the nitrogen present in flooded soil, mineralized from organic matter, fixed by algae, and heterotrophic bacteria, and supplementation. A correct estimate of the nitrogen supplies by the soil and its dynamics is

essential to calculate the quantity to be added and the moment of its application as fertilizer. The content of organic matter and its mineralization is an estimated indicator of the need for nitrogen supplementation (De Battista, 2006). However, the amounts of nutrients coming naturally from the soil, in general, are not sufficient to cover the needs of the crop. For each ton of rice 18.4 kg of N, 3.8 kg of P, and 21.9 kg of K are extracted from soil (Gamarra, 1996). The efficiency of nitrogen applied as fertilizer is very low. In most intensive agricultural production systems, over 50% and up to 75% of the N applied to the field is not used by the plant and is lost by leaching into the soil (Hirel *et al*., 2011). Nitrogen demand for rice crop is low at the beginning but increases drastically at the end of tillering, mainly after panicle initiation (**Table 6)**. Cornélio *et al*. (2007) while applications concentrated at panicle initiation has been noticed as the most efficient. Indeed, this management practice favors a higher yield of the crop, since in this period two components of the yield in the reproductive phase are defined: the number of spikelets per panicle and weight of the grain (BezerraBarreto, 2012). In a first approach the recommendation to farmers about the most appropriate time to apply at 50% at tillering and 50% at panicle initiation. With the application of fractionated nitrogen, greater efficiency is achieved (De Battista, 2006). During elongation of internodes, high nitrogen content makes the plants more susceptible to diseases, to overturning and may increase the number of empty grains (De Battista, 2006).

Tabla 6. Extraction of nutrients during the different development phases of rice plant. Source: Gamarra, 1996.

Development stages of the rice plant	Percentage of total extractions		
	N	P_2O_5	K_2O
Seedling to top tillering	37	33	36
Top tillering to panicula initiation	12	23	21
Panicula initiation to heading	31	34	0
Heading to maturity	20	10	43

The application of foliar fertilizers as a complement to traditional fertilization is a technology that has increased in the recent years, seeking better use of the resources available an improvement plant growth, yield, and grain nutritional quality. This improvement may result in similar results but using lower doses of urea-based fertilizer. The foliar spray used in the application of foliar fertilization allows the availability of nutrients at the time that plants requires, by reducing the delay time between the application and the absorption. Concentrated

N applications, near the stage of panicle initiation, were the most efficient, favoring a greater crop yield, regarding the number of spikelets per panicle and grain weight (Marzari, 2005). According to Ntanos and Koutroubas (2002), the use of foliar fertilizers applied near of the end of the cycle in rice provides a nutritional supplement during the translocation of photoassimilates to the grains, which is crucial for yield. Santos *et al*. (2007) observed that the foliar application of a product with 30% nitrogen, in the stages of tillering and panicle initiation, has comparative advantages with urea in coverage, since it reduces the sterility of the spikelets, and increases the weight of rice grains.

1.1.4. Rice derived food products

Currently, the domestic uses of rice in the West are limited to consumption as food and processed foods (mainly mixtures of flavored rice, cereals and rice cakes), beers, and pet's food. Waxi-type races are used in desserts, cakes, baby food sauces, and breakfast cereals (USDA, 2017). Rice with intermediate amylose content used in the development of canned soups and fermented rice cakes, while rice with high amylose content are used to make rice noodles (Juliano and Hicks, 1996). The rice bran is used as a defatted or complete ingredient in foods such as bakery products, breakfast cereals, and wafers as a protein supplement, binding ingredients for meats and sausages, and as a beverage base. The incorporation of rice protein concentrates has been studied in bread, sweet drinks, and baby foods (Prakash, 1996). Another co-product of rice being exploited hoardings is bran oil, a unique rich source of bioactive phytochemicals of commercial importance, most of them of interest in nutrition, pharmacy, and cosmetics. The unsaponifiable components of rice bran oil include mainly tocoles (vitamin E, 0.10-0.14%) and γ-oryzanol (esters of trans-ferulic acid with sterols and triterpene alcohols, 0.9-2.9%) (Lerma-García *et al*., 2009).

There are many advantages to the natural consumption of bran and co-products derived from rice, because they have high level of bioactive compounds, many of which have shown beneficial effects in humans. This fact is supported by the extensive literature exsiting (approximately 3600 citations in the Scopus database that include numerous reviews). The rice bran possesses antioxidant, antiallergic, anticancer, anticolesterol, antidiabetic and antimicrobial activities in cells, animals and humans. The costs are low and the global production of bran is very high (76 million tons). These findings suggest that rice bran could contribute to food security (Friedman, 2013).

1.1.5. Postharvest factors modifying the physical features and the proximate and phytochemical composition of rice-derived food products

Rice aging is a complicated process, which involves changes in the physical and chemical properties of the rice grain. These changes affect the sticking and gel properties, flavor and texture of cooked rice. Starch, protein and lipids are the main components of the grain that affect the quality of the cooking and the ingestion. During storage, structural changes occur but the total contents of starch, protein and lipids in the rice grain are not modified. Storage conditions are important in the aging process (Zhou *et al*., 2002).

The amylose content is considered the single most important characteristic to predict the cooking and processing behavior of rice which is directly related to water absorption, volume expansion, and sponginess of cooked grain. With storage the molecular weight of amylose decreases and that of amylopectin increases (Chastril, 1990). A decrease in the amount of soluble solids and amylose in the cooking water of rice has been observed. This is probably a consequence of the increased insolubility of rice starch and protein in water during aging, resulting in a slower cooking rate (Chastril, 1990). Regarding the protein and amino acid content of rice and coproducts, during storage, the content of free amino acids, increases while according to Chastril (1990), the protein content does not change significantly during storage, although its solubility decrease in parallel with, the solubility of albumin (water-soluble protein), mainly due to an increase in the number of disulphide bridges during storage. Within portingcomponents, the activity of the enzymes peroxidase and catalase is rapidly lost during storage of rice. In addition, upon this work, Dhaliwal *et al*. (1991) demonstrated that the alpha-amylase and beta-amylase activities of rice samples decrease significantly during storage while the activity of proteases, lipases, and lipoxygenase increase.

In respect to lipids, the changes observed during storage suggest that at least two processes are affecting this compounds, one involves the hydrolysis of lipids to produce free fatty acids; the other is the oxidation of lipids (including free fatty acids) giving rise to hydroperoxides. The storage conditions of temperature and light are particularly important for the speed of both reactions. The changes are greater in the outer layer, where the non-starch constituents are concentrated (Barber, 1972). Rice lipids are stable in the intact spheroid of the cell, but lipid hydrolysis begins when the lipid membrane is destroyed by phospholipases, physical damage or high temperature, as a consequence, and during storage, there are changes in the fatty acid profiles and an increase in free fatty acids. The fraction of neutral lipids is the most affected. Linoleic acid is released preferentially by lipase, which produces an increase in the proportion of oleic acid at the expense of linoleic acid (Zhou *et al.,* 2002).

According to the information available in the literature, the phytochemical composition of rice and coproducts are mainly represented by phenolic compounds. These compounds exert a significant effect on the properties of the cell wall that is mechanically strengthened by crosslinking. Phenolic acids are usually concentrated in the external aleurone layer of the seed, which is rich in arabinoxylans. The content of free phenolic acids increased during storage of milled rice. Tsugita *et al*. (1983) suggested that the bound phenolic acids were released by enzymatic and nonenzymatic reaction and that there were large increases in the concentration of *p*-hydroxybenzoic acid, vanillic acid, syrinic acid, caffeic acid, *p*-coumaric acid and ferulic acid when the rice was stored at 40 °C (80% RH) for 60 days compared to storage at 4 °C. Propanal, pentanal and hexanal were reported as the main carbonyl compounds that increase more during storage (Zhou *et al*., 2002).

1.2. CONNECTING AGRONOMICAL PRACTICES AND OXIDATIVE STRESS IN RICE PLANTS

1.2.1. General aspects and nutritional value

Oxidative stress has been mentioned as a common cellular and tisular condition enclosed to metabolic pathways in response to diverse biotic and abiotic stresses, and the regulation of oxidative stress has been mentioned as an indication of stress tolerance of plants according to different studies. In plant cells, ROS are produced continuously as a consequence of aerobic metabolism in all intracellular organelles, particularly in the chloroplast, mitochondria, and peroxisomes. The biotic and abiotic accelerates the generation of ROS including singlet oxygen ($^1O_2$), superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^\bullet$), thereby induced oxidative stress. Superoxide radical ($O_2^{\bullet-}$) is formed in many photooxidation reactions; Hydroxyl radical ($OH^\bullet$) is formed due to the reaction of H_2O_2 with $O_2^{\bullet-}$ (HaberWeiss reaction), singlet oxygen ($^1O_2$) is formed during photoinhibition, and photosystem II electron transfer reactions in chloroplasts. The ROS can produced potentially react with biomolecules namely, pigments, proteins, polyunsaturated fatty acids, DNA, and almost with all constituent of cells (Hasanuzzaman *et al*., 2013).

Rice plants are very sensitive to oxidative stress (Volkov *et al*., 2006) and given the toxicity of ROS, they need appropriate detoxification systems that allow the rapid elimination of reactive chemical species. These systems include several antioxidant enzymes and non-enzymatic compounds.

As a consequence, oxidative stress, according to Esfandiari *et al*. (2007), lipid peroxidation is linked to an increased activity of the antioxidant enzymes superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), catalase (CAT), peroxides (POX) among others, which is associated to an improved tolerance to oxidative stress. Besides, the activity of other intervening enzymes is modified in situations of oxidative stress. This is the case of dehydroascorbate reductase (DHAR), glutathione reductase (GR), glutathione S-transferase (GST), and monodehydroascorbate reductase (MDHAR). In heat stress tolerant cultivars, the activities of SOD, APX, CAT, GR and POX enzymes increased significantly in all stages of growth (Almeselmani *et al*., 2006).

In respect to non-enzymatic compounds, glutathione, ascorbate, flavonoids, carotenoids, and tocopherols are essential metabolites that regulate main functions of cells and play a fundamental role in the antioxidant defense (Ruiz-Sánchez *et al*., 2015). In plants glutathione is maintained exclusively in the reduced form. This exerts its antioxidant function by reacting with superoxide radicals, peroxide and singlet oxygen for the formation of oxidized glutathione (Ruiz-Sánchez *et al*., 2015). Malondialdehyde, a product of decomposition of hydroperoxides of polyunsaturated fatty acids, has been used very often as a suitable biomarker for lipid peroxidation (Bailly *et al*., 1996). It has been reported that under high temperature, the content of malondialdehyde, free proline, and soluble sugars in leaves increase significantly, while the chlorophyll content and photosynthetic rate of leaves were decreased (Wang *et al*., 2011). The plant phenolics develop an array of physiological functions to higher plants contribute to their capacity to survive and adapt to climatic disturbances (Adams-Phillip *et al*., 2010). In this regard, to date it has been developed numerous studies that correlate positively tolerance to temperature stress in plants with an increase in antioxidants. Indeed, under thermal stress, it has been found that plants accumulate a greater amount of non-enzymatic antioxidants (Hasanuzzaman *et al*., 2013). Malik, *et al*. (1999) found that a higher content of ascorbic acid was associated with a greater antioxidant capacity and a greater tolerance to cold in rice.

1.2.2. Impact of agronomical practices on rice productivity and composition

As detailed in section 1.1.3.1 Relation between abiotic and biotic stress and productivity of rice in Chapter I, page 18, to date, it has bin identified an array of environmental conditions that generate abiotic and biotic stress in rice cultivation. In this regard, in the recent years, several strategies have been set up to contrast the deleterious effects of abiotic and biotic stress, to crops productivity and composition, such as the application of exogenous protectants, namely

salicylic acid, jasmonic acid or its derivatives, proline, glycinebetaine, selenium, nitric oxide, silicon, and polyamines, (Hasanuzzaman *et al.,* 2013) or biological as mycorrhizae. These approaches have allowed notice that they are beneficial to protect plants against stress damage.

Concerning the application of exogenous protectants, salicylic acid (SA) is part of a large group of compounds synthesized in plants (Rangel Sánchez *et al*., 2010). White (1979) reported for the first time the participation of SA in disease resistance in experiments upon which aspirin (acetylsalicylic acid, an SA derivative) or directly SA were infected, into tobacco leaves of a resistant tobacco line and observed the production of proteins related to pathogenesis, also known as PR proteins, which are a heterogeneous group of proteins that are induced in plants by the infection of a pathogen.

Parallel to the production of pathogenesis-related (PR) protein, an increase in the resistance against tobacco mosaic virus infection was observed, which was manifested by a 90% reduction in the number of lesions in the tissues analyzed. Furthermore, in resistant tobacco plants, the concentration of endogenous SA increase about 40 times in leaves inoculated with the virus and approximately 10 times in non-inoculated leaves of the same plant. It has been reported that in many plants the treatment with SA or related compounds induces the expression of PR genes and/or resistance against pathogenic viruses, bacteria, and fungi (Vlot *et al*., 2009). SA seems to play an essential role in the signal transduction pathway that leads to the activation of genes that encode not only for PR proteins, but also for the establishment of the hypersensitive response (HR), considered a programmed cell death that is developed to delimit the area of infection of a pathogen, as well as in the acquired systemic resistance (RSA). A decrease in the activities of CAT, POX, SOD, and glutathione reductase enzymes was observed in plants treated with SA. The treatment of SA also reduces the level of reactive substances in rice (H_2O_2 and O_2^-), this providing to plants additional tolerance against oxidative stress generated by exposure to cadmium (Hayat *et al.,* 2010).

In respect to jasmonates (JA), these compounds constitute class of phytohormones derived from polyunsaturated fatty acids that play essential roles in plants, such as the regulation of development and growth, the response to environmental changes, and resistance to abiotic and biotic stresses.

The biosynthesis of JA starts in chloroplasts, and involves the release of α-linolenic acid (18:3 or 18:2) from the lipid membrane by phospholipases. Only α-linolenic (18:3) is used as a precursor of JA through one of the seven distinct branches of the lipoxygenase pathway. During signaling, JA interacts, either synergistically or antagonistically, with other hormones, such as

SA, gibberellin, ethylene, auxin, brassinosteroid, and abscisic acid, to regulate the expression of genes in regulatory networks, which confers physiological and metabolic adjustments in plants. Many reports indicated that JA plays critical roles in rice immunity against bacterial and fungal infections as well as against damage by herbivores. In bacterial and fungal diseases, such as blight caused by *Xanthomonas oryzae* pv. *Oryzae* and rice blast caused by *Pyricularia oryzae*, the exogenous application of JA is sufficient to induce rice resistance against infections, effectively reducing lesion symptoms (Liu *et al.*, 2015). The exogenous application of JA and JA-Me showed that in the treated plants, direct resistance against herbivorous insects is stimulated in a wide variety of crops. This direct resistance of the plants is manifested by the reduction of fecundity, growth and survival of insects (Eng Sánchez, 2008). Molecular assays developed to have revealed that the expression levels of several pathogenesis-related (PR) genes are up-regulated in rice upon treatment, confirming that JA functions as an important signaling molecule in pathogen resistance (Liu *et al.,* 2015).

Increased levels of proline may contribute, to enhanced biomass production, as reflected by a higher level of fresh shoots and root weight, of transgenic rice plants under water or saline stress conditions (Zhu *et al*., 1998). Wutipraditkul *et al*. (2015) investigated the effects of proline and/or glycine betaine application on growth, photosynthetic pigments, H_2O_2 content, and activities of antioxidant enzymes in rice under salt stress. The H_2O_2 content and the activities of superoxide dismutase, glutathione reductase, and ascorbate peroxidase, but not catalase, increased under salinity. Upon these work, it was demonstrated the addition of glycine betaine (1 mM) to rice plants with salt stress, significantly reduces the increase in H_2O_2 content compared to stressed salt-free rice plants, without the application of glycine betaine (Halford *et al.,* 2015). On the other hand, Djanaguiraman *et al*. (2010) found that the application of selenium in stressed rice plants decrease oxidative damage by improving antioxidant defense, which results in higher grain yield.

The establishment of harmonic processes between the plant and the arbuscular mycorrhizal fungi is generally associated with an improvement in tolerance to biotic stresses. Mycorrhizal induced resistance is especially important for the control of foliar pathogens, leaf cutters and necrotrophs. There has also been found that mycorrhizal plants are protected both locally and systemically and their protection is related with jasmonic acid levels at their tissues (Ramirez-Gomez and Rodriguez, 2012)**.** Ruiz-Sánchez *et al.* (2015) stressed mycorrhized and non-mycorrhized rice plants with *Rhizoglomus intraradices*. The results showed that the symbiosis reduces the accumulation of hydrogen peroxide and the oxidative damage to the lipids from an increase in the accumulation of the antioxidant glutathione. In this connection,

the use of mycorrhizal fungi contributes to saving water by maintaining and even increasing yields in agricultural crops.

In addition to agronomic management practices, overall, the increase in atmospheric CO_2 concentration result in a significant improvement of plants growth, total biomass at maturity, and grain yield for rice. The observed augment in biomass must be primarily to increases in tiller number and stem, root, and panicle weight. In addition, increases in CO_2 and/or temperature may also reduce protein content and overall nutritional quality. Because future climates may have higher levels of both CO_2 concentration and temperature, additional work is needed to test for rice cultivars that could maximize reproductive productivity as atmospheric CO_2 increases, even if air temperature rises concurrently (Ziska *et al*., 1997).

1.2.3. Rice derived food products

Yield is not the only crop parameter affected by abiotic stress. Climate change on crop composition is also important. The composition (quality) of a crop product affects its properties of manufactured products as well as their nutritional value, flavor, color, and aroma. Besides, biotic stress also affects food safety and regulatory compliance, because to contribute to the formation of undesirable contaminants, such as acrylamide, furan, and related products, and *trans* fatty acids being determined by the composition of the raw crop product. Composition is also affected by crop management, notably plant nutrition, with management factors interacting with the effects of the environment (Costa Crusciol *et al*., 2008).

Oxidative stress leads to a variety of physiological and biochemical changes which cause deterioration and cell death in grains and seeds. The deterioration is partially associated to the accumulation of free radicals produced as consequence of metabolic processes. The redox regulation can affect maturation of seeds, relatively to the accumulation of starch and storage proteins, and therefore grain quality. After harvest, seed storage subjects the lipids to slow and consistent attacks by oxygen, forming hydrogen peroxides, other oxygenated fatty acids and free radicals. Free radicals are unstable and can react and damage nearby molecules. Oxygenated fatty acids in the absence of enzymatic activity in the dry seed accumulate and damage cellular components. The accumulation of H_2O_2, which is a strong oxidant, leads to the disruption of cell membrane integrity that manifests itself in an increase in malondialdehyde content (Halford *et al.,* 2015).

Polyunsaturated fatty acids (PUFAs) are relatively unstable because they are subject to thermal oxidation during cooking and processing at high temperature, giving rise to lipid

peroxides. These lipid peroxides form polymers, responsible for a dark coloration, which can be toxic, and can degrade to product, causing rancidity, 'off' taste and odor, as well as furans. Oxidation also occurs during long-term storage, so a high PUFAs content would contribute to shorten the life of the oil. Same food processing practices prevent the oxidation of PUFAs by chemical hydrogenation of the double bonds, producing more stable saturated fatty acids. This is also an industrial practice solidify oils, making development to them suitable for the production of margarines. The problem with the chemical hydrogenation of PUFAs is that some of the double bonds remain unsaturated but change from the *cis* form, with the two hydrogen atoms attached to the carbon atoms involved in the double bond on the same side, to the *trans* form, with the two hydrogen atoms on the opposite side. *Trans* fatty acids that arise from the partial hydrogenation of vegetable oils are now considered as harmful as saturated fatty acids when consumed, and can therefore be considered another important class of processing contaminants (Halford *et al.,* 2015).

Rice bran constitutes approximately 10% of the dry matter of a seed and contains approximately 15-20% oil, of which a relatively large proportion (approximately 4%) relatively to other vegetable oils is unsaponifiable material. Rice bran has a short shelf life due mainly to the fact that it becomes rancid quickly during storage, especially in tropical countries due to the high temperatures. Hydrolytic rancidity is produced by the enzymatic activity of lipases, which causes rapid deterioration of the lipids present and an increase of free fatty acids, limiting the application of rice bran for the development of new food products. To avoid rancidity, lipase enzymes should be inactivated by stabilizing the bran. Different stabilization methods have been described so far including pH modification, chemical treatments, storage at low temperatures, microwaves, vaporization, and single screw extrusion (Guevara Guerrero *et al*., 2015).

Free amino acids, together with sugars, are the main determinants of the quality of processing and, in some cases, the safety of food. Free amino acids are present in plant tissues to enable protein synthesis. They accumulate at high concentrations in almost all tissue in response to an array of both biotic and abiotic stress factors, including nutrient deficiency, pathogen attack, toxic metals, drought, and salt stress. The amino acids found most frequently in stress situations are asparagine and proline (Harlford *et al*., 2015). Cereals and other plants interconvert monosaccharides, disaccharides and more complex carbohydrates, such as fructan to cope with the osmotic stresses caused by salt, freezing, and drought, as well as other stresses such as hypoxia and early senescence.The amino acids and free sugars combine during cooking, frying, and processing at high temperature, to produce a large number of compounds, including some that impart color, flavor, and aroma, as well as others that are potentially harmful to

health. The reaction in which free amino acids and sugars combines one to another to form these compounds is the Maillard reaction, a generic term for a series of non-enzymatic reactions that take place only at high temperatures (Harfold *et al*., 2015). While many of their products are highly desirable, some are not and can be classified as processing contaminants and are undesirable either, because they have deletions effect on the quality of the product or because they are potentially harmful (Curtis *et al.,* 2014). Maillard reaction products in cereals, that can be considered processing contaminants, include acrylamide and furan classified as possibly carcinogenic to humans by the International Agency for Research on Cancer. Among nowadays, these acrylamide is probably the most worrying to the food industry. While the risk posed by the presence of very low levels of acrylamide and furans in food remains unknown (Halford *et al.,* 2015), and observed to be further explored.

1.3. PLANT OXYLIPINS: PHYTOPROSTANES AND PHYTOFURANS

1.3.1. Structure and nomenclature

In humans and other mammals, oxidative stress is associated with the pathogenesis of several chronic diseases. Reactive oxygen species (ROS) are always generated in the cellular metabolism but intensifies in stress. These high levels of ROS exceed the antioxidant defenses and lead to oxidative damage of lipids, proteins, and nucleic acids. Polyunsaturated fatty acids (PUFAs) are extremely important compounds in all organisms. These fatty acids are important for modulating membrane fluidity, but can also be released from the membranes and used for the enzymatic synthesis of oxidized fatty acids (oxylipins) (Jahn *et al.,* 2008).

Unlike saturated and monounsaturated fatty acids, which are relatively inert under physiological conditions, PUFAs show a high reactivity and participate in the pathophysioological reactions of cells upon a number of biological functions. Therefore, they are substrates for enzymatic and non-enzymatic transformations that provide a variety of important signaling molecules, mediators and biologically active secondary metabolites, some of which function as defense signaling compounds (Jahn *et al*., 2008). When the oxidative reaction of ROS is directed against α-linolenic acid (ALA), the polyunsaturated fatty acid (PUFA) predominant in the cell membranes of plants, a broad spectrum of oxylipins are produced, which are called phytoprostanes (PhytoPs) and phytophurans (PhytoFs) (Collado- González *et al*., 2015c).

The cyclic PUFA metabolites appear to be found in all organisms (**Figure 10**). Some of them are enzymatically biosynthesized as prostaglandins and thromboxane in animals, (9S, 13S), 12-oxophytodienoic acid and jasmonic acids in plants, and as clavulones V and punaglandins VI in

marine invertebrates (Jahn *et al*., 2008). Isoprostanes, isothromboxanes, and isofurans are the cyclic compounds formed autoxidatively from arachidonic and eicosapentaenoic acids; neuroprostanes present in the human and animal brain, which are derived from docosahexaenoic acid also in mammals. In plants, the phytoprostanes are derived from α-linolenic acid (Jahn *et al*., 2008).

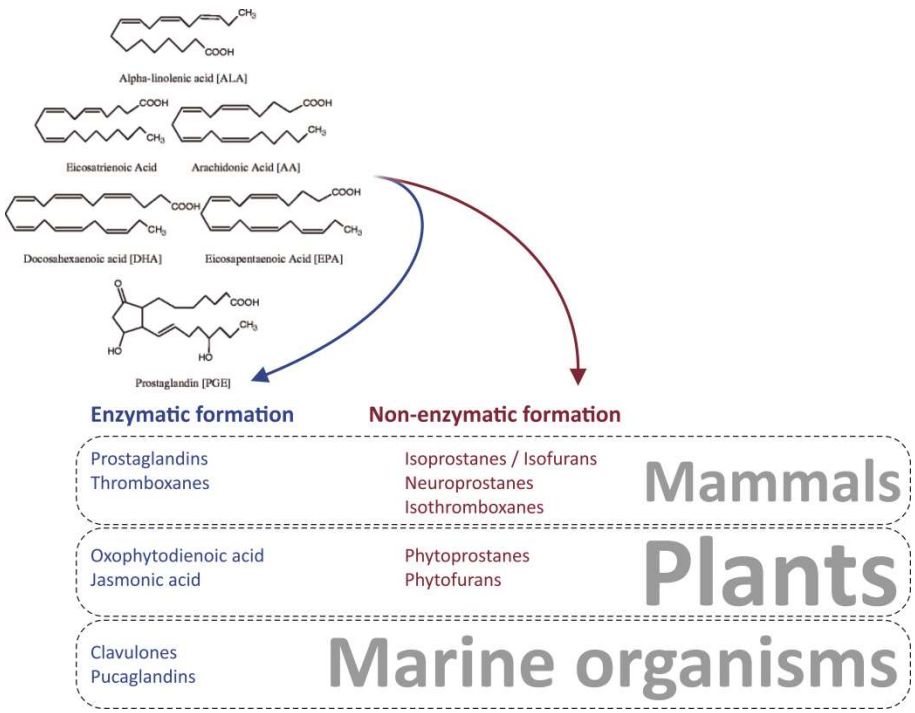


Figure 10. Cyclic PUFA metabolites-source and enzymatic or non-enzimatic formation. An example shows each compound type. Source: Jhan *et al*., 2008.

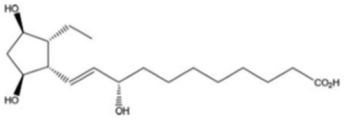
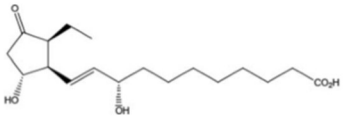
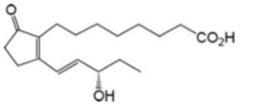
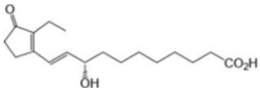
More recently, a new non-enzymatic route of formation of cyclic metabolites of PUFA was discovered recently, leading to 3-hydroxy-2, 5-disubstituted tetrahydrofuran structures, called isofurans (IsoFs) from arachidonic acid (AA), neurofurans (NeuroFs) from DHA, and dihomo-IsoFs fromf adrenic acid (AdA). Cuyamandous *et al*. (2015) investigated the occurrence of similar pathways in plants and successfully identified the production of phytofurans (PhytoFs). The PhytoFs biosynthetic pathway of ALA can generate two classes of PhytoFs (alkenyl and enediol), giving rice to total potential of 128 isomers.

Metabolites of PUFAs generated autoxidatively are a racemic mixture, being the main members of the individual classes' diastereomeric relatively to their congeners formed enzymatically (Jahn *et al*., 2008). It is known that they are extremely versatile natural products, unlike those synthetized enzymatically; oxylipins generated as a result of *in vivo* autoxidative conversion of PUFAs into cyclic products follows the generation of a much wider array of metabolites. The close constitutional similarity of cyclic PUFAs metabolites on the one hand, and

the diversity of their three-dimensionalshape arising from the configurational differences on the other, makes them an ideal “playground” to select functions in an evolutionary context (Jahn *et al*., 2008).

Different naming systems have been proposed to unequivocally identify the structural isomers of PhytoPs. Two systems were presented almost in parallel by Rokach and collaborators (Rokach *et al.*, 1997) and by Taber and collaborators (Taber *et al*., 1997). The Taber / Roberts nomenclature was approved by the IUPAC, and follows the convention of normal PG to assign the ring substitution pattern common to all carbocyclic regio and stereoisomeric metabolites of PUFAs. The diverse regioisomeric phytoprostane series are distinguished mainly by the position of the hydroxy group in the side chain. The Rokach nomenclature has the inherent merit of providing information on the formation of the cyclic PUFA metabolite. Mueller (2000) proposed a modified Rokach nomenclature; feature by the main advantage of considering only a common ring substitution pattern (in which the allylic hydroxyl fragment is included) and changes in the resulting side chains. This procedure leads to different names for the same structure with respect to the substitution ratio of ring to side chain. **Table 7** shows examples of compounds named by the three nomenclature systems. Future efforts should be directed towards a unified nomenclature that reflects the advantages of both predecessors.

Table 7. Denomination of four PhytoPs according to the three nomenclature systems. Source: Collado-Gonzalez *et al*., 2015c.

PhytoP	Taber/Roberts	Rokach	Muller
	9-F_1 PhytoP	Dinor-i$PF_{1\alpha}$ - II	PPF_1Type II
	9-D_1- PhytoP	Dinor-i$PE_{1\alpha}$- II	PPE_1Type II
	16-B_1- PhytoP	Dinor-i$PB_{1\alpha}$- II	PPB_1Type I
	9-L_1- PhytoP	Dinor-i$PB_{1\alpha}$- II	PPB_1Type II

1.3.2. Synthetic pathways

Non-enzymatic lipid peroxidation is initiated by the attack of ROS on ALA, producing a linolenate radical that is easily oxidized and cyclized to complex isomeric mixtures of two classes isomerics (series 9 and 16) of PhytoPs. The linolenate radical reacts with an oxygen molecule, forming a peroxyl radical of linolenate. α-linolenic acid peroxyl radical can also react internally, forming a cyclic peroxyl radical, which reacts spontaneously with a second oxygen molecule and is subsequently reduced to G1-PhytoP. This PhytoP decomposes spontaneously, forming malondialdehyde, alkanes and alkenes, or forms other PhytoP. The only requirement for the formation of PhytoP is the presence of ALA and molecular oxygen, which suggests that the formation of PhytoP does not necessarily requires the metabolic activity of living cells (Sattler *et al*., 2006). Under high oxygen tension, a new compound structure is sinthetized, which follows the same PhytoPs free radical pathway. Hense, the addition of molecular oxygen after the initial cyclization leads to the generation of furanic structures called PhytoFs. These were recently identified and quantified in seeds and nuts (Cuyamendous *et al*., 2015). **Figure 11** shows the representation of the structures of the two groups of metabolites generated as a result of the autoperoxidation of ALA.

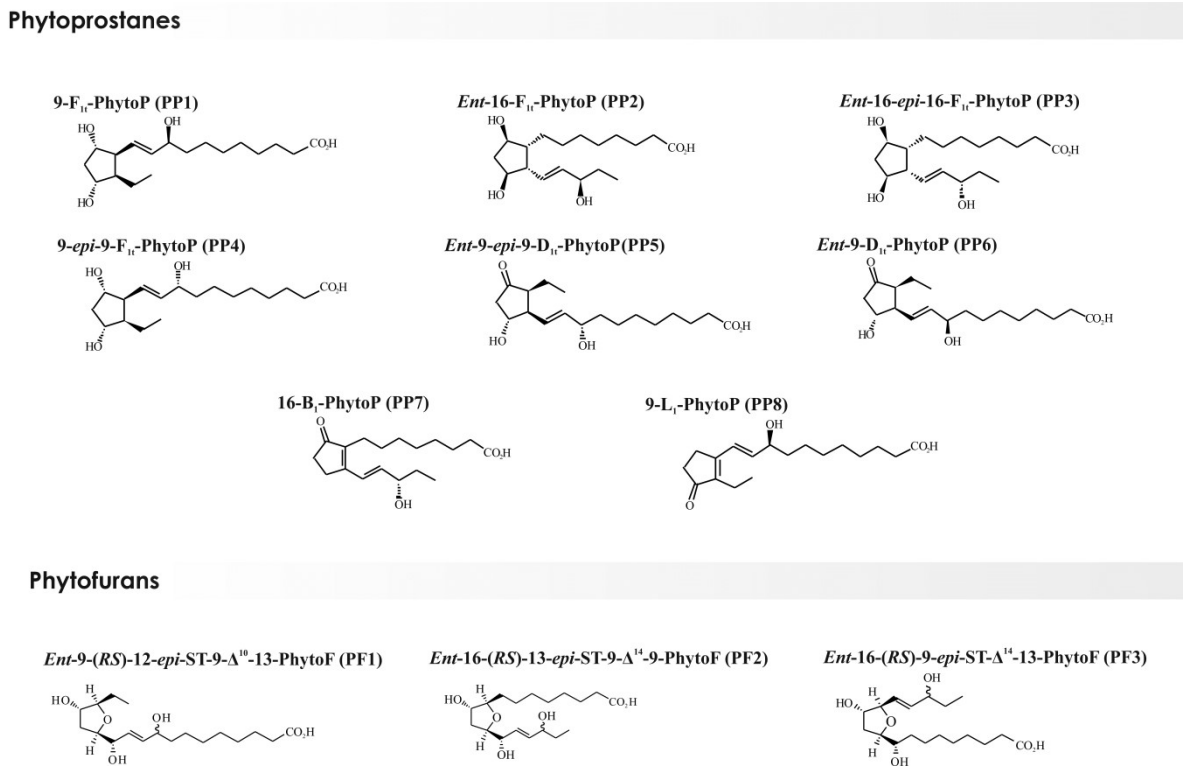


Figure 11. Chemical structures of the studied phytoprostanes and phytofurans. Source. Collado-González *et al*., 2015c.

1.3.3. Phytoprostanes and phytofurans occurrence and identified role in higher plants

Most phytoprostanes have been found to be esterified in the membranes of some plant species (Muller, 2004). Since PhytoPs shows some similarity with IsoPs and prostaglandins in humans, there is a growing interest in studying the profile and levels of PhytoPs in plant foods, as well as their response to cultural practices and / or environmental conditions (Collado-González *et al.,* 2015c).

From the first evidence of the formation of PhytoPs in plants provided by Parchmann and Mueller in 1998, their presence in a great variety of plant species and food products has been investigated to determine their PhytoPs content. Several series (A_1-, B_1-, D_1-, E_1-, F_1-and deoxy-J_1-PhytoPs series) of PhytoPs were found in *Arabidopsis thaliana* (leaves), *Betula pendula* (birch; leaves and pollen), *Brassica napus* (rape; seed oil), Glycine max (soya bean; seed oil and margarine), *Helianthus annuus* (sunflower; seed oil), *Hypericum perforatum* (saint johnswort), *Juglans regia* (walnut; seed oil), *Linum usitatissimum* (flax; seed oil), *Lycopersicon esculentum* (tomato; leaves), *Mentha piperita* (peppermint, leaves), *Nicotiana tabacum* (tobacco; leaves), *Olea europeae* (olive; fruit, flesh oil), *Prunus dulcis* (almond; kernel), *Rauvolfia serpentina* (Indian snakeroot; cell culture), *Salix alba* (white willow; leaves), *Tiliacordata/platyphyllos* (lime; tree flowers), *Valeriana officinalis* (valerian; root), and *Vitis vinifera* (grape; seed oil) (Thoma *et al*., 2004).

The content of PhytoFs has been less studied because of the lack of availabiliy of appropiate standards. Their occurrence has been reported in *Linum usitatissimum* (flaxseeds), *Salvia hispanica* L. (chia seed); *Pinus* sp. (pine, nuts) (Cuyamndous *et al.*, 2015), *Cucumis melo* (cantaloupe; leaves) (Yonny *et al*., 2016).

Traditionally, non-enzymatic lipid peroxidation products have been considered as toxic byproducts derived from the aerobic metabolism. Although it has been reported that PhytoPs have a wide range of biological activities in plant species, their exact biological functions in higher plants have not yet been elucidated in detail. From the information available, to date, it has been noticed that similar to IsoPs in mammals, PhytoPs represents a reliable marker of oxidative stress *in vivo* (Imbush and Muller, 2000). Besides, E_1- and F_1-PhytoPs have been found to occur ubiquitously in higher plants at basal levels, similar to their enzymatically biosynthesized congeners, 12-OPDA and JA (Jhan et al., 2008). Under oxidative stress, F_1-PhytoP levels increase dramatically and may exceed the levels of jasmonates in cells (Imbush and Muller, 2000). Thoma *et al.* (2004) proposed a model in which PhytoPs are components of an archaic sensory signaling system for oxidative damage that serves to protect plants from the

stress associated with increased production of free radicals. Analysis of gene expression of Arabidopsis (*Arabidopsis thaliana*) cell cultures treated with PPB_1-I or -II revealed that both regioisomers activated a massive detoxification and a defense response. Interestingly, it was found that the expression of several glutathione S-transferases (GST) increased with the application of one or both regioisomers of PPB_1 (16-B_1 PhytoPs) - and, therefore, can enhance the ability of the plant to inactivate and sequester reactive products of lipid peroxidation.

In both plants and animals, electrophiles have been shown to trigger the expression of antioxidative defense and electrophile detoxification genes. The induction of genes that are involved in protein rescue and apoptosis has been well established only in animals but may also occur in plants. The synthesis of secondary metabolites is apparently a plant-specific response to many electrophiles (Muller, 2004).

In addition, PhytoPs are not only considered components of an oxidant injury detection system but also excellent biomarkers of oxidative degradation in plant-derived foods (Collado-González *et al*., 2015c).

These findings highlight the need for a better understanding of the role of the metabolites of omega 3 PUFAs such as PhytoPs in the human diet. In the following years, efforts should be focused on assessing PhytoPs levels *in vivo* in humans, to demonstrate if they correlate with well-known disease symptoms, and if these symptoms can be alleviated with a vegetarian diet rich in omega 3 PUFAs.

1.4. TECHNOLOGICAL AND BIOLOGICAL INTEREST OF PHYTOPROSTANES AND PHYTOFURANS

1.4.1. Plant oxylipins as markers of plant food quality and safety

Several authors have proposed that PhytoPs are not only excellent biomarkers of the oxidative degradation of plant derived foodstuffs but also they coul be biologically active molecules, because they are components of an oxidant injury sensing, signaling system that induces several plant defense mechanisms (Imbuschand Mueller, 2000), while, qualitative and quantitative differences in the content of PhytoPs could indicate the influence of cultivar, oil extraction technology and / or storage conditions. The bioactivity, PhytoPs and PhytoFs is due to ther in structural suggested coincidences with mammals, oxylipins in which several biological activities have been demonstrated

Abiotic stress enclosed to specific growing conditions could modify quantitative and qualitative ocurrence of PhytoPs in plant species, which indicates that agronomic practices or environmental conditions would directly influence the quality of the final product in terms of organoleptical and compositional features.

Collado-González *et al.* (2016) studying the content of PhytoPs present in olive oil (*Olea europaea* L., cv. Cornicabra), revealed that their content increase when plants grow under irrigation deficit. In this regard, different behavior against the stress of different compounds has been described. For example, the content 9-epi-9-F_1t-PhytoP increase under irrigation deficits conditions, while 9-F_1t-PhytoP show dial not varied under these conditions (Collado-González *et al*., 2015b). The same working group also studied the concentration of PhytoPs in olives flesh harvested from plants grown with and without water stress. The results obtained indicate that the content of 9-F_1t-PhytoP and 9-epi-9-F_1t-PhytoP increase in olives flesh subjected to water stress. In there, there was an increase in the content of total PhytoPs, but a decrease in the range of compound within this class of oxylipins. They observed that this increase depends more on the duration than on the magnitude of the stress suffered (Collado-González *et al*., 2014). They also observed differences in the profile and level of PhytoPs among olive cultivars in similar edafoclimatic and cultural conditions. The total contents of PhytoPs were significantly different between the five cultivars studied ('Arbequina' = 'Picual', 'Arbequina'>'Hojiblanca', 'Picual' = 'Hojiblanca', 'Hojiblanca' = 'Cornicabra', 'Hojiblanca'>'Cuquillo', 'Cornicabra' = 'Cuquillo'). PhytoPs 9-F_1t-PhytoP, 9-epi-9-F_1t-PhytoP, *ent*-9-epi-9-D_1t-PhytoP, *ent*-9-D_1t-PhytoP, 16-B_1-PhytoP and 9- L_1-PhytoP were found in all cultivars (Collado-González *et al*., 2015a).

Carrasco-Del Amor *et al.* (2015) studied 11 almond cultivars regarding a range of growing factors conventional, ecological, rain-fed, and irrigation conditions. According to the results retieved by these authors, ecological system promotes the synthesis of D_1-phytoprostanes. Almonds from the rain-fed conditions had lower individual and total PhytoPs concentrations than those that were under irrigation conditions. In addition, different cultivars presented a difference in the level and profile of the PhytoPs present 9-F_1 PhytoP, 9-epi-9-F_1t- PhytoP, ent-16-epi-16-F_{1t}-PhytoPs, ent-16-F_{1t}- PhytoP, *ent*-9-D_{1t}- PhytoP, *ent*-9-epi- 9-D_{1t} have been identified 16-B_1-PhytoP, and 9-L_1-PhytoP. F_1-PhytoPs predominate and are identified in all almond cultivars. The L_1- PhytoPs are minor components, whereas the D_1-PhytoPs were only detected in the cultivars 'Colorada' and 'Avellanera' (Carrasco-Del Amor *et al*., 2015).

Yonny *et al.* (2016) studied PhytoPs and PhytoFs in leaves of melon plants with thermal stress. Their level of PhytoPs and PhytoFs in stressed plants was forms as significantly higher than in samples not sexpossed to stress. In addition, to strengthen the antioxidant defenses of

the melon plant, these authors tested foliar spraying using solutions of salicylic and gallic acid and extract of *Ilex paraguariensis*. In stressed samples treated with *I. paraguariensis*, the level of PhytoPs and PhytoFs appears significantly lower than in stressed samples without antioxidants.

The production of ROS through an oxidative burst has also been observed in plants as defense responses during pathogen infections (Bolwell, 1999). In this concern, *Toma et al.* (2003) demonstrated that several species of plants infected with the fungus *Botrytis cinerea* exhibited an increase in the levels of superoxide anions, hydrogen peroxide, final products of lipid peroxidation, such as aldehydes. These researchers observed that leaves infected with *B. cinerea* show up to increase of the level of all cyclic fatty acids.

Apart from the influence of the agroclimatic conditions in the level of plant oxylipins, **i**n many cases, thermal processing of food involves the use of techniques that produce chemical changes, such as oxidation. In this sense, the physicochemical conditions enclosed to the industrial production could be the key to understand the production and the level in final products of the different PhytoPs.

Carrasco-Del Amor *et al.* (2016) studied the composition and presence of PhytoPs in almonds in relation to packaging, temperature and storage and processing time. The most abundant PhytoPs belong to the F_{1t} series. PhytoPs levels increased significantly with storage time (3 and 6 months) and temperature. The total concentration of PhytoPs was higher in the air than in a vacuum-packed atmosphere. The processes of frying and roasting led to a strong reduction of the original concentration of most of the PhytoPs and promoted the synthesis of specific PhytoPs that are not detected in raw material. According to these data these compounds could be biomarkers of the degree of oxidative degradation during processing (Carrasco-Del Amor *et al.,* 2016).

Marhuenda *et al*. (2008) measured the content of PhytoPs in different stages of wine processing. Regarding this, the carbonic maceration must and aged must has lower concentrations of total PhytoPs than their respective finished wines. Indeed, the aging process of wine can be an important factor involved in the formation of PhytoPs. The data revealed two predominant classes of PhytoPs: The F_1 and D_1-PhytoPs series. Vinification and aging procedures for wine production seem to influence the final levels of PhytoPs in red wine and modify the profile of PhytoPs.

On the other hand, Collado-González *et al.* (2015d) studied the level of PhytoPs in oils with different processing. The results obtained reflect that the refined sunflower oil presents

more series of PhytoPs and an amount 20 and 8 times greater than two types of olive oil: Extra virgin and semi-refined olive oil. Again, the manufacturing process used could be the key for these different PhytoPs levels since most plant oils are subjected to a refining treatment.

1.4.2. Bioavailability and demonstrated functions of phytoprostanes and phytofurans, in biological systems: towards new promising bioactive compounds

Researchers have highlighted the importance of PhytoPs as bioactive lipid derivatives, not only in plant cells and tissues, but also in mammal's systems. It is believed that PhytoPs, derived from a non-enzymatic oxidation reaction, exert beneficial effects on the organism (Karg *et al.,* 2007; Minghetti *et al*., 2014). The most interesting aspect of PhytoPs is that they mimic the structure of other eicosanoids such as isoprostanes (IsoP) and prostanoids (prostaglandins and thromboxanes), compounds generated from arachidonic acid in mammals as a result of non-enzymatic reactions. In this regard, eicosanoids perform a wide range of biological activities in the human body, from pernicious actions (platelet aggregation or vasoconstriction) to beneficial effects related to the balance defense-physiology of the body. This fact could lead to the discovery of new properties of PhytoPs related to different physiological effects and disorders of human's body (Marhuenda *et al*., 2015).

In respect to bioavailability, metabolism, and biological effects of these compounds in humans, it must be taken into account that they can reach the gastrointestinal tract and interact with the intestinal microflora after food intake; which could give rise to addition compounds which distinct biological interest.

The importance of PhytoPs intake could also be related to neuroprotective effects. About this, Minghetti *et al.* (2014) demonstrated that B_1-PhytoPs were biologically active *in vitro* on immature cells of the central nervous system, exhibiting neuroprotective effects against oxidative injury induced by hydrogen peroxide, and promoting myelination through mechanisms that involve receptor activation. In addition, B_1-PhytoPs induced a moderate depolarization of mitochondrial inner membrane potential.

The bioavailability of PhytoPs has also been demonstrated *in vivo* with healthy humans. Barden *et al.* (2009) examined the effect of flax seed oil, which contains arachidonic acid; examined thirty-six non-smokers, 20 and 65 years old, consumed 9 g / day of flaxseed oil (62% ALA, 5.4 g / day) or olive oil (placebo) for 4 weeks. They observed the effect of a diet supplemented with flaxseed oil on the F_1-PhytoPs and F_2-IsoPs concentration in the urine and plasma of healthy men. Both plasma and urine analysis confirmed the absorption of PhytoPs by

the intestinal tract. The levels of esterified and non-esterified PhytoPs before the ingestion of flaxseed oil were higher in the plasma than in the urine. The higher plasma concentration of F_1-PhytoP in the flaxseed oil group probably resulted in an increase in plasma concentration of the ALA substrate and/or a higher content of PhytoPs from the flaxseed oil. In nutritional terms, additional studies would be necessary to know the physiological effects of PhytoPs in humans.

These PhytoPs can regulate inflammatory responses in dendritic cells. Karg *et al.* (2007) reported that phytoprostanes A_1 and B_1 inhibit the release of nitric oxide in RAW264.7 macrophages stimulated by lipopolysaccharides. Therefore, cardiovascular diseases could be improved by the effects of these compounds. In fact, Barden *et al*. (2009) related the intake of F_1-PhytoPs with the protective effects on the cardiovascular system.

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Objectives

CHAPTER II. OBJECTIVES

The rice production is a key worldwide agro-food production to meet the nutritional requirements of the growing human population. In this regard, the arable land is a more and more scarce resource due to the implantation of urban communities, as well as the degradation of soils suitable for agriculture. Besides, the models of planet air circulation predict an increase of the temperature affecting, fundamentally, to minimum temperatures. For these reasons, crops must to adapt to nutritional, hydric, saline, and/or thermal deficiencies.

Comprehensive studies on the mechanisms of plants to front these adverse situations have been carried out for decades. The results gathered from these studies suggest that tolerance to abiotic and biotic stress is a complex singularity involving multiple metabolic reactions connected to each other, synergistic and coordinated by the expression of gene pools.

The growing awareness of the population on the relevance of the nutritional, safety, and functional features of food motivatesadditional research towards obtaining new genetic resources that could be used as donors of traits contributing to high quality plant foods materials. In this regard, agronomic practices, such as fertilization and application of hormones could contribute to mitigate the damage caused by abiotic stress and deserve to be further explored to increase the productivity and quality in the short-term. In this frame, the present study pursues to shed some light on the relationship between yield and impact of agronomical practices by evaluating the evolution of the level of phytoprostanes and phytofurans in an array of rice genotypes.

2.1. OVERALL OBJECTIVES

The general objective of this work was to identify by first time, the occurrence and quantity of PhytoPs and PhytoFs in rice and the best agronomical and processing practices to achieve white rice, brown rice, and rice bran, as well as their flours, according to their profile and quantitative content of phytoprostanes and phytofurans, in diverse genotypes traditionally grown in Argentina. To fulfill this goal, the evolution of the level of these plant oxylipins was examined in rice crops treated with nitrogen, micronutrients, and salicylic acid by foliar fertilization in crops established in open field and under plastic covers.

In order to achieve this main objective, the following specific goals were also pursued:

- To determine the location, profile, and concentration, of phytoprostanes and phytofurans in an array of rice genotypes, by assessing their diverse presentation (white and brown rice, and rice bran) and the impact of producing rice flour on the level of plant oxylipins. Then, we will be able to identity the real utility of these compounds for the general dietary burden in humans.
- To study the effect of supplementation with nutrients and antioxidants in the mitigation of oxidative stress associated to rice crops monitored through the evaluation of the level of plant oxylipins;
- To identify differential responses of the diverse genetic materials resources to equal technological practices, regarding the level of phytoprostanes and phytofurans as markers of oxidative stress in plants;
- To study the existing relationships between the application of hormonal precursors (salicylic acid) by foliar fertilization and the level of phytoprostanes and phytofurans.

Results & Dicussion

CHAPTER III. Comparative study of the phytoprostanes and phytofurans content of *indica* and *japonica* rice's (*Oryza sativa* L.) flours

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3.1. INTRODUCTION

Plants and animals contain polyunsaturated fatty acids (PUFAs) in their membranes, which are co-responsible for modulating membrane fluidity, and can be released and metabolized enzymatically towards oxidized fatty acids, some of which act as defense signals in higher plants, *in vivo* (eg. jasmonates or antimicrobial lipids) (Muller, 2004). In addition to enzymatic oxidation, plant membranes (especially rich in linolenate) are prone to non-enzymatic oxidation that gives rise to phytoprostanes (PhytoPs). These compounds represent a broad range of biologically active oxidized lipids that are generated as a consequence of redox unbalance and oxidative stress.The discovery of PhytoPs has focused the interest of the research community because of their structural analogy regarding well established animal mediators of biological reactions such as isoprostanes (IsoPs) and prostanoids (prostaglandins and thromboxanes), also generated by non-enzymatic free radical attack and cyclooxygenase action on arachidonic acid, respectively (Collado-González *et al*., 2015).

The occurrence of PhytoPs and phytofurans (PhytoFs) in plant foods have been reported in the last two decades concerning variety of matrices including vegetable oils, seeds, and non-edible plant materials such as melon leaves (Carrasco-Del Amor *et al*., 2016; Yonny *et al*., 2016). These characterizations have provided important information on those matrices that seem to be the most relevant dietary source of such compounds, being their concentration related to the level of α-linolenic acid (C18:3 n-3, ALA). Besides of the dietary interest of these foods and foodstuffs as sources of PhytoPs and PhytoFs, the level of these compounds has been related with the plants response to abiotic stress (Collado-González *et al*., 2015).

Mammalians oxylipins, eicosanoids among others, are accepted to perform a wide range of functions in biological systems, from pernicious effects (platelet aggregation and vasoconstriction) to benefits related to the maintenance of the physiological-defensive balance (Patterson *et al*., 2012). Mirroring these effects in human cells, PhytoPs have been related to anti-inflammatory properties, and protection against neuronal lesions induced by pro-oxidant radicals (Minghetti *et al*., 2014). Apart from PhytoPs, under high oxygen tension another cyclic

structure of compounds referred as PhytoFs is generated upon additional oxidation after the initial endoperoxyde formation (Cuyamendous *et al*., 2015).

Thus, though to date the biological benefits associated to the consumption of plant foods have been attributed to the wide variety of compounds described in such matrices, the newly description of PhytoPs and PhytoFs allows to envisage that, at least in some extent, these compounds could contribute to the healthy attributions of plant foods. In this sense, further research on bioavailability and biological activity of PhytoPs and PhytoFs *in vivo* is currently in course (Leung *et al*., 2014), complementing the current trend on the evaluation of the value of PhytoPs and PhytoFs as markers of oxidation in foods (Carrasco-Del Amor *et al*., 2016). In this sense, to establish the actual interest of these compounds, firstly it should be established rationally their occurrence in the wide variety of foods integrating humans' diet.

Despite the interest of these molecules as potential bioactive compounds contributing to the healthy attributions of plant foods, to date there are not enough evidences on their bioavailability (Barden *et al*., 2009; Dupuy *et al*., 2016), and even less of the scope of their biological power, which has been mainly addressed to their capacity to polarize the immune response to a Th2 type (Tradidl-Hoffmann *et al*., 2005).

However, almost 20 years later of the first description (Parchmann *et al*., 1998), the scientific literature on PhytoPs and PhytoFs has been mainly focused on the evaluation of their occurrence in edible and non-edible higher plant species and manufactured products (Imbusch and Mueller, 2000; Thoma *et al*., 2004; Collado-González *et al*., 2015; Yonny *et al*., 2016). In this frame, rice (*Oryza sativa* L.) is one of the most important food crops around the world, being is the staple food for more than half of the world's population (Sasaki *et al*., 2000). *Indica* and *japonica* rice are the two main subspecies grown in tropical/subtropical environments and under mild climatic conditions, respectively. These subspecies differ clearly upon morphological, agronomic, physiological, and biochemical features, as well as in yield, quality, and resistance to stress (Yang *et al*., 2014). In addition, *indica* and *japonica* subspecies also diverge regarding their content of α-linolenic acid, precursor of PhytoPs and PhytoFs, which influences the distinct concentration of such compounds in different plant foods and foodstuffs (Imbusch and Mueller, 2000).

The rice plant is threshed to produce paddy rice, which is defatted to obtain brown rice. Then, brown rice is separated by a machine polished in bran (external parts of the grain including pericarp, tegmen, aleurone layer, and embryo) and polished rice (milled rice or white rice) that involves the internal parts of the grain, corresponding substantially to endosperm. In these

matrices, lipids are mainly concentrated in the series of rice bran (22.5%), brown rice (2.3%), and white rice (0.8%) (Juliano, 1983).

To date, their value as indicators of the quality of rice based foods and foodstuffs given their conditions of metabolites of oxidation of fatty acids has been already demonstrated; however, to gain a further insight on the biological interest of these compounds as markers in vitro as mediators of metabolic pathways that could be of interest for health requires of accurate determinations of their occurrence in plant foods. Hence, the objective of the present work was to assess, for the first time, different types of rice flours obtained from white and brown grains, and diverse rice bran of different *indica* and *japonica* cultivars on their concentration of PhytoPs and PhytoFs by UHPLC-ESI-QqQ-MS/MS. The value of these matrices as a dietary source of these largely unknown oxylipins need to be addressed in order to plan rationally nutritional trials devoted to shed some light on their actual biological activity.

3.2. MATERIALS AND METHODS

3.2.1. Chemical and reagents

The PhytoPs9-F_{1t}-PhytoP (PP1);*ent*-16-F_{1t}-PhytoP + *ent*-16-*epi*-16-F_{1t}-PhytoP (PP2); 9-*epi*-9-F_{1t}-PhytoP (PP3); *ent*-9-D_{1t}-PhytoP (PP4); *ent*-9-*epi*-9-D_{1t}-PhytoP (PP5); *ent*-16-B_1-PhytoP + 16-B_1-PhytoP (PP6); *ent*-9-L_1-PhytoP + 9-L_1-PhytoP (PP7); and the PhytoFs *ent*-16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF (PF1); *ent*-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF (PF2); *ent*-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF (PF3)(cited by elution order) (**Table 8**), were synthesized according to our previously published procedures (El Fangour *et al*., 2004; 2005; Cuyamendous *et al*., 2015), and provided by the *Institut des Biomolécules Max Mousseron* (IBMM) (Montpellier, France).

Hexane was obtained from Panreac (Castellar del Vallés, Barcelona, Spain), butylated hydroxyanisole (BHA) 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 from J.T. Baker (Phillipsburg, NJ, USA). Water was treated in a milli-Q water purification system from Millipore (Bedford, MA, USA). The SPE cartridges used was Strata cartridge (Strata X-AW, 100 mg/3 mL), which was acquired from Phenomenex (Torrance, CA, USA).

Table 8. Selected reaction monitoring of the phytoprostanes and phytofurans present in rice (*Oryza sativa* L.) flours

Code	Compound	Rt (min) [Z]	Precursor ion (*m/z*)	Product ion (*m/z*)	F (V)	CE (V)
Phytoprostanes						
PP1	9-F_{1t}-PhytoP	1.943	327.2	171.2	80	0
PP2	*Ent*-16-F_{1t}-PhytoP+ *Ent*-16-*epi*-16-F_{1t}-PhytoP	2.088	327.2	251.2	80	0
PP3	9-*epi*-9-F_{1t}-PhytoP	2.144	327.2	171.2	80	0
PP4	*Ent*-9-*epi*-9-D_{1t}-PhytoP	2.151	325.2	307.2	80	0
PP5	*Ent*-9-D_{1t}-PhytoP	2.510	325.2	307.2	80	0
PP6	16-B_1-PhytoP	3.224	307.2	235.2	70	0
PP7	9-L_1-PhytoP	3.481	307.2	185.2	70	
Phytofurans						
PF1	*Ent*-16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF	1.722	343.4	209.2	90	20
PF2	*Ent*-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF	1.711	343.3	237.4	115	25
PF3	*Ent*-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF	1.725	342.9	201.2	100	30

[Z]Rt, retention time; F, fragmentor; CE, collision energy.

3.2.2. Samples and standards preparation

Samples consisted in grain of 14 rice (*Oryza sativa* L.) cultivars, which were grown in Mid-East Argentina, in the Experimental Station 'Julio Hirschhorn', de La Plata (34°52'S, 57°57'W, 9.8m of altitude, Buenos Aires, Argentina). Climatic data were recorded during the growing season (October-2015/April-2016) including minimum and maximum temperatures (°C), relative humidity (%), and monthly rainfall (**Figure 12**).

The panel of rice cultivars included eight (8) of *indica* subspecies ('Cambá', 'Don Ignacio FCAyF', 'Don Justo FCAyF', 'El Paso 144', 'Guri INTA', 'IRGA 424', 'Nutriar FCAyF', and 'Puitá') and six(6)of *japonica* subspecies ('Amaroo', 'Arborio', 'Itapé', 'Quebracho FA', 'Yamani', and 'Yerua FA'). Seeds were harvested in dry land, manually at a rate of 350 seeds m^{-2} in lines at 0.20 m, in plots of 5 m^2. The trial was conducted with flood irrigation from the 30 days of the emergency. Harvesting and threshing were done manually; the grains were dried in an oven at 40 ± 1°C for 72 hours. Polishing was performed at room temperature from 100 grams of paddy rice.Husk and bran layers were removed using an experimental mill (Guidetti and Artioli Universal Type, Renazzo, Italy). Milling of rice materials was done at low speeds and during this process temperature was continuously monitored, avoiding augments higher than 1 °C, relatively to the initial conditions of the raw materials. Ground rice flour was obtained in a 0.4 mm mesh Cyclone Mill. Rice flour samples were stored at 4 °C, protected from light till analytical assessment.

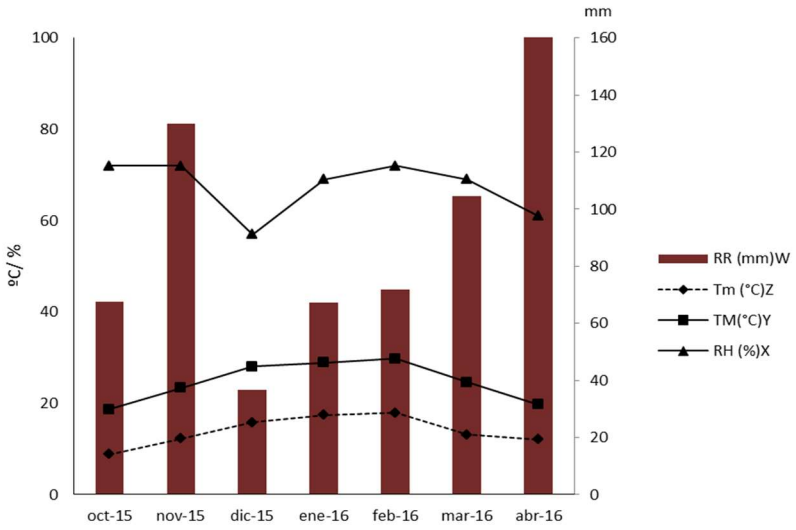

Figure 12. Climatic conditions in the experimental field (34˚52'S, 57˚57'W, 9.8 m of altitude) during the rice crop. [Z]Mean daily minimum air temperature; [Y]Mean daily maximum air temperature; Relative humidity; [W]Total monthly rainfall.

Stock solutions of PhytoPs and PhytoFs were prepared in methanol/water (50:50, v/v) to facilitate the ionization in the mass spectrometer at a concentration of 1000 nM for each compound and stored in Eppendorf tubes at -80°C. Five successive dilutions ¼ were freshly prepared each day of analysis to obtain standard solutions at the following concentrations: 250, 63, 16, 4, and 2nM, which were used for obtaining the calibration curves.

3.2.3. Extraction of phytoprostanes and phytofurans

The PhytoPs and PhytoFs present in rice flours were extracted following the methodology described by Leung *et al.* (2014) and Yonny *et al.* (2016) with minor modifications (Leung *et al.*, 2014; Yonny *et al.*, 2016). Shortly, samples (4 g) were crushed in a mortar and pestle with 10 mL of methanol (0.1 % BHA). The samples extracts were centrifuged at 2000g during 10 min, and supernatants were further processed upon solid phase extraction (SPE) using a Chromabond C_{18} column according to the procedure previously described (Collado-González *et al.*, 2015). Target compounds were eluted with 1 mL of methanol and dried using a SpeedVac concentrator (Savant SPD121P, Thermo Scientific, MA, USA). The dry extracts were reconstituted with 200 microliters of solvent A/solvent B (50:50, *v*/*v*), being solvent AmilliQ-water/acetic acid (99.99:0.01, *v*/*v*) and solvent B methanol/acetic acid (99.99:0.01, *v*/v). Reconstituted samples

were filtered through a 0.45-µm filter (Millipore, MA, USA) and immediately analyzed by UHPLC-ESI-QqQ-MS/MS (Agilent Technologies, Waldbronn, Germany).

3.2.4. UHPLC-ESI-QqQ-MS/MS analysis

Chromatographic separation of PhytoPs and PhytoFs was performed using a UHPLC coupled with a 6460 triple quadrupole-MS/MS (Agilent Technologies, Waldbronn, Germany), using the analytical column BEH C_{18} (2.1 x 50 mm, 1.7 µm) (Waters, Milford, M.A.). The column temperatures were 6 °C (both left and right). The mobile phases consisted of milliQ-water/acetic acid (99.99:0.01, *v*/*v*) (A) and methanol/acetic acid (99.99:0.01, *v*/*v*) (B). The injection volume and flow rate were 20 µL and 0.2 mL min^{-1} upon 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 for column equilibration. The spectrometric analysis was conducted in Multiple Reaction Monitoring mode (MRM) operated in negative mode, assigning preferential MRM transition for the corresponding analytes. The ionization and fragmentation conditions were: 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 product ions. Data acquisition and processing were performed using Mass Hunter software version B.04.00 (Agilent Technologies).

The quantification of PhytoPs and PhytoFs detected in rice grain and flour was performed using authentic standards according to standard curves freshly prepared as mentioned in the previous section. The selected reaction monitoring and chemical names were according to the nomenclature system of Taber *et al.* (1997) and are referred in **Table 8** (Taber *et al*., 1997).

3.2.5. Statistical analysis

All assays were developed in triplicate (n=3) and values were reported as means. To determine the effect of the type of flouron target compounds, One-Way analysis of variance (ANOVA)and multiple range test (Tukey's test) were carried out using Statgraphics Centurion XVI (StatPoint Technologies Inc., Warrenton, VA, USA). Significant differences were set at $p<0.05$. A Pearson's correlation analysis was developed to corroborate the relationship between both groups of compounds (PhytoPs and PhytoFs), concerning both individual and total compounds. The significance of the correlations found was set at $p<0.05$.

3.3. RESULTS AND DISCUSSION

For the evaluation of rice flours on the concentration of PhytoPs and PhytoFs, crops were set up in an experimental field of the Buenos Aires province, in Mid-East Argentina, and included 8 *indica* varieties ('Cambá', 'Don Ignacio FCAyF', 'Don Justo FCAyF', 'El Paso 144', 'Guri INTA', 'IRGA 424', 'Nutriar FCAyF', and 'Puitá') and 6 *japonica* cultivars ('Amaroo', 'Arborio', 'Itapé', 'Quebracho FA', 'Yamani', and 'Yerua FA'). The crops were developed under the climatic conditions specified in **Figure 12**, whichlead to the normal development of the rice crops although, given the role of PhytoPs and PhytoFs in stress response, contributed to define the final levels of the target compounds in the plant material.

3.3.1. Occurrence of phytoprostanes and phytofurans in rice flours

When analyzing the content of total PhytoPs and PhytoFs of rice flour obtained from grains of 14 rice cultivars by monitoring the relationship of the cultivar and the type of flour, it was found that total PhytoPs concentration was significantly higher in white and brown flours obtained from *japonica* varieties (12.23 and 16.14 ng g^{-1} dw, respectively) than in those of *indica* (a 36.7 and 6.1% lower, respectively). This trend was reverse when considering rice bran, which content in PhytoPs was 2.4% higher, on average, in plant material from *indica* subspecies than in those corresponding to *japonica* (**Table 9**).

The comparison of individual varieties concerning their content in total PhytoPs, noticed that, regarding white grain flour, the *indica* cultivar 'Guri INTA' and the *japonica* varieties 'Arborio' and 'Quebracho FA' presented the highest concentrations at average values around 22.02 ng g^{-1} dw (**Table 9**).

In a second group, the cultivars 'Camba' (*indica*), 'Itapé' (*japonica*), and 'Yamani' (*japonica*) were stressed with values ranging from 9.14 to 9.98 ng g^{-1} dw (a 66.0% lower than the formers, on average). Finally, the lowest concentration of total PhytoPs corresponded to the varieties 'IRGA 424' (*indica*) = 'Puitá' (*indica*) = 'Yerua FA' (*japonica*) (6.76 ng g^{-1} dw, on average) >'Nutriar FCAyF' (*indica*) = 'El Paso 144' (*indica*) = 'Amaroo' (*japonica*) (4.78 ng g^{-1} dw, on average) > 'Don Justo FCAyF' (*indica*) = 'Don Ignacio FCAyF' (*indica*), (2.80 ng g^{-1} dw, on average) (**Table 9**).

Table 9. Content of total phytoprostanes and phytofurans (both at ng g^{-1} dw) in three types of rice (*Oryza sativa* L.) grain flour genotypes evaluated belonging to *indica* and *japonica* subspecies.

Genotype	Total PhytoPs				Total PhytoFs			
	White grain flour	Brown grain flour	Rice bran	LSD	White grain flour	Brown grain flour	Rice bran	LSD
Oryza sativa L. subspecie *indica*								
'Camba'	9.98 b B[Z]	22.03 bc A	22.20 e A	1.18	0.89 c B	5.71 bc A	3.05 e B	0.57
'Don Ignacio FCAyF'	2.55 f B	20.38 bcd AB	74.75 bc A	1.86	0.27 ef B	5.41 bcd AB	8.97 cde A	0.24
'Don Justo FCAyF'	3.04 f B	22.53 b AB	39.58 cde A	7.74	0.36 e B	4.35 bcde A	1.59 e B	0.30
'El Paso 144'	4.83 ef B	16.39 de AB	118.00 a A	1.31	0.77 cd C	3.09 def B	27.74 a A	0.45
'Guri INTA'	22.47 a B	14.24 ef B	106.67 ab A	1.42	1.60 ab B	1.96 efg B	22.51 ab A	0.60
'IRGA 424'	7.35 cd B	10.14 f B	81.27 abc A	0.81	0.40 e B	1.41 fg B	15.39 bc A	0.53
'NutriarFCAyF'	4.94 ef B	20.75 bcd B	68.88 bcd A	0.01	0.41 e C	19.49 a A	7.98 cde B	2.36
'Puitá'	6.78 cd B	17.46 cde B	76.16 abc A	0.66	0.31 ef B	2.82 ef B	14.65 bcd A	2.93
Oryza sativa L. subspecie *japonica*								
'Amaroo'	4.58 ef B	13.32 ef B	60.22cde A	5.86	0.41e A	1.36fg A	7.47cde A	2.03
'Arborio'	21.86a B	9.51f C	65.65bcd A	0.91	1.79a B	0.00 g C	8.66cde A	0.21
'Itapé'	9.14 b C	32.89 a B	67.09 bcd A	1.24	1.45 b C	6.72 b B	15.08bc A	0.48
'Quebracho FA'	21.74a B	24.83b B	54.91 cde A	5.69	0.06e B	3.91cde AB	6.16cde A	1.24
'Yamani'	9.96 b B	21.65 bc AB	54.32 cde A	8.41	0.48 de B	4.00cde A	6.02cde A	0.81
'Yerua FA'	6.15 de B	12.65 ef AB	31.04 de A	0.52	0.35ef B	1.49fg B	3.79de A	0.34
LSD(p<0.05)	2.45	4.81	41.84		0.29	2.41	11.02	
Subespecie *indica*	7.79 B	17.99 B	73.44 A	3.90	0.63 C	5.53 B	12.74 A	1.29
Subespecie *japonica*	12.30 B	19.14 B	55.54 A	2.79	2.91 B	2.91 B	7.86 A	0.70
LSD (p<0.05)	1.35	1.31	5.49		0.11	0.93	1.57	
p-value	*** [Y]	N.s.	***		N.s.	N.s.	***	

[Z] Means (n=3) within a column followed by distinct lowercase letters (cultivar) or within a row (type of flour) are significantly different at p<0.001 according to Tukey's multiple range test. [Y] In the bottom of the table means of *indica* and *japonica* subspecies were compared resorting to paired t-test, significant differences were stated at p<0.001(***) and N.s., no significant.

In which respect to brown grain flours, the relative abundance of total PhytoPs in *indica* and *japonica* varieties evidenced that the concentration decreases as follows: 'Itapé' (*japonica*) (32.89 ng g^{-1} dw) > 'Quebracho FA' (*japonica*) = 'Don Justo FCAyF' (*indica*) = 'Camba (*indica*) = 'Yamani' (*japonica*) = 'NutriarFCAyF' (*indica*) = 'Don Ignacio FCAyF' (*indica*) (22.03 ng g^{-1} dw, on average) >'Puitá' (*indica*) = 'El Paso 144' (*indica*) = 'Guri INTA' (*indica*) = 'Amaroo' (*japonica*) = 'Yerua FA' (*japonica*)> 'IRGA 424' (*indica*) = 'Arborio' (*japonica*) (13.39 ng g^{-1} dw, on average) (**Table 9**).

Apart from the flour obtained from white and brown rice grains, rice bran that is constituted by the hard outer layers of grain, consisting of the combined aleurone and pericarp, exhibited a reverse trend for total PhytoPs. In this case, the highest concentration corresponded to the *indica* varieties 'El Paso 144' (118.00 ng g^{-1} dw) and 'Guri INTA' (106.67 ng g^{-1} dw), which

surpassed the levels observed in 'IRGA 424', 'Puitá', 'Don Ignacio FCAyF', 'Nutriar FCAyF', 'Itapé', 'Arborio', and 'Amaroo' by 34.0%, on average. The lowest concentration corresponded to the cultivars 'Quebracho FA', 'Arborio', 'Don Justo FCAyF', 'Yerua FA', and 'Camba' (22.20-54.91 ng g^{-1} dw, on average) (**Table 9**).

To the best of our knowledge, there is no previous description on the content of PhytoPs in rice flours before this study; however, some works have characterized other vegetable matrices, concerning the concentrations of these compounds, and showing 15.00-39.00 ng mL^{-1} (12.00-31.00 ng g^{-1}aprox) (Cuyamendous *et al*., 2015), 0.01-0.06 ng g^{-1} dw in algae (Barbosa *et al*., 2015), 5.81-9.99 ng g^{-1} in raw green table olive fruits (Collado-González, *et al*., 2014), and 40.25-238.09 ng g^{-1} in almonds (Carrasco –Del Amor *et al*., 2015). To date, the highest concentration of total PhytoPs was observed in fresh birch pollen with values of up to 1380.00 ng g^{-1} (Imbusch and Mueller, 2000),and in dry leaves of melon plants with a maximum values of 1600 ng g^{-1} dw (Yonny *et al*., 2016).Their level in almonds and sunflower oil is similar possibly because of its high lipid content with an average of 56.0% of the dry matter.Hence, when comparing the data retrieved from the present work with those in the literature, it is evidenced a higher amount of PhytoPs in rice flour than in algae, green olives, and olive oil, but minor than in almonds and melon leaves.

In addition to PhytoPs, recently has been describing another series of plant oxylipins, PhytoFs. These compounds are oxylipins sharing chemicals structure analogisms with PhytoPs generated favorably than PhytoPs by non-enzymatic oxidative reactions, but under higher oxygen pressure than PhytoPs.[25]The assessment of rice flours on PhytoFs noticed that the preponderance of *indica* and *japonica* subspecies on these compounds is closely dependent on the type of flour (**Table 9**). *Japonica* cultivars presented the highest concentration of total PhytoFs concerning white grain flour (2.91 ng g^{-1}dw), while *indica* subspecies were stressed as the one with the highest contents of PhytoFs regarding brown grain and rice bran (5.53 and 12.74 ng g^{-1} dw, respectively). In addition, the concentration of PhytoFs seems to be lower than the level of PhytoPs that has been attributed to the specific physico-chemical features of the panel of foods and foodstuffs evaluated, which account for very low water content (Albena *et al*., 2004), and the occurrence of lower oxidation conditions than the required for the synthesis of PhytoFs (Cuyamendous *et al*., 2017).

In addition to differences concerning the content of total PhytoFs between *indica* and *japonica* subspecies of rice, the evaluation of individual cultivars evidenced that higher concentrations of PhytoFs of rice grain flours are close dependent on the type of rice-derived plant material considered (white or brown grains or rice bran). In this sense, regarding white

grain flour, the highest concentrations corresponded to the *indica* cultivar 'Guri INTA', as well as the *japonica* varieties 'Arborio' and 'Itapé' (1.61 ng g^{-1} dw, on average), followed by 'Camba' and 'El Paso 144' (both belonging to the *indica* subspecies) (0.83 ng g^{-1} dw). On the opposite, the remaining cultivars were featured by similarly lower values ranging from 0.06 to 0.48 ng g^{-1}dw (**Table 9**).

When considering brown grain flour, the highest concentration of total PhytoFs were found in the cultivar 'Nutriar FCAyF' that displayed values of 19.49 ng g^{-1} dw, surpassing the following batch of samples formed by flour obtained of the *indica* cultivars 'Camba', 'Don Ignacio FCAyF', 'Don Justo FCAyF', and 'El Paso 144' and the *japonica* varieties 'Itapé', 'Quebracho FA', and 'Yamani' by 75.7%, on average. The lowest concentration of total PhytoFs corresponded to the cultivars 'Guri INTA', 'IRGA 424', 'Puitá', 'Amaroo', 'Arborio', and 'Yerua FA' (1.11 ng g^{-1} dw, on average) (**Table 9**).

Finally, in which respect to the concentration of PhytoFs in rice bran, the highest values wereobserved in the *indica* varieties 'El Paso 144' and 'Guri INTA' (25.13 ng g^{-1} dw, on average), followed by the also *indica* cultivars 'IRGA 424' and 'Puitá', as well as the *japonica* cultivar 'Itapé', which presented concentrations a 40.2% lower than the former, on average (**Table 9**).

To date, there are limited information on the occurrence of PhytoFs in plant materials and foods, concerning both profile and quantitative levels (Cuyamendous *et al*., 2017), which difficult to understand the actual scope of these compounds regarding their physiological role in plants, as well as to design experimental procedures addressed to retrieve mechanistic information on the biological interest of such bioactive molecules once ingested. Nonetheless, the comparison of results on the concentration of total PhytoFs in rice flours (0.06-27.74 ng g^{-1} dw) with data available in the literature allows to notice concentrations consistent with the levels reported in pine, walnuts, chia, and flax (0.30, 9.00, 6.00, and 0.70 ng g^{-1}, on average, respectively) (Cuyamendous *et al*., 2016), remaining in much lower concentrations than, for instance, melon leaves (130-4400 ng g^{-1}) (Yonny *et al*., 2016).

The higher concentration of PhytoPs and PhytoFs in whole grain and rice branin comparison with white and brown rice flours in addition to indicate the most adequate dietary source of these compounds (concerning rice) have a side consequence. In addition to be better sources of PhyoPs and PhytoFs, whole grain and rice bran can exert beneficial effects upon the gastrointestinal tract given their contribution to the proportion of dietary fiber. In fact, this specific composition (dietary fiber) could be related with the proper intestinal absorption of PhytoPs and PhytoFs (Brown *et al*., 2010). Although the fiber content is low in rice bran, it has

high oil content with an unusual high concentration of unsaponifiable healthy fatty acids that also are responsible for hypolipidemic effects (4.2%) (Seetharamaiah *et al.*, 1989). Hence, soluble fiber acquires gel-like consistency and helps to control glycaemia and excessive plasma cholesterol, which entails multiple metabolicconsequences.

3.3.2. Influence of rice flour type on the content of total phytoprostanes and phytofurans

As a general trend, when comparing the concentration of PhytoPs and PhytoFs in rice flours of diverse origins at different processing levels, it was noticed that the highest concentration corresponded to bran (22.20-106.67 and 1.59-27.74 ng g^{-1} dw of PhytoPs and PhytoFs, respectively), followed by brown grain flour (a 10.14-22.53 and 9.51-24.83 ng g^{-1} dw of PhytoPs and PhytoFs, respectively), whilst white grain flour was the one presenting the lowest levels (2.55-22.47 and 0.06-1.79 ng g^{-1} dw of PhytoPs and PhytoFs, respectively) (**Table 9**). The decreasing concentration of the target molecules recorded upon the present work (bran > brown grain flour > white grain flour) is in agreement with their content in polyunsaturated fatty acids (PUFAs) in general and specifically regarding ALA, according to previous descriptions available in the literature that describe the preponderant concentration of lipids in rice bran (22.5%), relatively to whole rice (2.3%) and polished rice (0.8%) (Juliano, 1983).

It is important to stress that lipids in the endosperm are present in different forms relatively to bran and germ. In addition, based on the association with starch (stabilized with Van der Waals contacts), rice lipids are often classified as starch and non-starch lipids (Morrison, 1995). Hence, non-starch lipids are found mainly in the lipid bodies of rice bran (aleurone layer) and germ (embryo), while starch lipids are associated with starch granules in the rice endosperm. This indicates that rice phospholipids may have significant impacts on traditional (storage, cooking, and food) and modern (glycemic index) concepts related to rice quality. Besides, although this fact has been broadly reported in the literature, much of the information available on the occurrence and distribution of phospholipids in rice, their effects on storage and food quality, and the implications for human health, is scattered with little consensus (Lui *et al.*, 2013). Hence, given the close relationship between the occurrence of PUFAs and PhytoPs/PhytoFs, in the next years research efforts should be focused on retrieving the exact connection between the phospholipids' distribution and the level of PhytoPs and PhytoFs, as well as their contribution to rice quality and eventually human health in the extent in which new data on bioavailability and pharmacokinetics become available.

3.3.3. Influence of rice flour type on the content of individual phytoprostanes and phytofurans

From a thoughtful analysis of the results obtained it is noticed that the relative occurrence of individual PhytoPs was dependent on the specific compounds evaluated, although as a general trend the highest concentration was observed in rice bran, followed by brown grain flour and white grain flour (**Figures 13** and **14**).

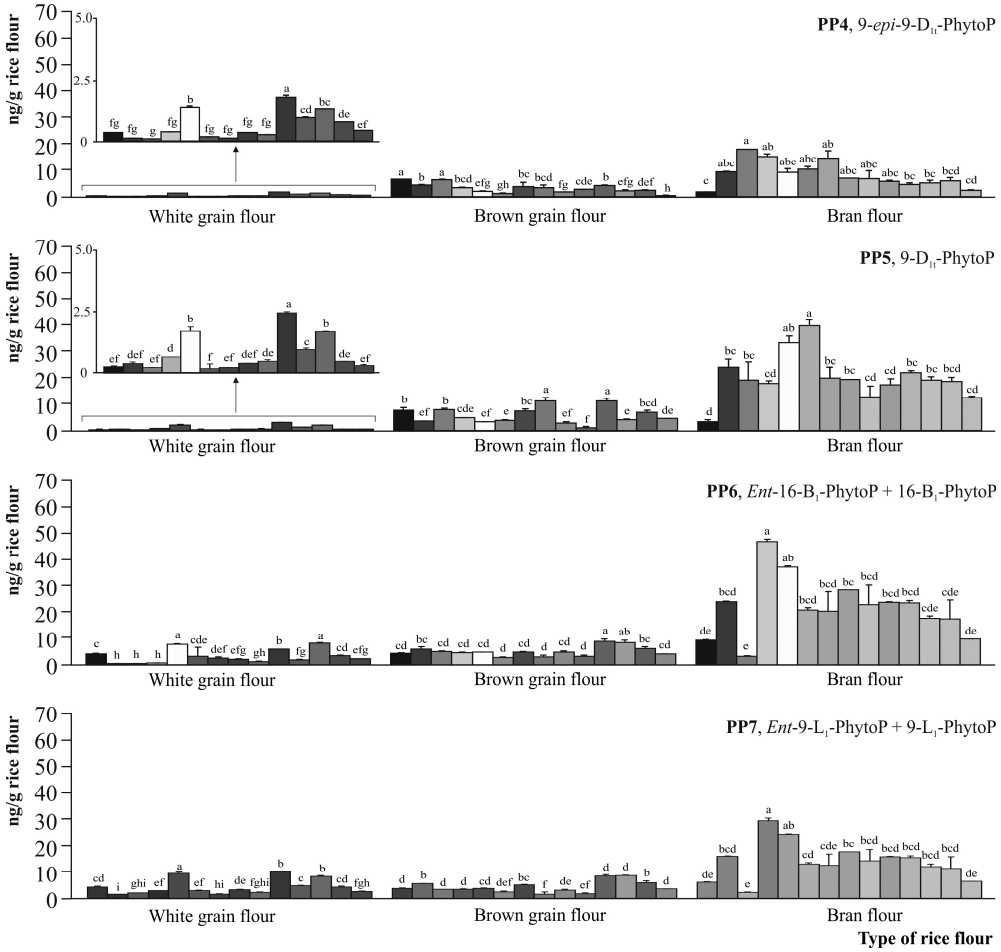

Figure 13. Comparison between rice subspecies and types of flour regarding individual phytoprostanes *Ent*-9-D_{1t}-PhytoP (PP4), *ent*-9-*epi*-9-D_{1t}-PhytoP (PP5), *ent*-16-B_1-PhytoP + 16-B_1-PhytoP (PP6), and *ent*-9-L_1-PhytoP + 9-L_1-PhytoP (PP7). Different lowercase letters indicate significant differences between varieties for the same flour type at $p<0.05$, according to the analysis of variance (ANOVA) and the multiple range test of Tukey.

This fact seems to be due to the higher exposition of bran (constituted by pericarp, seed coat, micelles, and aleurone layer to external aggressions, which enhance reactive oxygen species (ROS) production in the different compartments and thus, an increased formation of an array of lipid peroxidation products including PhytoPs and PhytoFs (Thoma *et al*., 2003; Cuyamendous *et al*., 2015).

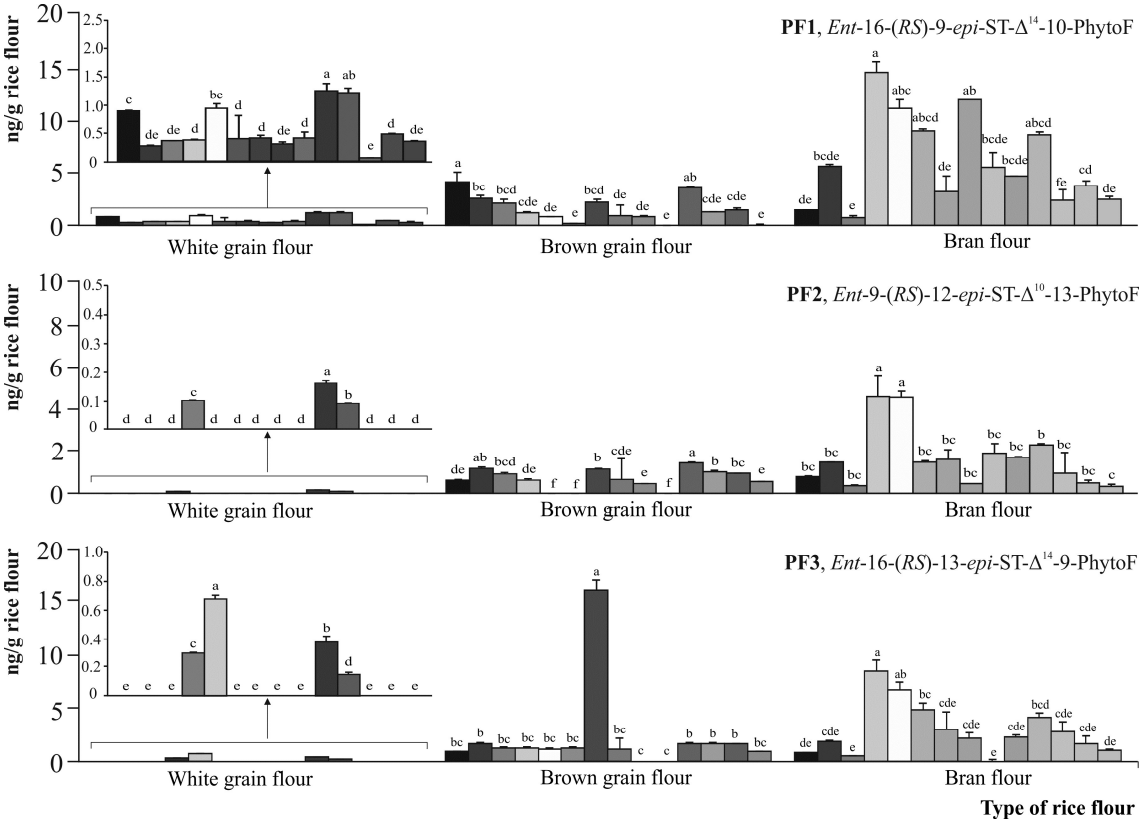

Figure 14. Comparison between rice subspecies and types of flour regarding individual phytofurans *ent*-16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF (PF1), *ent*-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF (PF2), and *ent*-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF (PF3). Different lowercase letters indicate significant differeces between varieties for the same flour type at $p<0.05$, according to the analysis of variance (ANOVA) and the multiple range test of Tukey

In addition, it has been found significant differences between cultivars belonging to both *indica* and *japonica* subspecies. The comparison of these two subspecies evidenced that individual PhytoPs in white grain flour were significantly higher in materials from *japonica* relatively to those belonging to *indica* subspecies (a 44.8% lower, on average), while in rice bran the trend was the opposite (23.3% lower in *japonica* cultivars than in the *indica* ones, on average). In which respect to brown grain flour, no differences were observed between both subspecies (**Figure 13**). When evaluating the distinct values of *indica* and *japonica* cultivars concerning their concentration of individual PhytoFs, it was observed concentrations a 38.2% higher in bran of *indica* subspecies than in those of *japonica* origin (**Figure 14**).

Among the individual PhytoPs and PhytoFs identified in the plant material evaluated in the present work (7 and 3, respectively), it was observed the presence of the PhytoPs *ent*-9-*epi*-9-D_{1t}-PhytoP, *ent*-9-D_{1t}-PhytoP, 16-B_1-PhytoP, and 9-L_1-PhytoP and the PhytoFs e*nt*-16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF, e*nt*-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF, and e*nt*-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF (**Table 8**, and **Figures 13** and **14**). When evaluating the concentration of such individual

compounds, regarding PhytoPs, it was stressed the preponderant occurrence of 16-B_1-PhytoP (52.79 ng g^{-1} dw, on average, in rice bran of the *indica* subspecies, var. 'El Paso 144' and 'Guri INTA'), *ent*-9-D_{1t}-PhytoP (28.98 ng g^{-1} dw, on average, in rice bran of the *indica* subspecies, var. 'Guri INTA' and 'IRGA 424'), and 9-L_1-PhytoP (27.04 ng g^{-1}, on average, in rice bran of the *indica* subspecies, var. 'El Paso 144' and 'Guri INTA'). The comparison of these concentrations with those in white and brown grain flours did not inform on significant differences between varieties, whilst all them displayed values much lower than rice bran (**Figure 13**). Given the lack of previous descriptions on the concentration of PhytoPs in rice and its derivatives, the development of a proper rational discussion should be done on additional food and foodstuffs matrices. So, these results are in agreement (in terms of concentration ranges) with the values reported previously in extra virgin oil, olive oil, and refined sunflower oil (Pinot *et al*.,2008). However, regarding the relative contribution of each individual compound, the profile of PhytoPs was discrepant relatively to vegetable oils and other plant matrices such almonds, melon leaves, algae, must, and wines, which were featured by the predominance of F_1-PhytoPs (Collado-González *et al*., 2015; Barbosa *et al*., 2015; Marhuenda *et al*., 2015; Yonny *et al*., 2016). The gap of proper matching on the PhytoPs profile of rice flours with previous descriptions available in the literature might be due to the specific physical features of the diverse plant matrices under comparison (moisture and fatty acids profile), but also by the differential processing procedures that may condition the transformation of PUFAs into this and other PhytoPs upon non-enzymatic oxidation reactions (Collado-González *et al.,* 2015). Given these differences, the rational attribution to these foods and foodstuffs of biological benefits based on the content of PhytoPs needs to be further demonstrated by studies devoted of the assessment of the molecular pathways affected in which these compounds could be involved.

Besides PhytoPs, upon non enzymatic reactions PUFAs can give rise to PhytoFs as a consequence of additional oxygenation after the initial endoperoxyde formation. These compounds, which assessment in plant material is in its infancy (Cuyamendous *et al*., 2015), were almost absent in white grain flour, especially concerning e*nt*-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF, and e*nt*-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF, while e*nt*-16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF was found in values ranging from 0.06 to 1.25 ng g^{-1} dw. The highest concentration of e*nt*-16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF in white rice flour corresponded to the *japonica* subspecies var. 'Arborio' and 'Itapé' that exhibited 1.23 ng g^{-1} dw, on average (**Figure 14**).

The evaluation of the concentration of PhytoFs in brown rice flour informed on no large differences with values within the range 0.00-4.21, 0.00-1.45, and 0.00-16.06 ng g^{-1} dw for e*nt*-16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF, e*nt*-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF, and e*nt*-16-(*RS*)-13-*epi*-ST-

Δ^{14}-9-PhytoF, respectively (**Figure 14**), without a consistent trend on the preponderance of any of the *indica* and *japonica* cultivars under study.

Finally, regarding the occurrence of PhytoFs in rice bran, as abovementioned it was observed a preponderance of the *indica* varieties in comparison with the *japonica* ones (38.2% lower on average for the three PhytoFs identified). In addition, in this flour type the highest concentrations of e*nt*-16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF, e*nt*-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF, and e*nt*-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoFcorresponded consistently to the varieties 'Quebracho FA', and 'Yamani', which were featured by the average concentrations 12.88, 4.62, and 7.63 ng g^{-1} dw, respectively (**Figure 14**), surpassing the amounts present in the remaining varieties by 61.3, 74.9, and 73.0%, on average, respectively. In this case, given that all varieties were processed by equal procedures, the differences observed seem to be a consequence of the biochemical composition of such materials.

The comparison of the features of rice bran concerning the occurrence of PhytoFs is defaulted by the gap existing in the literature on these compounds. Actually, to date, they have been described concerning 16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoFin pine nut, walnut, chia seed, and flax seed nuts, which has shown concentrations of 0.30, 9.00, 6.00, and 0.70 ng g^{-1}, respectively. Besides, the concentration ofe*nt*-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoFin walnuts and chia seeds was reported in levels up to 20-fold higher than in flaxseeds and pine (Cuyamendous *et al*., 2015). Both PhytoFs have been also detected in melon leaves at much higher concentrations with values ranging from 130 to4400 ng g^{-1} (Yonny *et al*., 2016), although the role of leaves as vegetative plant material could be responsible for such differences, requiring further analysis to set the link between plant physiology and the occurrence of PhytoPs and PhytoFs. In this sense, it is required to state that PhytoPs are components of an archaic signaling system that detects lesions by oxidants, and might contribute to protect plants from stress. In this connection, the different concentrations reported could be related to an adaptive response that would provide the plant with a survival advantage through detoxification and stress responses (Thoma *et al*., 2004).

3.3.4. Correlation between phytoprostanes and phytofurans

The analysis of correlation between the concentration of total and individual PhytoPs and PhytoFs, allowed to retrieve that the total concentration of both types of compounds are positively and significantly correlated (R^2 =0.8381, p<0.001). This correlation could be fueled by the fact that PhytoPs and PhytoFs share, in some extent, biosynthetic pathways, which give rise

to one or other type of oxidative metabolite upon non-enzymatic oxidation at different oxygen tension (Fessel *et al*., 2002; Cuyamendous *et al*., 2015).

In addition, a significant correlation has been observed between individual PhytoPs. In this sense, PhytoPs *ent*-9-*epi*-9-D_{1t}-PhytoP correlated with *ent*-9-D_{1t}-PhytoP (R^2 = 0.750, p<0.001) and 16-B_1-PhytoP (R^2 = 0.395, p<0.05), *ent*-9-D_{1t}-PhytoP with 16-B_1-PhytoP (R^2 = 0.415, p<0.01), and 16-B_1-PhytoPwith 9-L_1-PhytoP (R^2 = 0.462, p<0.01). Likewise, PhytoFs provided a positive and significant correlation with each other (**Table 10**). This can be attributed to their common origin as they result from the autooxidation of PUFAs, producing a linolenate radical that readily oxidizes and cyclizes to complex isomeric mixtures without enzymatic intervention and their ability to transform into one another PhytoPs (Fessel *et al*., 2002; Cuyamendous *et al*., 2015).

Table 10. Analysis of correlation between the individual phytoprostanes and phytofurans present in rice (*Oryza sativa* L.) flours.

	PP5[Z]	PP6	PP7	PF1	PF2	PF3
PP4	0.750***	0.395*	-0.027[N.s.]	0.537**	0.547**	0.349*
PP5	--	0.415**	0.026 [N.s.]	0.711***	0.654***	0.454**
PP6	--	--	0.462**	0.526**	0.681***	0.396*
PP7	--	--	--	0.220*	0.253*	0.181*
PF1	--	--	--	--	0.783***	0.510**
PF2	--	--	--	--	--	0.591**

[Z]**PP4**, *ent*-9-*epi*-9-D_{1t}-PhytoP; **PP5**, *ent*-9-D_{1t}-PhytoP; **PP6**, 16-B_1-PhytoP; **PP7**, 9-L_1-PhytoP; **PF1**, *ent*-16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF**; PF2**, *ent*-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF; **PF3**, *ent*-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF.[Y] N.s., no significant and significant at p<0.001(***).

3.4. CONCLUSIONS

In summary, to the best of our knowledge, this is the first work describing the presence of PhytoPs and PhytoFs in rice flours obtained from diverse *indica* and *japonica* cultivars, a crop as massive and important for human food ensuring then, a regular and abundant intake of these compounds in the worldwide diets. The PhytoPs *ent*-9-D_{1t}-PhytoP, 16-B_1-PhytoP, and 9-L_1-PhytoP and the PhytoFe*nt*-16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF appear as those contributing mostly to the total content of PhytoPs and PhytoFs in the studied food matrices, on which the study of the technological (safety and quality) and biological interest is in its infancy. The highest mean concentrations correspond to rice bran and the lowest to white grain flour. Besides, the

occurrence of PhytoFs exhibits average values of 1.77, 4.22, and 10.30 ng g^{-1}dw in rice bran, brown grain flour, and white grain flour, respectively. In this concern, the information obtained upon the detailed characterization performed in the present work will allow evaluate the toxicological and biological activity of PhytoPs and PhytoFs, and to clarify the extent in which these compounds could exert valuable biological activities upon *in vitro* (mechanistic) and *in vivo* (pharmacokinetic and bioavailability features), allocating the effort-focus on the chemical species of these compounds present in cereals. In this connection, in the near future, the rice flour compounds now identified and quantified will be candidate to be further evaluated through proper *in vivo* trials with experimental animals and humans that would contribute to elucidate how PhytoPs and PhytoFs impact health markers and prevent undesirable path physiological situations, co-working with additional bioactive compounds already evaluated in food and foodstuffs matrices.

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CHAPTER IV. Statement of foliar fertilization impact on oxidative biomarkers, yield, composition, and in rice (*Oryza sativa* L.)

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4.1. INTRODUCTION

Rice crops play a central role in the population nutrition of more than half world population (Calpe, 2006). In this regard, given the current growth pattern, it has been estimated that human world population could reach 9 billion people by 2050, which would require an augment of the agricultural productions (Hirel *et al.*, 2011). To meet this new situation, plant nutrition research is crucial to meet food quality and safety needs in the near future (Cakmak, 2002). The fact that at least 60% of the nowadays cultivated soils globally have several mineral problems, like toxicities or deficiencies, makes plant nutrition-based research a cutting-edge issue essential to meet the demand for massive increases in food production (Cakmak, 2002).

When analyzing the most important constraints associated to agricultural production to date, most yield losses and inappropriate nutrients concentrations are due to abiotic stress caused by drought, salinity, extreme temperatures, and soils acidity, which deteriorate the composition of soils and, consequently, the nutritional status of plants (Cakmak, 2006). Efficient and sustainable use of natural resources, along with appropriate agronomic practices, is key to overcome the challenges of the increasing world population and resources shortage. Under such challenging situation, enhancing crops yield per unit area is the main goal in the field (Ata-Ul-Karim *et al.*, 2016). In this frame, nitrogen (N) fertilization is a widely adopted practice in conventional agriculture. This is of especial relevance due to the management of irrigation water, responsible for the status of organic N into soils (concerning both concentration and molecular form), as well as the lack of consistent evidences on the optimal timing for its application (Quintero *et al*., 2009). As an alternative, foliar spraying provides nutrients availability at the exact time when they are required by plants, reducing the time-lapse between application and absorption. Hence, when applied at panicle initiation, foliar spraying provides plants the essential nutrients required for obtaining an optimal number of grains per panicle (Fageria, 2007; Waraich *et al.,* 2011). Besides, Waraich *et al*. (2011) demonstrated that the application of micronutrients reduces the toxicity of reactive oxygen species (ROS), due to an

increase in the concentration of antioxidant enzymes, contributing to the protection and maintenance of the integrity and stability of the cell membrane (Cakmak, 2000).

The production of ROS during photosynthesis is intensified due to a limited use of the light absorbed upon photosynthetic electron transport and fixation of CO_2 (Cakmak, 2006). These radicals are unstable and react rapidly with other compounds in the separate cell compartments, causing cellular or tissue damage. To avoid this negligible situation, plant cells account with molecular tools to combat excessive ROS, maintaining the redox balance within physiological limits. However, biotic and abiotic stress may disturb this balance (Hayat and Ahmad, 2007).

In many plant species, α-linolenic acid (ALA) constitutes the end point of fatty acid biosynthesis that is prone to react with ROS. These reactions consist of non-enzymatic peroxidations that give rise to phytoprostanes (PhytoPs), prostaglandin-like compounds found in plants as a result of autoxidation of ALA (Parchmann and Mueller, 1998). Moreover, recently, it has been reported a new class of plant oxylipins also generated non-enzymatically, phytofurans (PhytoFs), due to additional molecular oxygen (tension higher than 21%) after the initial formation of endoperoxydes (Cuyamendous *et al*., 2015).

Concerning the physiological role in plants, PhytoPs and PhytoFs have been noticed as signaling molecules, also responsible for the protection of plant cells against negligible reactions triggered under oxidative stress. In this regard, to date, remain a gap on the impact of management practices on their concentration of such molecules, as well as on the modification of their levels as a consequence of plants' stress (Collado-González *et al.,* 2015a; Yonny *et al*., 2016). Hence, to date, it has not been explored the extent, neither the sense, in which fertilization procedures may modulate the content of oxylipins in plants, which could have a critical impact on plants growth and productivity.

In this frame, foliar fertilization could contribute to change of yield and nutritional quality, as well as modifications of the level of PhytoPs and PhytoFs according to genotype. In this regard, the present study pursues to shed some light on these gaps of information by analyzing five genotypes of rice (*Oryza sativa* L.) featured by high protein concentrations on the grain concentration of protein and its correlation with the level of plants oxylipins.

4.2. MATERIALS AND METHODS

4.2.1. Chemical and reagents

The PhytoPs 9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, *ent*-16-F_{1t}-PhytoP, *ent*-16-*epi*-16-F_{1t}-PhytoP, *ent*-9-D_{1t}-PhytoP, *ent*-9-*epi*-9-D_{1t}-PhytoP, 16-B_1-PhytoP, 9-L_1-PhytoP and 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 (**Table 11**), were synthesized according to already published procedures (El Fangour *et al*., 2004; El Fangour *et al*., 2005; Pinot *et al*., 2008; Guy *et al*., 2015; Cuyamendous *et al*., 2015; Cuyamendous *et al*., 2017) and provided by the *Institut des Biomolécules Max Mousseron* (IBMM) (Montpellier, France).

Table 11. UHPLC-ESI-MS/MS-QqQ parameters for the quantification and confirmation of phytoprostanes and phytofurans in rice (*Oryza sativa* L.).

Compound	Retention time(min)	ESI mode	MRM transition (*m/z*)
Ent-16-*epi*-16-F_{1t}-PhytoP^{X}	1.583	Negative	327.1 > 283.2^{Z}
		Negative	327.1 > 225.1^{Y}
9-F_{1t}-PhytoP	1.631	Negative	327.2 > 273.1
		Negative	327.2 > 171.0
Ent-16-F_{1t}-PhytoP^{X}	1.712	Negative	327.2 > 283.2
		Negative	327.2 > 225.1
9-*epi*-9-F_{1t}-PhytoP	1.785	Negative	327.2 > 272.8
		Negative	327.2 > 171.0
Ent-9-D_{1t}-PhytoP	1.791	Negative	325.2 > 307.3
		Negative	325.2 > 134.7
Ent-9-*epi*-9-D_{1t}-PhytoP	2.022	Negative	325.2 > 307.2
		Negative	325.2 > 134.9
16-B_1-PhytoP	2.620	Negative	307.2 > 223.2
		Negative	307.2 > 235.1
9-L_1-PhytoP	3.079	Negative	307.2 > 185.1
		Negative	307.2 > 196.7
Phytofurans			
Ent-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF	0.906	Negative	344.0 > 300.0
		Negative	344.0 > 255.9
Ent-16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF	1.501	Negative	343.9 > 209.0
		Negative	343.9 > 201.1
Ent-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF	1.523	Negative	343.0 > 171.1
		Negative	343.0 > 97.2

^{Z} Quantification transition. ^{Y} confirmation transition. ^{X} coeluting diesteroisomers quantified together.

Hexane was obtained from Panreac (Castellar del Valles, Barcelona, Spain), butylated hydroxyanisole (BHA), and Bis–Tris (bis-(2-hydroxyethyl)-amino-tris (hydroxymethyl) methane)

were purchased from Sigma-Aldrich (St. Louis, MO, USA). All LC–MS grade solvents, methanol and acetonitrile, were purchased from J.T. Baker (Phillipsburg, NJ, USA). Water was obtained from a milli-Q water purification system from Millipore (Bedford, MA, USA). The solid phase extraction (SPE) cartridges used were Strata X-AW cartridges, (100 mg/3 mL) and were acquired from Phenomenex (Torrance, CA, USA).

4.2.2. Field experiment

The field trial was set up in the Experimental Station 'Julio Hirschhorn' of La Plata (34˚52'S, 57˚57'W, 9.8 m of altitude, Buenos Aires, Argentina) during the season 2016/2017. The soil was featured by 3.7% of organic matter (Walkey-Black method), 0.2% of total N (micro-Kjeldahl method), 18.0 ppm of P (Bray Kurtz method Nº1), and pH 6.2. The panel of rice genotypes included: One (1) featured by an average grain protein content, which is widely sown by farmers in the production area ('Guri INTA'), one (1) cultivar ('Nutriar FCAyF') characterized by having 30% more grain protein, and three (3) advanced breeding lines of high grain protein content ('H458-41-1-1-1', 'H475-3-1-1-2', and 'H484-9-1') from the 'Programa Arroz' of the Agronomy and Forestry Faculty (FCAyF).

The experimental setting was a randomized block design, with 3 replicates. Sowing was done manually in dry land, at a rate of 350 seeds m^{-2} in grooves at 0.20 m, in plots of 5 m^2. The trials were developed with flood irrigation after 30 days of emergence and weeds were controlled with sodium bispyribac. The treatments were Control (T0) and 0.3 L ha^{-1} foliar fertilizer (Nutri-Fort) in panicle initiation (T3). The product was diluted in water and applied as a spray with hand sprayed. The experimental plots were sprayed during late afternoon, when wind speed was less than 10 km h^{-1}. Nutri-Fort contains 7.0% Nitrogen, 2.0% Phosphorus assimilable, 2.2% soluble Potassium, 0.6% Calcium, 1.0% Magnesium, 0.1% Copper, 2.0% Iron, 0.7% Manganese, and ≤0.1% Zinc, and 1.0% Boron.

Climatic conditions during the field experiment set up at 34˚52'S, 57˚57'W, 9.8 m of altitude are summarized in **Figure 15**. The trial was sown in November 15th, 2016, the anthesis and harvest were in February 2nd and April 14th 2017, respectively. The temperatures recorded during seedling, establishment, and tillering meet the optimum values (25-31˚C), according to previous descriptions (De Datta, 1981), while during ripening, the temperatures recorded oscillated between 20 and 29 ˚C. Under these environmental conditions, the rice crops were developed properly.

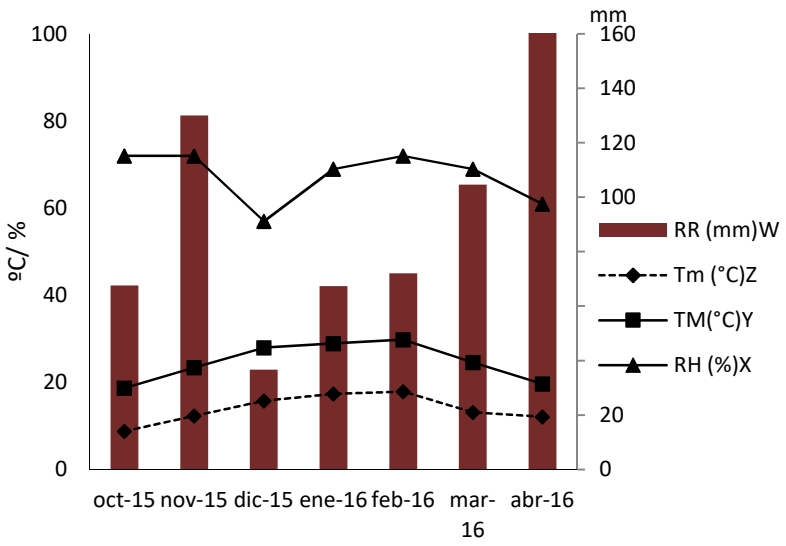

Figure 15. Climatic conditions in the experimental field (34˚52'S, 57˚57'W, 9.8 m of altitude) during the rice crop. [Z]Mean daily minimum air temperature; [Y]Mean daily maximum air temperature;Relative humidity; [W]Total monthly rainfall.

4.2.3. Samples and standards preparation

Harvest and threshing were done manually and, afterwards grains were dried in an oven at 40 °C until reaching 14% humidity. Dried grains were randomly weighed to record grain weight. Then, they were shelled to obtain whole grain. Samples were milled and sieved using cyclone mill 0.4 mm mesh and there were immediately stored at 4 °C and protected from light until analysis.

4.2.4. Determination of protein content

The protein content was evaluated on dry whole grain flour by micro-Kjeldahl method (Stuart, 1936). Shortly, sample (50 mg) was digested with 2 mL of 98% H_2SO_4 and a mixture of potassium sulfate/copper sulfate catalyst (10:1). Then, the mixture was heated until complete the digestion of the samples and cooled. Distilled water was added and steam distillation of NH_3 was carried out by the addition 10 mL of 40% sodium hydroxide in water (*w/v*) and collected in saturated boric acid solution (40 g L^{-1}). The acid neutralized by ammonia was evaluated with standardized 0.02 M HCl, using as indicator methyl red and bromophenol blue. The percentage of nitrogen was calculated using the equation "*% Nitrogen = ((mL standardized acid – mL blank) x N of acid) x 1.4007) / weight of sample in grams*". The factor 6.25 was applied for the determination of the protein concentration in rice samples.

4.2.5. Extraction of phytoprostanes and phytofurans

The PhytoPs and PhytoFs present in the rice flours were extracted following the methodology described by Pinciroli *et al.* (2017). The extracts obtained were further processed upon SPE, using a Chromabond C18 column according to the procedure previously described (Collado-González *et al.,* 2015b). PhytoPs and PhytoFs were eluted with 1 mL of methanol and dried using a SpeedVac concentrator (Savant SPD121P, Thermo Scientific, MA, USA). The dry extracts were reconstituted with 200 microliters of A-solvent/B-solvent (50:50, *v*/*v*), being solvent A milliQ-water/acetic acid (99.99:0.01, *v*/*v*) and solvent B methanol/acetic acid (99.99:0.01, *v*/*v*). Once reconstituted, samples were filtered through a 0.45-µm filter (Millipore, MA USA) and immediately analyzed by UHPLC-ESI-QqQ-MS/MS (Agilent Technologies, Waldbronn, Germany).

The quantification of PhytoPs and PhytoFs detected in rice grain and flour was performed using freshly standard curves of authentic standards. Stock solutions were prepared in milliQ-water/methanol (50:50, *v*/*v*) to facilitate the ionization in the mass spectrometer.

4.2.6. UHPLC-ESI-QqQ-MS/MS analysis

Chromatographic separation of PhytoPs and PhytoFs was performed using a UHPLC coupled with a 6460 triple quadrupole-MS/MS (Agilent Technologies, Waldbronn, Germany), using the analytical column BEH C_{18} (2.1 x 50 mm, 1.7 µm) (Waters, Milford, M.A.) applying the methodology and instrument settings described by Domínguez-Perles *et al.* (2018). The quantification of PhytoPs and PhytoFs in rice flour was performed using authentic standards, according to standard curves including five successive dilutions 1/4 (1.000, 0.250, 0.063, 0.016, and 0.004 µM), freshly prepared each day of analysis in MeOH/distilled-water (50:50, *v/v*), to facilitate the ionization in the mass spectrometer. The selected reaction monitoring and chemical names were according to the nomenclature system of Taber and are referred in **Table 11**.

4.2.7. Statistical analysis

All assays were developed in triplicate (n=3) and values were reported as means ± standard deviation (SD). To determine the effect of the foliar fertilization, an analysis of variance (ANOVA) was carried out using treatment of fertilization and genotypes as sources of variation. It expresses the values of *F*-ratio and *p*-value. The F-statistic is a ratio of two variances. When

significant interactions were found, it was carried out a One-Way ANOVA and the means were compared by multiple range tests (Tukey's test). The analyses were carried out using Statgraphics Centurion XVI (StatPoint Technologies Inc., Warrenton, VA, USA). Significant differences were set at $p<0.05$.

4.3. RESULTS AND DISCUSSION

To evaluate the effect of foliar fertilization on yield and quality parameters of rice flours, as well as on the concentration of PhytoPs and PhytoFs as indicators of plants stress induced by these agricultural practices, crops were set up in an experimental field of the Buenos Aires province, in mid-east Argentina, and included two cultivars ('Guri INTA' and 'Nutriar FCAyF') and 3 advanced breeding lines ('H458-41-1-1-1', 'H475-3-1-1-2', and 'H484-9-1').

4.3.1. Effect of foliar fertilization on grain yield and quality

In order to understand the behavior of the rice genotypes under study, regarding the impact of foliar fertilization on productivity, grain yield, 1000-whole grain weight, and protein content were monitored. **Table 12** shows the detail of these parameters in the 5 genotypes and two treatments of foliar fertilization. From the results retrieved, it was noticed that for none of the varieties there was significant interaction of fertilization treatment by genotype ($p>0.05$), exception made of the protein content in the variety 'Guri-INTA' that experienced a significant augment from 8.8 to 9.3% under foliar fertilization.

When comparing yield, 1000-whole grain weight, and protein content of the five genotypes under study, it was found that the average yield under control conditions was 961.22 g m^{-2}, being not observed statistically significant differences between genotypes ($p>0.05$). This trend was also observed when monitoring the effect of foliar fertilization on yield that revealed that, even though the treatment applied increased by 7.2% the average yields means of all five genotypes, the differences observed were no statistically significant (**Table 12**). These results are in agreement with the information available in the literature, since to date, most authors have found positive responses to foliar fertilization. In this connection, the application of N concentrated near of panicle initiation was demonstrated as the most efficient practice, which favors the formation of a higher number of spikelets per panicle (Bezerra Barreto *et al*., 2012).

Table 12. Mean values of yield (g m^{-2}), and 1000-whole grain weight (g) in the 5 genotypes upon 2 foliar fertilization treatments.

Genotype	Yield			1000-whole grain weight		
	No fertilization	Foliar fertilization	*p*-value	No fertilization	Foliar fertilization	*p*-value
'Guri INTA'	915.56 ± 115.92[Z] a	955.56 ± 101.18 a	N.s.[Y]	18.42 ± 0.12 d	18.32 ± 0.16 d	N.s.
'Nutriar FCAyF'	840.00 ± 103.92 a	860.0 ± 214.89 a	N.s.	19.59 ± 0.06 c	19.48 ± 0.29 c	N.s.
'H458-41-1-1-1'	963.33 ± 4.71 a	1060.0 ± 113.92 a	N.s.	22.81 ± 0.34 b	22.73 ± 0.08 b	N.s.
'H475-3-1-1-2'	953.33 ± 81.10 a	953.33 ± 59.25 a	N.s.	23.98 ± 0.45 a	23.53 ± 0.22 a	N.s.
'H484-9-1'	966.67 ± 90.18 a	1144.44 ± 40.73 a	N.s.	23.56 ± 0.44 ab	23.88 ± 0.16 a	N.s.
p-Value	N.s.	N.s.		***	***	

[Z] Means ± SD (n=3) within a column followed by distinct letters are significantly different at $p<0.05$ according to Tukey's multiple range test. The effect of foliar fertilization was compared for each genotype by paired *t*-test. [Y] N.s., no significant; *$p<0.05$; ***, $p<0.001$.

Besides, Bhuyan *et al.* (2012) reported yield increases of up to 9.3% when applying urea, using the technique of foliar fertilizations. However, although a clear trend was found, the present study failed in identifying significant differences as a consequence of foliar fertilization that could be due to the application of the minimum dose recommended by the manufacturer, which could be insufficient.

Regarding 1000-whole grain weight, it was revealed that foliar fertilization induced significant differences between genotypes (**Table 12**). Hence, the higher value corresponded to 'H475-3-1-1-2', H458-41-1-1-1', and 'H484-9-1' (23.41 g, on average) that surpassed the values recorded for 'Guri-INTA' and 'Nutriar FCAyF' by 19.00 g on average. These data revealed that grain weight is a phenological feature weakly influenced by the environmental conditions. Indeed, rice grains size is physically restricted by the size of the hull and its weight, under most conditions, is a very stable varietal characteristic (Yoshida, 1983). These results were in agreement with Bezerra Barreto *et al.* (2012) that studied the effect of three seasons of application of N and four doses of N on five rice cultivars. Besides, Midorikawa *et al*. (2014) evaluated the effect of the spraying with ammonium chloride on the soil surface at a rate of 8 g m^{-2} on the 'Niponbare' cultivar and Figueroa (2009) studied the effect of *Fertideg* foliar fertilizer on rice cultivars. Upon none of these works it was reported differences in the weight of the grains due to the treatments applied. Possibly, the relative contribution of grains per panicle to grain yield is moderate, while that of grain weight is very low (Koutroubas and Ntanos, 2003).

The evaluation of the impact of foliar fertilization on the protein content evidenced that only 'Guri INTA' responded to the treatment applied increasing protein significantly in fertilized

plants (by 6.4%, *p*=0.0273) compared to unfertilized controls (**Table 12**). Nowadays, it is already known that foliar fertilization produces contradictory effects regarding protein content in rice grain, since it depends on several factors, such as the moment of application and the initial nutrition status according to the soil features (Quintero *et al*., 2009). In this regard, several authors have reported critical differences among cultivars featured by average protein content, as a consequence of foliar fertilization upon field trials developed in soils with similar composition (De Battista, 2006, Midorikawa *et al.,* 2014). On the other hand, Bezus *et al*. (2007) and Vidal *et al.* (2010), evaluated genotypes featured by high protein content, and did not observe a statistically significant response to the nitrogen fertilization applied. Additional studies by the same authors evidenced that the response varies according to the genotype, which has been justified because the applied N can be directed to other destinations different from grain, as yield or biomass, for which it is necessary to evaluate higher doses and other moments of application (Vidal *et al*., 2005; Vidal *et al.,* 2006).

Significant differences were observed between genotypes under both no fertilization and foliar fertilization conditions (**Figure 16**). Genotypes featured by high protein content ('Nutriar FCAyF', 'H458-41-1-1-1', 'H475-3-1-1-2', and 'H484-9-1') presented, on average, 11.1% dw, while 'Camba INTA' revealed a significantly lower content (9.1%).

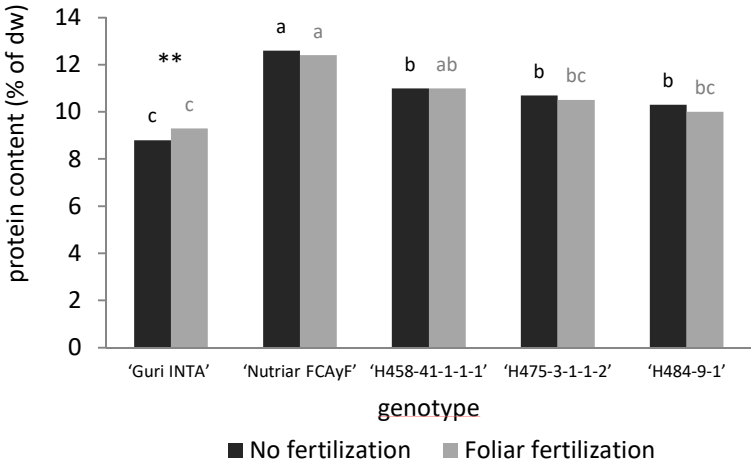

Figure 16. Mean values of protein content (% of dry matter) in the 5 genotypes upon 2 foliar fertilization treatments. [Z] Means ± SD (n=3) within a same treatment (same colors) followed by distinct letters are significantly different at *p*<0.05 according to Tukey's multiple range test.**significantly different at *p*<0.05 according to compared for each genotype by paired *t*-test.

Thus, from these results, it was noticed that 'Nutriar FCAyF' was the genotype displaying the highest protein content. Indeed, this variety has been obtained and registered by the Rice Program, as a high-protein in grain variety that could contribute to improve the nutritional properties of this cereal.

4.3.2. Total and individual phytoprostanes in rice grain

The evaluation of the abundance of total PhytoPs in the genotypes studied evidenced that their concentration decreased as follows: 'H475-3-1-1-2' (509.25 ng g^{-1} dw) > 'Guri INTA' (396.92 ng g^{-1} dw) > 'Nutriar FCAyF' (385.23 ng g^{-1} dw) > 'H484-9-1' (346.43 ng g^{-1} dw) > 'H458-41-1-1-1' (309.16 ng g^{-1} dw). In this regard, recently, Pinciroli *et al.* (2017) evaluated the concentration of total PhytoPs in an array of rice varieties by studying 14 genotypes. Upon this work, the highest concentration of total PhytoPs in whole grain flour corresponded to the variety 'Arborio' and the lowest to 'Itape', with values of 9.51 and 32.89 ng g^{-1} dw, respectively. Concerning additional plant materials, and in order to get a rational understanding on the level of these compounds in rice, it is required to notice the abundance of total PhytoPs in other plant materials. In this regard, to date, it has been described the level of total PhytoPs in green olives (6.0-87.9 ng g^{-1} fw), vegetable oils (1.9-119.2 ng mL^{-1}), wines and musts (134.1-2160.0 and 21.4-447.1 ng mL^{-1}, respectively), and in 24 species of macroalgae (0.0-113.8 ng g^{-1} dw) (Collado-González *et al*., 2014; Barbosa *et al*., 2015; Marhuenda *et al*., 2015; Collado-González *et al*., 2015c; Domínguez-Perles *et al*., 2018). Nonetheless, to date, the highest concentration of total PhytoPs has been described in fresh birch pollen and dry leaves of melon plants, with values up to 1380.0 and 1600.0 ng g^{-1}, respectively (Imbusch and Mueller, 2000; Yonny *et al*., 2016). The comparison of the level of PhytoPs in the diverse matrices allowed describing that the total amount in rice grains is in the range of the major part of vegetable foods, which revealed this matrix as an especially interesting source of dietary PhytoPs given the high implantation in diets of rice and rice flours.

Apart from total PhytoPs, when profiling the individual compounds within this oxylipins class, it was revealed the presence of seven individual PhytoPs: 9-F_{1t}-PhytoP, *ent*-16-F_{1t}-PhytoP + *ent*-16-*epi*-16-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, *ent*-9-*epi*-9-D_{1t}-PhytoP, *ent*-9-D_{1t}-PhytoP, 16-B_1-PhytoP, and 9-L_1-PhytoP (**Table 11**). In respect to the abundance of the individual PhytoPs present in concentrations higher than the LOQ, they could be order according to lowering average concentrations under control conditions as follows: 16-F_{1t}-PhytoP+*ent*-16-*epi*-16-F_{1t}-PhytoP (213.25 ng/g dw) > 9-F_{1t}-PhytoP (125.57 ng g^{-1} dw) > *ent*-9-*epi*-D_{1t}-PhytoP (22.66 ng g^{-1}

dw) > 16-B_{1t}-PhytoP (12.61 ng g^{-1} dw) > 9-L_{1t}-PhytoP (8.69 ng g^{-1} dw) > 9-*epi*-9-F_{1t}-PhytoP (3.44 ng g^{-1} dw) > *ent*-16-*epi*-16-F_{1t}-PhytoP (3.08 ng g^{-1} dw).

These results were in agreement with previous studies on the level of PhytoPs in a range of rice flours, upon which was stated the predominant occurrence of 16-B_1-PhytoP + ent-16-B_1-PhytoP (52.79 ng g^{-1} dw), *ent*-9-D_{1t}-PhytoP (28.98 ng g^{-1} dw) and *ent*-9-L_1-PhytoP + 9-L_1-PhytoP (27.04 ng g^{-1}) in rice bran of the *indica* subspecies (Pinciroli *et al.*, 2017). In addition, when evaluating the information available in the literature on the presence of individual PhytoPs in edible and non-edible plant material, it was stated that *ent*-16-F_{1t}-PhytoP and *ent*-16-*epi*-16-F_{1t}-PhytoP were also the most abundant compounds in dry melon leaves (1160.0 ng g^{-1}) (Yonny *et al*., 2016) and aged wine (133.8 ng mL^{-1}) (Marhuenda *et al*., 2015). On the other hand, in rice grains, 9-F_{1t}-PhytoP ranked second, while this PhytoP has been described as the most abundant in olive oil (5.2 ng mL^{-1}) (Collado-González *et al.,* 2015a), almonds (42.4 ng g^{-1}) (Carrasco-Del Amor *et al*., 2016), macroalgae (152.6 ng g^{-1}) (Barbosa *et al*., 2015), and in grape musts (32.7-13.5 ng g^{-1}) (Marhuenda *et al*., 2015). The PhytoPs 16-B_1-PhytoP and 9-L_1-PhytoP resulted in low in carbonic maceration wine (4.11 ng mL^{-1}, on average) (Marhuenda *et al.* 2015) and macroalgae (0.10 ng g^{-1}, on average) (Barbosa *et al.*, 2015), while *ent*-9-*epi*-9-D_{1t}-PhytoP has been described in low concentrations olive oil (Collado-González *et al*., 2015a) and almonds (Carrasco-Del Amor *et al*., 2015), and reported as almost absent in melon leaves (Yonny *et al*., 2016). It is important to notice that certain agronomic and industrial procedures may enhance the PhytoPs content of fruits and derived vegetable oils. In this way, it may be possible to "functionalize" plant products with higher concentrations of these compounds, which are readily bioavailable and absorbed by the human body (Karg *et al*., 2009).

The relative abundance of the individual PhytoPs in the plant materials evaluated so far is of special relevance and indicates the challenge of getting a further insight in the biological function of specific classes of PhytoPs, upon dedicated *in vivo* assays in the frame of animal experimental models and clinical trials. So, in this regard, it is of special relevance the works developed by Barden *et al*. (2009) that measured, by using GC-MS, the content of F_1-PhytoPs in human plasma and urine samples after consuming flaxseed oil for four weeks. This nutritional trial allowed them to describe an augment of the plasma concentration of F_1-PhytoPs; however, the pharmacokinetic curve and the physiological effects on the human body remain underexplored (Collado-González *et al.*, 2015d), and require further characterization in the near future, suggesting that the assessment of new food matrices deserves to be developed to identify optimal dietary sources of these compounds.

4.3.3. Total and individual phytofurans in rice grain

When profiling PhytoFs in the five varieties of rice included in the present work, the 3 phytofurans monitored (e*nt*-16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF, e*nt*-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF, and e*nt*-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF (**Table 11**)) were found in levels higher than the limit of detection (LOD) of the method. The occurrence of PhytoFs in plant material is a consequence of non-enzymatic oxidation reactions under higher oxygen tension, relatively to the conditions required for the synthesis of PhytoPs (Cuyamendous *et al*., 2017).

The concentration of PhytoFs of the treatments and the genotypes studied decreased as follows: e*nt*-16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF (19.18 ng g^{-1} dw) > e*nt*-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF (10.48 ng g^{-1} dw) > e*nt*-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF (3.46 ng g^{-1} dw) (**Figure 14**). This decreasing order coincides with that reported by Pinciroli *et al*. (2017) upon the analysis of different types of flour and rice varieties under matching agro-climatic conditions. Moreover, these data are also in agreement with Yonny *et al.* (2016) that observed e*nt*-16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoFs the most abundant PhytoF in dry melon leaves, while e*nt*-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF was not detected.

Total PhytoFs were found in the following order of abundance: 'Guri INTA' (38.01 ng g^{-1} dw) > 'H458-41-1-1-1' (37.11 ng g^{-1} dw) > 'Nutriar FCAyF' (35.92 ng g^{-1} dw) > 'H475-3-1-1-2' (35.24 ng g^{-1} dw) > 'H484-9-1' (19.34 ng g^{-1} dw).

To date, there is limited information on the occurrence of PhytoFs in plant materials and vegetable foods, concerning both profile and quantitative presence (Cuyamendous *et al*., 2017). Among few articles providing this information, Pinciroli *et al.* (2017) revealed differences between flour types and rice varieties featured by concentrations ranging between 0.06 ng g^{-1}dw (for white grain flour obtained of the variety 'Quebracho FA') and 27.70 ng g^{-1} dw (bran rice of the cultivar 'Guri INTA'). Moreover, Cuyamendous *et al.* (2016) reported the occurrence of these compounds in pine, nuts, chia, and flaxseeds (0.30, 9.00, 6.00, and 0.70 ng g^{-1}, respectively); while Yonny *et al.* (2016) found much higher concentrations in melon leaves (130.00 – 4400.00 ng g^{-1} dw). However, it has to be noticed that the concentration of PhytoFs in the plant material evaluated has been reported in levels significantly lowers relatively to those describe for PhytoPs, which could be related with the specific physico-chemical features of rice and rice flour, accounting for very low water content (Albena *et al.,* 2004).

4.3.4. Influence of foliar fertilization in phytoprostanes and phytofurans concentration

When analyzing the effect of foliar fertilization on the concentration of PhytoPs and PhytoFs it was revealed that this practice modified their concentration significantly, exception made of *ent*-9-*epi*-9-D_{1t}-PhytoP that remained unaltered in comparison with no treated controls. Overall, upon this work, it was noticed that foliar fertilization causes a decrease of the concentration of plant oxylipins in the five genotypes evaluated.

Similarly, according to diverse authors, PhytoPs and PhytoFs are generated in plant species as a consequence of non-enzymatic peroxidation reactions catalyzed by free radicals (ROS) (Imbusch and Mueller, 2000). Actually, biotic and abiotic stress alters the balance between the generation of ROS and the scavenging capacity, which entails the disturbance of the redox homeostasis in cells (Das and Roychoudhury, 2014). In this frame, probably, foliar fertilization could contribute to prevent crops from a situation of nutritional stress. Indeed, increasing evidence suggests that a proper mineral-nutrient status of plants contribute to enhance plants resistance to environmental stress factors (Cakmak, 2005). In addition, according to Cakmak (2002), fertilization increases the antioxidant defense mechanisms, resulting in a reduced photo-oxidation of the chloroplast pigments and the stability of cell membranes. In this regard, this author suggested that the improvement of the nutritional status of plants, regarding the mineral nutrients Zn, K, Mg, and Bo, might contribute to lower the production of ROS and thereby, to minimize the activity of NADPH oxidases, while maintaining the photosynthetic transport of electrons (Cakman, 2002; Cakman, 2006). This is further supported by the fact that micronutrients integrate the isoforms of the set of enzymes responsible for detoxification reactive oxygen species like CuZn-SOD, Mn-SOD, and Fe-SOD (Cakmak, 2000). These antioxidants neutralize ROS contributing to maintain the integrity of the chloroplast membrane in plants (Waraich *et al*., 2011). This protection from the oxidative damage generated by the adequate supply of minerals could be the cause of the lower generation of oxylipins.

Hence, the quantification of individual PhytoPs in grain of the five rice genotypes, grown under control and foliar fertilization conditions, revealed significantly different concentrations in control plants relatively to those fertilized. In this regard, resorting to the analysis of variance (ANOVA), it was observed that foliar fertilization is enclosed to significant and varied effects on the levels of diverse plant oxylipins. **Table 13** shows the mean concentrations of the compounds (9-*epi*-9-F_{1t}-PhytoP, *ent*-9-*epi*-9-D_{1t}-PhytoP, *ent*-9-D_{1t}-PhytoP, *ent*-16-F_{1t}-PhytoP + *ent*-16-*epi*-16-F_{1t}-PhytoP) that did not show significant *foliar fertilization x genotypes* interaction, which exhibited equal modifications of their concentrations in all genotypes, while the compounds featured by a significant *foliar fertilization x genotypes* interaction (9-F_{1t}-PhytoP, 16-B_{1t}-PhytoP,

9-L_{1t}-PhytoP, *ent*-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF, *ent*-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF) are referred in **Table 11**.

In respect to the compounds that presented no significant *foliar fertilization x genotype* interaction (**Table 13**), the foliar fertilization decreased significantly (p<0.05) the concentration of 16-F_{1t}-PhytoP + *ent*-16-*epi*-16-F_{1t}-PhytoP (from 249.08 to 177.63 ng g^{-1} dw, on average), 9-*epi*-9-F_{1t}-PhytoP (from 3.42 to 2.73 ng g^{-1} dw, on average), *ent*-9-*epi*-9-D_{1t}-PhytoP (from 3.86 to 3.01 ng g^{-1} dw, on average), and *ent*-9-D_{1t}-PhytoP (from 25.00 to 20.32 ng g^{-1} dw, on average), e*nt*-16-(*RS*)-9-*epi*-*ST*-Δ^{14}-10-PhytoF (from 21.05 to 17.32 ng g^{-1} dw, on average). On average, it was observed a 20.0% decrease, being *ent*-16-F_{1t}-PhytoP + *ent*-16-*epi*-16-F_{1t}-PhytoP (28.7%, on average) and *ent*-9-*epi*-9-D_{1t}-PhytoP (12.0%, on average) the most and less sensitive PhytoPs to foliar fertilization, respectively (**Table 13**).

Table 13. Content of phytoprostanes (ng g^{-1} dw) without significant interaction foliar fertilization treatment x genotype (p<0.05).

Genotype	16-F_{1t}-PhytoP + *ent*-16-*epi*-16-F_{1t}-PhytoP	*9-epi*-F_{1t}-PhytoP	*Ent*-9-*epi*-9-D_{1t}-PhytoP	*Ent*-9-D_{1t}-PhytoP
'Guri INTA'	233.12 ± 93.99z a	2.69 ± 0.29 b	3.43 ± 1.36 a	19.98 ± 3.07 bc
'Nutriar FCAyF'	217.96 ± 82.47 a	2.66 ± 0.26 b	3.36 ± 1.87 a	18.31 ± 2.15 bc
'H458-41-1-1-1'	151.45 ± 57.21 a	2.83 ± 1.00 b	3.18 ± 1.25 a	23.33 ± 5.79 b
'H475-3-1-1-2'	254.97 ± 116.34 a	5.27 ± 2.05 a	4.62 ± 2.01 a	36.01 ± 6.10 a
'H484-9-1'	209.28 ± 76.69 a	1.93 ± 0.84 b	2.60 ± 0.91 a	15.68 ± 2.58 c
F-ratio$^{p\text{-value}}$	1.30 $^{N.s.}$	7.84***	1.23 $^{N.s.}$	30.42***
Foliar fertilization treatment				
No fertilization	249.08 ± 86.07 a	3.42 ± 1.77 a	3.86 ± 1.66 a	25.00 ± 8.82 a
Foliar fertilization	177.63 ± 78.19 b	2.73 ± 1.26 a	3.01 ± 1.41 a	20.32 ± 7.19 b
F-ratio$^{p\text{-value}}$	5.52*	2.84 $^{N.s.}$	2.04 $^{N.s.}$	13.12**
Interaction genotype x foliar fertilization treatment				
F-ratio$^{p\text{-value}}$	0.53 $^{N.s.}$	0.41 $^{N.s.}$	0.10 $^{N.s.}$	0.87 $^{N.s.}$

Means (n=3) within a column followed by distinct letters are significantly different at p<0.05 according to Tukey's multiple range test. N.s., no significant; **, p<0.01; ***, p<0.001.

For the compounds exhibiting significant *foliar fertilization x genotypes* interaction, the genotypes behaved differentially against the application of foliar fertilization (**Table 14**).

Table 14. Content of phytoprostans (ng g^{-1} dw) with significant interaction foliar fertilization treatment x genotype.

Genotype	9-F_{1t}-PhytoP		16-B_1-PhytoP		9-L_1-PhytoP	
	No fertilization	Foliar fertilization	No fertilization	Foliar fertilization	No fertilization	Foliar fertilization
'Guri INTA'	115.36 ± 15.79 cd	110.92 ± 10.77 cd	18.08 ± 0.07 ab	11.37 ± 1.84 de	11.75 ± 0.24 ab	7.94 ± 1.68 cdef
'Nutriar FCAyF'	121.98 ± 29.79 bcd	131.07 ± 23.71 bc	9.25 ± 0.83 ef	10.22 ± 0.65 de	6.37 ± 0.51 efg	7.01 ± 0.56 defg
'H458-41-1-1-1'	133.29 ± 7.13 abc	67.10 ± 33.12 d	18.88 ± 1.30 a	13.85 ± 3.44 cd	13.84 ± 0.76 a	9.80 ± 2.80 bcd
'H475-3-1-1-2'	189.79 ± 26.81 a	175.53 ± 9.85 ab	16.58 ± 1.12 abc	14.11 ± 0.21 bcd	11.09 ± 0.94 abc	9.66 ± 0.53 bcde
'H484-9-1'	128.27 ± 6.05 bc	82.40 ± 10.75 cd	7.85 ± 0.52 ef	5.89 ± 1.16 f	5.42 ± 0.58 fg	4.04 ± 0.67 g
p-Value	**	***	***	**	***	**
Interaction foliar fertilization treatment x genotype						
F-ratio$^{p\text{-value}}$	3.69***		6.26***		4.11**	

Means ± SD (n=3) different letters are significantly different at p<0.05 according to Tukey's multiple range test. ** p<0.01; ***, p<0.001. ANOVA multifactor statistical analysis.

The concentration of 9-F_{1t}-PhytoP decreased as consequence of the application of foliar fertilization in the advanced line 'H458-41-1-1-1', while for 'Guri INTA', 'Nutriar FCAyF', 'H475-3-1-1-2', and 'H484-9-1' the diminution was no significant. The concentrations of 16-B_1-PhytoP and 9-L_1-PhytoP decreased due to the application of foliar fertilization in 'Guri INTA' and 'H458-41-1-1-1', while in the other genotypes included in this study no significant modifications were found (**Table 14**).

The quantification of individual PhytoFs in the grain of the five rice genotypes revealed different behaviors regarding *foliar fertilization x genotype* interaction. In this respect, *ent*-16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF did no exhibit a significant interaction as its concentration was modified in equivalent extent in the 5 genotypes with average values of 21.05 and 17.32 ng g^{-1} dw for the unfertilized and fertilized samples, respectively. In contrast, the compounds *ent*-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF and *ent*-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF were affected by significant *foliar fertilization x genotype* interaction (**Figure 17**).

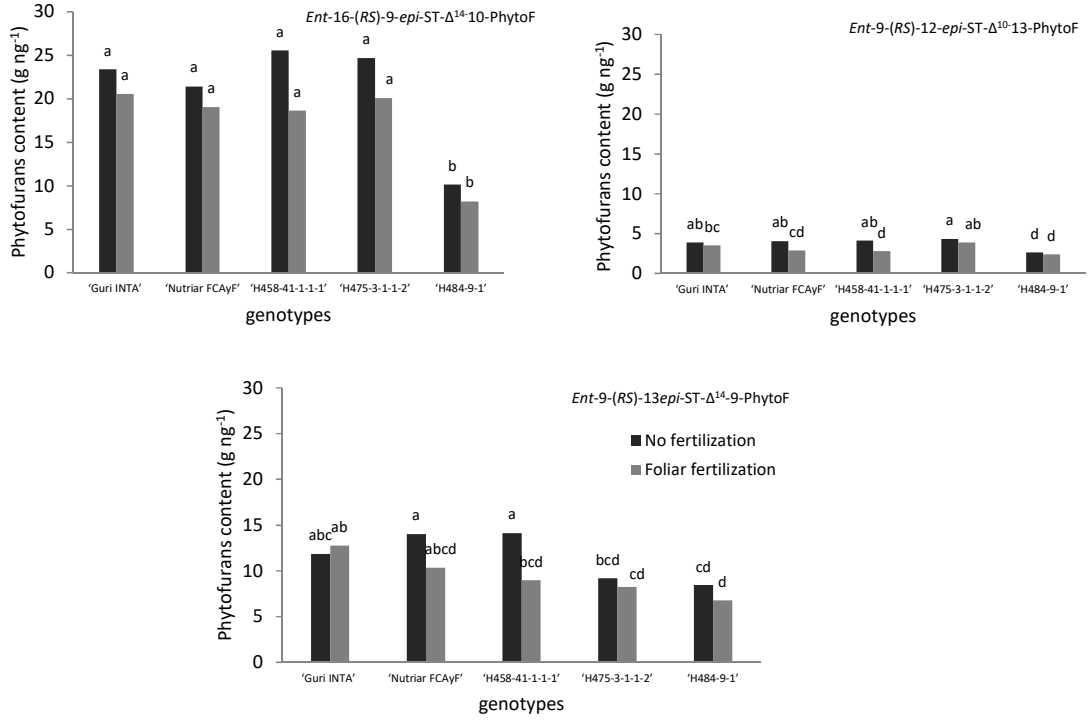

Figure 17. Content of phytofurans (ng g^{-1} dw) with and without significant interaction foliar fertilization treatment x genotype. [Z] Means ± SD (n=3) within a same treatment (same colors) followed by distinct letters are significantly different at $p<0.05$ according to Tukey's multiple range test.

Hence, the concentration of *ent*-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF decreased in 'Nutriar FCAyF' and 'H458-41-1-1-1' genotypes, while *ent*-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF was only significantly reduced in 'H458-41-1-1-1'. In the rest of the genotypes, the concentration of these compounds was not modified significantly by the application of foliar fertilization. Summarizing de effect of this fertilization approach, the mean decrease of concentrations was by 17.7, 18.8 and 18.4% for the compounds *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, respectively.

These results corroborate previous descriptions reporting that some PhytoPs can modify their presence due to stress situations *in vivo* or to the conditions during storage or processing (Collado-González *et al.,* 2015b). In this regard, in melon leaves exposed to thermal stress, the compound that showed the greatest variations was 9-F_1-PhytoP (Yonny *et al.,* 2016). The concentration of the 9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, and 16-B_1-PhytoP compounds in fresh olives increased when olive trees developed under different situations of water deficit (Collado-González, *et al*., 2015a). In the same way, *ent*-9-*epi*-9-D_{1t}-PhytoP and *ent*-9-D_{1t}-PhytoP concentrations in fresh almonds increased during storage (Carrasco-Del Amor *et al*., 2016). Thus, seems clear that so far, there is limited knowledge about the response of plants to diverse environmental situations in respect to the levels of oxylipins.

When evaluating the influence of the genotype on the variation of the concentration of PhytoPs and PhytoFs due to stress, it was noticed that the diverse genotypes showed differences in the concentrations of these compounds, as well as in their response to fertilization. Hence, the line 'H475-3-1-1-2' presented high concentrations of most compounds monitored, while the concentration of the treated and untreated samples was not modified, with the only exception 9-epi-F_{1t}-PhytoP and *ent*-9-epi-9-D_{1t}-PhytoP. Conversely, line 'H458-41-1-1-1' showed a decrease in the concentration of the treated samples with respect to those untreated, demonstrating differential genetic response, according to the varietal resistance against stress situations. In this regard, Bezerra Barreto *et al*. (2012) proved that genotypes have different tolerance to nutritional stress. Both the absorption of nutrients and the effects of their deficiencies can be genotype-dependent (Cakmak, 2000). White and Broadley (2005) reported recent advances in the development of new genotypes with high levels of micronutrients, suggesting that integration of plant nutrition research with plant genetics and molecular biology is indispensable in developing plant genotypes with high genetic ability to adapt to soils featured by deficient or toxic micronutrient levels and to allocate more micronutrients into edible plant products such as cereal grains (Cakmak, 2002).

4.4. CONCLUSIONS

This study is the first providing valuable information on the effect of specific rice crop management on the expression of plant oxylipins (phytoprostanes and phytofurans), in a specific way, in diverse genotypes and advanced lines when applying foliar fertilization. As expected, and given the minimum doses applied, the effect of the treatments was majorly reflected by modifications of the levels of these compounds than by changes in productivity parameters, such as yield and grain quality. Eventually, this practice can induce a reduction of plants stress in fertilized samples, as demonstrated the generation of ROS inducing non-enzymatically production of plant oxylipins was lower compared with non-treated plants. It would be possible that obtaining deep knowledge on mineral nutrients metabolism and its effect on oxidative stress allow getting rice varieties featured by enhanced tolerance to nutritional stress or with better efficiency in the response to agricultural practices, such as foliar fertilization. Agronomic biofortification, grain enrichment with micronutrients in crop cereal, along with breeding/genetic engineering, would be a useful strategy to solve health problems related to the minerals deficiency in humans, without affect detrimentally the environment. The fertilizer strategy could be a quick solution to the problem of easy application and affordable in low-income populations and can be considered an important complementary approach for long-term improvement programs.

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CHAPTER V. IMPACT ON PHYTOPROSTANE AND PHYTOFURAN (STRESS BIOMARKERS) OF CONCENTRATION SALICYLIC ACID AND GROWING ENVIRONMENT ON *Oryza sativa* L.

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5.1. INTRODUCTION

Rice (*Oryza sativa* L.) is a tropical or subtropical crop featured by high adaptability to diverse environmental conditions (Arguissain, 2006). High temperature impairs grain filling, leading to loss of yield for crops, such as rice through decreases in grain size, number, and quality. Thus, considering recent climate change (global warming), an understanding of the physiological processes responsible for these effects deserve to be further evaluated (Yamakawa and Hakata, 2010). In this regard, studies on *japonica* varieties of rice developed in Japan have provided information on the optimum air temperature for ripening that ranges between 20 and 25 °C (Yamakawa *et al.,* 2007; Yamakawa and Hakata, 2010), while air temperature above 30 °C can cause deleterious effects on yield and nutritional quality, as well as to modify physicochemical features of the grain (De Datta, 1981; Wu *et al*., 2016). Indeed, in the last decades, it has been described varietal differences regarding the sensitivity of rice plants to high temperatures (De Datta, 1981).

Under normal physiological conditions, reactive oxygen species (ROS) are continuously produced in plants as a consequence of the cellular metabolism, especially in the frame of the photosynthetic process (Holuigue *et al*., 2007). Most of these stress conditions either increase light-driven electron transport or restrict CO_2 availability, augmenting ROS production in the chloroplast, associated to the Photosystems I and II, as well as in peroxisomes, associated to photorespiration (Holuigue *et al*., 2007). These radicals are very unstable and react rapidly with other groups or substances leading to cell or tissue injury. One of the main targets of the attack of free radicals are the membrane lipids, which results in the initiation of the enzymatic lipid peroxidation (Anderson *et al*., 1998). In many plant species, it is known that there is a non-enzymatic pathway through which ROS react, fundamentally, with α-linolenic acid (ALA) in cell membranes, giving rise to phytoprostanes (PhytoPs). Apart from PhytoPs, under higher oxygen tension, a new structure of compounds referred as phytofurans (PhytoFs) is generated, upon additional oxidation after initial cyclization (Cuyamendous *et al*., 2015).

Salicylic acid (SA) is a plant hormone that participates in various processes: germination, cell growth, floral induction, absorption of ions, respiration, rate of transpiration, net photosynthesis, stomatal conductance, senescence, response to abiotic stress, and essential in thermogenesis, as well as in resistance to diseases (Hayat *et al*., 2010). The SA seems induce plant responses to stress situations upon an array of mechanisms. The idea of interplay between SA and ROS, and the influence of such interaction in the stress defense reactions was firstly proposed more than 25 years ago (Chen *et al*., 1993). In the recent years, the most accepted hypothesis on the role of SA in plants exposed to stressing factors is that this compound is essential for modulating the redox balance, thus protecting plants from oxidative stress (Holuigue *et al*., 2007). The treatment of SA also minimizes the level of reactive compounds (H_2O_2 and $O_2^{\cdot-}$) in rice, providing additional resistance against the oxidative stress generated by exposure to cadmium to plants. Hence, upon the work developed by Hayat *et al.* (2010), it was demonstrated a decrease in the activities of the enzymes catalase, peroxidase, superoxide dismutase, and glutathione reductase in plants treated with SA (Hayat *et al*., 2010).

The literature contains evidences indicating that SA, together with ROS, which accumulate in stressed cells, are essential signaling molecules involved in triggering local defense responses or activating transcription of defense genes involved in the systemic stress response. Indeed, studies with transgenic materials have allowed to notice that SA induces the expression of pathogenesis related (PR-1) genes, responsible for an enhance resistance to disease, while regulates the expression of TOP2 gene, which encodes for topoisomerase II. This is closely related to the plant resistance against abiotic stress (Janda *et al*., 2007).

This research seeks to deepen the current understanding of the interrelation of environments and hormonal factors with the qualitative and quantitative presence of PhytoPs and PhytoFs, resorting to UHPLC-ESI-QqQ-MS/MS in seven genotypes belonging to the *japonica* rice variety from the rice breeding program of the Agriculture and Forestry Sciences Faculty, of the National University “de la Plata” (Argentina), which are featured by excellent agronomic features within a small group of genotypes of the commercial type length-width.

5.2. MATERIAL AND METHODS

5.2.1. Chemical and reagents

The PhytoPs 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; *ent*-9-epi-9-D_{1t}-PhytoP; 16-B_1-PhytoP; 9-L_1-PhytoP; and 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 procedures described in the literature (El Fangour *et al.,* 2004; Oger, Brinkmann, Bouazzaoui, Durand, & Galano, 2008; Pinot *et al*., 2008; El Fangour, Guy, Vidal, Rossi, & Durand, 2005; Guy, Flanagan, Durand, Oger, & Galano, 2015; Cuyamendous *et al.,* 2015; Cuyamendous *et al*., 2017). Hexane was obtained from Panreac (Castellar del Valles, Barcelona, Spain), butylated hydroxyanisole (BHA), bis–Tris (bis (2-hydroxyethyl) amino-tris (hydroxymethyl) methane), and salicylic acid (SA) were purchased from Sigma–Aldrich (St. Louis, MO, USA) and all LC–MS grade solvents, methanol and acetonitrile were from J.T. Baker (Phillipsburg, NJ, USA). Water was treated in a milli-Q water purification system from Millipore (Bedford, MA, USA). The Strata solid-phase extraction (SPE) cartridges used (Strata X-AW, 100 mg/3 mL) were acquired from Phenomenex (Torrance, CA, USA).

5.2.2. Plant samples and crop management

The field trial was set up in the Experimental Station 'Julio Hirschhorn' located in La Plata (34º52'S, 57º57'W, 9.8 m of altitude, Buenos Aires, Argentina). This field experiment included the widespread variety in the producing area 'Yerua PA' (Yerua), and the advanced lines 'R/03-5x desc/04-52-1-1' (L1), 'R/03-5x desc/04-45-1-1'(L2), 'Amaroo x desc /08-1-1-1-2' (L3), 'R/03-5x desc/04-27-3-1' (L4), 'R/03-5x desc/14-1-1-1' (L5), and 'H489-5-1-2' (L6), all them belonging to the subspecie *japonica*. The rice varieties were sourced by the Rice Breeding Program of the Agriculture and Forestry Sciences Faculty, of the National University "de la Plata". The experimental design included randomized blocks with 3 repetitions. Dry-land seeding was done manually at a rate of 350 seeds m^{-2} in 0.20 m lines within plots of 5 m^2. The assay was conducted applying flood irrigation for 30 days after emergence. Weeds were controlled with bispiribac sodium. Three experimental conditions were included in the assay; no SA application (SA0, control condition), application of 1 mM SA (SA1), and application of 15 mM SA (SA15). These treatements were sprayed 10 and 17 days after anthesis. The SA solutions were prepared in distilled water with 50 ppm Tween-20 surfactant. The plots were sprayed at late afternoon hours when wind speed was less than 10 km hr^{-1}.

Additionally, to SA applications, the arable areas evaluated were crop covered with plastic of 100 µm thickness, between the second SA application and harvest (EP) or as open crop (E). Temperature values were recorded by an XR440 Pocket TM data logger (Pace Scientific Inc. Charlotte, NC, USA) every 30 minutes during the ripening. Two temperature sensors (PT940, Pace Scientific Inc) were located in the canopy, at the panicles height (0.90 m of the ground) in both EP and E crop systems.

Each rice variety samples were collected and threshers manually at maturity according to standard of industrial processes. The kernels were dried in an oven at 40 °C, until 14.0% de humidity. Paddy grain samples (100 g) were flaked to obtain brown rice. Brown rice flours were obtained by milling in a Cyclone Mill and passing the powder through 0.4 mm mesh; afterwards samples were stored at 4 °C, protected from light, until analysis.

5.2.3. Extraction of phytoprostanes and phytofurans

The PhytoPs and PhytoFs present in the rice flours were extracted following the methodology described by Pinciroli *et al.* (2017). The extracts obtained were further processed upon SPE, using a Chromabond C18 column according to the procedure previously described (Collado-González *et al*., 2015b). PhytoPs and PhytoFs were eluted with 1 mL of methanol and dried using a SpeedVac concentrator (Savant SPD121P, Thermo Scientific, MA, USA). The dry extracts were reconstituted with 200 microliters of A-solvent/B-solvent (50:50, *v*/*v*), being solvent A milliQ-water/acetic acid (99.99:0.01, *v*/*v*) and solvent B methanol/acetic acid (99.99:0.01, *v*/*v*). Once reconstituted, samples were filtered through a 0.45-µm filter (Millipore, MA USA) and immediately analyzed by UHPLC-ESI-QqQ-MS/MS (Agilent Technologies, Waldbronn, Germany).

The quantification of PhytoPs and PhytoFs detected in rice grain and flour was performed using freshly standard curves of authentic standards. Stock solutions were prepared in milliQ-water/methanol (50:50, *v*/*v*) to facilitate the ionization in the mass spectrometer.

5.2.4. UHPLC-ESI-QqQ-MS/MS analysis

Chromatographic separation of PhytoPs and PhytoFs was performed using a UHPLC coupled with a 6460 triple quadrupole-MS/MS (Agilent Technologies, Waldbronn, Germany), using the analytical column BEH C18 (2.1 x 50 mm, 1.7 µm) (Waters, Milford, M.A.). The column temperatures were 6 ºC (both left and right). The mobile phases consisted of milliQ-water/acetic acid (99.99:0.01, *v*/*v*) (A) and methanol/acetic acid (99.99:0.01, *v*/*v*) (B). 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 for column equilibration. The spectrometric analysis was conducted in multiple reaction monitoring (MRM), operated in negative mode, assigning quantification and confirmation MRM transition for each analyte, (**Table 15**), which were presviously reported by Collado-González *et al*. (2015) and Domínguez-Perles *et al*. (2018).

Table 15. UHPLC/MS/MS parameters for the quantification and confirmation of phytoprostanes and phytofurans in rice.

	Compound	Retention time (min)	ESI mode	MRM transition (*m/z*)
Phytoprostanes				
PP1[Z]	9-F_1t-PhytoPX	1.631	Negative	327.2 > 273.1Y
			Negative	327.2 > 171.0X
PP2	*Ent*-16-F_1t-PhytoP	1.712	Negative	327.2 > 283.2
			Negative	327.2 > 225.1
PP2	*Ent*-16-epi-16-F_1t-PhytoP[X]	1.583	Negative	327.1 > 283.2Z
			Negative	327.1 > 225.1Y
PP3	9-*epi*-9-F_1t-PhytoP	1.785	Negative	327.2 > 272.8
			Negative	327.2 > 171.0
PP4	*Ent*-9-*epi*-9-D_1t-PhytoP	2.022	Negative	325.2 > 307.2
			Negative	325.2 > 134.9
PP5	*Ent*-9-D_1t-PhytoP	1.791	Negative	325.2 > 307.3
			Negative	325.2 > 134.7
PP6	16-B_1-PhytoP	2.620	Negative	307.2 > 223.2
			Negative	307.2 > 235.1
PP7	9-L_1-PhytoP	3.079	Negative	307.2 > 185.1
			Negative	307.2 > 196.7
Phytofurans				
PF1	*Ent*-16*(RS)*-9-*epi*-ST-Δ^{14}-10-PhytoF	1.501	Negative	343.9 > 209.0
			Negative	343.9 > 201.1
PF2	*Ent*-9*(RS)*-12-*epi*-ST-Δ^{10}-*13-PhytoF*	0.906	Negative	344.0 > 300.0
			Negative	344.0 > 255.9
PF3	*Ent*-16*(RS)*-13-*epi*-ST-Δ^{14}-9-PhytoF	1.523	Negative	343.0 > 171.1
			Negative	343.0 > 97.2

[Z] PP1, 9-F_{1t}-PhytoP; PP2, *ent*-16-F_{1t}-PhytoP + *ent*-16-*epi*-16-F_{1t}-PhytoP; PP3, 9-*epi*-9-F_{1t}-PhytoP; PP4, ent-9-*epi*-9-D_{1t}-PhytoP; PP5, *ent*-9-D_{1t}-PhytoP; PP6, 16-B_1-PhytoP; PP7, 9-L_1-PhytoP, PF1, *ent*-16(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF; PF2, *ent*-9(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF; and PF3, *ent*-16(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF. Quantification transition. [Y] confirmation transition. [X] coeluting diasteroisomers quantified together.

The ionization and fragmentation conditions were: 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 product ions. Data acquisition and processing were performed using Mass Hunter software version B.04.00 (Agilent Technologies).

5.2.5. Statistical analysis

All extractions were performed in triplicate (n=3) and data were expressed as means ± SD. All statistical tests were performed at a 5% significance level using Statgraphics Centurion XVI (StatPoint Technologies Inc., Warrenton, VA, USA). All data were subjected to one-way analysis of variance (ANOVA). When statistical differences were identified, variables were compared using multiple range tests (Tukey's test).

5.3. RESULTS AND DISCUSSION

To evaluate the effect of the environmental conditions and SA spraying on the stress level of plants of different rice genotypes upon the modification of the concentration of PhytoPs and PhytoFs, a field trial was set up in an experimental field in the province of Buenos Aires, in the central-east Argentina, which included the genotypes 'Yerua PA', 'R/03-5x desc/04-52-1-1', 'R/03-5x desc/04-45-1-1', 'Amaroo x desc /08-1-1-1-2', 'R/03-5x desc/04-27-3-1', 'R/03-5x desc/14-1-1-1', and 'H489-5-1-2', all them belonging to the subspecies *japonica*. The average air temperature of the canopy, during ripening in the two environments (EP, covered with plastic and E, open field), were 20.3 °C (range 9.4-44.0 °C) and 21.8 °C (range 9.4-44.0 °C), respectively (**Figure 18**). The optimum temperatures for the rice during the ripening they oscillate between 20 and 29 ºC (De Datta, 1981). Thus, given the temperature conditions recorded during the development of the field trial, the crops set up showed a normal growth.

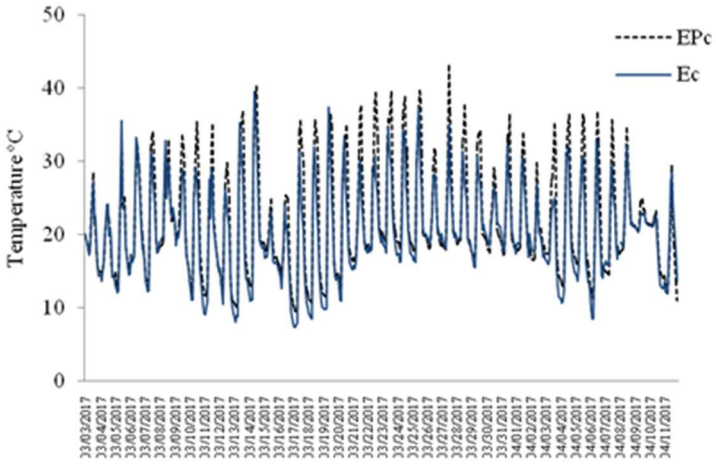

Figure 18. Air temperature (Ta) of canopy during ripening (registered with datalogger XR440 Pocket Logger ™, Pace Scientific Inc). **EPc**: Air temperature recorded by sensor under plastic cover, **Ec**: Air temperature recorded by sensor located in the open field.

5.3.1. Occurrence of total and individual phytoprostanes and phytofurans in diverse genotypes of rice

In respect to PhytoPs, the relative abundance of total compounds in the genotypes studied evidenced a decrease of the concentration of these compounds as follows: 'Yerua' (1713.2 ng g^{-1} dw) > 'R/03-5x desc/04-52-1-1' (L1) (1453.2 ng g^{-1} dw) > 'H489-5-1-2' (L6) (425.9 ng g^{-1} dw) > 'R/03-5x desc/04-45-1-1' (L2) (420.4 ng g^{-1} dw) > 'R/03-5x desc/04-27-3-1' (L4) (368.0 ng g^{-1} dw) > 'R/03-5x desc/14-1-1-1' (L5) (257.6 ng g^{-1} dw) > 'Amaroo x desc /08-1-1-1-2' (L3) (180.2 ng g^{-1} dw).

These concentrations were lower than those observed in different rice genotypes belonging to the subspecies *indica*, as they ranged between 509.3 and 309.2 ng g^{-1} dw. However, other authors have found modifications in the concentration of total PhytoPs according to the variety. In this regard, Barbosa *et al*. (2015), upon a study including 24 species of *macroalgae*, found the highest values in the species *Saccharina latissima* and the lowest in *Asparagopsis armata* (13.8 and 0.1 ng g^{-1} dw, respectively). Additionally, when studying 11 diverse almond cultivars, grown under rain-fed and conventional conditions, Carrasco-Del Amor *et al*. (2015) described maximum values in the cv. 'Blanqueta' and minimus in the cv. 'Garriges', with values of 238.1 and 40.3 ng g^{-1}, respectively. Finally, in 2017, Pinciroli *et al*. studied 14 genotypes of rice, revealing the highest concentration of total PhytoPs in the subespiecies 'Itape' (variety *japonica*), with values of 9.2, 32.9, and 67.1 ng g^{-1} for white grain flour, brown grain flour, and bran flour, respectively. In this work, upon profiling PhytoPs in rice flour from the seven varieties under study, independently on the growing environmental condition (open field or plastic cover) and salicylic treatment, it was observed the following decreasing average concentration of individual PhytoPs: *ent*-16-F_{1t}-PhytoP + *ent*-16-*epi*-16-F_{1t}-PhytoP (PP2) (333.8 ng g^{-1} dw, on average) > 9-F_{1t}-PhytoP (PP1) (172.5 ng g^{-1} dw, on average) > 9-*epi*-9-F_{1t}-PhytoP (PP3) (164.0 ng g^{-1} dw, on average) > *ent*-9-D_{1t}-PhytoP (PP5) (30.5 ng g^{-1} dw, on average) > 16-B_1-PhytoP (PP6) (6.4 ng g^{-1} dw, on average) > 9-L_1-PhytoP (PP7) (4.5 ng g^{-1} dw, on average) > *ent*-9-*epi*-9-D_{1t}-PhytoP (PP4) (4.1 ng g^{-1} dw, on average) (**Table 16**). Indeed, this trend was also observed when evaluating the isolated impact of the salicylic acid treatments on the concentration of these plant oxylipins (**Figure 19**).

Table 16. Average values of each phytoprostane and phytofuran in rice (*Oryza sativa* L.) genotypes.

Genotype	Phytoprostanes							Phytofurans		
	PP1 [y]	PP2	PP3	PP4	PP5	PP6	PP7	PF1	PF2	PF3
'Yerua'	353.3 ± 33.3 a	803.8 ± 67.3 a	445.1 ± 73.5 a	6.3 ± 0.7 a	75.3 ± 9.6 a	17.7 ± 1.3 a	11.8 ± 0.8 a	21.0 ± 3.2 a	5.2 ± 0.5 a	12.0 ± 1.3 a
L1	238.0 ± 25.5 b	732.3 ± 65.0 a	396.7 ± 34.8 a	5.8 ± 0.8 b	54.0 ± 4.1 a	9.5 ± 1.1 b	7.1 ± 0.5 b	11.9 ± 1.3 b	3.3 ± 0.3 b	6.9 ± 0.5 b
L2	91.9 ± 10.8 c	222.6 ± 27.6 b	70.6 ± 7.4 b	4.1 ± 0.4 c	23.1 ± 1.3 c	4.6 ± 0.4 c	3.6 ± 0.2 c	9.4 ± 0.7 c	2.1 ± 0.3 c	5.4 ± 0.2 c
L3	70.4 ± 13.1 c	58.6 ± 7.8 c	31.9 ± 5.1 b	2.5 ± 0.3 d	11.4 ± 1.1 c	3.4 ± 0.6 c	2.0 ± 0.2 e	6.8 ± 1.1 d	1.7 ± 0.3 c	3.7 ± 0.4 d
L4	43.2 ± 16.1 c	186.2 ± 36.9 cb	84.8 ± 27.7 b	3.0 ± 0.4 d	15.8 ± 2.7 c	2.9 ± 0.6 c	2.3 ± 0.3 de	5.2 ± 0.8 d	1.5 ± 0.2 c	2.8 ± 0.3 de
L5	71.8 ± 18.8 c	124.5 ± 17.4 cb	40.4 ± 6.9 b	3.4 ± 0.4 cd	13.4 ± 1.6 c	2.4 ± 0.3 c	1.7 ± 0.2 e	4.9 ± 0.8 d	1.4 ± 0.2 c	2.3 ± 0.3 e
L6	107.3 ± 20.7 c	209.0 ± 23.8 b	78.5 ± 3.5 b	3.9 ± 0.4 c	20.2 ± 2.2 c	4.0 ± 0.4 c	3.2 ± 0.2 cd	8.9 ± 0.6 c	2.1 ± 0.2 c	5.1 ± 0.2 c
p-value (ANOVA)	***	***	***	*	***	*	***	*	*	***

[y] PP1, 9-F_{1t}-PhytoP; PP2, *ent*-16-F_{1t}-PhytoP + *ent*-16-*epi*-16-F_{1t}-PhytoP; PP3, 9-*epi*-9-F_{1t}-PhytoP; PP4, *ent*-9-*epi*-9-D_{1t}-PhytoP; PP5, *ent*-9-D_{1t}-PhytoP; PP6, 16-B_1-PhytoP; PP7, 9-L_1-PhytoP, PF1, *ent*-16(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF; PF2, *ent*-9(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF; and PF3, *ent*-10.66(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF. [z] Means ± SEM (n=3) for average data from all genotypes within a column followed by distinct letters are significantly different at $p<0.05$ according to the Tukey's multiple range test. [y] N.s., no significant, $p<0.05$(*), $p<0.01$(**), and $p<0.001$(***).

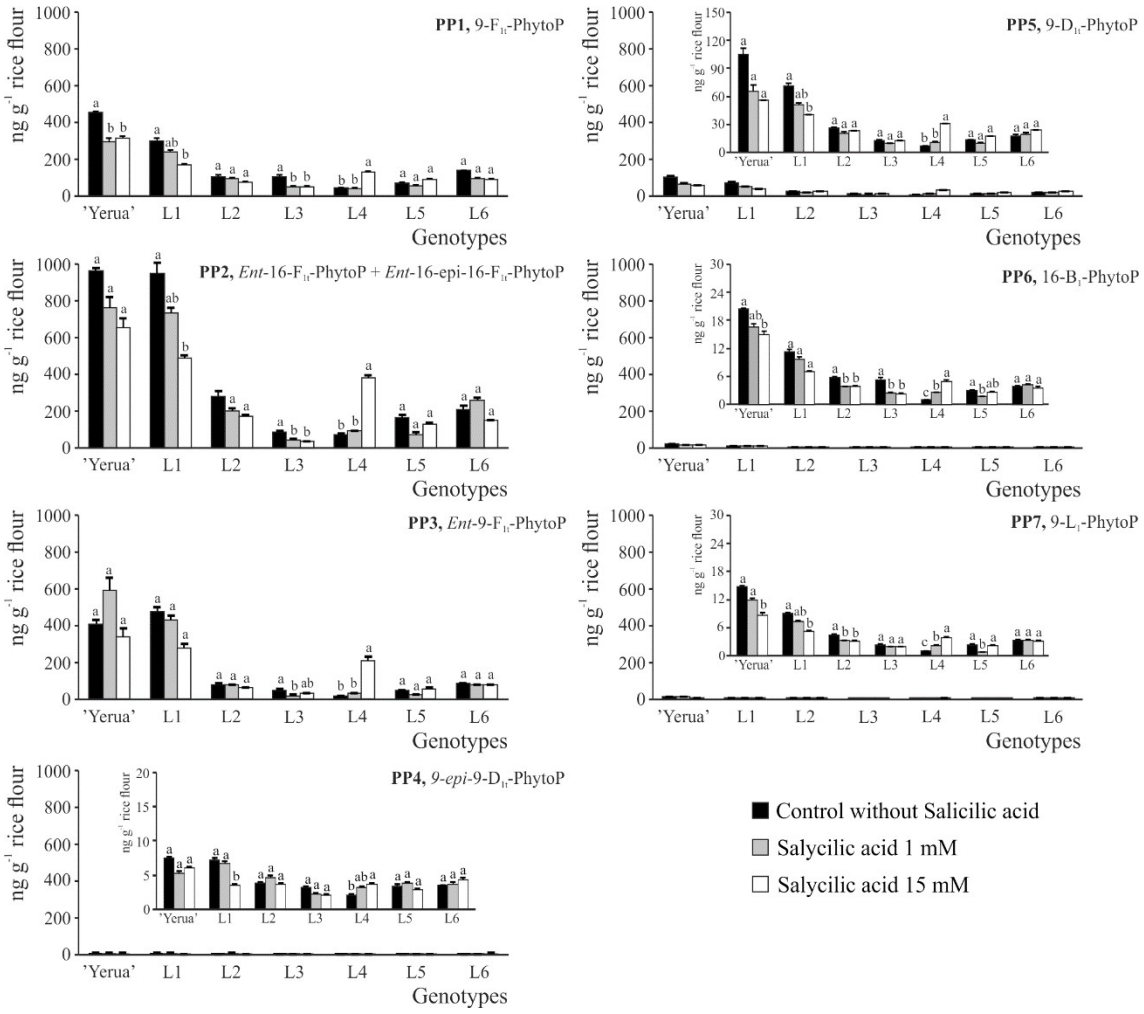

Figure 19. Comparison between different of salicylic acid suplementation in the seven varieties of rice Yerua PA' (Yerua), 'R/03-5x desc/04-52-1-1' (L1), 'R/03-5x desc/04-45-1-1'(L2), 'Amaroo x desc /08-1-1-1-2' (L3), 'R/03-5x desc/04-27-3-1' (L4), 'R/03-5x desc/14-1-1-1' (L5), and 'H489-5-1-2' (L6) for the PhytoPs 9-F_{1t}-PhytoP (PP1), *Ent*-16-F_{1t}-PhytoP + *Ent*-16-*epi*-16-F_{1t}-PhytoP (PP2), 9-*epi*-9-F_{1t}-PhytoP (PP3), *ent*-9-*epi*-9-D_{1t}-PhytoP (PP4), *ent*-9-D_{1t}-PhytoP (PP5), 16-B_1-PhytoP (PP6), and 9-L_1-PhytoP (PP7). Different lowercase letters indicate significant differences between SA suplementation for the same genotype at $p<0.05$, according to the analysis of variance (ANOVA) and the multiple range test of Tukey.

When comparing the the abundance of the separate PhytoPs in the variety 'Yerua' and the six advanced genotypes evidenced that, the overall analysis, developed independently on the environmental and salicylic acid treatments effects, evidenced that, on average, 'Yerua' displayed the highest concentration 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-*epi*-9-D_{1t}-PhytoP, *ent*-9-D_{1t}-PhytoP, 16-B_1-PhytoP, and 9-L_1-PhytoP (353.3, 803.8, 445.1, 6.3, 75.3, 17.7, and 11.8 ng g^{-1} dw), on average, respectively), surpassing the variety that exhibited the second higher concentration, the advanced genotype L1, from 7.9 to 46.3%, and the advanced genotypes L2 to L6, which remained in similar lower levels, from 46.3 to 86.2% (**Table 16**).

The comparison of the results obtained with other plant foods suggests that F_1-PhytoPs series is also the most abundant in dry melon leaves Sweet Ball cv. Early Spring cv. (2740.0 and 2340.0 ng g^{-1}, on average, repectively) (Yonny *et al.,* 2016), in aged wine and grape musts (210.7 and 18.0 ng mL^{-1}, on average, respectively) (Marhuenda *et al*., 2015), in olive oil (8.1, on average) (Collado-González *et al*., 2015c), in almonds (90.6 ng g^{-1}, on average) (Carrasco-Del Amor *et al*., 2015), and in *macroalgae* (2.7 ng g^{-1}, on average) (Barbosa *et al*., 2015).

Apart from PhytoPs, the evaluation of the array of rice grains on the occurrence of plant oxylipins was also focused on PhytoFs. The average concentration of the individual PhytoFs in the separate genotypes of rice evidenced, the following decreasing levels: *ent*-16(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF (PF1) (9.7 ng g^{-1} dw) > *ent*-16(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF (PF3) (5.5 ng g^{-1} dw) > *ent*-9(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF (PF2) (2.5 ng g^{-1} dw) (**Figure 20**). These values matched with that reported by Pinciroli *et al*. (2017) in an array of diverse rice flours obtained from varieties belonging to *japonica* and *indica* subspecies. Again, when comparing the concentration of PhytoFs in the range of rice grain under study, it was noticed 'Yerua' was featured by the highest content of *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 (21.0, 5.2, and 12.0 ng g^{-1} dw, respectively), followed by the advanced genotypes L1, L2, and L6 (10.1, 2.5, and 5.8 ng g^{-1} dw, on average, respectively). The remaining genotypes L3, L4, and L5 were found in similar lower levels with average the conectrations 5.6, 1.5, and 2.9, ng g^{-1} dw, respectively (**Table 16**).

In all cases, significant differences between genotypes were revealed (**Table 16**). These results are in agreement with those reported by a range of authors, who argue that there are genetic varietal differences in response to high critical temperatures (De Datta, 1981, Enyedi e*t al.*, 1992), according to their ability to regulate redox homeostasis in cells.

Pinciroli *et al.* (2017) upon the study of diverse genotypes of rice discerned the lower concentration of total PhytoFs, on average, of the 14 genotypes 0.7, 4.4, and 10.7 ng g^{-1} dw, regarding white grain flour, brown grain flour, and bran flour. Furthermore, PhytoFs have been also studied in few additional plant matrices. In this regard, Cuyamendous *et al*. (2016) reported the occurrence of these compounds in pine, nuts, chia, and flaxseeds (0.30, 9.00, 6.00, and 0.70 ng g^{-1}, respectively) and Yonny *et al.* (2016) described their occurrence in leaves of melon in which they are found at the concentration 2355.0 ng g^{-1} dw.

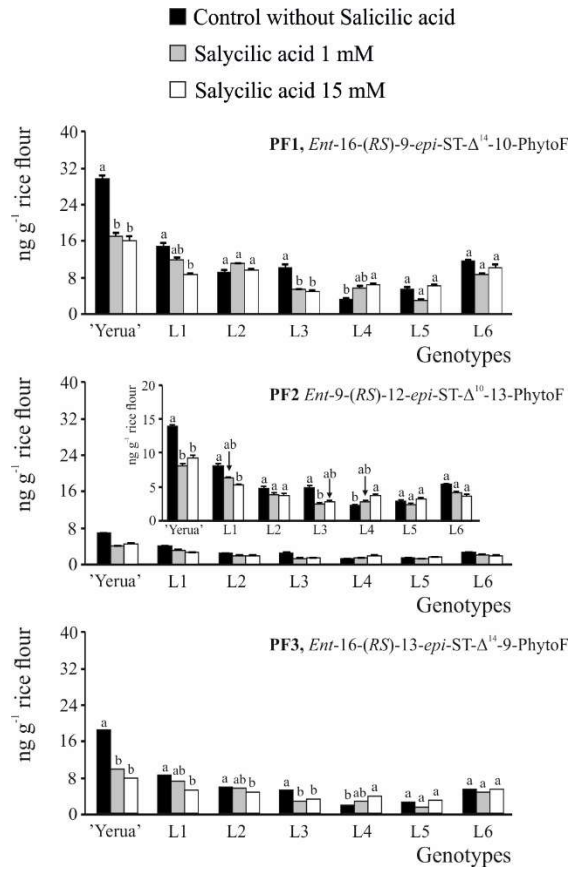

Figure 20. Comparison between different of salicylic acid suplementation in the seven varieties of rice Yerua PA' (Yerua), 'R/03-5x desc/04-52-1-1' (L1), 'R/03-5x desc/04-45-1-1'(L2), 'Amaroo x desc /08-1-1-1-2' (L3), 'R/03-5x desc/04-27-3-1' (L4), 'R/03-5x desc/14-1-1-1' (L5), and 'H489-5-1-2' (L6) for the PhytoFs *ent*-16(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF (PF1), *ent*-9(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF (PF2), and *ent*-16(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF (PF3). Different lowercase letters indicate significant differences between SA suplementation for the same genotype at $p<0.05$, according to the analysis of variance (ANOVA) and the multiple range test of Tukey.

5.3.2. Environmental effect on the level of phytoprostanes and phytofurans in rice

The average temperatures during ripening were 20.3 and 21.8 °C in the E and EP systems, respectively, while the results retrieved revealed plastic covered providing less stressful growing conditions to the genotypes assessed. Rice is a tropical or subtropical species that in the experimental procedure described in the present work was grown under suboptimal conditions, in terms of thermic supply. The warm environment provided by the plastic cover was found favorable for the development of all genotypes. Heat stress is often considered an increase in temperature beyond a threshold level for a period of time that induces irreversible damage to plant growth (Le Gall *et al*., 2015). Previous studies carried out by Yoshida and Hara (1977) and Yamakawa *et al*. (2007) demonstrated that optimal air temperature for rice ripening, in plants belonging to *japonica* varieties, ranges from 20 to 25 °C, while higher or lower temperatures

may reduce significantly grain yield. Hence, so that damages by high temperatures turn out to be severe, these must last in the time. For instance, a period of 20 days with temperatures above 27 °C, during ripening, seems to be enough to decrease grain production, grain weight, and protein content, as well as to augment chalkiness (Wakamatsu *et al*., 2007). On the other hand, 15 days at 30 °C has been demonstrated as a growing environmental condition capable to reduce significantly rice yield and appearance quality, while change grain shape (Wu *et al.,* 2016). Besides, higher temperatures (35 °C for 5 days) cause damage in the phenological phases and infertility of the spikelets in rice plants (Yoshida and Hara, 1977). In our study, high temperatures were recorded during an 8 and 6 hours in EP and E environments, respectively (**Figure 18**). These conditions caused no harmful effect, contrary to what was expected accordingly to the information available in the literature.

When evaluating the differential response of rice genotypes to the environment in crops set up under plastic cover (EP) or open field (E) conditions, concerning the level of individual PhytoPs and PhytoFs, it was found that, overall, EP growing rice reduced significantly (p<0.05) the concentration of most of the stress biomarkers analyzed (by 3.9%, on average). This decrease was statistically significant for 9-F_{1t}-PhytoP (from 153.2 to 133.9 ng g^{-1} dw (a 12.7% lower)) and *ent*-9(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF (from 2.6 to 2.3 ng g^{-1} dw (a 10.4% lower)) (**Table 17**). In this regard, the reduction in the photosynthetically active radiation (PAR) in EP was approximately 76% with respect to E (Data not shown). It is possible that the electron transport chain was not saturated with light; therefore, a lower amount of ROS was produced, and consequently lower content of plant oxylipins. This result matchs with the behavior of the content of melatonin in most pepper cultivars observed by Riga *et al*. (2014). In addition, in the work carried out by Yonny *et al.*, (2016) on melon leaves, the compound that showed the most significant changes of concentration as a consequence of different environments was 9-F_1-PhytoP. In this study, the plastic cover raised temperatures to values of ranging between 35 and 45 °C, inducing severe heat stress and the subsequent increase of the PhytoPs and PhytoFs concentrations. Possibly the augment of ROS due to the growing temperatures caused greater lipid peroxidation of ALA and thus, a proportional change of the level of cyclic metabolites.

Table 17. Average values of each phytoprostane and phytofuran in all rice genotypes grown in open field and under plastic cover, and exposed to 0, 1 and 15 mM salicylic acid (SA0, SA1, and SA15, respectively).

Factors	Phytoprostanes							Phytofurans		
	PP1 [y]	PP2	PP3	PP4	PP5	PP6	PP7	PF1	PF2	PF3
Environments										
Open field (E)	153.5 ± 27.8 [z]	335.2 ± 74.1	168.0 ± 51.5	4.2 ± 0.5	30.4 ± 5.6	6.2 ± 1.3	4.5 ± 0.6	10.3 ± 1.3	2.6 ± 0.4	5.5 ± 0.7
Plastic cover (EP)	133.9 ± 23.2	332.5 ± 68.3	159.9 ± 40.8	4.0 ± 0.4	30.5 ± 7.0	6.5 ± 1.2	4.5 ± 0.8	9.6 ± 1.5	2.3 ± 0.3	5.4 ± 1.0
p-value (*t*-test)	**	N.s.	N.s.	N.s.	N.s.	N.s.	N.s.	N.s.	*	N.s.
Salicylic acid treatment										
SA0	174.7 ± 20.4 a	396.0 ± 57.8 a	165.4 ± 24.4 a	4.4 ± 0.3 a	35.8 ± 4.1 a	7.3 ± 0.9 a	5.3 ± 0.6 a	12.0 ± 1.0 a	3.0 ± 0.3 a	6.8 ± 0.6 a
SA1	124.9 ± 17.1 b	314.2 ± 50.6 b	176.6 ± 43.9 a	4.2 ± 0.3 a	26.6 ± 4.2 b	5.9 ± 0.8 b	4.3 ± 0.6 b	9.0 ± 0.7 b	2.1 ± 0.2 b	4.9 ± 0.4 b
SA15	131.5 ± 12.3 b	291.5 ± 27.5 b	150.0 ± 14.4 a	3.7 ± 0.2 a	28.9 ± 1.9 b	5.7 ± 0.5 b	4.0 ± 0.3 b	8.8 ± 0.7 b	2.3 ± 0.2 b	4.7 ± 0.3 b
p-value (ANOVA)	**	**	N.s.	N.s.	**	**	**	**	**	**

[y] PP1, 9-F_{1t}-PhytoP; PP2, *ent*-16-F_{1t}-PhytoP + *ent*-16-*epi*-16-F_{1t}-PhytoP; PP3, 9-*epi*-9-F_{1t}-PhytoP; PP4, *ent*-9-*epi*-9-D_{1t}-PhytoP; PP5, *ent*-9-D_{1t}-PhytoP; PP6, 16-B_1-PhytoP; PP7, 9-L_1-PhytoP, PF1, *ent*-16(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF; PF2, *ent*-9(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF; and PF3, *ent*-16(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF. [z] Means ± SEM (n=3) for average data from all genotypes and growing envrionment within a column followed by distinct letters (for SA treatments) are significantly different at $p<0.05$ according to the Tukey's multiple range test. The effect of growing environments on the level of individual phytoprostanes and phytofurans were compared for average data from all genotypes and salicylic acid treatments by paired *t*-test. [y] N.s., no significant, $p<0.05$(*), and $p<0.01$(**).

5.3.3. Effect of supplementation with salicylic acid on phytoprostane and phytofuran levels in rice

When analyzing the impact of the treatment with two levels of salicylic acid (1 and 15 mM (SA1 and SA15, respectively)) of rice crops set up on the variety 'Yerua' and the six advanced genotypes (lines L1 to L6) on the content of total PhytoPs and PhytoFs of rice grains, significant effects were identified concerning almost all PhytoPs and PhytoFs. In general, a decreasing concentration of the biomarkers monitored was observed due to the exogenous application of SA (SA1 and SA15), relatively to the control (SA0). The concentration of 9-*epi*-9-F_{1t}-PhytoP and *ent*-9-*epi*-9-D_{1t}-PhytoP did not change significantly due to SA supplementation (**Table 17**), while 9-F_{1t}-PhytoP, *ent*-16-F_{1t}-PhytoP + *ent*-16-*epi*-16-F_{1t}-PhytoP, *ent*-9-D_{1t}-PhytoP, 16-B_1-PhytoP, and 9-L_1-PhytoP, as well as the 3 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 lowered significantly after supplementing with SA at both dosages (SA1 and SA15) (from 174.7 to 131.5, from 396.0 to 291.5, from 35.8 to 28.9, from 7.63 to 5.7, from 5.3 to 4.0, from 12.0 to 8.8, from 3.0 to 2.3, and from 6.8 to 4.7 ng g^{-1} dw, respectively), without presenting differences one to another (**Table 17**). This result suggests that even at low doses, the exogenous application of SA (with hormonal functions in the frame of plant physiology) is featured by strong radical scavenging capacity. Indeed, SA plays a crucial role in the control of cellular redox homeostasis in plants (Holuigue *et al*., 2007). Regarding this, Clarke *et al*. (2004) demonstrated that SA-dependent signaling plays a role in the maintenance of basal thermotolerance. Additionally, both authors observed lower electrolyte leakage after heat stress, together with the induction of pathogenesis-related proteins in 3-week-old *Arabidopsis* leaves with SA applications. This fact was further supported by studies conducted on *Cicer arietinum* plants, upon which was revealed that the application of SA significantly reduced the cell membrane lesions induced by heat stress (Hayat *et al*., 2010).

Although heat stress was not at extreme values in the experimental work developed, the decrease of the level of PhytoPs and PhytoFs upon the application of SA suggests that this could improve the adaptation of rice plants to augmented levels of ROS and leakage of electrolytes generated during temperature increase or excesses of radiation. This may allow a modular redox balance and thus, protect plants from oxidative stress. Hence, according to Hayat *et al*. (2010), SA effectively alleviated the toxic effects generated in plants as a consecuence of the exposition to abiotic stress factors.

The concentration of the compounds 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 *ent*-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF responded

differentially, depending on the growing conditions, being revealed as compounds with significant sensitivity to the interaction *environments x SA* (**Table 18**). Hence, it was noticed a decrease of the concentration of the PhytoPs 9-F_{1t}-PhytoP, *Ent*-16-F_{1t}-PhytoP + *Ent*-16-*epi*-16-F_{1t}-PhytoP, 9-epi-9-PhytoP, and 9-D_1-PhytoP, and the PhytoF *ent*-16(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF, upon the interaction between the application of both 1 and 15 mM SA and the growing environments (EP and E). In addition, interactions between environment and genotype, for 9-F_{1t}-PhytoP, *ent*-9-*epi*-9-D_{1t}-PhytoP, 16-B_1-PhytoP, *ent*-16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF, and *ent*-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF was identified (**Table 18**). However, the most significant interaction corresponded to SA and genotype that affected all genotypes evaluated with a degree of statistical significance of $p<0.001$. This result encourages analyzing the differeces concerning the level of total and individual PhytoPs and PhytoFs upon the application of the separate concentration of SA (SA1 and SA15).

In the sequence of the findings reported so far, when analyzing the evolution of total PhytoPs and PhytoFs regarding the different applications of SA, it was found that total PhytoP concentrations, calculated as the average of all genotypes, were 772.9 (SA0), 769.7 (SA1), and 563.4 (SA15) ng g^{-1} dw and of 752.2 (SA0), 543.8 (SA1), and 650.7 (SA15) ng g^{-1} dw in E and EP environments, respectively. On the other hand, regarding total PhytoFs, the concentrations observed were 21.0 (SA0), 17.0 (SA1), and 17.2 (SA15) ng g^{-1} dw and 22.4 (SA0), 15.0 (SA1), and 14.4 (SA15) ng g^{-1} dw in E and EP, respectively. These results suggest more significant interactions between the application of salicylic acid and the growing environment (E and EP) for the application of 1 and 15 mM SA for PhytoPs and PhytoFs, respectively, with a decrease of 29.3 and 16.3% of the total concentration, respectively.

The impact of hormones can be beneficial or toxic, depending on the concentration in which they are present in plant tissues. According to Janda *et al*. (2007), the effect of SA on heat tolerance in mustard plants depends on the concentration. Thus, in this work, SA only exhibited a protective effect at low concentrations (0.01-0.1 mM). In addition, Clarke *et al*. (2004) showed that pretreatment with SA at doses of 0.5 or 1.0 mM promotes basal thermotolerance in *Arabidopsis* plants.

Table 18. *P-value* corresponding to the interactions between genotype, growing environment, and salicylic acid treatments, evaluated in the study.

Factors and interactions	Phytoprostanes							Phytofurans		
	PP1[Z]	PP2	PP3	PP4	PP5	PP6	PP7	PF1	PF2	PF3
Interactions										
Environment x salicilic acid	*[Y]	*	**	N.s.	**	N.s.	N.s.	N.s.	N.s.	***
Environment x genotype	***	N.s.	N.s.	*	N.s.	*	N.s.	*	*	N.s.
Salicilic acid x genotype	***	***	***	***	***	***	***	***	***	***
Environments x salicylic acid x genotype	N.s.	**	***	N.s.	***	N.s.	**	*	N.s.	***

[Z]PP1, 9-F_{1t}-PhytoP; PP2, *Ent*-16-F_{1t}-PhytoP + *Ent*-16-*epi*-16-F_{1t}-PhytoP; PP3, 9-*epi*-9-F_{1t}-PhytoP; PP4, *ent*-9-*epi*-9-D_{1t}-PhytoP; PP5, *ent*-9-D_{1t}-PhytoP; PP6, 16-B_1-PhytoP; PP7, 9-L_1-PhytoP, PF1, *ent*-16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF; PF2, *ent*-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF; and PF3, *ent*-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF. [Y]N.s., no significant, $p<0.05$ (*), and $p<0.001$ (***).

5.3.4. Differential response of rice genotypes to treatments

In **Figures 19** and **20** are referred the modification of the concentrations of individual PhytoPs and PhytoFs through the increase of the concentration of SA applied to the crops. In general, the behavior was similar for all the compounds studied, inducing supplementation with SA an almost invariable reduction of the individual PhytoPs and PhytoFs concentration, exception made of 'R/03-5x desc/04-27-3-1' that presented higher levels of PhytoPs and PhytoFs in plants treated with 15 mM SA (**Figures 19** and **20**).

In parallel, the *environment x SA x genotypes* interaction was also analyzed (**Table 18**). Sorting all possible combinations, from the lowest to the highest concentration of PhytoPs and PhytoFs, the lowest concentration values corresponded to the advanced lines 'Amaroo x desc /08-1-1-1-2', 'R/03-5x desc/04-27-3-1', 'R/03-5x desc/14-1-1-1' for all SA doses assayed in the two environments.

The divergence between genotypes in their response to stress can be attributed to the efficiency in neutralizing ROS. According to Enyedi *et al.* (1992), survival in stressful conditions depends on the ability of the plants to perceive a stimulus, generate and transmit signals, and instigate biochemical changes that adjust the metabolism accordingly.

Nowadays, there is a broad body of literature on the effect of abiotic stress on secondary metabolites, including PhytoPs. Collado-González *et al*. (2016) studied the content of PhytoPs present in oil from *Olea europaea* L., cv. Cornicabra, and observed that their content increased in plants grown in deficit irrigation. The qualitative and quantitative differences in the content of PhytoPs indicate a decisive effect of the cultivar, oil extraction technology and/or storage conditions, prone to autooxidation (Collado-*González et al*., 2015c). These authors studied the concentration of PhytoPs in olives pulps harvested from plants grown with and without water stress. The results obtained indicated that the content of 9-F_{1t}-PhytoP and 9-*epi*-9-F_{1t}-PhytoP increases in the pulp of olives subjected to water stress. The total content of PhytoPs varied in the five cultivars studied (Collado-González *et al.,* 2015a). Carrasco-Del Amor *et al.* (2015) studied 11 almond cultivars in an agricultural system (conventional or ecological) and in rain-fed and irrigation conditions. Almonds from trees fed by rain had lower individual and total PhytoPs concentrations than those that were under irrigation. Yonny *et al.* (2016) studied PhytoPs and PhytoFs in leaves of melon plants with heat stress. Their levels in stressed plants were significantly higher than in samples not subjected to stress although they did not observe differences between varieties in the behavior.

Hence according to previous reports in the literature, jointly with the results obtained in the present work, it follows that there are qualitative and quantitative differences in the content of PhytoPs that indicates the influence of the variety, technology and/or storage conditions of plant matrices. Hence, the conversion of PUFAs into autoxidative *in vivo* allows the generation from a much wider range of metabolites that are also rich in biochemistry and versatile (Jahn *et al*., 2008). Given this complexity, from agronomical point of view in connection with food science and technology, future research is required to study how the production of PhytoPs is affected by different types of abiotic stress in plants (Collado-González *et al.,* 2015b).

It is important note that certain agronomic and industrial procedures may enhance the PhytoPs content of plant foods. In this way, it may be to possible to "functionalize" plant products with higher concentrations of these compounds, which are readily bioavailable and absorbed by the human body (Collado-González *et al*., 2015). Interestingly, upon this work, it was revealed that the concentration of these compounds in fresh plant material was more than two orders of magnitude higher than that of F_2-isoprostanes (F_2-IsoP) derived from arachidonic acid, in mammalian tissues. Regarding this, Karg *et al.* (2007) demonstrated that that F_1-PhytoPs are bioaccessible and bioavailable. Closely linked with this and in respect to their functionality, recently, it has been stated possible functions as anti-inflammatory and apoptosis-inducing compounds, by acting as to cyclopentenone prostaglandin-like molecules (Jahn *et al*., 2008). It is expected that in the next few years, these sterified derivatives could be accurately identified, resorting to spectrometric tools, and synthesized. Gaining this knowledge and reagents would help to assess their occurrence in cells and tissues after oral administration and to obtain a further insight in their bioactive capacities upon a wide range of cellular models (Jahn *et al.,* 2008).

The possibility of having genetic materials more tolerant to heat stress allows the expansion of areas suitable for rice cultivation or adapted to climate changes that are expected in the near future, without changes in production costs and ensuring sustainability and environment protection.

5.4. CONCLUSIONS

The information obtained in the present work on the behavior of PhytoPs and PhytoFs against differential environmental conditions and exogenous application of particular plant hormones contributes to understand the rice plants mechanisms against abiotic stress, as well as to the extent in which PhytoPs and PhytoFs contribute to defense and signaling activities. In

this regard, the variations found in the level of plant oxylipins in rice plants grown under differential conditions, suggest that these metabolites are generated in situations of stress conditioned by the presence of ROS and respond to the effects of plant hormones. This information would allow you to understand more clearly its functions in plant metabolism. Supplementation with SA could be a promising management practice in warmer years, which will be more and more frequent in the coming years due to the climate change, mitigating the damaging effects of heat stress. In this sense, the information obtained will help to understand the physiological tools of higher plants to stand protected against external agressions, which is essential to obtain, through bioengineering approaches, more resistant varieties upon breeding, which would increase the arable field suitable for cultivation, the production, and the overall nutritional and physical quality. Simultaneously new knowledge would contribute to maintain production costs and guarantee the sustainability of resources and environment.

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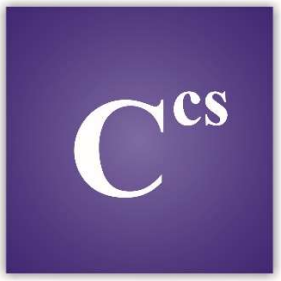

Conclusions

CHAPTER VI. CONCLUSIONS

The studies carried out in this Doctoral Thesis have led to the following general conclusions:

1. Supplementation with nutrients and antioxidants significantly decreases the concentration of phytoprostanes and phytofurans, further demonstrating that this technological practice contributes to mitigate the level of oxidative stress caused as a consequence of the augment of reactive oxygen species produced in situations of abiotic stress;
2. The assessment of phytoprostanes and phytofurans as biomarkers of nutritional stress in the frame of foliar fertilization revealed that metabolites are more sensitive than other indicators of plants performance and plant-foods quality;
3. Even in minimum doses, the application of fertilizers by foliar administration modify the level of plant oxylipins (phytoprostanes and phytofurans) in a higher and more significant extent than productivity parameters, such as yield and grain quality, thus, confirming the close relationship of the concentration of the compounds with the metabolic network responsible for tolerance to stress;
4. Plant oxylipins in rice are preferably located in the external layers or pericarp of the rice grains where lipids are concentrated, while the profile and quantitative level of phytoprostanes and phytofurans strongly depends on the genetic resource considered;
5. The high level of phytoprostanes and phytofurans in the co-products of the rice industry (bran rice and brown flour) supports their application as a dietary source of these compounds to take advantage of their biological properties and healthy attributions, providing interesting side applications as functional foods and ingredient, of these underexploited foodstuffs.