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Dissecting Rice Pearl Character, an Important Added Value in High-Quality Temperate Mediterranean *Japonica* Cultivars

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Abstract: Rice holds an important sociocultural meaning in Europe, and especially in the gastronomy of its Mediterranean regions, as it is used for world-famous recipes such as *Risotto* in Italy and *Paella* in Spain. *Paella* is prepared with highly appreciated pearled (white-core) rice cultivars such as Bomba or Montsianell, while *Risotto* is prepared with white-belly Carnaroli cultivar among others. Pearled rice grains have a limited and enclosed translucent zone which is physicochemically different from stress-induced chalky grains present in any rice cultivar at a low rate, and whose opaque area covers at least three quarters of the grain surface. We have studied for the first time the physicochemical aspects of grains from pearled white-belly, white-core and crystalline rice grains of Mediterranean *japonica* rice cultivars in comparison with their defective stress-induced chalky grains in order to shed some light on their differences. Spanish Bomba and Montsianell white-core (pearled) cultivars have similar physicochemical behaviours but are clearly different from white-belly Carnaroli cultivar. Furthermore, their pearled fractions differ in some traits from stress-induced chalkiness, especially in terms of amyloplastic integrity, relative amylose content and relative storage protein content. This study establishes some physicochemical differences between white-belly, white-core and stress-induced defective chalky grains and will guide future studies to unravel this much-appreciated pearl character in the Mediterranean gastronomy.

Keywords: rice; *japonica*; white-belly; white-core; chalky grains

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1. Introduction

Rice (*Oryza sativa* L.) contributes to 20% of the total calories consumed worldwide [1] and comprises half of the daily calories consumed in some areas [2]. The European contribution to global rice production is very small, although rice production in Europe holds an important sociocultural and ecological value [3]. Some Mediterranean countries have developed world-famous rice dishes such as *Risotto* in Italy or *Paella* in Spain that have co-evolved together with certain traditional *japonica* rice cultivars. In Spain, *japonica* rice cultivars such as Montsianell or Bomba are highly appreciated since they are less sticky and more prone to absorb flavours from the broth and sofrito sauce that they are cooked in, thanks to the presence of the white-core "pearled" character, which is a limited and enclosed translucent zone in the centre of pearled cultivars' rice grains [4,5]. On the contrary, Carnaroli and other Italian *Risotto* rice cultivars are highly appreciated for their creaminess, which is linked to the presence of white-belly character in the grains, an opacity that emerges on a lateral surface of the grain [6].

On the other hand, stress-induced defective rice grains show a similar opaque area that covers at least three quarters of the grain surface. These stress-induced defective rice grains are known as chalky, floury or withe grains, appearing in small proportions of both pearled or crystalline rice batches, especially when plants have been stressed by heat [7,8], wind [9], shade [10] or even nitrogen deficiency [8]. These defective chalky grains are considered unprofitable because of their undesirable appearance and low palatability [11,12] and easily break during milling. In most rice-producing countries, the term "chalkiness" is used to define any non-perfect crystalline rice grain, extending the term to white-belly and white-core pearled grains [13–15]. In addition, milling non-crystalline cultivars needs to be carefully tuned in order to avoid grain breakage and reductions in head rice recovery [16]. On the contrary, white-belly and white-core rice cultivars are considered as high quality and have a key added value in the Italian and Spanish rice industries, being the central rice exports from Europe.

Unfortunately, the white-core and white-belly character from Mediterranean non-crystalline *japonica* cultivars has not been studied as profusely as other rice quality character because, as said, it is not considered of interest for breeding purposes in the main rice-producing countries. In addition, some published studies and reviews concerning chalkiness do not distinguish white-core and white-belly added value cultivars and those varieties prone to suffering stress-induced chalkiness [6,8,11,13,14,17–20], or they have only studied chalky mutants of non-pearled cultivars instead of pearled cultivars per se. Furthermore, most studies have focused on Asian *indica* or *japonica* cultivars, with research undertaken with Mediterranean pearled cultivars practically non-existent.

Regarding genetics, the presence of pearled character has not been linked to a single gene for all *japonica* and *indica* cultivars. Instead, many QTLs and some genes have been associated with the presence of pearl or chalkiness [21]. In contrast, some physical and compositional parameters such as the crystallinity degree [18] and the amylose and protein content [6], as well as scanning electron microscopy studies [22], have been performed in relation to pearled and chalky grains.

The aim of this study is to shed some light on the Mediterranean *japonica* white-core and white-belly rice pearled cultivars character by studying physicochemical aspects of pearled, crystalline and chalky grains of Mediterranean *japonica* rice cultivars for the first time.

2. Materials and Methods

2.1. Plant Material

Dehulled and milled rice grains from five commercial Mediterranean *japonica* rice cultivars (Montsianell, Olesa, Guadiamar, Bomba and Carnaroli) were used along with chalky grains from each cultivar. The first four cultivars were provided by Càmara Arrossera del Montsià (Amposta, Spain), while the last cultivar was provided by Società Italiana de Sementi (San Lazzaro di Savena, Italy). Montsianell and Bomba white-core Spanish cultivars and Carnaroli white-belly cultivar possess pearled grains, while Olesa and Guadiamar possess crystalline grains. Chalky grains (80–100% opacity) from the rice cultivars were obtained from the automated colour sorter rejection process, a step during the rice milling process that detects abnormal grains and impurities, in which chalky grains belong. Pearled and crystalline rice fractions were obtained by manual dissection of the pearled grains by using a scalpel (Figure 1). At least three biological replicates of each group were used in all experiments except for the physical measurements.

2.2. Sample Preparation

Polished grains were used for grain physical measurements. Rice flour was obtained by milling the samples in a TissueLyser II (QIAGEN, Venlo, The Netherlands) at 120 Hz for 60 s. Four flour fractions were obtained from each pearl (white-core or white-belly) cultivar: the whole pearl grain, the whole stress-induced chalky grain, the crystalline fraction and the pearl fraction. Pearl and crystalline rice grain fractions were obtained by manual dissection using a scalpel. For the crystalline rice grain cultivars, two flour fractions were obtained corresponding to whole crystalline grains and whole chalky grains. Starch grains were isolated from the flour by alkaline extraction of proteins and defatting was performed with methanol.

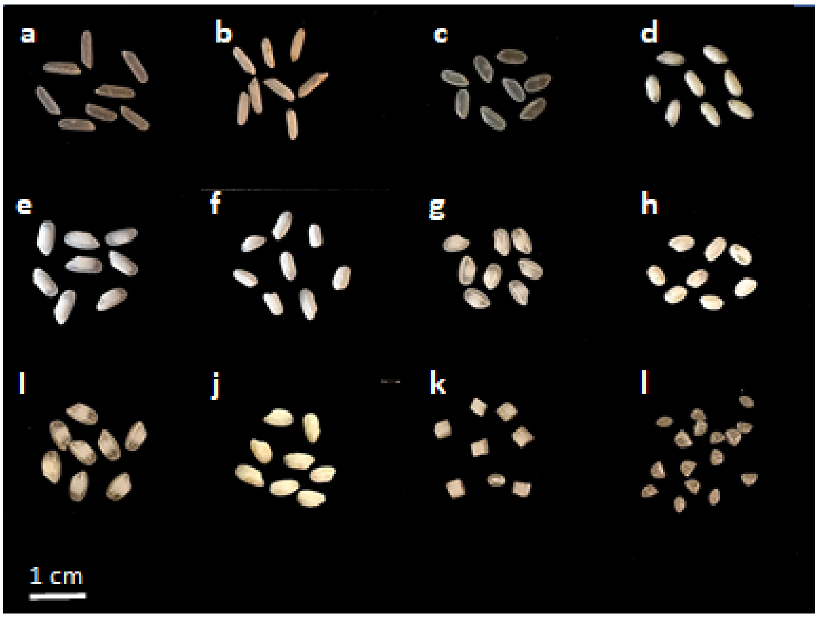

Figure 1. Milled rice grains photographs of the different cultivars used in this study. (**a**,**b**), Olesa and chalky Olesa; (**c**,**d**), Guadiamar and chalky Guadiamar; (**e**,**f**), Carnaroli and chalky Carnaroli; (**g**,**h**), Bomba and chalky Bomba; (**i**,**j**), Montsianell and chalky Montsianell; (**k**,**l**), pearl and crystalline fractions of the rice grains.

2.3. Physical Measurements

A total of 100 rice grains of each cultivar were randomly selected and weighed, in groups of 10, for estimating the average weight. Morphometric descriptors such as area, perimeter, length, width, length/width ratio and percentage of the pearl region to the rice grain whole area were determined by an imaging analysis system. Then, 100 pearl rice grains, in groups of 20, were placed over a white light transilluminator to discriminate between the pearl and translucent fractions of the grains. The images were captured using a Panasonic Lumix DC-GH5 camera and were digitally processed using the IMAT software developed at the Centres Científics i Tecnològics from the Universitat de Barcelona (CCiTUB, Barcelona, Spain). The grain profiles were auto-recognised and the pearl profiles were manually selected. The reported values correspond to the average of 100 grains. Grain type was determined according to the IRRI [23] and European Union [24] classifications.

2.4. X-ray Diffraction

X-ray diffraction patterns (for starch crystalline structure analysis) were obtained using starch in powder form and whole rice grains in a PANalytical X'Pert PRO MPD θ/θ powder diffractometer (Malvern Panalytical Ltd., Malvern, The Netherlands) with Cu Kα radiations (λ = 1.5418 Å), at 45 kV and 40 mA, in transmission configuration with a focalising elliptic mirror and a 1D PIXcel detector (active length 3.347°), 2θ scans from 4 to 50° 2θ with a step size of 0.039° and a measuring time of 350 s per step. Starch samples were placed in circular sample holders sandwiched between two sheets of 3.6 μm width polyester films. Grain samples were placed on the goniometric head of the capillary sample stage, which acted as a capillary. A crystallinity index was calculated by determining the proportion of the crystalline area to the total diffraction area, in the main angular range from 7 to 28° 2θ. Analysis was performed in triplicate for each of the treatments (Figure 2).

2.5. Scanning Electron Microscopy (SEM)

Pearl rice grains, crystalline rice grains and chalky rice grains of each cultivar were cut transversally in half with a scalpel to expose the fractured surface of the starchy endosperm. The specimens were mounted (applied) on the aluminium stubs using double-sided adhesive tape and the cracked surface was carbon silver-coated under vacuum conditions. The specimens were observed using a Scanning Electron Microscope (SEM, JSM-7001F JEOL, Japan) at an accelerating voltage of 5–15 kV, at the Centres Científics i Tecnològics de la UB (CCiTUB, Barcelona, Spain). SEM analysis was performed using three biological replicates with at least four technical repetitions.

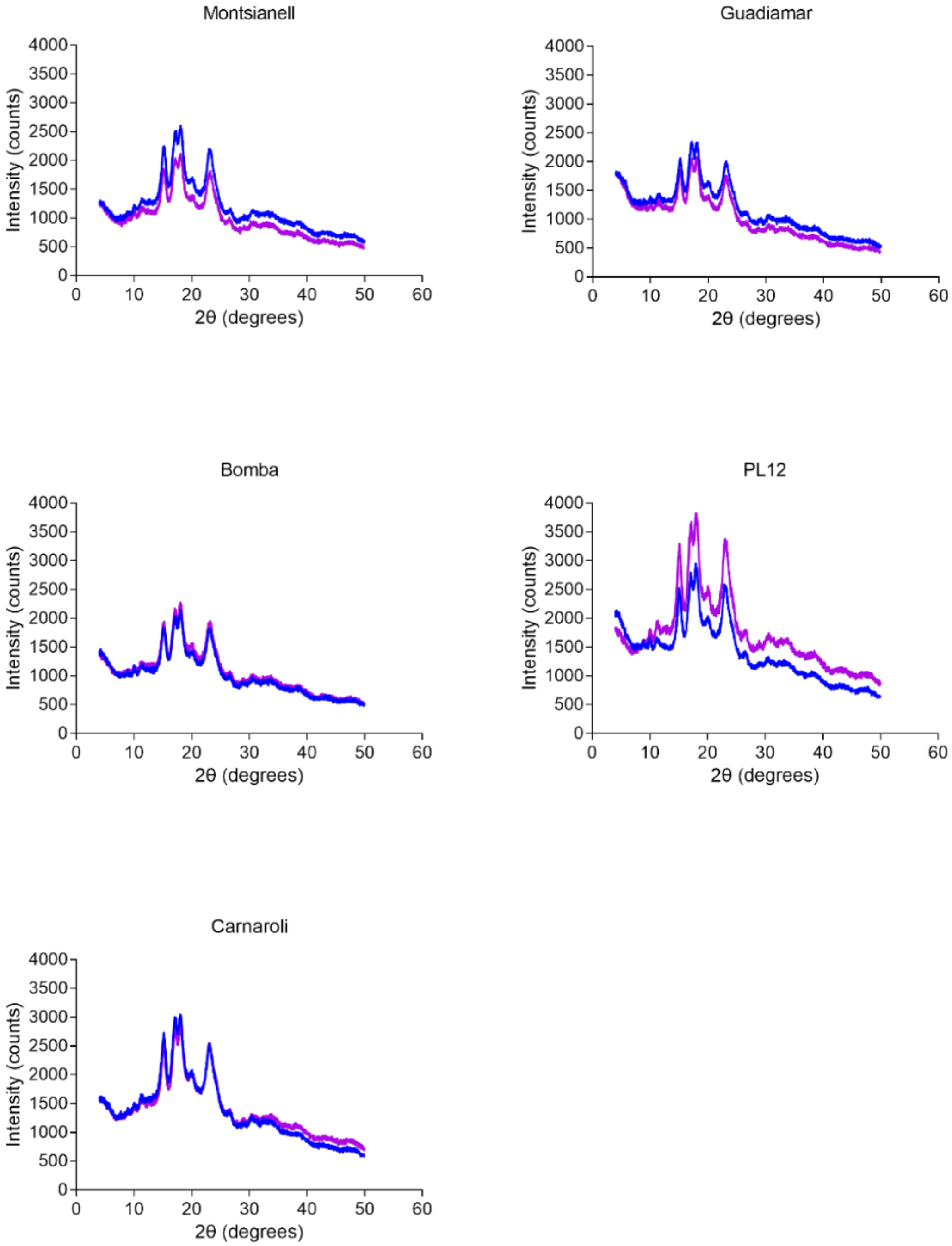

Figure 2. Diffractograms for all the cultivars and grain types studied. The pearled cultivars are on the left and the crystalline cultivars are on the right. Whole grain diffractograms are represented with a purple line and chalky grain diffractograms are represented in blue.

2.6. Amylose Content Determination

The amylose content was determined using flour samples from different grain fractions, depending on the cultivar. As mentioned above, the flour was obtained by milling the samples in a TissueLyser II (QIAGEN, Venlo, The Netherlands) at 120 Hz for 60 s. For the pearled cultivars, the quantification was performed in four different fractions: perfect grains, chalky grains, the crystalline fraction and the pearled fraction, with the last two obtained by dissecting the pearled regions using a scalpel. For crystalline cultivars, since they present uniform grains with no distinguishable pearl, only whole grains and chalky grains were used. The amylose content was determined using a colorimetric method which is based on amylose–iodine complex formation adapted from [15]. Analysis was conducted using the four flour fractions of pearl rice grains and the flour of the crystalline and chalky rice grains. Four biological samples with three technical repetitions were taken.

2.7. SDS-Polyacrylamide Gel Electrophoresis (PAGE)

Proteins were extracted from the same flour samples used for the amylose content. In a 50 mL tube, 500 mg of sample flour was mixed with 140 µL 40 mM phenylmethylsulphonyl fluoride (PMSF) and 10 mL of extraction buffer (0.7 M sucrose, 0.5 M Tris-base, 50 mM EDTA (pH 8.0), 0.1 M KCl (*w*/*v*), 5 mM HCl (*v*/*v*) and 0.328 M β-mercaptoethanol in miliQ water). The mixture was centrifuged (25,000× *g* 4 °C for 20 min) and the supernatant was transferred to another fresh tube. Three times the volume of cold precipitation buffer (0.1 M Nh_4OAc and methanol, 10 mM β-mercaptoethanol and methanol) was added to the recovered phenol phase. After mixing by inversion, it was left incubating overnight at −20 °C and the pellet was recovered and washed twice with 1.8 mL precipitation buffer by centrifugation (7200× *g* 4 °C for 5 min). The pellet was then dried in an Eppendorf 5301 Concentrator Speed Vac system (Eppendorf, Hamburg, Germany) for 3 min and resuspended in 1.5 mL of lysis buffer (9.5 M urea, 40 mM tris-base and 15 mM DTT in distilled water). The protein concentration was calculated using the Bradford method.

Protein separation was performed with Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) gels using a method based on Laemmli [16]. The stacking gel was 6% acrylamide and the resolving gel was 15% acrylamide. A prior assay was performed with a 12% resolving gel, but the separation of the prolamin band was not as clear as with the 15% gels. Quantification of SDS-PAGE bands was performed using ImageJ [17]. The previously obtained gel images were subjected to a colour inversion and the integrated density was calculated for each of the relevant bands, together with the whole lanes densities. The percentage content of each protein fraction was calculated as the proportion between the integrated density of each fraction and the integrated density of the whole lane. Three replicates per cultivar and fraction were performed.

2.8. Statistical Analysis

All statistical analyses for physical measurements, X-ray diffraction and amylose and protein determination were performed with Minitab 17 Statistical Software (Minitab Inc., State College, PA, USA). Datasets were subjected to a one-way analysis of variance (ANOVA) with 95% confidence and significant differences were established by using Tukey post-hoc analysis. Datasets lacking variance homoscedasticity were subjected to a Welch ANOVA with 95% confidence and significant differences were established by using Games–Howell post-hoc analysis.

3. Results

3.1. Physical Measurements

Different physical measurements were determined for all cultivars. The classification of each rice cultivar was established from the grain area, pearl area, length, width and weight parameters (Table 1).

Carnaroli and Montsianell cultivars showed the highest grain area values (greater than 14 mm^2), being significantly different from the other cultivars, which ranged from 11 to 12 mm^2. The averaged pearled area percentages were also significantly different between cultivars, with Carnaroli having the highest percentage of pearled (white-belly) area (44.27%) and Bomba having the lowest (19.47%) among the studied pearled cultivars. The averaged pearled area was more than twice in Carnaroli compared to Bomba cultivar, indicating a high variability within the pearl character. The long-grain cultivars Carnaroli and Olesa shared a grain length group greater than 6 mm length, with Guadiamar and Montsianell of approximate 5.5 mm length, and Bomba in a third group with less than 5 mm length, matching the cultivars' respective long-, medium- and short-grain classifications. There was only a small difference between the two classification systems in the case of Guadiamar, which is considered a Long A-type in the EU classification because its grain length is greater than 6.00 mm and it has a length/width ratio of less than 3, although it would be considered medium-grained according to the IRRI classification due to its length of between 5.51 and 6.60 mm and length/width ratio of between 2.1 and 3.0. The grain width was also significantly different among all cultivars. The cultivars having the heaviest and the lightest grains were Carnaroli and Olesa, respectively, although both were long-grained.

Table 1. Physical parameters of the cultivars studied, presented as mean ± SEM. Different letters associated with a measurement indicate significant differences ($p < 0.05$).

	Montsianell	Bomba	Carnaroli	Guadiamar	Olesa
Grain area (mm^2)	14.10 ± 0.12 b	11.28 ± 0.09 d	14.75 ± 0.12 a	11.79 ± 0.08 c	11.61 ± 0.09 cd
Pearl area (%)	31.63 ± 0.75 b	19.47 ± 0.81 c	44.27 ± 0.85 a		
Perimeter (mm)	15.46 ± 0.07 b	13.87 ± 0.06 d	16.78 ± 0.09 a	14.67 ± 0.06 c	16.87 ± 0.10 a
Length (mm)	5.51 ± 0.02 b	4.95 ± 0.02 c	6.32 ± 0.03 a	5.52 ± 0.02 b	6.38 ± 0.04 a
Width (mm)	3.17 ± 0.02 a	2.92 ± 0.02 b	2.79 ± 0.02 c	2.66 ± 0.01 d	2.07 ± 0.01 e
Length/width	1.74 ± 0.01 d	1.70 ± 0.01 e	2.27 ± 0.02 b	2.08 ± 0.01 c	3.09 ± 0.02 a
Weight (mg)	26.83 ± 0.37 a	20.31 ± 0.38 b	26.93 ± 0.19 a	20.66 ± 0.19 b	18.99 ± 0.21 c
Grain classification (EU/IRRI)	Medium/Medium bold	Short/Short medium	Long A/Medium	Medium/Medium bold	Long B/Long slender

3.2. X-ray Diffraction Patterns and Crystallinity

Diffractograms were obtained from perfect and chalky grains for all cultivars (Figure 2). All cultivars had very similar diffractograms for each type of grain, with variations in their intensity. Their peaks were placed close to 15°, 17°, 18° and 23° θ and a small (weak) shoulder peak at 20° 2θ. The shape of the patterns was almost identical for all cultivars. Concerning the white-core pearled cultivars, the Montsianell stress-induced chalky grains diffractogram showed a slightly higher intensity (with a maximum value of 2600 counts) than the normal white-core Montsianell grains measurement. In the case of Bomba, both diffractograms practically matched, and it was the cultivar that had the lowest maximum intensity, with a value of 2200 counts. Carnaroli white-belly cultivar also had the most intense chalky diffractogram of all the cultivars, with a maximum value of 3000 counts, and its normal grain diffractogram matched the chalky diffractogram almost completely. Considering the crystalline cultivars, Guadiamar had a chalky grain diffractogram with a maximum count value of 2350, which was slightly higher than the normal crystalline grain diffractogram, while Olesa showed by far the highest intensities and the highest diffractogram differences in terms of intensity. The maximum value of the most intense diffractogram, the perfect grain one, was 3800. On the other hand, the chalky diffractogram had a maximum value of 2950. Other authors found peaks exactly in the same degrees [25], being relative intensities lower in stress-induced chalky grains from crystalline cultivars than in perfect ones [25]. In our case, Olesa long-grained, stress-induced chalky grains clearly result in a lower intensity diffraction pattern compared to normal grains matching the results, while Montsianell pearled cultivar and Guadiamar crystalline cultivar behaved the opposite. On the other hand, white-belly Carnaroli cultivar and white-core Bomba pearled cultivar yielded similar intensities between stress-induced chalky and normal grains. This indicates that the optical properties of stress-induced chalky grains can be similarly lower or higher than perfect grains, depending on the cultivar.

Crystallinity percentages were calculated from the area contained beneath the peaks of the diffractograms and are shown in Table 2. Similar to the diffractograms, no significant differences were observed in the crystallinity values within the same cultivar, except for Olesa, whose diffractograms' intensities varied.

Table 2. Crystallinity percentages for the cultivars studied. Values are presented as mean ± SEM. Different letters within a cultivar indicate significant differences ($p < 0.05$).

	Montsianell	**Bomba**	**Carnaroli**	**Guadiamar**	**Olesa**
Perfect grain (%)	45.12 ± 1.08 a	45.48 ± 2.31 a	40.02 ± 0.86 a	54.22 ± 3.97 a	43.92 ± 1.45 b
Chalky grain (%)	40.93 ± 1.44 a	48.57 ± 0.92 a	38.53 ± 1.19 a	50.34 ± 3.03 a	53.21 ± 2.04 a

3.3. Scanning Electron Microscopy (SEM)

Transverse sections of both perfect and chalky grains of each cultivar were observed with SEM. For the pearled cultivars, images were taken from both distinguishable parts of the grain, the pearl and the crystalline fraction. In the case of crystalline cultivars, as far as their grains are not pearled, the pictures were representative of the whole grain area. Chalky grains were also photographed for each cultivar. Many images were captured, with the most representative for each cultivar and grain fraction selected. The images are shown in Figure 3.

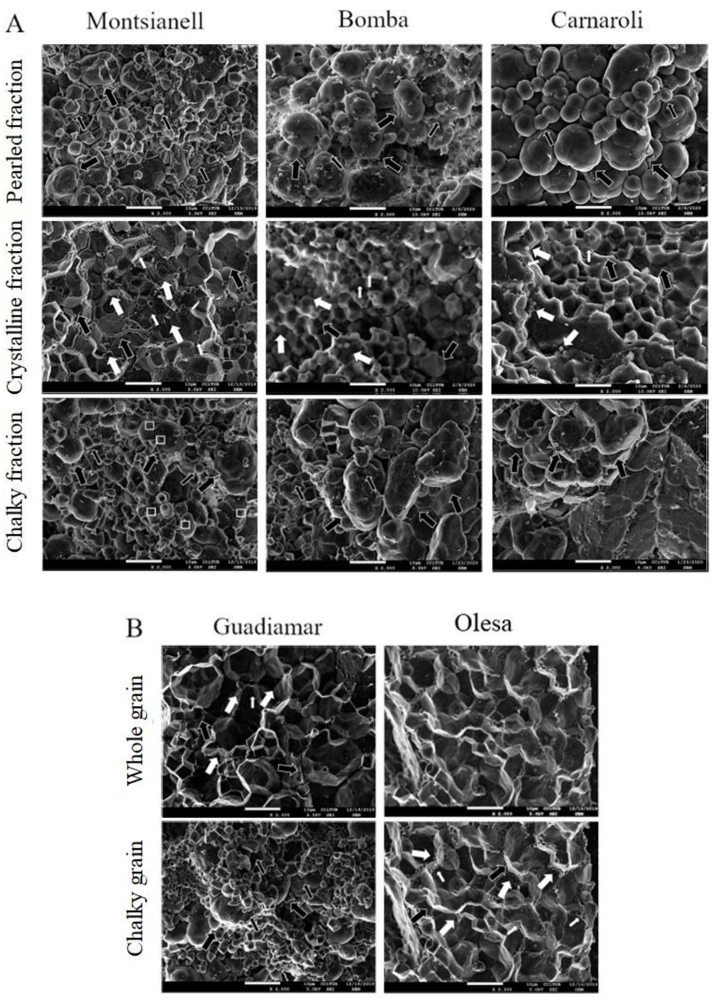

Figure 3. (**A**). SEM images of sections of the pearl fraction and the chalky fraction from the normal perfect grain, and sections of the chalky grain of each pearled cultivar. (**B**). SEM images of sections of the normal perfect grain and sections of the chalky grain of each crystalline cultivar. Large black arrows point to amyloplasts, small black arrows point to free starch granules, large white arrows point to protein bodies and small white arrows point to traces of protein bodies. White squares enclose micropores derived from α amylase attacks.

Among the pearled cultivars (Montsianell, Bomba and Carnaroli), the SEM images showed that there is a clear difference between the pearled parts and the crystalline parts of the grain in all three cultivars. The pearled parts displayed disorganised starch granules and amyloplast distribution, with many empty spaces between the starch granule groups. The amyloplasts were rounded and seemed to have broken easily into separate starch granules when the grain was cut. These isolated starch granules presented an irregular shape. Despite the similar arrangements of the pearled parts in all three cultivars, Carnaroli showed the greatest amyloplastic integrity when cut. In contrast, the crystalline zones presented perfectly arranged starch granules in the amyloplasts, with polyhedral shapes. There were almost no loose starch granules, and protein bodies and the space artefacts left behind as a consequence of their loss during sectioning their slots could be observed. Regarding the chalky grains of the pearled cultivars, their appearance was practically the same as the pearl parts, with disorganised and irregular amyloplasts and starch granules. In parallel with the pearl parts, the Carnaroli grains seemed to preserve greater amyloplast integrity.

The appearance of the crystalline parts of the pearled grains was similar to the non-pearled (crystalline) cultivars Guadiamar and Olesa, with the polyhedral starch granules perfectly arranged inside the amyloplasts and surrounded by protein bodies or the space artefacts left behind as a consequence of their loss during sectioning. The chalky grains of both crystalline cultivars appeared the same as the chalky grains from the pearled cultivars.

3.4. Amylose Content Determination

The amylose content was determined from the different fractions, depending on the type of grain (Figure 4). In the case of the pearled cultivars, no significant differences were observed between the amylose content of the whole grain flours and the pearled and crystalline fractions. On the other hand, significant differences were found between these three fractions and the chalky grain for Montsianell and Bomba. Regarding Carnaroli, no significant differences were observed between chalky flour, whole grain flour, the flour of the crystalline fraction and the flour of the pearled fraction, although the amylose content in the chalky grains was lower than that in the perfect grains and their fractions. Considering the crystalline cultivars, a significantly lower amylose content percentage was observed in chalky grains for both Guadiamar and Olesa cultivars.

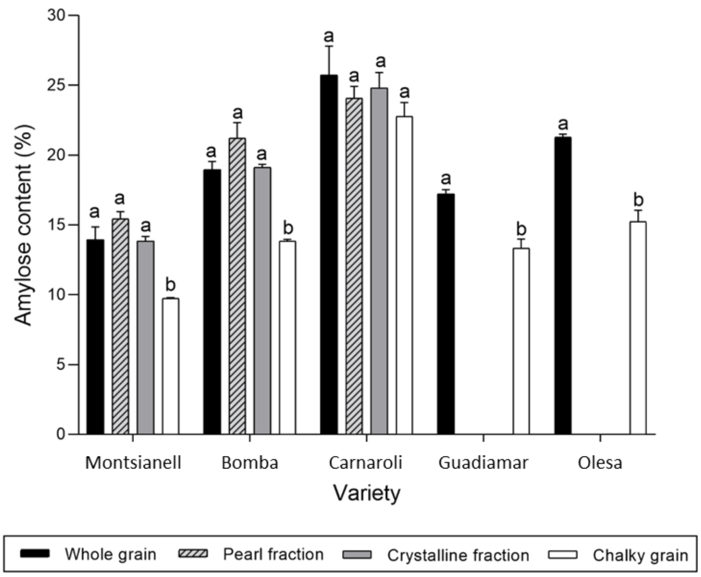

Figure 4. Percentage amylose content for each cultivar and fraction. Black bars represent the amylose content in normal perfect grains, dashed grey bars represent the content of the pearl fraction in the pearled cultivars, grey bars represent the content of the crystalline fraction in the pearled cultivars and white bars represent the content for chalky grains in all cultivars. Values are presented as mean ± SEM. Different letters within a cultivar indicate significant differences between groups ($p < 0.05$).

3.5. Determination of Relative Storage Protein Content

SDS-PAGE 15% acrylamide gels were used in order to properly separate and quantify the different storage protein fractions. A densitometry analysis was conducted to quantify the relative percentages of each protein fraction relative to the total storage protein content, which is presented in Table 3. The globulin fraction was not considered because its band was difficult to resolve in any of the gels.

Table 3. Relative percentages of various storage protein fractions of the rice grains for each cultivar. Values are presented as mean ± SEM. Different letters between groups within a cultivar indicate significant differences for that specific fraction ($p < 0.05$). WG = whole grain. PF = pearl fraction. CF = crystalline fraction. CG = chalky grain.

		Glutelin Precursor (%)	Glutelin Acidic Subunit (%)	Glutelin Basic Subunit (%)	Prolamins (%)
Montsianell	WG	3.24 ± 0.09 b	21.83 ± 0.88 a	16.09 ± 0.66 a	11.58 ± 0.32 a
	PF	3.43 ± 0.02 b	21.50 ± 0.72 a	16.91 ± 0.52 a	12.09 ± 0.25 a
	CF	3.28 ± 0.05 b	21.70 ± 0.61 a	16.83 ± 0.60 a	11.69 ± 0.34 a
	CG	4.13 ± 0.11 a	20.31 ± 0.30 a	15.17 ± 0.36 a	9.51 ± 0.07 b
Bomba	WG	3.00 ± 0.34 a	15.70 ± 0.38 a	13.42 ± 0.29 a	11.67 ± 0.53 a
	PF	2.85 ± 0.25 a	16.59 ± 0.46 a	14.55 ± 0.15 a	11.47 ± 0.89 a
	CF	3.26 ± 0.3 a	17.69 ± 1.14 a	15.81 ± 0.98 a	13.92 ± 1.70 a
	CG	3.77 ± 0.18 a	17.56 ± 1.08 a	15.68 ± 0.86 a	12.18 ± 1.43 a
Carnaroli	WG	1.71 ± 0.36 a	16.00 ± 0.23 a	10.64 ± 0.73 a	11.80 ± 0.48 a
	PF	1.67 ± 0.34 a	16.19 ± 0.23 a	10.90 ± 0.56 a	11.86 ± 0.35 a
	CF	1.59 ± 0.29 a	15.72 ± 0.55 a	10.40 ± 0.05 a	12.32 ± 0.69 a
	CG	1.56 ± 0.21 a	16.27 ± 0.70 a	10.51 ± 0.22 a	11.46 ± 0.90 a
Guadiamar	WG	3.46 ± 0.10 b	19.91 ± 0.32 a	18.15 ± 0.39 a	14.12 ± 0.25 a
	CG	3.88 ± 0.03 a	19.03 ± 0.24 a	17.56 ± 0.06 a	11.87 ± 0.24 b
Olesa	WG	3.36 ± 0.12 a	21.53 ± 0.58 a	16.17 ± 1.02 a	14.13 ± 0.64 a
	CG	3.61 ± 0.03 a	19.92 ± 0.41 a	15.64 ± 0.49 a	12.11 ± 0.30 b

Among the pearled cultivars, no clear pattern could be found in any of them in terms of quantities of the different protein groups in each fraction. For Montsianell, the quantity of the glutelin precursor and prolamins were significantly different between normal (3.24% and 11.28%, respectively) and chalky grains (4.13% and 9.51%, respectively). In addition, Montsianell's chalky grains had the lowest acidic and basic glutelin subunit relative quantities, although they were not statistically significant. In the case of Bomba, no significant differences were observed between perfect and chalky grains, but the measurements followed some trends. The highest value of the glutelin precursor was found in Bomba's chalky grains, similar to Montsianell. In both cultivars, the highest glutelin subunit values were present in the crystalline part and the lowest values were found in the perfect grains. The lowest value for prolamins was from the pearled fraction, while the highest corresponded to the crystalline fraction. In the case of Carnaroli, different distribution patterns of the protein groups were found, although no significant statistical differences were found. Contrary to Montsianell and Bomba, the glutelin precursor in Carnaroli was lowest in the chalky grain and highest in the perfect grain. For the glutelin acidic subunit fraction, the chalky grain had the highest value and the crystalline fraction of the pearled grain had the lowest. The highest and the lowest values in the glutelin basic subunits corresponded to the pearled and crystalline fractions, respectively. For the prolamins, the lowest value belonged to the chalky grain, as it also occurred in Montsianell.

The crystalline cultivars Guadiamar and Olesa had similar variation in the distribution patterns of their protein fractions. For the glutelin precursor, the lowest value was present in the whole grain (3.46% and 3.36%, respectively) and the highest in the chalky grain (3.88% and 3.61%, respectively), with significant differences between the two grain types observed in Guadiamar. For all three glutelin subunits and the prolamins, the highest value was recorded in the perfect grains and the lowest in the chalky grains, with significant differences for the prolamins in both of the crystalline cultivars.

4. Discussion

This experiment has taken multiple approaches in examining the properties of rice pearl in Mediterranean cultivars because of the important cultural and gastronomic role that rice plays in Italy and Spain. The disregard for the pearl character in many rice-consuming countries, alongside confusion of the terms pearl/chalky, has limited the progress in fully understanding the causes of the pearled phenotype [5]. During the past 10 years, an increasing number of publications have studied the lack of rice transparency, but most have focused on chalkiness and *indica* cultivars. This experiment is pioneering the study of rice pearl and chalkiness in Mediterranean temperate *japonica* cultivars that are widely consumed throughout Mediterranean Europe and also exported.

The physical measurements defined the diversity of the grain types examined in this experiment. Another relevant aspect about the pearled cultivars was the significant differences between cultivars related to the percentage that the pearl occupies in the grain, again confirming the diversity of this character.

The amylose/amylopectin ratio influences the physicochemical properties of a certain starch, affecting both gelatinisation and retrogradation of starch from various botanical sources [26–28]. During gelatinisation, starch granules swell and form gel particles. In general, starch granules are rich in amylopectin, with the layers interlaced with strands of open amylose chains. Upon swelling, linear molecules of amylose diffuse out of the swollen granules making up the continuous phase (network) outside the granules [29]. It is well known that sticky starches usually swell to a greater extent than their non-sticky counterparts [30]. Amylose has been proposed to act as a restraint to swelling [29]. The averaged amylose percentages vary for each cultivar [31], which was also observed in our studied varieties.

Montsianell is a low-sticky *japonica* cultivar despite having relatively low amylose content, while Carnaroli results were ideal to attain the expected creaminess of the *Risotto* recipes despite having the highest amylose content. Lower amylose content was found in chalky grains compared to normal grains (Figure 2), being statistically significant in all cultivars except in the case of Carnaroli, where there was only a tendency. This fact matches the results obtained by various authors when measuring the amylose content in normal and chalky grains of some rice cultivars [18–20]. Patindol and Wang [18] proposed that there is a mechanism related to chalky grains that favours glucan branching over elongation, explaining the lower quantity of amylose or the higher amount of amylopectin.

Interestingly, pearled fractions showed higher amylose content than the chalky grains (Figure 2), being statistically significant in the case of Bomba and Montsianell, demonstrating that pearled fractions are different than chalky grains at least in terms of amylose content. This suggests a different explanation for the formation of chalky grains and pearled grains. Indeed, Lin et al. [6] reported that the amylose content is not related to the presence of pearl per se.

X-ray diffraction was used in order to determine the degree of crystallinity of each cultivar because it is the most widespread technique to study starch structure. All diffractograms of each cultivar and grain type followed an A-type pattern, with peaks close to 15°, 17°, 18° and 23°, as expected for cereal starches [31]. Other authors have also found an A-type pattern when studying rice starches [18,32,33]. Considering the crystallinity percentages obtained in the current work, the range seemed to vary more depending on the cultivar than if the grain was pearled or crystalline. Variations in the crystallinity percentages for different rice cultivars have previously been reported [18,32]. The percentage of crystallinity in chalky grains did not follow a fixed pattern, being higher than the perfect grains (non-chalky) for some cultivars (i.e., Bomba, Olesa) and lower than the values for the normal grains (i.e., Montsianell, Carnaroli, Guadiamar). Patindol and Wang studied the degree of crystallinity in perfect and chalky grains of six crystalline cultivars and found that this value was always higher in the chalky grains than in the perfect grains of each cultivar [18]. This premise does not match our results for the white-core Montsianell cultivar and the crystalline Guadiamar cultivar, while no differences were found for the white-core cultivar Bomba and white-belly cultivar Carnaroli. Interestingly, Olesa crystalline cultivar behaved the opposite, having a statistically significant lower intensity in chalky grains. Patindol and Wang also found an inverse relationship between crystallinity and amylose content, a result that also does not match ours; for example, the highest amylose values belonged to Carnaroli, whereas its crystallinity percentages were not the highest among the cultivars tested [18]. Nonetheless, other authors such as Wani et al. have refuted a significant effect of amylose content on the crystallinity percentage [31]. These discrepancies might be due to further and more complex aspects that determine the degree of crystallinity besides amylose content, such as the proportion of short-side chained and long-side chained amylopectin, as proposed by Singh et al. [34].

Moving onto SEM, this technique has been used to study pearled or chalky areas in rice grains relative to crystalline fractions or grains [6,20,22,35], obtaining pictures that match the general appearance of both our pearl/chalky and crystalline images. In all these studies, the pearl/chalky areas present irregular and round-shaped amyloplasts and starch granules, with spaces between them, and an irregular distribution, which are similar to our images. Ratanasumawong et al. [17] studied the water diffusion in pearled and crystalline rice cultivars and found a faster diffusion in the pearled grains due to the spaces between the amyloplasts and the disorganised structure in the pearled tissue. This explains why the pearled grains take up the flavour of the frying sauce "sofrito" or broth that the grains are cooked in.

Another noticeable aspect of our images is how the protein bodies were more visible in the crystalline tissues than in the pearl/chalky tissues. Our measurements of storage protein content (storage proteins are contained in the protein bodies) were relative measurements and cannot be translated into conclusions about their distribution. Xi et al. [22] proposed a weaker adhesion of the protein bodies in pearl/chalky tissues due to the many spaces between the amyloplasts. A plausible explanation is that the protein bodies present in the surface may have fallen out during sectioning of the grain during sample preparation. In our images of crystalline tissues, many traces of protein bodies were observed, which also indicates loss during sample preparation, although many protein bodies were still present due to the more organised structures of the amyloplasts and the more regular arrangement of the protein bodies between them. Lin et al. [6] also reported observable protein bodies or traces in crystalline tissues but not in pearled tissues. Li et al. [35] analysed the quantity of protein bodies in a pearled and a crystalline rice cultivar, and found a decreased concentration of PB-Is and PB-IIs in the pearled cultivar in comparison to the crystalline cultivar. A combination of poor PB adhesion and low quantities of PB could explain the lack of visible protein bodies in our pearl/chalky images.

In the majority of cases, the relative content of the different storage protein fractions seemed to have a heterogeneous distribution, but there were some exceptions. Of all the fractions, the prolamin content was the lowest in the chalky grains in all cultivars except Bomba. Indeed, Lin et al. [6] found a lower quantity of prolamins in the pearl fraction than in the crystalline fraction of a pearled mutant. Prolamins are contained in PB-Is [36], so a measurement of the quantity of PBs in the different fractions, as performed by Li et al. [35], would be an interesting future experiment to undertake.

Our results also showed a higher level of pro-glutelins or glutelin precursors in the chalky grains of Montsianell, Bomba, Guadiamar and Olesa. Wang et al. (2010) created a mutant of the OsRab5a gene, which codes for a GTPase responsible for vesicle trafficking. This mutant abnormally accumulated pro-glutelins and had a chalky grain appearance. Liu et al. [37] created a mutant of the OsVPS9A gene, which codes for an activator of OsRab5a, and it also produced an abnormal accumulation of pro-glutelins and chalky endosperm. Zhu et al. [38] created a mutant that accumulated glutelin precursors by altering the OsNHX5 gene, which codes for a $Na+/H+$ antiporter and alters vesicle trafficking, and this originated a chalky endosperm in the grain. Thus, the association between glutelins and chalkiness seems relatively strong.

Lin et al. [6] performed an iTRAQ analysis on a pearled mutant and found that the biggest group of differentially expressed proteins in the mutant belonged to the metabolism proteins. The same authors also reported an alteration in vesicle trafficking and therefore protein trafficking to explain the rice pearl. Wada et al. [8] affirmed that under heat stress, crystalline cultivars begin to form pearl due to the disruption of protein synthesis, with a consequent inhibition of PB synthesis. These different experiments cannot be directly correlated with our study because they were performed using mutants, some of them chalky, some pearled, or with stressed crystalline cultivars. Nevertheless, it seems clear that the protein contents and their related metabolism and trafficking may be associated with the presence of the rice pearl.

5. Conclusions

By evaluating existing studies and supporting those with novel findings, this study has taken the first step in dissecting the causes of rice pearl in Mediterranean temperate *japonica* cultivars, establishing a clear distinction between the desired white-core "pearled" and white-belly grain phenotypes and the unwanted stress-induced chalky grain phenotype. Due to differences between the stress-induced chalky, white-core and white-belly grains in the amylose, diffractograms and protein measurements, we can conclude that their origins or causes must not be the same. The white-belly cultivar Carnaroli showed higher amyloplastic integrity in chalky or pearled grains and higher amylose content in chalky grains than fractions than any other cultivar. In addition, Carnaroli had lower amylose content in the pearled fraction than white-core cultivars Montsianell and Bomba, which showed higher amylose content. Furthermore, Carnaroli was the only cultivar having a lower glutelin precursor in the chalky grains, while all other cultivars had higher glutelin precursors in chalky grains. Thus, Spanish Bomba and Montsianell white-core cultivars have similar physicochemical behaviours but are clearly different from white-belly Carnaroli cultivar, while their pearled fractions differ in some traits from stress-induced chalkiness. It is likely that our traditional Mediterranean cultivars carry different mutations in one or more genes that confer the specific high-quality white-core pearled phenotype and the high-quality white-belly phenotype. Ultimately, this should lead to the creation of a marker profile applicable to genotyping and breeding improved-quality rice cultivars.

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