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Continuous Flooding or Alternate Wetting and Drying Differently Affect the Accumulation of Health-Promoting Phytochemicals and Minerals in Rice Brown Grain

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Abstract: Climate changes impose adoption of water-saving techniques to improve the sustainability of irrigated rice systems. This study was aimed, by a two-years side-by-side comparison, at verifying the hypothesis whether "Alternate Wetting and Drying" (AWD) affects the concentrations of health-related compounds and minerals in brown grains of three japonica rice (*Oryza sativa* L.) cvs ('Baldo', 'Gladio', and 'Loto') usually grown in temperate areas in continuous flooding (CF). Due to the rotational turns in water distribution imposed by local authorities and to the weather behavior, different AWD timing and severity occurred in the two years of the study. AWD induced in both seasons yield losses in 'Baldo' and 'Gladio' but not in 'Loto'. In the brown grains of 'Loto', AWD increased the concentrations of total tocols, γ-oryzanol, flavonoids, and the antioxidant activity. AWD affected the concentrations of minerals, particularly increasing copper, cadmium and nickel, and decreasing manganese, arsenic and zinc. In the sensitive cultivars, 'Baldo' and 'Gladio', AWD seems to affect plant yield, rather than for severity of the dry period, for prolonged absence of ponded water that exposes plants to cooler temperatures. The selection of suitable cultivars, like 'Loto', tolerant to AWD-related stresses, could combine environmental, yield-related, and nutritional benefits improving the product quality.

Keywords: *Oryza sativa* L.; japonica ssp.; grain ionome

1. Introduction

Where it is environmentally and socio-economically possible, rice (*Oryza sativa* L.) is grown under continuous soil flooding (CF) in the so-called lowland rice systems. Therefore, even if the average value of physiological water productivity of rice is comparable to that of the other major C3 cereal crops, higher total inputs of water are required, so that about 40% of the global irrigation freshwater is used in rice paddy fields [1–4]. Global warming and competition between lowland rice and other seasonal crops, together with industrial and civic requirements for water, may cause physical and/or economic water shortages for rice cultivation [5]. It is expected that by 2050 several million hectares of currently lowland irrigated rice systems will experience water scarcity [3]. A further critical issue in lowland rice systems is the establishment of ideal conditions for emission into the atmosphere of greenhouse gases, mostly methane [6,7]. It follows that the development of water-saving techniques is essential to improve the sustainability of irrigated rice systems [3]. Among them, "Alternate Wetting and Drying" (AWD) is a management practice developed by the International Rice Research Institute (IRRI) [8] that is becoming increasingly widespread.

In the CF condition, excluding a short period to allow weed control, constant pond water is maintained in the field until the pre-harvest drainage. In AWD, rice paddies are intermittently submerged and dried so that the upper soil layers switch from anaerobic to aerobic conditions several times during the crop growing season. In order to avoid yield reductions due to drought stress responses, in AWD the field is usually re-submerged before the values of the soil water potential (Ψ_w) in the rooting zone become lower than −20 kPa [9]. Despite the original protocol issued by IRRI [10], several variants of paddy field water management are reported as AWD; they differ in number, severity, and timing (referred to the crop phenological phases) of the dry periods, resulting in different effects on yield and water use efficiency [9]. Several studies demonstrated that AWD could markedly (up to 90%) reduce the global warming potential of the gaseous emissions from rice fields and improve water use efficiency up to more than 60% [9,11]. Depending on severity and duration of soil dryness, number of the dry-wetting cycles along the season, cultivar adopted, and local weather trends, AWD does not affect [12], lowers [13] or even increases [14] yield compared to CF. Recently, it has been put in evidence that soil properties and particularly duration and degree of soil drought induced by the dry periods are the most effective factors that influence yield under AWD [9]. A study on a large panel of rice accessions usually grown under CF also suggested genotypic influences on growth and yield performance under AWD [15].

Although it is well known that the soil water status during rice growth might affect qualitative traits of the grains [16,17], to our best knowledge only a recent paper [18] reported on the possible effects of AWD on grain quality and nutritional value.

Due to the presence in the bran of several bioactive molecules able to protect against age-related pathologies [19], the dietary consumption of brown grain rice is advised, expanding the market of this commodity. The accumulation of vitamins, health-promoting metabolites and mineral microelements in the grain is regulated by genetic and environmental factors as well as by their interactions [19,20]. Conventional and modern breeding tools, specific agronomic techniques and postharvest treatments offer opportunities to improve the nutritional value of rice grain. Nevertheless, the actual environmental sustainability of these solutions is not always ascertained [21].

Aim of this work was to verify, by a side-by-side comparison, the hypothesis whether AWD, as influenced by the different environmental conditions in different seasons, could affect the concentrations of metabolites with health-related properties, minerals and toxic trace elements in the brown grain of three temperate japonica rice cultivars usually grown in Italy under CF. The three cultivars selected belong to different market segments: 'Baldo', largely used for the typical Italian dish risotto, 'Gladio', particularly suited for parboiling, and 'Loto', with several applications in the industrial production of rice-derived foods.

The study was conducted by relying entirely on the water supply (i.e., soil water status) available in the two years considered (2012 and 2013), as made possible uniquely by the weather conditions and the rotational turns in water distribution. In fact, the in advance-fixed irrigation turns stated in most of the European rice areas by the local Authorities that manage the distribution of water among fields can limit the possibility to reflood promptly when the soil Ψ_w drops below the value commonly considered as the threshold for a yield-safe AWD [3,9].

2. Materials and Methods

2.1. Experimental Design, Growth Conditions, and Grain Processing

Field trials were carried out during the 2012 and 2013 growing seasons at the CREA-Research Centre for Cereal and Industrial Crops in Vercelli (Italy; coordinates 45°19’204” N, 8°22’25,35” E-WGS84). Air temperatures and rainfall were recorded daily over the growing period. The soil of the field used is classified as the loam type; its physico-chemical characteristics, reported in Supplemental Table S1, were evaluated according to the Italian official methods for soil analyses. The field was divided by embankments in three areas and two of them, non-adjacent and hydraulically independent, were subjected to two different water regimes: Continuous flooding (CF) and alternate wetting and drying (AWD). Three temperate japonica rice cultivars (‘Baldo’, ‘Gladio’, and ‘Loto’) were grown under CF or AWD adopting a completely randomized block design with three replicates per watering condition. Six trial tensiometers (2710ARL series, Soilmoisture Equipment Corp., Goleta, CA, USA) were applied to the AWD area to record the value of soil Ψ_w at a depth of −20 cm at intervals of about 72 h. On May 10th, 2012 and May 14th, 2013, seeds were drill seeded into dry soil to a depth of 2 cm by using a Wintersteiger plot seeder machine (Plotseed S; Wintersteiger Italia Srl., Badia, Italy). Each plot consisted of three 170-cm long rows, 10-cm spaced. In both years, when plants were at the three leaflets stage (21 days and 25 days after sowing in 2012 and 2013, respectively), their number per row was reduced to 60 (180 plants/plot) by hand-thinning and both areas were flooded to obtain little more than 5 cm of pond water. In the CF water regime, flooding was maintained at this level up to the pre-harvest drainage, imposed on August 24th and September 16th in 2012 and 2013, respectively (5–10 cm water). In AWD in 2012, after the soil drainage, water was re-supplied a first time after the value of soil Ψ_w had reached a critical value lower than −30 kPa, and two additional times when Ψ_w dropped to about −20 kPa. In 2013 no irrigation intervention was necessary since, due to abundant rainfalls, the values of soil Ψ_w fluctuated between 0 kPa and the minimum value of −8 kPa. Fertilization (0.1 t ha^{-1} 23-0-10 nitrogen-phosphorus-potassium, NPK) was performed at pre-sowing and no additional nitrogen input was applied to the crop; weeds were manually removed.

Panicles were hand-harvested from each plot when they visually reached the R9 stage [22]. The grains harvested from each plot were air-dried, weighed and randomly divided into batches of 50, 100, or 300 units. The 300-seed batches were ground to a fine powder by an agate porcelain spheres RetschTM Mixer Mill MM200 (Retsch GmbH, Haan, Germany). Aliquots (about 350 mg) of the powder were immediately frozen in liquid N_2 and stored at −80 °C.

2.2. Physiological and Yield Performances

Panicle initiation timing was defined as the day when three out of 10 main stems per plot had a panicle 1 to 3 mm long. Days to flowering (DF) refers to the number of calendar days between the date of sowing and the date of flowering, as defined when at least 50% of the plants of the plot had extruded more than 1/3 of the panicle. Days to maturity (DM) was calculated as the number of days from sowing until at least 50% of the panicles had a dry (straw-colored) rachis for 2/3 of their length. Days from flowering to maturity (DFM) were calculated as DM-DF.

Flag leaf chlorophyll and nitrogen nutritional status were estimated 10 days after flowering as chlorophyll (CHL) and nitrogen balance (NBI) indexes using the Dualex Scientific+^{TM} (Force-A; Dynamax Inc., Houston, TX, USA) sensors [23].

The number of fertile tillers and panicles per linear meter was evaluated at plant maturity for each plot. All seeds harvested from each plot were weighed and the linear yield per meter was calculated. Three 100-seed samples obtained from each plot were weighed and scanned to obtain images subsequently analyzed by the WinSeedlePro V.201 software package (Regent Instruments Inc., Quebec, Canada) to determine the average width and length of the seeds.

2.3. Brown Grain Nutritional Traits

The brown grain apparent amylose content (AAC) was estimated as previously described [24] by using a FIAstar^{TM} 5000 Auto-Analyzer (Foss Italia Srl., Padova, Italy). Three samples of 50 seeds from each plot were analyzed. The results were expressed with reference to a calibration curve constructed by the Foss Infratec^{TM} SoFIA software and obtained by using standard rice samples (CRM 465: 15.4%, CRM 466: 23.1%, and CRM 467: 27.7% of AAC) supplied and certified by Foss. Both experimental and reference analyses were repeated twice.

The N-protein total content was evaluated on 1-g samples of brown grain powder (see above) by a macro-Kjeldhal procedure [25] using the Tecator^{TM} Kjeltec^{TM} System (Foss Italia Srl.).

The total flavonoids concentrations and total antioxidant capacity were determined in samples (about 1 g) of brown grain powder extracted and analyzed according to methods previously described [26,27]. Briefly, samples were extracted twice with 3 mL of methanol added with 1% (v/v) HCl at room temperature (RT) for 24 h, filtered through a Millipore filter (Merck SpA, Milano, Italy) with a 0.2-µm nylon membrane under vacuum at 23 °C, and finally centrifuged at 20000× *g* for 10 min. Total flavonoids were determined colorimetric ally in 0.5 mL of the methanolic extracts and quantified based on a standard curve of rutin. To determine the total antioxidant capacity, an $ABTS^+$ (2,2′-azino-bis [3-ethylbenzothiazoline-6-sulphonic acid]) solution (3.9 mL, A_{734} = 0.700) was added to 0.1 mL of the methanolic extract and mixed thoroughly. The reaction mixture was kept at RT for 6 min, and the A_{734} was immediately recorded. A Trolox standard solution in 80% (v/v) ethanol was prepared and assayed under the same conditions. Results were expressed as Trolox equivalent antioxidant capacity (TEAC, mmol Trolox equivalents kg^{-1} brown grain powder).

The concentrations of tocotrienols (T3: αT3, γT3, and δT3) and of tocopherols (Ts: αT, γT, and δT) in the brown grain were detected by a one-step equilibrium extraction procedure coupled with reversed-phase HPLC. Briefly, aliquots (350 mg) of brown grain powder were vortex-mixed in 10 mL of methanol and treated according to the method described previously [28]. The analyses of the extracts were conducted using the Agilent 1260 Infinity Quaternary LC System (Agilent Technologies Italia SpA, Cernusco s/N, Italy), equipped with a Sentry guard-column (Nova-Pak C18, 4 µm, 3.9 mm × 20 mm, Waters SpA, Sesto S.G., Italy), a Nova-Pak C18 column (4 µm, 3.9 mm × 150 mm, Waters) and an Agilent G134F UV/Vis absorbance detector in series coupled with the Agilent G1321B Fluorescence Detector A. After sample injection, a mobile phase consisting in 45/45/5/5 (v/v/v/v) acetonitrile/methanol/isopropyl alcohol/aqueous acetic acid was flushed for 6 min at a flow rate of 0.8 mL min^{-1}. Afterwards, the mobile phase was changed to 25/70/5 (v/v/v) acetonitrile/methanol/isopropyl alcohol over the next 10 min and held for 12 min before being returned to the initial conditions. Both Ts and T3s were detected by fluorescence at the excitation and emission wavelengths of 298 nm and 328 nm, respectively, and the γ-oryzanol components were determined by the UV/Vis detector at 325 nm. The chromatograms were recorded and processed with a LC/MSD ChemStation Rev.B.04.03 (Agilent Technologies). All compounds were confirmed by chromatographic comparison with the respective standards and their concentrations in the brown grain extracts quantified using external calibration curves. T3s and Ts standards were purchased from Supelco Analytical and Sigma Aldrich (Sigma Aldrich Srl., Milano, Italy). Standards of the three major γ-oryzanol components, namely *cyclo*artenyl ferulate (CAF), 24-methylen*cyclo*artenyl ferulate (24 Me-CAF), and campesteryl ferulate (CSF), were purified and generously provided by Dr. A. Tava (CREA-FLC, Fodder and Dairy Productions Research Centre, Lodi, Italy).

The phytic acid brown grain contents were determined in 350-mg powder samples according to the literature [29]. The results were expressed as phytic acid concentrations (g kg^{-1} of brown grain powder) referring to a Na-phytate (Sigma Aldrich) standard curve (0–60 mg L^{-1}).

2.4. Brown Grain Ionome

Brown grain powder (350 mg) samples were digested in Teflon tubes filled with 10 mL of 65% (v/v) HNO_3 by a microwave digester system (MULTIWAVE-ECO, Anton Paar Italia Srl., Rivoli, Italy) by applying two-steps power ramps (Step 1: To 500 W in 10 min, maintained for 5 min; Step 2: To 1200 W in 10 min, maintained for 15 min). After 20 min cooling, the mineralized samples were transferred into polypropylene test tubes and diluted 1:20 with MILLI-Q water (Merck). Concentrations of the considered elements (arsenic (As), calcium (Ca), cadmium (Cd), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), nickel (Ni), phosphorus (P), and zinc (Zn)) were measured by inductively coupled plasma-mass spectrometry (ICP–MS; Bruker AURORA M90 ICP–MS, Bruker Daltonik GmbH, Leipzig, Germany). An aliquot of a 2 mg L^{-1} internal standard solution (^{72}Ge, ^{89}Y, and ^{159}Tb) was added to both samples and multi- and single- (for As and P) element calibration standards to a final concentration of 20 μg L^{-1}. Possible polyatomic interferences were removed by the collision–reaction-interface with an H_2 flow of 70 mL min^{-1}. Accuracy was evaluated using a certified reference material (rice flour-NIST SRM 1568b; National Institute of Standards and Technology-NIST, Gaithersburg, MD; Supplemental Table S9).

2.5. Statistical Analysis

Descriptive statistical analyses were carried out by Sigma Plot for Windows version 11.0 (Systat Software, Inc., San Jose, CA, USA). Quantitative results were presented as means of the values obtained from each of the three plots; the value of each plot was obtained from a variable number of analytical repeats (see above for the specific analyses). For each parameter, minimum and maximum values and SD are reported in Supplemental Material. The data were also subjected to multiway ANOVA analysis (variables: Cultivar, year, and water regime) conducted in the freely available R environment with the Foreign, Agricolae, and Emmeans packages. The homogeneity of the variances was checked with a Levene's test. A Tukey post-hoc test was carried out with Bonferroni correction, and significance was set at $p < 0.05$.

3. Results

3.1. Weather Conditions and AWD Timing and Severity

The two growing seasons during which the study was carried out resulted quite different in rainfall and temperature ranges (Figure 1a–d). In 2013, in the time span May–September, the total rainfall was nearly two-fold than in 2012 (491 mm and 264 mm, respectively); therefore, in 2013 the drop of soil Ψ_w to particularly negative values during all the AWD dry periods was prevented (Figure 1f).

In the second year, no additional irrigation intervention after the first one was necessary to re-supply water to the plots after drainage. Moreover, the air temperatures, on an average basis, were lower in 2013 than in 2012, and this difference was particularly apparent at the flowering stage of the plants, when minimum and maximum temperatures were, as an average, 3 °C and 5 °C, respectively, lower (Figure 1c,d). In both years, plants experienced two dry periods (soil $\Psi_w < 0$ kPa at a depth of −20 cm) during the vegetative phase (Figure 1e,f). These resulted longer in 2012 than in 2013 (10 days and 14 days vs. 6 days and 9 days, respectively), and lower values of soil Ψ_w at −20 cm (minimum −32.0 kPa and −20.5 kPa vs. −7.0 kPa and −7.5 kPa) were reached before the soil was again saturated, by either irrigation in 2012 or rainfall in 2013. During the reproductive stage, after flowering and before the pre-harvest drainage, plants experienced an additional dry period whose duration was longer (10 days vs. 8 days) and severity higher (up to −18.0 kPa and −8.0 kPa) in 2012 than in 2013. Once again, in the second year this dry period was interrupted by rainfall, whereas in the first year the AWD plots were irrigated (Figure 1e,f). In both years, flowering of all three cultivars occurred under saturated soil conditions.

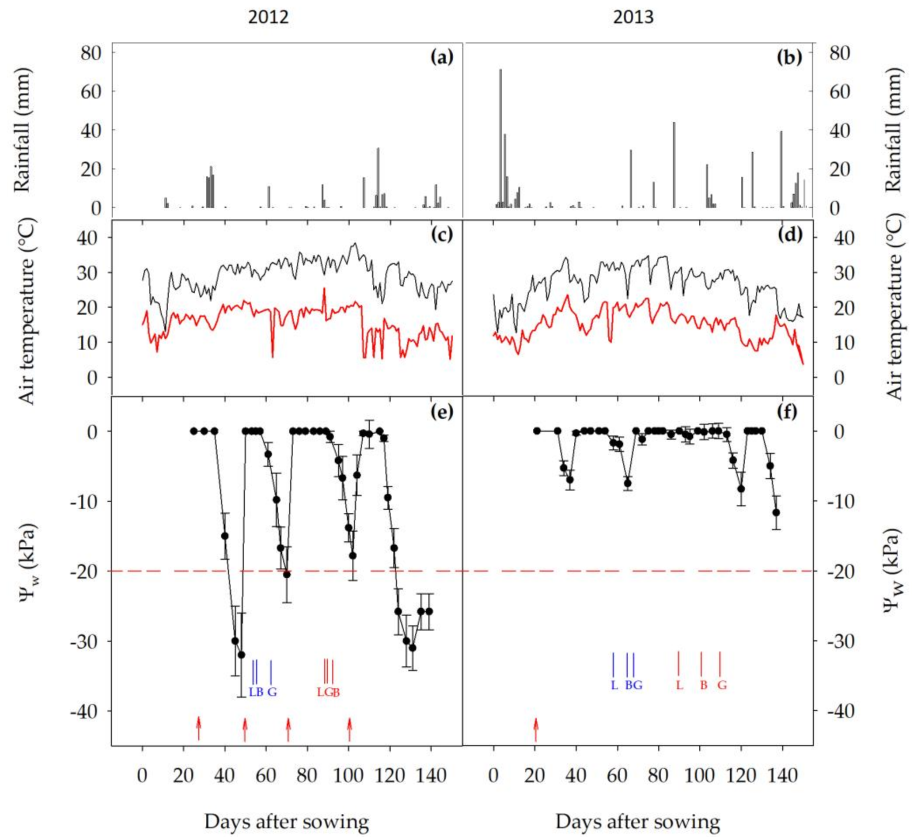

Figure 1. Values of rainfall (**a**,**b**), air temperature (**c**,**d**), and soil water potential (Ψ_w; **e**,**f**) in the two growing seasons 2012 (**a**,**c**,**e**) and 2013 (**b**,**d**,**f**); c and d: Black lines: Maximum values; red lines: Minimum values; e and f: Red arrows indicate field flooding in the AWD field trials; blue vertical lines indicate the panicle initiation day of 'Baldo' (B), 'Gladio' (G), and 'Loto' (L); red vertical lines indicate the flowering days of 'Baldo' (B), 'Gladio' (G), and 'Loto' (L). The red dotted horizontal line indicates the –20 kPa value usually considered in the literature as a threshold for reflooding [9]. Ψ_w values are the means ± SD of six measurements.

3.2. Trait Differences

The results of the statistical analysis on the significance of variance estimates related to cultivar, year, water regime, and their interactions on the traits considered are reported in Supplemental Table S2. Remarkable significant differences ($p < 0.001$ and $p < 0.01$) were observed, for most of the traits considered, among the cultivars (phenological and yield-related traits; apparent amylose and N-protein contents; health-related and antioxidant compounds, with the exceptions of phytic acid, αT, and CAF; all minerals with the exception of Zn and As, whose contents were significantly affected by the water regime and by the year) and between the two water regimes (phenological and yield-related traits, with the exception of fertile tillers m^{-1}, grain width, and flowering-maturing time that varied among cultivars and/or between years; health-related and antioxidant compounds, with the exceptions of phytic acid, αT, and CAF; all minerals with the exception of Fe and Mg). Relevant differences, even if less numerous, were also found between the two seasons (phenological and yield-related traits, with the exception of weight of 100 seeds, and grain length and width; health-related and antioxidant compounds, i.e., total tocols, δT3, all tocopherols, and CSF; minerals, with particular regard to Zn, As, and Cd).

Remarkable interactions between and among the variables were also observed for most traits (Supplemental Table S2). Such a complex picture in phenotypic variability arose from effects ascribable to water regime, intrinsic differences in the genetic, phenotypic and agronomic characteristics of the rice cultivars considered, and from the natural seasonal weather patterns that affected the AWD condition.

3.3. Plant Phenology, Yield, Grain Morphology, and Quality

In 2012 the flowering times for the three cultivars were not significantly different in the two water regimes (Table 1, Table S3), whereas in 2013 plants grown under AWD showed a significant flowering delay compared to CF. Moreover, in 'Gladio' the flowering time was affected by the season under both water regimes, whereas in 'Baldo' and in 'Loto' a season effect was observed only in plants grown under AWD.

Neither the water regime nor the season affected the flowering-maturing time of each of the three cultivars (Table 1, Table S3). Table 1 highlights that under CF the linear yield in 'Baldo' was always higher than in 'Gladio' and 'Loto'. Indeed, 'Baldo' suffered significant yield reductions in both years (−21% and −15%) when grown under AWD, whereas 'Gladio' showed a reduction only in 2013 (−32%), and 'Loto' never showed AWD-related yield reduction.

For the different yield components, Table 1 indicates that: a) Concerning the number of fertile tillers per meter in the three cultivars, no significant effects were observed in the two growing seasons and in the two different water regimes; b) concerning the number of seeds per panicle, only 'Gladio' showed a reduction in AWD and the differences, more apparent in 2013 (−36%) than in 2012 (−22%), were significant in both years; no effect of the season resulted in the comparison within the same water regime; c) concerning the weight of 100 grains, in 2013 it was always reduced by AWD in all three cultivars, whereas in 2012 this reduction was significant only in 'Baldo'.

For 'Gladio' (in 2012 and 2013) and 'Baldo' (in 2013) seeds harvested from plants grown in AWD were shorter than those grown in CF (Table 1), whereas no year effects for grain size could be observed within each cultivar under the same water regime. The seed width did not change significantly in any cultivar in the two different years and under the two water management regimes (see also Table S3).

Table 1. Plant phenology, yield and grain morphology of three rice cultivars grown under continuous flooding (CF) or alternate wetting and drying (AWD).

Trait	Year	cv. 'Baldo'		cv. 'Gladio'		cv. 'Loto'	
		CF	AWD	CF	AWD	CF	AWD
Flowering time, d	2012	91^{bcd}	91^{bcd}	83^{e}	88^{cde}	87^{de}	87^{de}
	2013	96^{b}	105^{a}	94^{bc}	110^{a}	85^{e}	97^{b}
Flowering-maturing time, d	2012	56^{a}	52^{abc}	45^{bc}	42^{c}	50^{abc}	49^{abc}
	2013	59^{ab}	58^{ab}	57^{abc}	48^{abc}	62^{a}	63^{a}
Linear yield, kg m^{-1}	2012	319^{ab}	254^{cd}	201^{ef}	165^{f}	222^{de}	191^{ef}
	2013	327^{a}	276^{bc}	247^{cd}	169^{f}	261^{cd}	264^{cd}
Fertile tillers m^{-1}, n	2012	66^{c}	53^{c}	81^{abc}	74^{abc}	92^{abc}	67^{bc}
	2013	65^{c}	84^{abc}	93^{abc}	119^{a}	86^{abc}	112^{ab}
Seeds $panicle^{-1}$, n	2012	114^{ab}	100^{bcde}	107^{abc}	84^{fg}	92^{def}	85^{fg}
	2013	115^{a}	95^{cdef}	103^{abcd}	66^{e}	86^{efg}	76^{ge}
Weight of 100 grains, g	2012	3.70^{a}	3.20^{b}	2.12^{f}	1.90^{fg}	2.65^{de}	2.46^{e}
	2013	3.65^{a}	3.05^{bc}	2.11^{f}	1.71^{g}	2.82^{cd}	2.51^{e}
Grain length, mm	2012	7.26^{ab}	7.09^{abc}	7.46^{a}	6.77^{bcd}	6.53^{de}	6.40^{de}
	2013	7.55^{a}	6.89^{bcd}	7.45^{a}	6.82^{bcd}	6.59^{cde}	6.26^{e}
Grain width, mm	2012	3.28^{a}	3.20^{a}	2.19^{d}	2.46^{cd}	3.00^{ab}	2.76^{bc}
	2013	3.23^{a}	2.99^{ab}	2.11^{d}	2.21^{d}	2.99^{ab}	2.88^{abc}

Data are the means of values from each plot ($n = 3$). For each trait, values with same letter(s) are not significantly different at $p < 0.05$, as resulted by a Tukey post-hoc test with a Bonferroni correction.

In the early phases of seed development and filling, when the flag leaf was exerting its maximum source activity, plants experienced in both years the third dry soil period (Figure 1). Since the stay green and physiological status of flag leaf may impact on grain starch and protein accumulation [12], we measured two parameters (chlorophyll and nitrogen balance indexes), indicative of the flag leaf physiological status, as well as the apparent amylose and the N-protein contents in the mature seeds, in the two years and in the two water regimes. The results are reported in Table 2 and Table S4.

Table 2. Chlorophyll (CHL) and nitrogen balance (NBI) indexes in the flag leaf, apparent amylose and N-protein contents in brown grains of three rice cultivars grown under continuous flooding (CF) or alternate wetting and drying (AWD).

Trait	Year	cv. 'Baldo'		cv. 'Gladio'		cv. 'Loto'	
		CF	AWD	CF	AWD	CF	AWD
CHL	2012	36.52^{ab}	23.10^{b}	29.08^{ab}	27.56^{ab}	30.27^{ab}	33.03^{ab}
	2013	30.71^{ab}	30.50^{ab}	41.12^{a}	31.43^{ab}	38.04^{a}	33.20^{ab}
NBI	2012	28.93^{a}	16.47^{b}	20.93^{ab}	20.27^{ab}	23.05^{ab}	23.48^{ab}
	2013	17.73^{b}	20.31^{ab}	25.84^{ab}	19.69^{ab}	25.91^{ab}	22.15^{ab}
Apparent amylose content (%)	2012	18.57^{cd}	16.91^{def}	24.36^{a}	21.30^{bc}	14.22^{f}	16.28^{def}
	2013	17.52^{de}	19.06^{cd}	22.91^{ab}	25.86^{a}	16.94^{def}	14.76^{ef}
N-protein content (%)	2012	7.14^{abc}	6.81^{bc}	6.67^{bc}	7.11^{abc}	7.90^{ab}	7.41^{abc}
	2013	6.14^{c}	8.33^{a}	7.60^{ab}	7.31^{abc}	7.53^{ab}	8.22^{a}

Data are the means of values from each plot (n = 3). For each trait, values with same letter(s) are not significantly different at $p < 0.05$, as resulted by a Tukey post-hoc test with a Bonferroni correction.

Very similar values of CHL and NBI indexes were observed in all three cultivars independently of season and water regime. The only exception was represented by NBI in 'Baldo' in 2012, when in AWD a significant decrease (−43%) compared to CF was recorded.

Under CF the brown grain apparent amylose content (AAC) in the three cultivars was inherently different, with the highest values observed in both 2012 and 2013 in 'Gladio'. The AAC of the grain was in both seasons not significantly different between the irrigation regimes in 'Baldo' and 'Loto'. In 'Gladio' under AWD a reduction (−13%) in AAC was observed in 2012. Concerning the N-protein contents, only in 'Baldo' in 2013 a significant water regime-related difference (+36% in AWD) was observed.

3.4. Concentrations of Health-Promoting Phytochemicals in the Grains

Major antioxidant compounds in brown grain rice are flavonoids, tocols, and γ-oryzanol [30]. Since both genetic and environmental factors drive the accumulation of these molecules in the grain [19,20], we investigated whether adoption of the AWD water regime determined any change in their concentrations. The results are reported in Table 3 and Table S5. The concentrations of total flavonoids in the brown grains resulted rather similar among the cultivars, the two seasons and the two water regimes. In 2012 in 'Baldo' (+55%) and in both years in 'Loto' (+25% and +38%) higher values were observed in AWD compared to CF.

Table 3. Total concentrations of health-promoting compounds and antioxidant activity in brown grains of three rice cultivars grown under continuous flooding (CF) or alternate wetting and drying (AWD).

Trait	Year	cv. 'Baldo'		cv. 'Gladio'		cv. 'Loto'	
		CF	AWD	CF	AWD	CF	AWD
Total Flavonoids, g RE kg^{-1}	2012	1.34^{f}	2.08^{b}	2.01^{bc}	2.52^{a}	1.67^{de}	2.09^{b}
	2013	1.44^{ef}	1.93^{bcd}	1.86^{bcd}	2.39^{a}	1.75^{cd}	2.41^{a}
Total Tocols, mg kg^{-1}	2012	14.26^{d}	33.85^{ab}	15.54^{d}	14.28^{d}	11.14^{d}	27.40^{bc}
	2013	17.03^{d}	28.62^{b}	18.61^{cd}	19.42^{cd}	17.34^{d}	38.58^{a}
γ-oryzanol, mg kg^{-1}	2012	54.10^{cd}	57.00^{cd}	44.21^{d}	57.57^{cd}	49.95^{cd}	91.87^{ab}
	2013	60.44^{bcd}	78.47^{abc}	42.09^{d}	33.87^{d}	60.43^{bcd}	92.97^{a}
Phytic acid, g kg^{-1}	2012	10.03^{a}	10.49^{a}	10.82^{a}	11.30^{a}	10.71^{a}	10.23^{a}
	2013	10.44^{a}	10.87^{a}	8.63^{a}	9.12^{a}	9.71^{a}	10.29^{a}
Antioxidant Activity, mmol TEAC kg^{-1}	2012	5.22^{de}	5.39^{bcde}	6.24^{ab}	6.15^{abc}	3.92^{fg}	4.80^{ef}
	2013	4.91^{e}	5.33^{cde}	6.31^{a}	5.92^{abcd}	3.40^{g}	4.71^{ef}

RE, rutin equivalents; and TEAC, trolox equivalent antioxidant capacity. Data are the means of values from each plot (n = 3). For each trait, values with same letter(s) are not significantly different at $p < 0.05$, as resulted by a Tukey post-hoc test with a Bonferroni correction.

Rice brown grains are rich in tocols, the family of chroman-6-ol compounds collectively known as vitamin E [30]. In plants grown under CF the total tocols concentrations were very similar among the cultivars and not subjected to season variability. In AWD, in both 2012 and 2013, marked increases in the brown grain total tocols concentrations were observed in 'Baldo' (+137% and +68%) and in 'Loto' (+146% and +122%). Rice γ-oryzanol is a mixture of ferulate esters of triterpene alcohols with antioxidant properties. Its concentrations, similar in the three cultivars under CF and never conditioned by a seasonal effect, were significantly higher in AWD only in 'Loto' in both 2012 (+84%) and 2013 (+54%). Phytic acid is considered an antinutritional phytochemical since it binds with high affinity mineral nutrients limiting their absorption in mono-gastric animals and humans; nevertheless, at the cellular level it exerts beneficial effects by counteracting the generation of hydroxyl radicals via the Fenton-type reaction [31]. Although the concentrations of phytic acid in rice brown grain are regulated by genetic and environmental factors [30], none of the variables considered in our investigation resulted to affect them. Concerning the total antioxidant activity, significant differences resulted related to the genotype in plants grown under CF: The lowest activity was always observed in 'Loto' with progressively increasing values in 'Baldo' and in 'Gladio'. An AWD-induced increase was observed only in 'Loto' in 2013 (+39%; Table 3 and Table S5).

The analysis of the components of the tocol family, i.e., tocotrienols (αT3, γT3, and δT3) and tocopherols (αT, γT, and δT; Table 4 and Table S6) showed that, consistent to the literature [30], γT3 was by far the most abundant tocol in the brown grain, representing about 60%–70% of the total tocols (Table 3) measured under CF in the three cultivars.

Table 4. Concentrations of the single tocochromanols in brown grains of three rice cultivars grown under continuous flooding (CF) or alternate wetting and drying (AWD).

Tocochromanol	Year	CF	AWD	CF	AWD	CF	AWD
αT3, mg kg^{-1}	2012	0.67^{cd}	1.39^{cd}	0.35^{d}	0.56^{cd}	3.95^{b}	2.25^{bc}
	2013	0.50^{d}	5.95^{a}	0.56^{cd}	0.68^{cd}	0.55^{cd}	3.30^{b}
γT3, mg kg^{-1}	2012	11.08^{bc}	23.64^{a}	10.27^{bc}	10.17^{bc}	6.84^{c}	25.60^{a}
	2013	10.55^{bc}	16.06^{b}	13.39^{b}	14.55^{b}	12.80^{b}	27.80^{a}
δT3, mg kg^{-1}	2012	0.53^{de}	1.69^{c}	0.27^{e}	1.13^{cde}	0.89^{cde}	2.71^{b}
	2013	1.55^{c}	1.44^{c}	1.38^{cd}	0.98^{cde}	1.29^{cd}	3.88^{a}
αT, mg kg^{-1}	2012	0.09^{c}	0.04^{c}	0.67^{bc}	0.39^{bc}	0.21^{c}	0.84^{bc}
	2013	0.77^{bc}	1.46^{ab}	0.98^{bc}	0.74^{bc}	0.87^{bc}	2.25^{a}
γT, mg kg^{-1}	2012	0.09^{e}	2.45^{b}	0.96^{cd}	0.88^{cd}	0.93^{cd}	3.49^{a}
	2013	2.47^{b}	1.19^{c}	1.00^{cd}	1.04^{c}	0.46^{de}	0.83^{cd}
δT, mg kg^{-1}	2012	0.92^{c}	0.27^{d}	0.11^{d}	0.09^{d}	0.31^{d}	1.39^{b}
	2013	0.26^{d}	2.23^{a}	0.20^{d}	nd	nd	nd

αT3, γT3, and δT3: α-, γ-, and δ-tocotrienols; αT, γT, and δT: α-, γ-, and δ-tocopherols. Data are the means of values from each plot (n = 3). For each trait, values with same letter(s) are not significantly different at $p < 0.05$, as resulted by a Tukey post-hoc test with a Bonferroni correction. nd: Not detectable.

Concerning the specific effect of the water regime on γT3, AWD determined a strong increase in 'Loto' in both 2012 (+274%) and 2013 (+117%). A similar effect was observed in 'Baldo' in 2012 (+113%), whereas in 'Gladio' the AWD water regime did not induce any effect. Concerning αT3, in 2013 strong increases under AWD were observed in 'Baldo' (by about 10-fold) and in 'Loto' (by about five-fold). In 'Gladio' the concentrations of αT3 did not change in the two water regimes in both years. The concentrations of δT3 were higher under AWD in 'Baldo' in 2012 (+219%) as they were in 'Loto' in both seasons (+204% in 2012 and +200% in 2013). The concentrations of tocols (αT, γT, and δT) were very low and, in a few cases, not detectable. As already observed also for T3s, in 'Gladio' the concentrations of all Ts did not change under AWD in both years. In 'Baldo', the concentrations of αT, γT, and δT, although changed under AWD, did not show a univocal trend to either increase or decrease in the two seasons. In 'Loto' all three tocols increased under AWD. The greatest part of the effect of AWD on total tocochromanols in both 'Baldo' and 'Loto' (Table 3 and Table S5) seemed due to the increases in T3s, in relation also to their higher (particularly of γT3) concentrations. Table 5 reports the relative amounts of the three main ferulate esters detected in the brown grain of the three cultivars: Cycloartenyl ferulate (CAF), 24-methylencycloartenyl ferulate (24Me-CAF), and campesteryl ferulate (CSF), which account for 85% of the total γ-oryzanol concentration in rice grain [30]. The highest concentrations were always observed for 24Me-CAF, followed by CSF and CAF. In the extracts from brown grains of the three rice cultivars considered in the present study, these three major γ-oryzanol components were detected (Table 5 and Table S7) and the total γ-oryzanol content was estimated as their sum (Table 3).

In all three cultivars the concentrations of CAF did not change in AWD compared to CF in both years. 24-methylencycloartenyl ferulate and CSF also showed a similar behavior in 'Baldo' and 'Gladio' in both seasons. In 'Loto' the concentrations of both compounds significantly increased under AWD in 2012 (+139% for 24Me-CAF and +57% for CSF) and in 2013 (+71% for 24Me-CAF and +64% for CSF). These values were consistent with the AWD effects observed in 'Loto' for γ-oryzanol (+84% in 2012 and +54% in 2013; Table 3).

Table 5. Concentrations of the γ-oryzanol complex components in the brown grain of three rice cultivars grown under continuous flooding (CF) or alternate wetting and drying (AWD).

Component	Year	cv. 'Baldo'		cv. 'Gladio'		cv. 'Loto'	
		CF	AWD	CF	AWD	CF	AWD
CAF, mg kg^{-1}	2012	5.59^{ab}	5.65^{ab}	6.34^{ab}	4.18^{ab}	4.00^{ab}	4.89^{ab}
	2013	3.83^{ab}	6.11^{ab}	4.94^{ab}	1.40^{b}	7.14^{a}	9.05^{a}
24Me-CAF, mg kg^{-1}	2012	31.05^{bcd}	34.26^{bcd}	23.24^{cd}	26.94^{cd}	25.05^{cd}	59.85^{a}
	2013	32.89^{bcd}	40.20^{abc}	21.62^{cd}	15.03^{d}	29.16^{cd}	49.97^{ab}
CSF, mg kg^{-1}	2012	17.42^{c}	15.40^{c}	16.37^{c}	15.44^{c}	17.82^{c}	27.97^{b}
	2013	23.27^{bc}	33.23^{ab}	15.24^{c}	15.50^{c}	24.43^{bc}	40.09^{a}

CAF, *cyclo*artenyl ferulate; 24Me-CAF, 24-methylen*cyclo*artenyl ferulate; CSF, campesteryl ferulate. Data are the means of values from each plot (n = 3). For each component, values with same letter(s) are not significantly different at $p < 0.05$, as resulted by a Tukey post-hoc test with a Bonferroni correction.

3.5. Effects of AWD on Grain Ionome

The concentrations of mineral nutrients and trace elements in brown grains from the three cultivars in either CF or AWD were compared in both years; the results are reported in Table 6 and Table S8.

The concentrations of four major macro-elements, i.e., K, P, Ca, and Mg, were in most cases significantly affected by neither the season nor the irrigation regime, even if under AWD a slight tendency to a reduction could be observed. An exception was represented by 'Baldo' in 2012, that showed an increased concentration of K in AWD with respect to CF. For the essential mineral nutrient Fe, a genotype-related difference was observed: In 'Baldo' the concentrations of Fe in CF were generally lower than in 'Gladio' and 'Loto'. No change in Fe concentrations under the two different water regimes could be observed in any of the three genotypes and in any season. Concerning other essential mineral nutrients, for Mn in all three cultivars its concentrations in the brown grain of plants grown in AWD were reduced compared to CF in both years. The concentrations of Zn were diminished under AWD in all three cultivars only in 2013, whereas in 2012 no change was observed. On the contrary, the concentrations of Cu and Ni were in both years increased by AWD in all three cultivars; in particular, for the latter element the effect was quite strong, in the range between +200% and +650%.

The accumulation in brown grains of Cd and As, two toxic trace elements of major concern for the safety risks of rice consumers, had opposite behaviors in the two water regimes. In fact, in both years, the concentrations of Cd in the brown grain resulted higher in AWD than in CF. The most dramatic increase (+850%) was observed in 'Baldo' in 2012. In the three cultivars, the increases in Cd concentrations observed in AWD were lower in 2013 than in 2012. The concentrations of As resulted always strongly diminished under AWD. Within each genotype and water regime, the concentrations of As tended to be lower in 2013 than in 2012.

Table 6. Brown grain ionome of three rice cultivars grown under continuous flooding (CF) or alternate wetting and drying (AWD).

Element	Year	cv. 'Baldo'		cv. 'Gladio'		cv. 'Loto'	
		CF	AWD	CF	AWD	CF	AWD
K, g kg^{-1}	2012	2.72^{def}	3.04^{abc}	2.86^{bcdef}	2.74^{def}	3.13^{a}	2.91^{abcde}
	2013	2.81^{cdef}	2.67^{f}	3.09^{ab}	2.70^{ef}	2.94^{abcd}	2.71^{def}
P, g kg^{-1}	2012	3.50^{efg}	3.62^{defg}	4.05^{bc}	4.01^{bc}	4.22^{ab}	3.97^{bcd}
	2013	3.33^{g}	3.44^{fg}	4.47^{a}	3.80^{cdef}	3.86^{bcde}	3.77^{cdef}
Ca, mg kg^{-1}	2012	80.21^{abc}	66.18^{e}	68.32^{de}	73.75^{cde}	82.72^{abc}	79.60^{abcd}
	2013	75.11^{bcde}	65.95^{e}	85.94^{ab}	80.61^{abc}	87.53^{a}	78.80^{abcd}
Cu, mg kg^{-1}	2012	2.80^{de}	4.90^{a}	3.31^{cde}	5.10^{a}	3.32^{cde}	4.82^{a}
	2013	2.39^{e}	4.30^{abc}	3.70^{bcd}	4.81^{a}	2.73^{de}	4.42^{ab}
Fe, mg kg^{-1}	2012	5.65^{d}	6.22^{cd}	10.12^{ab}	9.83^{ab}	8.73^{abc}	7.68^{bcd}
	2013	7.61^{bcd}	8.24^{abcd}	10.63^{a}	9.20^{ab}	9.01^{ab}	10.60^{a}
Mg, g kg^{-1}	2012	1.16^{f}	1.27^{def}	1.33^{bcd}	1.31^{cde}	1.49^{a}	1.44^{ab}
	2013	1.20^{ef}	1.18^{f}	1.47^{a}	1.32^{cd}	1.38^{abcd}	1.41^{abc}
Mn, mg kg^{-1}	2012	15.05^{c}	7.94^{e}	17.00^{bc}	14.69^{c}	19.54^{ab}	11.31^{d}
	2013	15.62^{c}	8.23^{de}	17.91^{abc}	10.25^{de}	21.12^{a}	16.51^{bc}
Ni, mg kg^{-1}	2012	0.40^{ef}	1.40^{cd}	0.71^{e}	2.16^{ab}	0.19^{f}	1.26^{d}
	2013	0.21^{ef}	1.58^{cd}	0.49^{ef}	2.57^{a}	0.25^{ef}	1.79^{bc}
Zn, mg kg^{-1}	2012	22.64^{ab}	24.38^{a}	22.16^{abc}	23.63^{a}	25.13^{a}	23.96^{a}
	2013	24.28^{a}	19.24^{c}	24.00^{a}	20.18^{bc}	22.62^{ab}	19.14^{c}
As, µg kg^{-1}	2012	148.00^{ab}	79.60^{de}	170.21^{a}	63.42^{e}	146.19^{abc}	86.09^{de}
	2013	107.38^{cd}	18.68^{f}	109.71^{bcd}	10.75^{f}	119.43^{bcd}	20.84^{f}
Cd, µg kg^{-1}	2012	10.52^{g}	99.92^{a}	26.28^{de}	53.26^{b}	12.83^{fg}	43.48^{bc}
	2013	19.52^{efg}	39.14^{cd}	12.83^{fg}	25.17^{ef}	11.63^{g}	29.93^{de}

Data are the means of values from each plot (n = 3). For each trait, values with same letter(s) are not significantly different at $p < 0.05$, as resulted by a Tukey post-hoc test with a Bonferroni correction.

4. Discussion

Among the agronomic techniques able to improve the sustainability of the rice systems, AWD complies the increasing requirement of water saving practices. Nevertheless, knowledge of the possible effects of the soil water status induced by this irrigation regime on the qualitative traits of the rice grain is still scanty.

The present work intended to deepen the knowledge on this topic by verifying the hypothesis whether AWD, as influenced by the different environmental conditions experienced in different seasons, could affect the concentrations of metabolites with health-related properties, minerals and toxic trace elements in the brown grain of three temperate japonica rice cultivars.

The very different weather conditions in 2012 and 2013 and the rotational turns in water distribution imposed by the local Irrigation Water Managers determined two very different AWD conditions (Figure 1) than can be classified as severe AWD and mild AWD [9] in 2012 and 2013, respectively.

In rice, panicle initiation and flowering are the developmental stages most sensitive to environmental conditions, and particularly to low soil Ψ_w and air temperature. Although the phenological patterns of the three cultivars are different ('Loto' and 'Gladio' early cultivars, 'Baldo' a late one), in 2012 both panicle initiation and flowering of all cultivars occurred in flooded soil condition. In 2013 all cultivars reached the panicle initiation stage during a period of mild soil drying and flowered in flooded soil condition. In this context, it appears reasonable to exclude that the differences concerning the yield responses to AWD of the three cultivars ('Baldo' and 'Gladio' sensitive, 'Loto' insensitive) are primarily due to differences in the soil water content during critical developmental phases. The yield losses in 'Baldo' and 'Gladio' were not related to the severity of the AWD drying periods, since in 'Gladio' the negative effect was even more pronounced in the mild AWD condition of 2013. The yield components were affected differently by AWD in the three cultivars: The number of seeds per panicle, particularly in 'Gladio', and the weight of the grains, particularly in 'Baldo', contributed to a greater extent to the observed yield losses in AWD. Seeds harvested from 'Gladio' grown in AWD were smaller because they were basically shorter (Table 1).

The negative effects of AWD on yield and its components, when present, seemed related rather to the cooler temperatures during the growing periods than to the severity of soil drying (Figure 1 and Supplemental Figure S1). In 2013, in fact, independent of the value of soil Ψ_w, the concomitant low temperatures and absence, in AWD, of ponded water with its thermal buffering effect exposed more severely the plants to the effects of the daily temperature excursions, that negatively affect yield [32]. In the same year, the generalized delay in the flowering time of the three cultivars in AWD in comparison to CF shifted the grain-filling period towards cooler days. Since the overall length of the filling time (Table 1) of each cultivar was unchanged, the temperature factor could explain the greater effect on grain weight recorded in 2013 in the two sensitive cultivars (Table 1).

Possible negative effects of AWD on yield have been ascribed to decreased leaf photosynthetic rate and general metabolic activity, influenced by the leaf N nutritional status [12,33]. The Dualex®CHL and NBI indexes were similar in AWD and in CF (Table 2); therefore, we might reasonably exclude that in our conditions AWD determines early leaf senescence and/or N deprivation, capable to affect the leaf source function for the developing grains.

Apparent amylose content and N-protein concentration are indicators of rice grain quality, affected by genetic and environmental determinants, like temperature and N availability during the grain-filling period [34]. These parameters were not modified substantially by AWD in any cultivar (Table 2), allowing us to exclude effects on the sink strength of the developing grains [12].

Flavonoids, tocols, γ-oryzanol, and phytic acid are major antioxidants in rice brown grain [30]. The concentrations of total flavonoids in AWD tended to increase (Table 3). The essentially constant values of CHL and NBI indexes suggest, in agreement with Cerovic et al. [23], that the flavonoid concentrations in the flag leaf did not change in any genotype and water management regime, allowing to hypothesize that the microclimate condition established by AWD might stimulate the biosynthesis of flavonoids in the grains.

Under CF, the total tocols concentrations in the brown grain were close to the lowest limit of the range (15–60 mg kg^{-1}) reported for non-pigmented rice varieties [30]. The antioxidant properties of tocols contribute to stress tolerance in plants, explaining their accumulation under environmental constraints [35,36]. In 'Baldo' and 'Loto', AWD markedly increased the total tocols concentrations and seemed to increase all T3s, with particular regard to γT3 (the most abundant tocol in brown grain). In 'Gladio', lack of effect of AWD on total tocols concerned all of their components (Tables 3 and 4). In our material, the increases in the concentrations of Ts and T3s in AWD were particularly apparent in 'Loto' and essentially absent in 'Gladio' (Table 4). This effect may be related to a higher general sensitiveness of this genotype to the stressful environment of AWD; it may be hypothesized that the growth condition generated by this water regime somehow affects different specific regulatory points of the tocols metabolic pathway, known to be finely tuned [37,38].

Rice γ-oryzanol is a mixture of 25 so far identified ferulate esters of triterpene alcohols with antioxidant properties [26]. Out of the three cultivars studied, only 'Loto' showed a marked AWD-induced enhancement of total γ-oryzanol (Table 3) mainly due to a significant increase in 24-MeCAF (Table 5), consistent to what described for rice brown grain under different stressful environmental conditions [35]. A wide genetic variability in the environment-related accumulation of γ-oryzanol is described in several japonica rice cultivars [39], possibly explaining the peculiar behavior observed in 'Loto'.

The antioxidant activity of rice brown grain usually falls in the range 0.2–20 mmol TEAC kg^{-1} [30]. Only in 'Loto' this activity was increased in AWD (Table 3), consistent with the effects of this water regime on the antioxidants levels. It is interesting to stress that 'Loto', insensitive to AWD in terms of yield (Table 1), showed a higher capability to respond, through the accumulation of antioxidant compounds, to the probable stress conditions induced by this water regime.

Phytic acid is acknowledged to have antinutritional but also antioxidant properties [31,40]. In our material, the levels of phytic acid were not affected by AWD in any cultivar and in any year (Table 3), according to the general lack of effect of the water regime on the concentrations of total P (Table 6).

Whilst excellent source of carbohydrates, rice grains are poor in inorganic micronutrients whose dietary supply dramatically drops after processing to produce white grains. Consumption of brown rice could enhance the intake of essential inorganic micronutrients, but precautions should be taken since toxic trace elements can also accumulate in the bran. On this basis, several efforts to increase the presence of beneficial microelements (e.g., Fe and Zn) and limit the accumulation of hazardous ones (e.g., As and Cd) in rice grains have been made by applying specific fertilization practices, exploiting the existing natural variation within germplasm, or using transgenic approaches [41]. Since also the soil redox conditions affect the solubility and availability for plants of several mineral elements, AWD, by altering, compared to CF, the soil redox status, may represent an interesting strategy to control the concentrations of healthy/hazardous mineral nutrients in the grain. In our material, AWD did not substantially modify the accumulation of K^+, Ca^{2+}, and Mg^{2+} even if soil aerobiosis is expected to diminish their release into the soil solution, on the contrary favored by anaerobiosis in prolonged flooding [42]. In spite of the aerobic/anaerobic fluctuations in the soil, since the amounts of these cations are always relatively high in relation to the plant requirements, changes in their uptake and accumulation are not expected.

The ionomic results indicate that, in our conditions, AWD, compared to CF, caused changes in the grain concentrations of those elements whose redox status and/or solubility are a function of the soil redox status. In major detail, alternation from anaerobic to aerobic soil conditions would determine: i) shifts of Cu^{2+}, Ni^{2+}, and Cd^{2+} from their insoluble sulfide salts to the soluble sulfate ones [43–45]; ii) shifts of the As(III) water-soluble, mobile species to As(V), strongly adsorbed on soil (hydr)oxides and poorly mobile [46] and iii) changes in the balance between soluble Mn^{2+} salts and insoluble Mn oxides [47]. The dynamics of Fe and Zn species as a function of the soil redox conditions and the interaction of these elements with the root system are quite complex and can alter their bioavailability [47,48], explaining the null or weak effect of the aerobic periods on their accumulation in the grain.

For a few elements, our results are different from those recently reported by Xu and coworkers [18], who compared CF with a moderate soil dry irrigation. In particular, for Mn and Zn their results are essentially consistent with ours, whereas these authors reported that Cu and Fe concentrations decreased in the water-saving regime and in our conditions the Cu concentrations increased and Fe did not show any change (Table 6). These discrepancies may be possibly due to differences in the soil physico-chemical properties and in the rice genotypes considered.

Different effects of AWD on the concentrations of Cd and As are reported in the literature. The behavior observed in our conditions (increased Cd accumulation and decreased As accumulation in AWD in both years) is in agreement with the results reported by Norton et al. [49], but is different, for what concerns Cd, from the results by Xu et al. [18], who did not observe any effect of AWD, and, for what concerns As, from the results of Carrijo et al. [50], who observed effects on As accumulation only in severe AWD. Most probably these differences depend on the different timing and severity of the imposed AWD condition.

For both Cd and As we observed also a strong seasonal effect. The higher Cd concentrations in AWD in 2012 compared to 2013 may be due to the different duration and severity of the third dry period, which occurred during the grain-filling phase. Indeed, in rice the largest fraction of Cd in the grain derives directly from the roots through a xylem–phloem bypass in the upper node [51]. Under -oxic conditions, the lower availability of Mn in the soil induces overexpression of the NRamp5 Mn transporter, which is also the main route for the entry into the root of Cd [51], whose solubility is enhanced in aerobic soils. This -oxic condition was of longer duration and higher severity in 2012 than in 2013, explaining the significant season effect reported for Cd. For As, to our best knowledge such a detailed picture is not reported. We might only speculate that the general lower accumulation of As in AWD in 2013 was due to multiple, possibly interacting factors, like the metabolism of As-reducing microorganisms, O_2 availability, soil pH and redox conditions, involved in the equilibrium of As(V)/As(III) [52] and all possibly affected by the different weather conditions.

Consistent with their low concentrations in the soil used in our experiments (Supplemental Table S1), the levels of Cd (particularly under AWD) or As (particularly under CF) in the brown grain of each cultivar were much lower than the maximum EU limits (0.2 mg kg^{-1} for both) for commercialization of white rice. Therefore, according to our results, consumption of brown rice would not constitute a hazard for the consumer's safety for what concerns Cd and As. Different considerations should be made for rice grown on soils with higher levels of the available fractions of these elements: AWD would be counterproductive for Cd but advisable for As.

Finally, considering the allergenic risks related to Ni and the increase in its levels observed in all cultivars under AWD, attention should be devoted to the consumption of brown rice from plants grown under AWD.

5. Conclusions

Due to the consumer's increasing preference towards healthy eating habits, the brown rice market is currently expanding justifying the adoption of practices able to improve its nutritional quality while also preserving the environment.

AWD induced in the rice varieties considered an improvement of the nutritional quality of the brown grain (higher concentrations of health-promoting molecules like components of vitamin E, γ-oryzanol and flavonoids), which could indeed represent a plus in the rice market, with marked ('Baldo' and 'Gladio') or null ('Loto') effects on yield.

Concerning inorganic microelements for human nutrition, AWD did not, or only slightly, alter the grain density of the most important ones, i.e., Fe and Zn.

AWD exerted opposite effects on potentially toxic/allergenic elements, favoring the accumulation of Cd or diminishing that of As in the grain. This factor must be considered where soils and/or surface water contain these toxic elements. The marked increases in Ni levels observed in AWD should also be considered carefully when addressing to consumers who require Ni-free food.

AWD seemed to slow down the rate of plant growth and development through exposure to cooler temperatures for the absence of ponded water in the field. In temperate rice growing areas, the choice of rice cultivars for AWD should therefore rely on the ones suited better to low temperatures than to drought. In our conditions, 'Loto' showed the best maintenance of yield and grain nutritional quality under AWD.

The present study offers a promising perspective for combining the environmental-friendly water saving technique AWD with improved rice grain nutritional quality.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/9/10/628/s1. Figure S1: Growing degree days (GDD) for the three rice cultivars during the two growing seasons 2012 and 2013, Table S1: Physico-chemical characteristics of the soil in the paddy fields where rice plants were grown, Table S2: Significance of variance estimates related to rice cultivar, year, water regime and their interactions on plant phenology, yield, grain morphology, and brown grain quality traits, Table S3: Variability indexes of the results concerning plant phenology- and yield-related traits and grain morphology of three rice cultivars grown under continuous flooding (CF) or alternate wetting and drying (AWD), Table S4: Variability indexes of the data concerning chlorophyll (CHL) and nitrogen balance (NBI) indexes in the flag leaf, apparent amylose and N-protein contents in brown grains of three rice cultivars grown under continuous flooding (CF) or alternate wetting and drying (AWD), Table S5: Variability indexes of the results concerning the total concentrations of health-promoting compounds and antioxidant activity in brown grains of three rice cultivars grown under continuous flooding (CF) or alternate wetting and drying (AWD), Table S6: Variability indexes of the results concerning the concentrations of the single tocochromanols in brown grains of three rice cultivars grown under continuous flooding (CF) or alternate wetting and drying (AWD), Table S7: Variability indexes of the results concerning the concentrations of the γ-oryzanol complex components in brown grains of three rice cultivars grown under continuous flooding (CF) or alternate wetting and drying (AWD), Table S8: Variability indexes of the results concerning brown grain ionome of three rice cultivars grown under continuous flooding (CF) or alternate wetting and drying (AWD), Table S9: Recovery values for the ICP analysis of grain ionome contents.

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