Article

Fertilizer Deep Placement Significantly Affected Yield, Rice Quality, 2-AP Biosynthesis and Physiological Characteristics of the Fragrant Rice Cultivars

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Abstract: The management of fertilizers in a context of climate change and the preservation of the environment is strongly related to the regulation and accumulation of 2-acetyl-1-pyrroline (2AP) in fragrant rice. However, the feasibility of such management strategies in terms of enhancing the accumulation of 2AP has not yet been explored in aromatic cultivars. Here, we investigated the impact of the application of two fertilizers at three depth (surface, 5 cm and 10 cm) levels of placements to improve the aromatic rice quality, including such aspects as the 2AP content-, protein-, amylose- and yield-related traits. For this purpose, two known rice cultivars, Basmati 385 (B-385) and Yunjingyou (YJY), were grown in pots during 2019 and 2020 under fluctuating climates. The deep application of fertilizer at 10 cm significantly affected the 2AP content with such values as 127.53 µg kg^{-1} and 111.91 µg kg^{-1} obtained for Fragrant Fertilizer (FF) and Urea in B-385 cultivar, and 126.5 µg kg^{-1} and 114.24 µg kg^{-1} being observed for FF and Urea in YJY, respectively, during 2019. In addition, values of 108.41 µg kg^{-1} and 117.35 µg kg^{-1} were recorded for FF and Urea in B-385, while 125.91-µg kg^{-1} and 90.71-µg kg^{-1} were measured for FF and Urea in YJY, respectively, during 2020. Similarly, B-385 had better 2AP content and yield-related traits, as well as amylose content and cooked rice elongation, as compared to the YJY rice cultivar. The 2AP accumulation and its related biochemical parameters, and their relationships in different plant tissues at different growth stages under FF and Urea treatments, were also improved. Further, the 2AP content and the P5C activity demonstrated strong correlations during the grain filling periods in both fragrant rice cultivars. In conclusion, our findings have the potential to provide useful information to farmers and agriculture extension workers in terms of the saving of fertilizers and the improvement of rice grain quality under fluctuating climate conditions.

Keywords: climate change; fertilizers; 2AP biosynthesis; fragrant rice

1. Introduction

Rice (*Oryza sativa* L.) feeds two-thirds of the world's population as a staple food [1]. It is the main export product of several countries; thus, it contributes to international trade gains, as well as feeding more than 60% of the population of China and contributing up to 40% of the total national grain production [2]. With an annual production of 782 million tonnes, rice is grown globally over an area of 167.13 million hectares [3]. Aromatic rice has

a higher grain quality than non-aromatic rice, and consumers prefer aromatic rice due to its pleasant smell [4]. Aromatic rice plays an important role in international rice markets, and the global demand for aromatic rice is increasing [5]. Rice cultivation faces many problems such as abiotic stresses, climate change, disease infestations, the non-availability of quality seeds, low nitrogen utilization efficiency (NUE) and low ratios between the fertilizer inputs and rice quality. The use of fertilizers in agriculture is associated with water pollution and greenhouse gas emissions. As practices and programs to reduce the application of fertilizer continue to be developed, inefficient fertilizer use persists and little is known about the relationship between deep fertilizer inputs, climate change, environmental preservation and the quality of crops, particularly aromatic rice [6]. All of these are considered to be the main reasons for the low average yield and quality of rice, which make it difficult to meet consumer needs [7].

In China, high rates of fertilizer are applied to obtain high yields of cereal crops [8]. Within the limits of the ground conditions, the application of fertilizer is the most common and effective way to improve productivity, as it increases yield and improves rice quality to some extent [9]. In addition, fertilizers have a significant effect in terms of the promotion of tiller development [10]. The participation of mechanization in rice production systems presents an opportunity to solve problems associated with rice production and climate change [11]. Direct seeding (DSR) and transplanting are the main rice cultivation techniques that require efficient management of the fertilizer input in order to optimize the yield and quality of the rice [12]. Fertilizer broadcasting, in which farmers often broadcast fertilizers manually on the wet surface of puddles containing soils, is one of the main traditional methods of rice cultivation in southern China, but it often leads to a poor distribution of these fertilizers, as well as uneven nutrition of plants and water pollution [13]. This practice also causes the volatilization of ammonia, in addition to denitrification, surface runoff and leaching in paddy cropping systems, resulting in low N recovery efficiency (NRE) [8]. Many experiments have shown that the deep placement of N fertilizer can effectively increase the grain yield of rice, in addition to its quality, including such aspects as fertilizer use efficiency and environmental preservation [14,15]. Thus, it seems imperative to bring the fertilizers to a reasonable depth in order to allow their effective use by rice plants.

Urea and Fragrant Fertilizer are two important fertilizer types that can affect crop growth, yield and quality [16]. It has been reported that Urea can regulate the protective activities of enzymes in rice [9,17]. Compared to commercial compound fertilizers, the use of fragrant rice fertilizer (15% N, 4% P_2O_5, 7% K_2O, 25% organic matter and 2% $ZnCl_2$) can also improve rice growth, in terms of grain yield and quality, by controlling the release of N, P and K contents in soils [18,19]. An efficient supply of fertilizers, at a reasonable depth, is necessary to meet the growing global demand for quality rice [20]. However, better use of fertilizers could minimize negative impacts on the environment and multiply farmers' profits. For example, Park et al., 2019 [21] reported that the aroma, sweetness, whiteness, stickiness and glassiness of cooked milled rice are inversely proportional to the percentage of nitrogen in the cereals. It was argued that the application of 10 t/ha of farmyard manure or 80 or 120 kg of N ha-1 increased the head rice recovery and alkaline value of fragrant rice varieties [22]. In addition, the application of nitrogen fertilizer increased the grain length, length:width ratio, grain length after cooking and aspect ratio. Manganese, the application of nitrogen, salinity, temperature during the ripening phase and shading during grain filling stage [23–27] have all been shown to affect the biosynthesis of 2AP in rice. Amylose can be used as a criterion to determine the quality of rice grains because it is a major component of rice starch [28]. The elongation of cooked rice is also used as a factor to determine the quality of rice grains [20]. Little or no research has been conducted on the impact of different depths of fertilizer application on 2AP, in addition to the percentage of amylose content, the elongation of cooked rice, characteristics related to plant growth and other factors in determining the quality of rice.

This two-year pot study evaluated the impacts of two different fertilizers' application methods (broadcasting, 5 cm and 10 cm) on the growth-, 2AP- and yield-related traits

and quality of two fragrant rice cultivars. We hypothesized that: (i) fertilizer application methods would affect the growth, 2AP, yield and grain quality traits; (ii) rice cultivars would differ in terms of their growth, 2AP, yield and quality aspects; and (iii) deep placement (5 cm or 10 cm) would result in better growth, 2AP, yield and grain quality traits compared to conventional fertilizer broadcasting methods. The results will help to decide the best fertilizer placement method and means of quality improvement in transplanted fragrant rice under adverse climate conditions.

2. Materials and Methods

2.1. Plant Materials and Experimental Details

One indica rice cultivar (Basmati 385 (B-385)) and one japonica rice cultivar (Yunjingyou (YJY)) were used in this study. The rice cultivars were obtained from the College of Agriculture, South China Agricultural University (SCAU), Guangzhou, China. Before sowing under greenhouse conditions, the seeds were soaked in water for 24 h at room temperature (25 °C). Then, the pre-germinated seeds were hill-seeded. These experiments were conducted from March 2019 to July 2019 and from March 2020 to July 2020 at the Experimental Research Farm, College of Agriculture, South China Agricultural University, China (23°09′ N, 113°22′ E and 11 m from mean sea level). The sprouted seeds were sown in PVC trays for the nursery, then placed in a puddled field and covered with a sheet of plastic. Seedlings were transplanted into a plastic pot, of 30 cm diameter and 29 cm height, containing 10 kg sandy loam soil. The soil-filled pots, with four hills per pot and three seedlings per hill, were transplanted with uniform and stable rice seedlings that were 22-days old. Soil and climate parameters were measured for each season during the two years of the experiment (Table 1 and Figure 1).

Table 1. Physical and chemical properties of the soil.

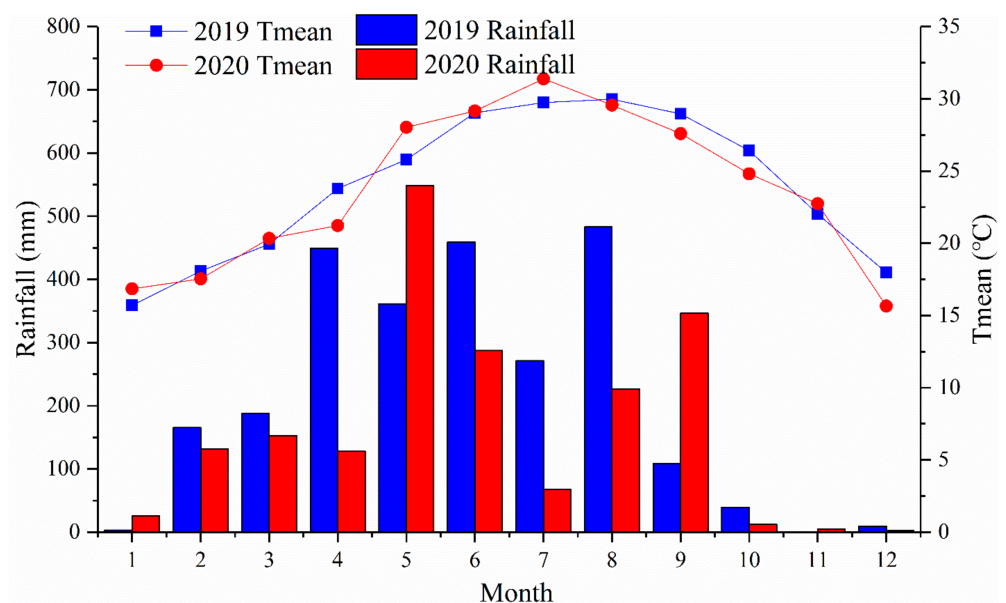| Year	Organic Content g·kg⁻¹	Total N g·kg⁻¹	Total P g·kg⁻¹	Total K g·kg⁻¹	Available N mg·kg⁻¹	Available P mg·kg⁻¹	Available K mg·kg⁻¹	pH
2019	21.52	2.96	14.12	16.22	364.32	34.23	43.21	5.74
2020	22.68	3.41	14.10	16.88	371.33	32.98	41.95	5.70

Figure 1. Temperature and rainfall trend during 2019 and 2020 at SCAU experimental farm.

2.2. Experimental Designs and Treatments

The pots used for some treatments contained soil with 3 g of Fragrant Fertilizer (FF) (15% N, 4% P_2O_5, 7% K_2O, 25% organic matter and 2% $ZnCl_2$). Other treatments' pots contained 3 g Urea (U) and 46% N (46N-0P-0K), with the following formula: CO $(NH_2)_2$. Twenty-one days after transplantation, an additional 2 g of FF and 2 g 46% N Urea was added to each treatment pot following the same treatment protocols. A randomized complete block design (RCBD) with eight replicates in total was used for this experiment. Six fertilizer placement treatments were applied: surface Fragrant Fertilizer (FFS), deep Fragrant Fertilizer at 5-cm depth (FF5), deep Fragrant Fertilizer at 10-cm depth (FF10), surface Urea (US), Urea at 5-cm depth (U5) and Urea at 10-cm depth (U10). Each cultivar received the fertilizer at different levels of input; the levels of B-385 (FFS), B-385 (FF5), B-385 (FF10), B-385 (US), B-385 (U5) and B-385 (U10) were equivalent to YJY (FFS), YJY (FF5), YJY (FF10), YJY (US), YJY (U5) and YJY (U10) FF. The placement was conducted by making holes with depths of 5 cm and 10 cm in different pots. The fertilizers were placed according to the treatments provided on the surface, at a depth of 5 cm and 10 cm, with a tubule pierced with many small holes.

2.3. Sampling and Data Collection

The number of panicles was counted by monitoring their appearance until maturity by taking an average per pot. During tillering, heading and at maturity, the leaves, sheath and stem (green) were sampled. For each harvest treatment, the grains were sampled. The collected samples were separated and immediately immersed in liquid nitrogen and stored in the laboratory at $-80\,°C$ until biochemical analysis were performed. The grain samples, which were intended for analysis of the percentage amylose content, percentage of elongation of cooked rice and protein content, were left at room temperature ($25\,°C$) for three months before grinding.

2.4. GC-MS Analysis for the Determination of 2-Acetyl-1-pyrroline

GCMS-QP 2010 (GCMS-QP 2010 Plus, Shimadzu Corporation: Kyoto, Japan) was used for GC-MS analysis. The specifications were as follows: the chromatographic column SH-Rxi-5Sil MS was 30 m long and 0.25 mm in diameter, the film thickness was 0.25 L and the heating temperature was $220\,°C$ when at its maximum; helium, with a high purity of 99.999%, was used as the carrier gas. In addition, splitless injection, at a constant pressure, was used, and the injection volume used was 2 μL. The electron bombardment ion source was used for mass spectrometry and $200\,°C$ was the temperature of the ion source. The ionization energy was 70 Ev. The interface temperature was $250\,°C$; the quadrupole was $150\,°C$ with full scan mode and an m/z scan mass range of 35 to 160. Three repetitions of the samples were used in this study. A quantity of 2 g of powdered sample was transferred to a 20 mL bottle and then 10 mL of dichloromethane (CH_2Cl_2) was added. The samples were then transferred to an ultrasonic cleaner (KQ-800ES from KUNSHAN ULTRASONIC INSTRUMENT CO. LTD: Shanghai, China.) and set at a temperature of $0\,°C$ for 4 h. These samples were transferred to a 10 mL conical flask before the addition of 4 g of anhydrous sodium sulfite (Na_2SO_3). After 30 s, 1 mL of the supernatant was taken and then transferred to a vial with a disposable micropipette. This was followed by the addition of 2 μL of 2,3,6-trimethylpyridine ($1000\times$ dilution with CH_2Cl_2) as an international standard. The vials were transferred to the GC-MS machine for analysis. The GC Solution 2.3 software was used for data analysis and the retention time of 2AP was 14.8 min [20].

2.5. Determination of Elongation of Cooked Rice and Percentage of Amylose Content

The elongation of cooked rice is the difference between the length of the grain measured before and after cooking. The Microtek ScanMaker i800 plus scanner (Microtek International, Inc. Hsinchu, Taiwan) was used to perform the measurements. Ten grains of milled rice were measured for each replicate. Each grain was then transferred to a PCR tube containing 150 μL of distilled water. The PCR plate containing the rice grains was then

placed in a PCR thermal cycler and the rice grains were individually baked for 30 min at a block temperature of 99 °C. The cooked rice grains were removed from the PCR plate and placed on filter paper. After drying for 5 min at room temperature, they were re-measured. Ten cooked grains were measured again simultaneously, and this made it possible to control the variability of the moisture content, which could have resulted from unequal standing times between measurements. In order to determine the elongation percentage of cooked rice for each treatment, the following formula was used:

$$\% \text{ E} = \frac{(\text{ACML} - \text{BCML})}{\text{BCML}} \times 100 \qquad (1)$$

%E is the elongation percentage; ACML is After Cooking Mean Length and BCML is Before Cooking Mean Length [28].

To determine the amylose content percentage, we used the FOSS INFRATECTM 1241 ANALYSER (FOSS Nils DK-3400 Copenhagen, Denmark) (part no: 10,014,925) with a temperature range of 0–42 °C, which was regulated by the machine. A quantity of 250 g of milled rice sample from each treatment was used in three replicates to determine the amylose content, which was immediately read after each operation.

2.6. Determination of Fragrance-Related Enzymes (Proline, Δ 1Pyrroline-5-carboxylate Synthetase (P5CS), Δ 1-Pyrroline-5-carboxylate (P5C))

The proline contents were estimated according to [29]. Quantities of 0.3 g of grains of each treatment were homogenized in 5 mL of 3% sulfosalicylic acid, boiled for 10 min in water and then cooled down. Two milliliters of the filtrate were mixed with ninhydrin reagent (3 mL) and glacial acetic acid (2 mL). The mixture was again placed in boiling water for 30 min and then cooled down in an ice bath before being extracted with 4 mL of toluene. The mixture was centrifuged at $4000 \times g$ for 5 min. The toluene extraction and the absorbance of the red chromophore were measured at 520 nm and the proline contents were estimated by comparison with a standard curve and expressed as micrograms per gram ($\mu g \cdot g^{-1}$ fresh weight (FW)).

Δ 1-pyrroline-5-carboxylate synthetase (P5CS) activity was determined by referring to the method of [30,31]. The reaction solutions contained 10 mM ATP, 20.0 mM MgCl$_2$, 50 mM Tris–HCl buffer, 50 mM sodium glutamate, 100 mM hydroxamate-HCL and 0.5 mL of enzyme extract. The prepared mixture was kept in a 37 °C water bath for 5 min, and then the reaction was terminated by the addition of 0.5 mL of a stop buffer (2.5% FeCl 3 and 6% TCA, dissolved in 100 mL of 2.5 M HCl). The P5CS activity was expressed as $U \cdot g^{-1} \cdot FW$. The absorbance was measured at 535 nm.

The P5C concentration was determined according to a previously described method [2]. Fresh leaves or rice seed (100 mg) were ground into powder with liquid nitrogen and resuspended in 375 μL of extraction solution containing 50 mM Tris-HCl (pH 8.0), 10% glycerol, 1%triton X-100 and 1%β-mercaptoethanol. The mixture was vortexed for 1 min and kept in an ice bath for 2 min. The above procedure was repeated 5 times and then the mixture was allowed to stand for 30 min. After centrifugation at $14,000 \times g$ for 30 min at 4 °C, the supernatant was added to a mixture containing 500 μL of 10% trichloroacetic acid and 125 μL of 40 m Mo-aminobenzaldehyde. The absorbance was measured at 440 nm after the reaction, and the P5C concentration was expressed as $\mu mol \ g^{-1}$ FW.

2.7. Rice Quality

To calculate the rate of brown rice, approximately 120 g of rice samples were weighed and husked by the SDL-A rice huller to remove the glume before weighing. In addition, to calculate the rate of milled rice, an SDJJ 100 milled rice mill was used to remove the husk from the brown rice, which was then weighed. The milled rice was removed with a 1.0 mm round whole sieve, and the head rice was weighed out then its percentage was calculated. The SC-E Rice Appearance Quality Analyzer measured the length and width of the head

rice (vendor: Wanshen, Hangzhou, China). The specific operation was determined with reference to GB/T17891-2017 high-quality rice [32].

2.8. Statistical Analysis

Analysis of variance (ANOVA) and correlation coefficients were performed using Statistix version 8 (Analytical, Tallahassee, FL, USA). The differences amongst means were separated by using the least significant difference (LSD) test at the 5% significance level.

3. Results

3.1. Effects of Fertilizer Placement on the Yield of Fragrant Rice and Its Components

Grain yield and its components varied with different fertilizer application treatments, in both rice genotypes, for both the 2019 and 2020 seasons (Table 2). Compared to the differences between the various grain yield components among all the treatments, FF10 and U10 treatment showed the highest grain yield in both B-385 and YJY across the two years.

Table 2. Effects of different fertilizer placements on the yield of fragrant rice and its components.

Growing Season	Variety	Treatment	Number of Filled Grains per Panicle	Effective Panicle Number (pot^{-1})	1000-Grain Weight (g)	Grain-Filling (%)	Yield pot^{-1} (g)
2019	B-385	FFS	128.22 a	25 a	24.10 a	49.84 b	25.18 b
		FF5	114.32 b	23 b	19.46 b	41.39 ab	22.28 ab
		FF10	122.28 a	24 a	23.42 ab	54.07 a	28.71 a
		US	102.03 b	26 ab	20.82 ab	56.05 a	27.19 b
		U5	117.31 b	24 b	19.50 b	45.70 b	25.37 c
		U10	132.79 a	28 a	24.78 a	54.69 a	33.11 a
	YJY	FFS	113.25 b	27 b	22.57 a	55.26 a	27.70 ab
		FF5	111.10 b	27 b	19.64 b	50.94 b	23.03 b
		FF10	130.07 a	28 a	24.57 a	58.05 a	33.31 a
		US	127.38 b	28 b	23.36 b	61.87 b	32.35 ab
		U5	123.43 b	27 c	21.85 c	58.94 b	27.20 b
		U10	133.04 a	30 a	24.33 a	76.54 a	36.35 a
2020	B-385	FFS	124.22 b	27 b	24.10 b	59.84 b	29.18 a
		FF5	119.32 c	27 b	20.46 c	53.39 c	22.28 b
		FF10	129.28 a	28 a	27.42 a	71.07 a	31.71 a
		US	122.03 a	28 ab	22.82 ab	68.05 b	33.19 ab
		U5	112.31 b	27 b	20.50 b	59.70 cb	29.37 b
		U10	122.79 a	29 a	26.78 a	75.69 a	36.89 a
	YJY	FFS	114.25 b	27 b	20.57 b	53.26 ab	29.70 a
		FF5	112.10 b	27 b	19.64 b	50.94 a	23.03 b
		FF10	131.07 a	28 a	22.57 a	55.05 a	30.31 a
		US	127.38 ab	28 b	22.36 b	61.87 b	26.35 b
		U5	123.43 c	28 b	20.85 cb	50.94 c	21.20 c
		U10	133.04 a	31 a	24.83 a	76.54 a	31.35 a

Different small letters (a, b, c, ab, cb) in each column indicate significant differences among different treatments $p < 0.05$ within a variety.

The 1000-grain weight was significantly affected by fertilizer application. In 2019, the maximum mean of 24.10 g/pot^{-1} and 23.42 g/pot^{-1} for FFS and FF10 were recorded for B-385. In addition, for YJY, the highest mean values of 22.57 g/pot^{-1} and 24.57 g/pot^{-1} were reported for FFS and FF10, respectively. Additionally, 24.78 g/pot^{-1} and 24.33 g/pot^{-1} for U10 were the best means reported for B-385 and YJY, respectively. In 2020, the highest mean values of 27.42 g/pot^{-1} and 22.57 g/pot^{-1} for FF10 were recorded for B-385 and YJY, respectively. In addition, the maximum means of 26.78 g/pot^{-1} and 24.83 g/pot^{-1} were recorded for U10 in B-385 and YJY, respectively (Table 2).

The results of the interactions between the two varieties and the placement of fertilizers, from 2019 to 2020, showed a significant effect on the number of effective panicles, as well

as the 1000-kernel weight, percentage kernel filling and yield. The interaction between the variety and depth of fertilizer application had no significant effect on the number of grains filled per panicle (Table 3).

Table 3. The interactions between variety and fertilizer placement on yield and its components.

	F Value	Number of Filled Grains per Panicle	Effective Panicle Number (pot^{-1})	1000-Grain Weight (g)	Grain-Filling (%)	Yield (pot^{-1})
	Variety	NS	**	**	**	**
2019	Fertilizer Placement	NS	NS	**	NS	NS
	Variety X Fertilizer Placement	NS	NS	**	**	**
	Variety	NS	**	**	**	*
2020	Fertilizer Placement	NS	NS	**	*	NS
	Variety X Fertilizer Placement	NS	NS	**	*	*

NS: no Significant difference; ** and * indicate significant difference at 1% and 5% level, respectively.

3.2. Rice Quality and Its Components

As shown in Table 4, the protein content of rice grains was significantly affected by different depths of fertilizer input during 2019 and 2020. Interestingly, for the two years, the B-385 cultivar protein level was found to be higher under treatments U10 and FF10, followed by US and FFS treatment. Likewise, during the same period, for the YJY variety, the U10 and FF10 treatments, followed by FFS and US, respectively, obtained higher protein levels. The amylose content rate followed the same trend as the protein content rate during the two year-long culture periods; for the two varieties of U10 (FF10 and FFS) as well as for those of US (B-385 and YJY), the rates were higher. As compared to the protein and amylose content, the level of green grains was significantly higher under the U5 and FF5 treatments for both B-385 and YJY. No significant differences were observed in the rate of brown rice for both B-385 and YJY. The different fertilizer application depths also significantly affected the rate of milled rice and the head rice rate; however, during the year 2019, no significant difference was observed in the head rice rate for B-385 variety. In addition, the chalk white and chalkiness attributes were significantly affected by different treatments of fertilizer application for the two varieties (Table 4).

In 2019, for B-385, in terms of the protein content percentage, we noted a difference of 0.60% and 0.30%, respectively, for FF10 and FFS as compared to FF5. It was also noted that there was a difference of 0.63% and 0.33%, respectively, for U10 and US as compared to U5. For the YJY cultivar, a difference of 0.13% and 0.6%, respectively, for FF10 and FFS, as compared to FF5, was observed, and a difference of 0.87% and 0.5%, respectively, was noted for U10 and US as compared to U5. In 2020, for the B-385 cultivar, a difference of 1.5% and 0.04%, respectively, for FF10 and FFS, as compared to FF5, was noted, with a difference of 1.5% and 0.64%, respectively, for U10 and US as compared to U5 treatment. For YJY, we noted a difference of 1% and 0.23%, respectively, for FF10 and FFS as compared to FF5, and a difference of 1.16% and 0.66% g, respectively, for U10 and US as compared to U5 (Table 4).

In 2019, for B-385, in terms of amylose content, we noted a difference of 3.07% and 0.97%, respectively, for FF10 and FFS compared to FF5. It was also noted that there was a difference of 0.87% and 0.48%, respectively, for U10 and US as compared to U5. For cultivar YJY, a difference of 1.67% and 0.23%, respectively, for FF10 and FFS, as compared to FF5 was observed, and a difference of 0.66% and 0.16%, respectively, was noted for U10 and US as compared to U5. In 2020, for cultivar B-385, a difference of 1.34% and 0.57%, respectively, for FF10 and FFS, as compared to FF5, was noted, with a difference of 2.46% and 1%, respectively, for the U10 and US treatment as compared to U5. For YJY, we noted a difference of 2.37% and 0.67%, respectively, for FF10 and FFS as compared to FF5, and a difference of 0.6% and 0.4%, respectively, for U10 and US as compared to U5 (Table 4).

Table 4. Effects of different placements of fertilizers on rice quality.

Growing Season	Variety	Treatment	Brown Rice Rate (%)	Milled Rice Rate (%)	Head Rice Rate (%)	Green Grain Rate (%)	Protein Content (%)	Amylose Content (%)	Chalk White	Chalkiness
2019	B-385	FFS	83.15 a	71.82 a	52.52 ab	14.35 b	8.50 ab	15.87 b	15.31 a	7.18 bc
		FF5	82.72 a	70.90 a	49.25 b	26.57 a	8.20 b	14.90 c	10.30 b	5.32 c
		FF10	85.34 a	70.53 a	57.91 a	14.82 b	8.80 a	17.97 a	13.35 ab	13.33 a
		US	84.78 a	71.91 a	56.56 b	25.41 b	8.60 b	16.70 b	14.7 ab	10.25 a
		U5	83.72 a	69.57 a	53.74 bc	31.62 a	8.27 c	16.22 c	14.55 b	5.48 b
		U10	85.68 a	71.71 a	59.54 a	12.51 c	8.90 a	17.09 a	19.82 a	9.96 ab
	YJY	FFS	74.64 a	68.70 b	58.22 a	17.26 b	8.43 ab	16.43 b	22.30 a	11.66 ab
		FF5	73.49 a	65.09 c	56.70 a	42.55 a	8.37 b	16.20 b	21.31 a	9.94 c
		FF10	78.63 a	69.90 a	47.57 b	7.91 c	8.50 a	17.87 a	20.11 ab	13.83 a
		US	76.26 a	69.87 ab	48.61 b	24.94 b	8.03 b	16.43 b	18.72 ab	6.30 c
		U5	72.55 a	65.36 c	56.93 a	37.72 a	7.53 c	16.27 bc	22.13 a	13.07 a
		U10	79.27 a	70.08 a	58.58 a	20.72 c	8.40 a	16.93 a	18.71 b	9.67 b
2020	B-385	FFS	82.74 a	67.70 ab	53.76 a	15.74 b	7.37 b	17.80 b	8.36 b	2.57 b
		FF5	80.87 a	63.29 b	53.36 a	20.54 a	7.33 b	17.23 bc	9.83 b	3.76 b
		FF10	85.39 a	71.63 a	55.88 a	10.48 c	8.83 a	18.57 a	12.16 a	4.00 a
		US	84.97 a	68.79 ab	58.27 a	9.86 b	8.07 b	17.07 c	14.69 a	7.22 ab
		U5	81.42 a	64.70 c	46.89 b	15.36 a	7.43 c	15.07 b	12.05 a	5.28 b
		U10	86.85 a	72.69 a	48.40 b	7.53 c	8.93 a	17.53 a	19.31 a	7.49 a
	YJY	FFS	74.61 a	67.92 ab	48.77 ab	12.93 b	6.23 b	16.07 b	16.04 a	7.59 b
		FF5	75.03 a	65.66 c	54.29 a	31.19 a	6.00 c	15.40 c	19.44 a	7.86 b
		FF10	77.57 a	70.75 a	52.01 b	8.11 c	7.00 a	17.77 a	6.52 b	9.34 a
		US	77.06 a	68.56 b	60.43 ab	20.53 a	6.83 b	16.07 b	6.91 b	3.07 b
		U5	75.68 a	65.75 b	57.11 ab	22.17 a	6.17 c	15.67 c	11.95 a	4.76 a
		U10	79.04 a	73.53 a	64.20 a	18.52 b	7.33 a	16.27 b	12.05 a	4.75 a

Different small letters (a, b, c, ab, cb) in each column indicate significant differences among different treatments *p* < 0.05 within a variety.

The results of the interactions between the two cultivars and fertilizer placement in 2019 showed a significant effect on the green kernel rate, percentage of protein content, percentage of amylose content, and chalk white and chalky attributes. No significant effect of the interactions between varieties on the rate of brown rice was observed in 2019. In addition, in 2020, the analysis of the interactions revealed a significant effect for the rate of green grain, percentage of protein content, percentage of amylose content, and chalk white and chalky attributes. However, there was no significant effect caused by these interactions on the rate of brown rice over the same period (Table 5).

Table 5. The effects of interactions between varieties and fertilizer placement on fragrant rice quality.

	F Value	Brown Rice Rate (%)	Milled Rice Rate (%)	Head Rice Rate (%)	Green Grain Rate (%)	Protein Content (%)	Amylose Content (%)	Chalk White	Chalkiness
2019	Variety	NS	**	NS	**	**	**	**	NS
	Fertilizer Placement	*	NS	**	**	**	**	*	**
	Variety X Fertilizer Placement	NS	*	NS	**	**	**	**	**
2020	Variety	NS	NS	**	**	**	**	**	**
	Fertilizer Placement	NS	**	*	**	**	**	**	*
	Variety X Fertilizer Placement	NS	NS	NS	NS	**	**	NS	NS

NS: no Significant difference, ** and * denote significant difference at the 1% and 5% levels, respectively.

3.3. Effects of Fertilizer Application on Cooked Rice Elongation

The results (2019 and 2020) shown in Figure 2a illustrate that the grains of B-385 grown at 10-cm depth under FF treatment had 6.35% and 21.83% higher cooked rice elongation compared to those corresponding to the surface input and 5-cm depth, respectively. The cooked rice elongation of the same variety collected at the maturity stage of rice plants grown under Urea treatment, at the surface, 5-cm and 10-cm depths, were 4.49 mm, 4.06 mm and 4.90 mm, respectively (Figure 2b). These results show that the grains of B-385 grown under Urea treatment at 10-cm depth had 17.14% and 8.37% higher cooked rice elongation than the surface and 5-cm applications, respectively. Figure 2c reveals that the elongation of cooked rice in grains of YJY, harvested from rice plants grown under FF treatment, at the surface, 5-cm and 10-cm depths, were 3.81 mm, 3.09 mm and 3.99 mm,

respectively. The additional analyses of the results showed that grains harvested under a 10-cm depth regime had 4.51% and 22.56% greater cooked rice elongation than the grains harvested under the 5 cm and surface conditions, respectively. Furthermore, the cooked rice elongation in grains of the same variety harvested from rice plants grown with Urea under the surface placement, and at 5-cm and 10-cm depths, were 3.60 mm, 3.30 mm and 4.04 mm, respectively (Figure 2d). These results show that YJY grains harvested at 10-cm depth with Urea had 10.89% and 18.32% higher cooked rice elongation compared to grains harvested with the 5 cm and surface placement depths, respectively. The results of *T* Student's tests showed significant differences in the cooked rice elongation for the grains of B-385 and YJY at different levels of intake for the two types of fertilizer (FF and Urea).
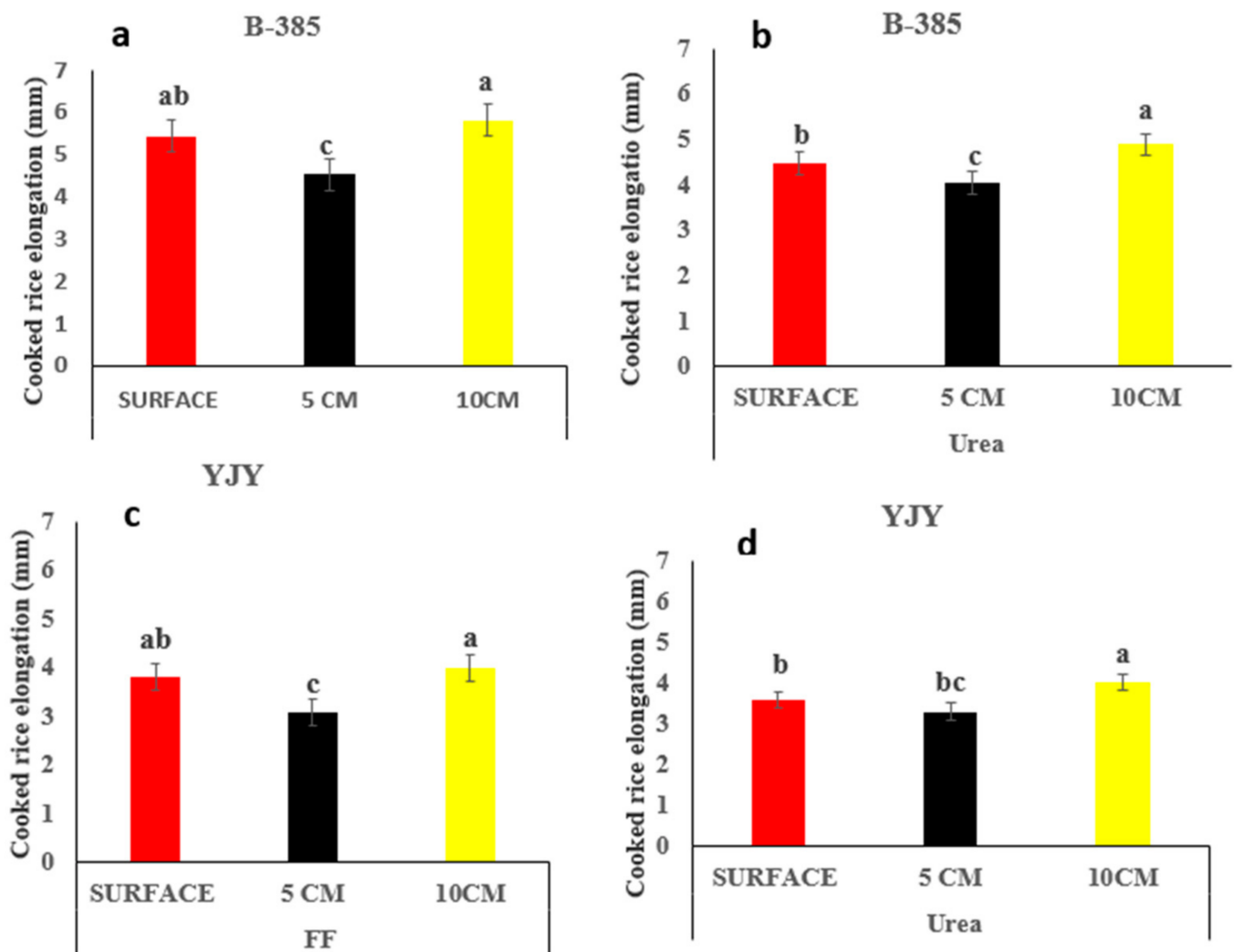

Figure 2. The effects of fertilizer application on cooked rice elongation in the grains of B-385 and YJY: (**a**) B-385 cooked rice elongation under different applications of FF; (**b**) B-385 cooked rice elongation under different applications of Urea; (**c**) YJY cooked rice elongation under different applications of FF; (**d**) YJY cooked rice elongation under different applications of Urea. (a, b, c, ab, cb) on each barre indicate significant differences among different treatments $p < 0.05$ within a variety.

3.4. Application of 2AP and Fertilizers

Different rice cultivars and fertilizer application depths, and their interactions, significantly affected the 2AP content at different stages of growth and in different parts of the plant, including the grains (Figures 3 and 4). During the two years of the study, we noticed significant differences between all treatments with B-385 and YJY at the tillering,

heading and maturity stages, but these differences were not observed in the stem. At the tillering stage, the highest 2AP content in the leaves, sheath and stem was recorded for the surface placement, whereas the lowest values of the 2AP content were noted for the 10-cm placement of fertilizers (Figures 3 and 4). Contrarily, at the heading stage, in the leaves and sheathes, the highest values were recorded for the 10-cm placement as compared to those of the other treatments (5 cm and surface placement), but there were no significant differences observed in the stems at this stage of plant growth. Similarly, at maturity, in leaves and grains, the highest values for 2AP content were recorded for the 10-cm placement as compared to the 5 cm and surface placements (Figures 3 and 4). In the two years, the highest 2AP content was registered for Fragrant Fertilizer compared to Urea Fertilizer for both rice cultivars under different placement conditions (Figures 3 and 4).
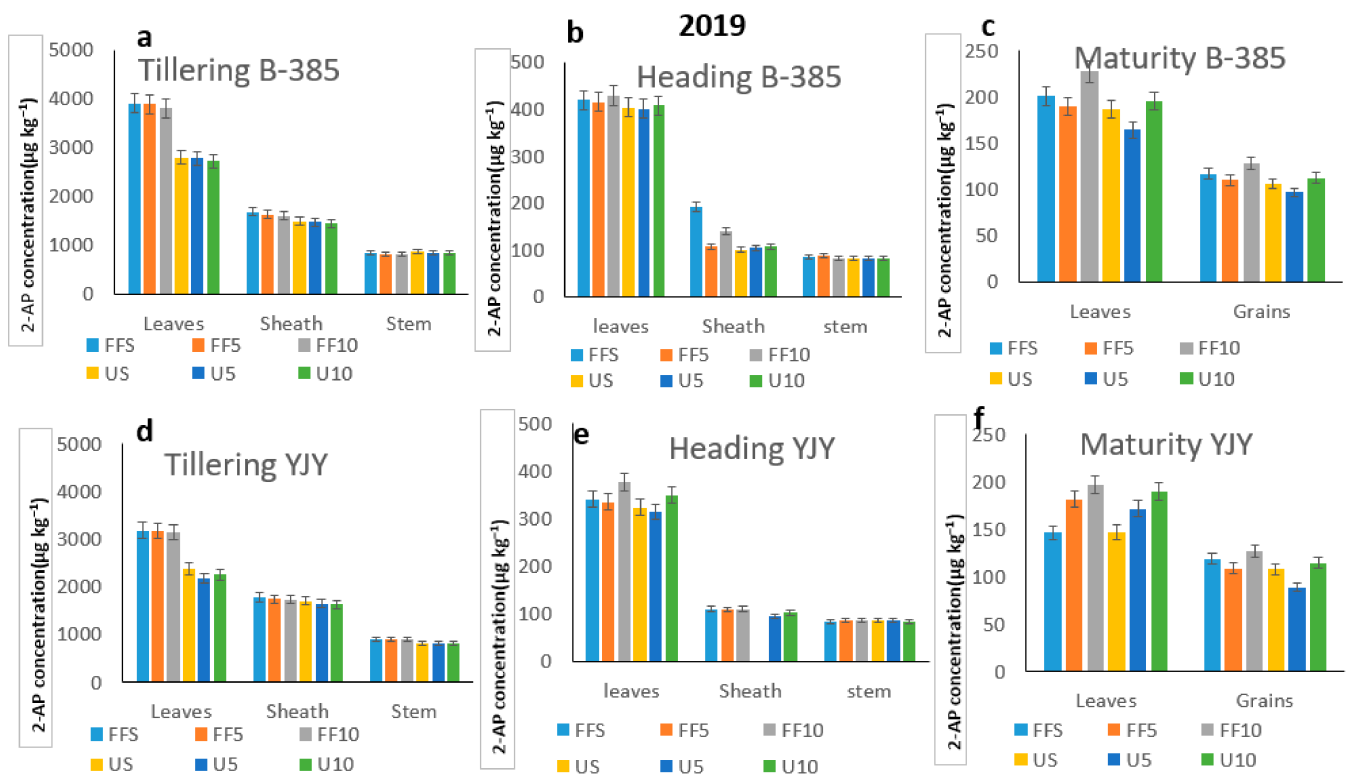

Figure 3. AP contents (μg kg^{-1}) of different cultivars under fertilizer application in 2019: (**a**) AP content (μg kg^{-1}) of B-385 rice cultivar under fertilizer application at the tillering stage; (**b**) AP content (μg kg^{-1}) of B-385 rice cultivar under fertilizer application at the heading stage; (**c**) AP content (μg kg^{-1}) of B-385 rice cultivar under fertilizer application at maturity; (**d**) AP content (μg kg^{-1}) of YJY rice cultivar under fertilizer application at the tillering stage; (**e**) AP content (μg kg^{-1}) of YJY rice cultivar under fertilizer application at the heading stage; (**f**) AP content (μg kg^{-1}) of YJY rice cultivar under fertilizer application at maturity.

According to the interactions, the two cultivars were significantly affected by the application of fertilizer, in terms of 2AP content, at different stages of growth (tillering, heading and maturity) and in different parts of the plant (leaves, sheaths and grains), except for the stem (Table 6). With regard to the interactions caused by variations in the application of the X fertilizer, B-385 and YJY were significantly affected by the placement of fertilizers, in terms of the 2AP content, during the two years. The 2AP content, cultivars and rice growth stages were significantly affected by differences in the application of fertilizer (surface, 5 cm and 10 cm) and their interactions are detailed in Table 6.
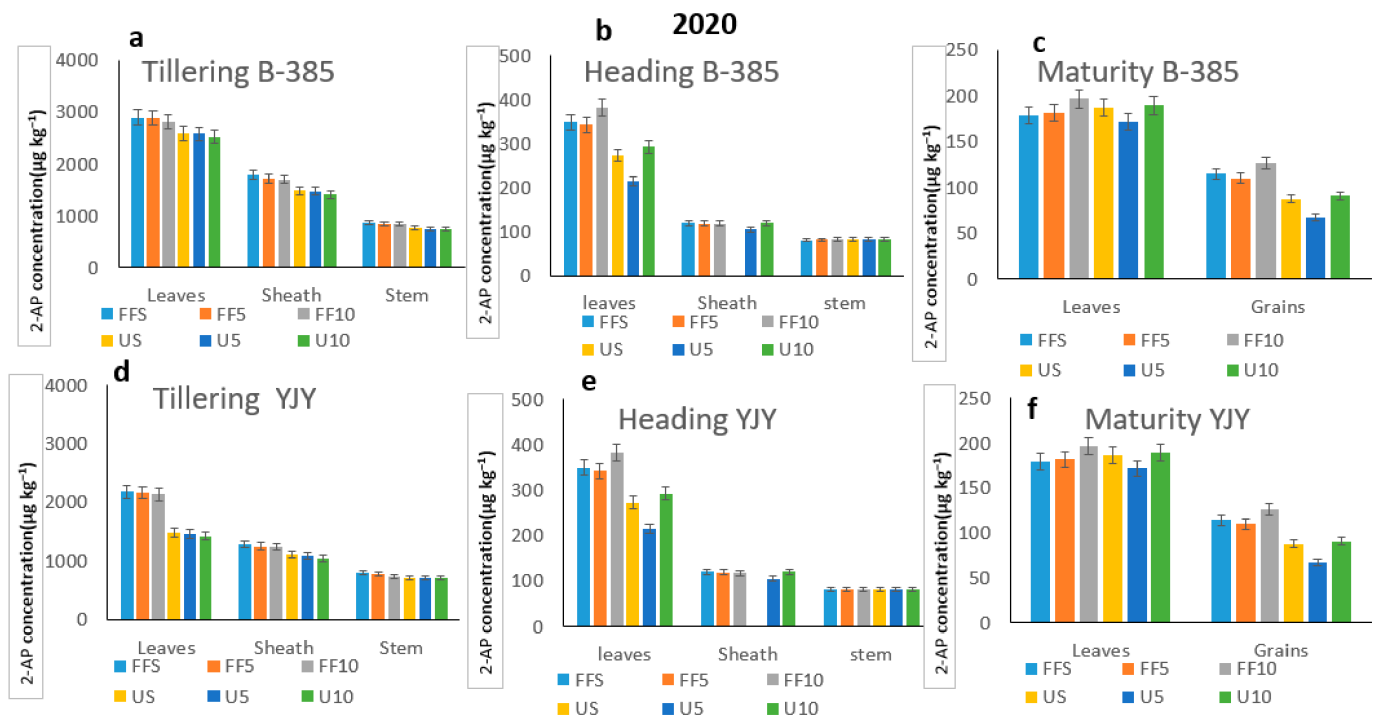
Figure 4. AP contents (µg kg^{-1}) of different cultivars under fertilizer application in 2020: (**a**) AP content (µg kg^{-1}) of B-385 rice cultivar under fertilizer application at the tillering stage; (**b**) AP content (µg kg^{-1}) of B-385 rice cultivar under fertilizer application at the heading stage; (**c**) AP content (µg kg^{-1}) of B-385 rice cultivar under fertilizer application at maturity; (**d**) AP content (µg kg^{-1}) of YJY rice cultivar under fertilizer application at the tillering stage; (**e**) AP content (µg kg^{-1}) of YJY rice cultivar under fertilizer application at the heading stage; (**f**) AP content (µg kg^{-1}) of YJY rice cultivar under fertilizer application at maturity.

Table 6. The effects of the interactions between variety and fertilizer placement on AP content (µg kg^{-1}).

F Value		Tillering			Heading			Maturity	
		Leaves	Sheath	Stem	Leaves	Sheath	Stem	Leaves	Grains
	Variety	*	*	NS	*	**	NS	**	**
2019	Fertilizer Placement	*	**	NS	**	*	NS	*	**
	Variety X Fertilizer Placement	*	**	NS	*	*	NS	*	**
	Variety	*	*	NS	*	**	NS	**	**
2020	Fertilizer Placement	*	**	NS	**	*	NS	**	**
	Variety X Fertilizer Placement	**	**	NS	**	*	NS	**	*

NS: no Significant difference; ** and * indicate significant difference at the 1% and 5% levels, respectively.

3.5. Aroma-Related Enzymes

Over two year-long periods of cultivation, during the tillering stage of all the treatments, the leaves showed no significant differences in terms of the enzymes (P5C, P5CS, and Proline) involved in the biosynthesis of 2AP (Figures 5 and 6). On the contrary, over the same period, at both the heading and maturity stages, and between all the treatments for both the B-385 and YJY rice cultivars, the leaves showed significant differences in terms of the activities of the three enzymes (P5C, P5CS, and Proline) involved in the biosynthesis of 2AP (Figures 5 and 6). Furthermore, for both B-385 and YJY, the application of fertilizer at 10 cm was higher compared to those other treatments (5 cm and surface placement). Similarly, the P5C activity, P5CS activity and Proline content were significantly affected by the application of fertilizer in grains: FF10 > FF5 > FFS for Fragrant Fertilizer, and U10 > U5 > US for Urea (Figures 5 and 6).
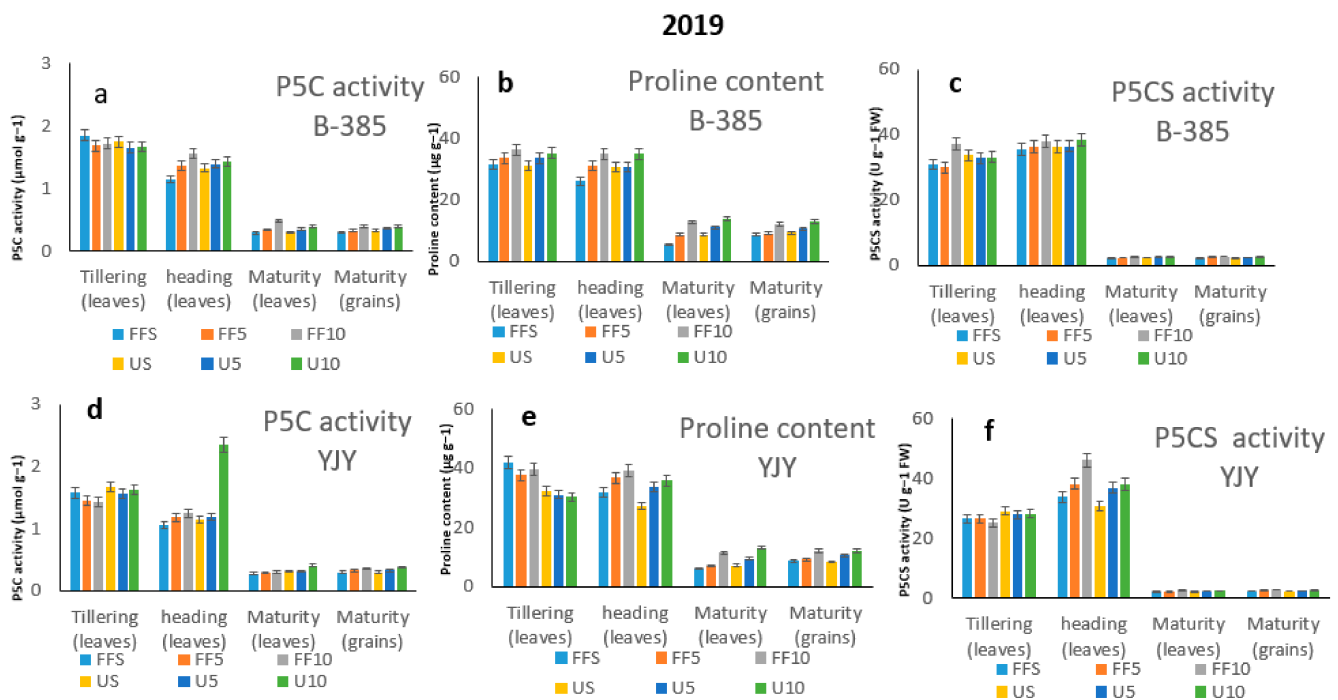
2019

Figure 5. Aroma-related substances' activities in the leaves and grains at different growing stages under fertilizer application in 2019. (**a**) P5C activity (μmol g^{-1}) in B-385 rice cultivar under different fertilizer application; (**b**) Proline content (μg g^{-1}) in B-385 rice cultivar under different fertilizer application; (**c**) P5CS activity (U g^{-1} FW) in B-385 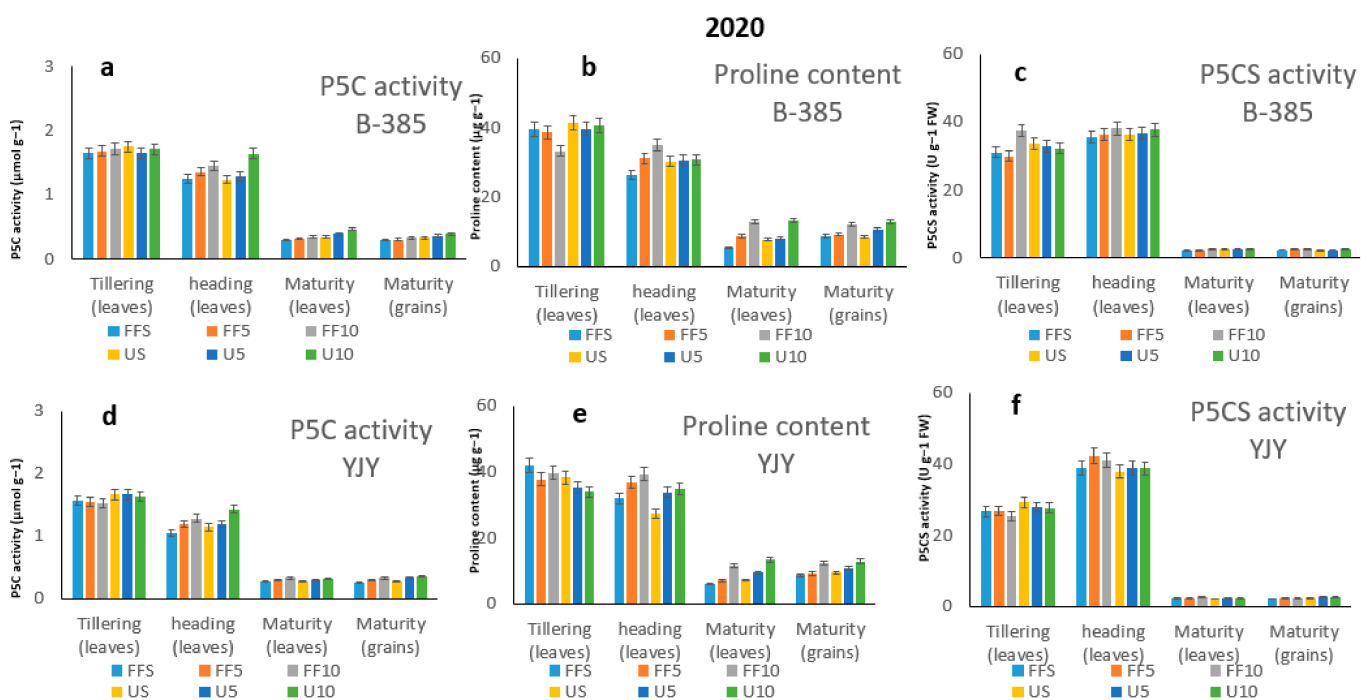rice cultivar under different fertilizer application; (**d**) P5C activity (μmol g^{-1}) in YJY rice cultivar under different fertilizer application; (**e**) Proline content (μg g^{-1}) in YJY rice cultivar under different fertilizer application; (**f**) P5CS activity (U g^{-1} FW) in B-385 rice cultivar under different fertilizer application.

2020

Figure 6. Aroma-related substances' activities in the leaves and grains at different growing stages under fertilizer application in 2020. (**a**) P5C activity (μmol g^{-1}) in B-385 rice cultivar under different

fertilizer application; (**b**) Proline content (μg g^{-1}) in B-385 rice cultivar under different fertilizer application; (**c**) P5CS activity (U g^{-1} FW) in B-385 rice cultivar under different fertilizer application; (**d**) P5C activity (μmol g^{-1}) in YJY rice cultivar under different fertilizer application; (**e**) Proline content (μg g^{-1}) in YJY rice cultivar under different fertilizer application; (**f**) P5CS activity (U g^{-1} FW) in B-385 rice cultivar under different fertilizer application.

4. Discussion

The effects of the deep application of fertilizer on yield and quality traits in rice production were previously reported [8,33–37]. Differences in rice cultivars and the methods of fertilizer application (in terms of depth), as assumed, led to considerable variation in growth, yield and quality traits (aroma, starch, amylose percentage and cooked rice elongation) [38]. The results obtained for each of the two years differed; however, we observed the same trend and, according to the climate aspect (Figure 1), the results for each of the two years (2019 and 2020) of the experiment showed equal effects regarding the placement of fertilizers. Differences between years might be attributed to variations in climatic conditions at the experimental site.

The depth of the fertilizer application significantly affected the yield and its components for the two rice varieties over the two crop years. In addition, the application of fertilizers (at 10 cm) was remarkably distinguished by a better score in terms of the number of filled grains per panicle, the effective panicle number, and the 1000-Grain, Grain-Filling and yield values, as compared to other two depths of application (Table 2). Many researchers considered the deep placement of fertilizer to be the most efficient and promising method to reduce total nitrogen losses and increase the nitrogen use efficiency (NUE) and grain yield of wetland rice [39,40]. It was observed that the deep placement of fertilizer caused significant improvements in grain yields as compared with broadcasting in the wet season, which was ascribed to the increase in the production of tillers and the numbers of panicles [15]. In the present study, we also found that the input depth of 10 cm, for both types of fertilizer, significantly increased the grain yield of the two varieties of rice as well as the number of spikelets per panicle and the percentage of grain filling (Table 2) as compared to the surface and 5-cm placements. This study found a significant positive correlation between variety and fertilizer placement in terms of the grain yield, 1000-Grain and Grain-Filling values (Table 2). Deep fertilizer placement would have led to a reduction in NH4+ N concentrations in soil and floodwaters with the application of hydrolyzed fertilizers due to the soil urease activity being lower than the surface diffusion [37,39].

In terms of the rice quality and its components, no significant interaction was observed for brown rice and milled rice (Table 5). However, the head rice rate, green grain rate, protein content, amylose content, chalk white and chalkiness attributes were significantly affected by the placement of the fertilizers (Table 4). We further observed that for both varieties overall, for the two years of production, 10-cm deep placement of fertilizers gave greater results compared to the other placement depths, which was in agreement with the results described by Mo., et al. [16]. The dynamics of the deep placement of fertilizers during rice growth period could substantially affect the head rice rate, green grain rate, protein content, amylose content, chalk white and chalkiness attributes of aromatic rice grains [40–43]. Therefore, rice quality and its components could be modulated with the application of fertilizer at the 10-cm depth [44]. The possible reason for the improvement in the quality of rice and its components with the 10-cm deep application could be better nitrogen uptake and lower leaching and evaporation losses as compared to the input of fertilizer at the surface and at 5-cm depth below ground level.

It has been established in several studies that rice consumers prefer grains with significant elongation after cooking and a high percentage of amylose content [28,45,46]. In our study, we observed that both cultivars (B-385 and YJY) exhibited different responses to the depth of fertilizer application. The surface supply and the 10-cm deep supply had a higher elongation percentage of cooked rice compared to the surface supply (Figure 2a–d). The main reason could be that the deep placement of the fertilizers (10 cm) could have

increased the number of productive panicles, which would have led to better rice growth and better quality traits [40]. However, the application of fertilizers at 5-cm depth was not beneficial to the plant; this could be explained by the fact that at this depth, the roots of the plants did not have enough nutrients for their growth, which would have had a significantly negative effect on the growth and quality parameters of the plant. Hence, it was found that the deep placement of fertilizers allowed a higher N supply to be maintained in deep soil layers than N broadcast for 52 days during rice growth [47]. This is in agreement with our results with regard to the high values obtained for the elongation percentage of the cooked rice under the fertilizer treatment at 10-cm depth (Figure 2). This could be explained by the fact that fertilizers, when located at a depth of 10 cm, are immediately available for uptake, and nitrogen loss would be considerably limited, thus promoting the efficient nutrition of the rice plant [48]. However, information on the effect of different fertilizer application depths on the elongation percentage of cooked rice as a key factor of rice quality is still very scarce. Our work showed that a 10-cm depth of fertilizer input for the two cultivars increases the percentage of elongation of cooked rice. Sun, Potcho and Zhou [20,49,50] mentioned that the application of fertilizers at a depth of 10 or 15 cm has advantages not only in terms of the effective growth of the rice plants but also in helping to improve the quality of the rice, based on such aspects as cooked rice elongation.

In order to elucidate the effect of the application of fertilizers on the accumulation of 2AP, we observed a difference in the plant tissues under study (leaves, stems, sheaths and grains) and the results corroborate those of [51], who reported that the flavor of the grains depends on the depth of fertilizer application. Here, we confirmed that the variation in 2AP content at different stages and in different tissues, including grains, is dependent on differences in the depths of fertilizer application. Our study found that there was no significant difference for the accumulation of 2AP in the stem during either the tillering stage or the heading stage. In addition, at the tillering stage, the biosynthesis of 2AP in the leaves and sheath followed a trend characterized by a high concentration in the contribution of the surface application, followed by those of 5 cm and finally 10 cm depths (Figures 3 and 4). However, at the heading and maturity stage, the accumulation of 2AP followed the opposite trend for the leaves, sheath, stem and grains. Thus, we noted that the 2AP is more concentrated at 10-cm deep application compared to other treatments. It was pointed out that the 2AP biosynthesis takes place from an ascending line in the plant [52], and this is in agreement with our results. The work of Dias [53] underlined that the accumulation of 2AP is subject to several factors such as the application of fertilizers. This confirms the results of our study as we noted significant correlations between cultivars and different fertilizer placements (Figures 3 and 4) (Table 6). Interestingly, we observed that the content of 2AP in grains at the maturity stages increased in the 10-cm deep placement treatments (Figure 4 and 6) (Table 4). Correlation analysis indicated that the 2AP content of matured grains showed a significantly positive correlation with the placement of cultivars and fertilizers during the two growing years (Table 6). Correlation with cultivars and fertilizer placement (Table 6) suggests that 2AP may possibly be transported from leaves to grains [27,54,55].

Valuable studies have reported some precursors in the 2AP biosynthetic pathways. For example, Meijuan Li and Huang [25,56] found that proline is the most important precursor and that it is directly involved in the formation of 2-AP; moreover, the relationship of P5C and P5CS was found to be relevant to the constitution of the perfume [57]. The fragrance of aromatic rice is affected by environmental factors, particularly the application of nitrogen. The enhancement in 2AP content in grains was found to be associated with improved proline content in grains [58]. Moreover, the application of nitrogen also has a positive influence on the accumulation of proline and 2AP contents. Yang [25] reported that more proline was found in the aromatic rice grains, and that this was consistent with the higher total nitrogen and proline contents. The proline, P5C and P5CS contents were significantly affected by the application of fertilizer in both cultivars throughout all the stages (Figures 5 and 6). In this study, the deep application of fertilizers regulated P5C,

P5CS and proline in the leaves and grains at the tillering, heading and maturity stages during the two year-long growing seasons, following the rainfall trend (Figure 1). This shows that under the current conditions of climate change, the 10 cm intake depth is better adapted to the growth and quality traits of aromatic rice [59]. In addition, it should be noted that the activity of the enzymes P5C and P5CS, as well as the proline contents, followed the same trend as the 2AP content in different plant tissues (leaves, grains) of the rice plant at the tillering, heading and maturity stages of the grains (Figures 4–6). Overall, the application of fertilizers at depths of 10 cm has a regulatory effect on the yield, the elongation of cooked rice, and the amylose content and formation of 2AP, which are due to the absorption of nutrients in sufficient quantities, thereby promoting photosynthesis, the accumulation of biomass, and the general quality parameters of rice and related attributes.

5. Conclusions

Overall, the deep application of fertilizers improved the growth traits of rice plants, as well as increasing the grain yield and some parameters related to the quality of fragrant rice grains. These findings showed how the deep placement of fertilizers at 10 cm was related to the presently occurring climate change as a function of precipitation over the two years of observation (2019, 2020), and this opens up the possibility of further research on the residual effect of deep fertilizer application and its impact on soil pollution and environmental preservation. The application of fertilizers at a reasonable depth has a significantly positive impact on the 2AP content and the activities of the enzymes involved in 2AP formation such as P5C, P5CS and proline. However, in order to reveal the basic mechanism of 2AP transport during fertilizer delivery practices, a lot of work is required at the molecular and physiological levels.

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