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Effect of Timing and Duration of Salt Treatment during Growth of a Fragrant Rice Variety on Yield and 2-Acetyl-1-pyrroline, Proline, and GABA Levels

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ABSTRACT: In greenhouse experiments, Aychade, a fragrant rice variety, was grown under one level of salt solution (EC of 3800 ± 400 $\mu S \cdot cm^{-1}$) sufficient to induce salt stress in rice. Timing and duration of salt solution application varied according to the growth stages. 2-Acetyl-1-pyrroline (2AP), a characteristic flavor compound of fragrant rice as well as biogenetically related compounds, proline, and γ-aminobutyric acid (GABA) were quantified. Salt treatments induced 2AP synthesis in the leaves, but the increase was often higher in the vegetative phase. This increase was correlated with proline level but not with that of GABA. Interestingly the grains from all the salt treated plants contained significantly higher levels of 2AP (733−998 $\mu g \cdot kg^{-1}$) than those from the control (592 $\mu g \cdot kg^{-1}$). The highest 2AP synthesis occurred when the plants were subjected to salt treatment during whole vegetative or reproductive phases. However in the latter case crop yield decreased significantly.

KEYWORDS: *Oryza sativa L., Aychade, fragrant rice, greenhouse, salinity treatment, 2-acetyl-1-pyrroline, GABA, proline*

INTRODUCTION

Fragrant rice is highly appreciated due to its pleasant and characteristic flavor. Many studies have established that 2-acetyl-1-pyrroline (2AP) is responsible for the distinct popcornlike flavor of fragrant rice varieties.[1−5] Its synthesis in rice was found to increase under osmotic stress, i.e., drought and salinity. Drought during the period of milky consistency of starch in the grain led to an increase in 2AP concentration in the mature grain.[6] The concentration of 2AP in the grains was found to increase when fragrant rice cultivars were grown in the field with high salinity level.[7]

Under salt stress many plants like rice tend to accumulate proline[8,9] and γ-aminobutyric acid (GABA).[10] Experiments with seedlings and callus of a fragrant rice variety (Khao Dawk Mali 105) showed that proline was the main amino acid precursor of 2AP.[11] Besides, GABA is one of the metabolites in the biosynthetic pathway of 2AP through proline.[12] The effect of salinity on 2AP concentration in rice leaves was studied but without considering the relation between proline and GABA levels.[13] One paper reports this relation in rice under salinity in the field experiments but only in the grains.[7] No clear correlation was found between 2AP, proline, and GABA levels. The aim of the present study was to better understand this relation both in the leaf tissues and in the grains under salinity through controlled conditions in a greenhouse.

Salinity during rice cultivation is associated with reduced yield in rice.[14−18] It has been proposed that salinity stress before flowering would increase 2AP level in the grains.[7] However almost nothing is known about the effect of timing and duration of salinity at different rice growth stages both on 2AP concentration and on yield. This aspect was also studied to have some insight into a possible management of salinity during rice cultivation to obtain grains of high 2AP concentration.

MATERIALS AND METHODS

Chemicals. Proline and GABA were purchased from Sigma. 2-Acetyl-1-pyrroline (2AP) and its deuterium-labeled analogue, 2-acetyl-1-d_2-pyrroline (2AP-d_2) were synthesized by our group.[19] Briefly starting material was L-glutamic acid. Its cyclization and acetylation yielded *N*,5-diacetylpyrrolidin-2-one **1**. Deactylation of **1** gave 5-acetylpyyrolidin-2-one **2**. Reduction of **2** by $LiAlD_4$ and $LiAlH_4$ yielded 2-(1-hydroxyethyl)pyrrolidine (d_3) **3** and 2-(1-hydroxyethyl)-pyrrolidine **4**, respectively. The oxidation of **3** and **4** led to 2AP-d_2 and 2AP, respectively. Strategy of synthesis allowed introducing two deuterium atoms on the α carbon to the nitrogen atom of 2AP. Gas chromatography positive chemical ionization tandem mass spectrometry (GC−PCI-MS−MS) analysis confirmed the identity of 2AP and 2AP-d_2.[19]

Greenhouse and Salinity Treatment Conditions. Soil was prepared from a mixture of fertile soil (from Lavalette field, Montpellier, France) (75%) and natural compost (25%) (a blend of decomposed remains of peat mosses (*Sphagnum* sp.) and herbs). Each pot (2 L; 20 cm high) was filled with 1500 g of the prepared soil (16 to 17 cm of pot's height) and 4 g of a chemical fertilizer (Basacote Plus 6 M from Compo France SAS ; N:P:K ratio of 11:9:19). Physicochemical characteristics of the soil before sowing and during rice production are given in Table 1.

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Table 1. Physicochemical Characteristics of the Soil Sample from Three Different Periods during Rice Growth[a]

characteristic	before sowing	BT[b] stage (T0)	FLO[c] stage (T0)
organic matter (%)	5.95 ± 0.28	7.24 ± 0.34	7.14 ± 0.34
organic carbon (%)	3.45 ± 0.17	4.20 ± 0.20	4.14 ± 0.20
total nitrogen (%)	1.39 ± 0.08	1.71 ± 0.09	1.59 ± 0.09
C:N ratio	24.80 ± 1.99	24.61 ± 2.15	25.96 ± 2.22
cation exchange capacity (mequiv/100 g)	11.47 ± 0.95	14.02 ± 1.11	13.87 ± 1.10
saturation rate (%)	>100	>100	>100
pH	8.01 ± 0.07	7.86 ± 0.08	7.86 ± 0.08

[a]Data are means ± SD (three replicates). [b]Beginning tillering stage. [c]Flowering stage.

The rice cultivar was Aychade, an improved fragrant temperate japonica type (*Oryza sativa* L.) from the Camargue area in France.[7] Four growth phases were defined (Figure 1): (i) the early vegetative phase from the sowing to beginning tillering stage (BT) (about 30−35 days after sowing (DAS); (ii) the vegetative phase from the BT stage to the panicle initiation stage (PI) (about 60−65 DAS) which is subdivided into the vegetative I phase, from BT to middle tillering (MT), and the vegetative II phase, from MT to PI; (iii) the reproductive phase from the PI stage to the flowering stage (FLO) (about 80−85 DAS); and (iv) the ripening phase from the FLO stage to harvest time (about 120−125 DAS).

The experiments were conducted in a greenhouse at Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD) in Montpellier (France). Two seeds of cv. Aychade per pot were sown on June 3 in 2010 (a total of 330 pots). Seeds were grown under controlled conditions (12 h photoperiod, 28/20 °C day/night, and relative humidity of 70−80%). Pots were irrigated every two days for one week with 100 mL of reverse osmosis water (RO water). In the second week only one seedling was kept in each pot. The selection was done to obtain as much as possible homogeneous seedlings between pots. Pots were then placed until the BT stage (33 DAS) in the trays filled with RO water (50 L per tray) up to 5 to 6 cm of the height of the pots. The plants were then subjected to five treatments (T0−T4) to study the salinity effect. Each treatment comprised 66 pots, a total of 330 pots. T0 treatment was the control and received RO water. T1 to T4 treatments received saline solution (30 mM NaCl) at different growth stages for different durations as follows (Figure 1): T1 received saline solution from the beginning tillering (BT) to the middle tillering stage (MT) (so-called vegetative I phase) for 14 days, T2 treatment from the MT to the panicle initiation stage (PI) (vegetative II phase) for 14 days, T3 treatment from the BT to the PI stage (whole vegetative phase) for 28 days, and T4 treatment from the PI to the flowering stage (FLO) (reproductive phase) for 21 days. Prior to the addition of NaCl solution (30 mM) in the trays, pots were rinsed twice over two days with salt solution (300 mL of salt solution per pot each time). RO water from trays was replaced by NaCl solution (30 mM) (50 L per tray). At the end of salt treatment, the pots were leached out with RO water four times over two days (300 mL of RO water per pot each time). Saline solution in the tray was then replaced by RO water. Electrical conductivity (EC) was measured using the instrument CyberScan PC300 from Eutech Instruments Europe B.V. (The Netherlands) (Figure 2). The EC of the NaCl solution was 3800 ± 400 $\mu S \cdot cm^{-1}$, while that of RO water was less than 500 $\mu S \cdot cm^{-1}$. The EC of the saline solution and RO water in the trays was checked at least twice a week. If necessary the EC was adjusted with the addition of saline solution or RO water in the control trays was renewed. Note that following the flowering stage all the trays were maintained empty from 110 DAS to harvest (124 DAS), which occurred on October 4 in 2010.

Sampling and Measurement. Samplings from the MT, PI, FLO, and harvest stages were made at 47, 61, 82, and 124 DAS, respectively (Table 2). Each treatment had at least three plant replicates, and the number of plants sampled per treatment was from 9 to 18. The number of plants sampled per growth stage was from 45 to 90.

At the MT, PI, and FLO stages, number of tillers, plant height, and % dry weight of the leaves were measured. At the harvest stage, number of panicles, panicle length, thousand grain weight (TGW), and dry weight of grains and panicles were also determined.

Samples (0.5 g leaves, 10 grains) were dried at 70 °C in an oven to a constant weight for % dry weight measurement. The rest of the sample was immediately frozen in liquid nitrogen and stored at −20 °C prior to 2AP, proline, and GABA analysis.

Determination of 2AP Concentration. All rice samples (leaves and grains) were evaluated for 2AP concentration by a stable isotope dilution assay (SIDA) involving solid-phase microextraction (SPME) combined with GC−PCI-MS−MS.[19]

Each leaf sample (ca., 5 g) was cut and frozen with liquid nitrogen and powdered by a mortar. Unpolished grains (ca. 5 g) were frozen with liquid nitrogen and then ground finely by using an analytical crusher (IKAWERKE, Staufen, Germany). The samples were immediately analyzed for 2AP. 0.5 g of leaves or grains was placed in a 10 mL glass vial containing 4 mL of sodium carbonate buffer pH 9.2 (0.1 M) and 8 μL of 2AP-d_2 solution (24.42 $\mu g \cdot mL^{-1}$ in ethanol) as an internal standard. The vial was then closed with a PTFE septum and magnetically agitated (800 rpm) at 80 °C. After 5 min of equilibration, the SPME fiber (Stable-Flex divinylbenzene/Carboxen/polydimethylsiloxane 50/30 μm fiber, Supelco, Bellefonte, PA, USA) was introduced into the headspace (80 °C) and kept for 20 min. We checked using leaf tissues and grains from a nonfragrant variety (Gladio × Fidji K2) that at 80 °C and alkaline pH of the medium 2AP was not generated from possible precursors. After extraction, the analytes from SPME fiber were desorbed in the injection port (splitless, 5 min, 250 °C) of CP-3800 gas chromatograph equipped

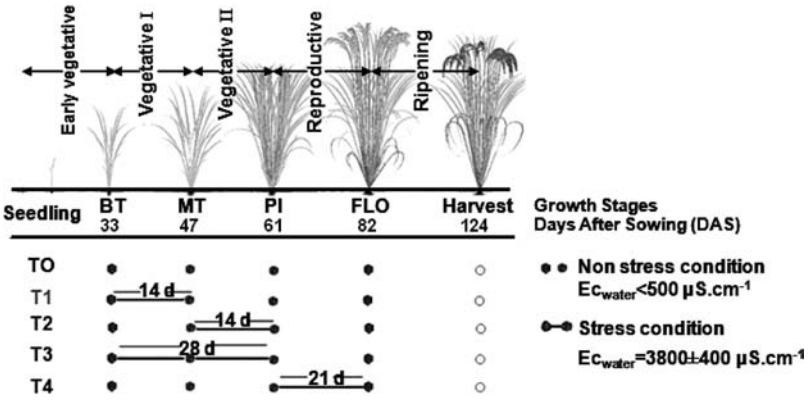

Figure 1. Experimental design for saline solution application during rice plant growth: BT = beginning tillering stage; MT = middle tillering stage; PI = panicle initiation stage; FLO = flowering stage; d = days; T0−T4 = treatments T0−T4.

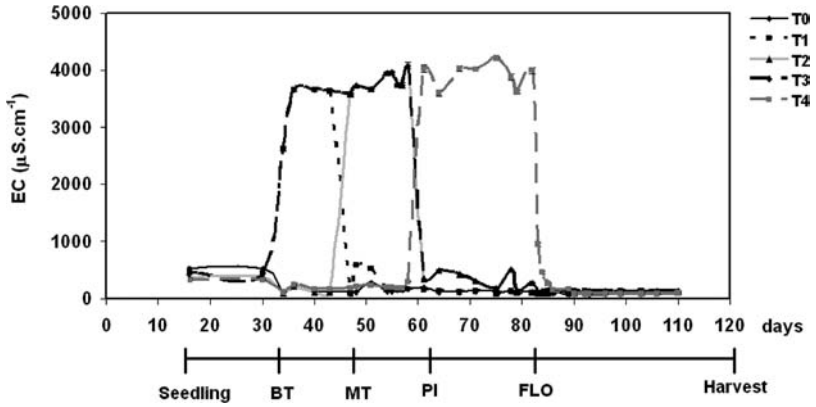

Figure 2. Changes in electrical conductivity (EC in $\mu S \cdot cm^{-1}$) of aqueous solution from trays during rice plant cultivation. T0–T4: treatments T0–T4.

Table 2. Number of Plants Sampled on Each Growth Stage from Salinity Treatments

stages	MT[a]	PI[b]	FLO[c]	harvest
(1) sampling (DAS)	(47)	(61)	(82)	(124)
(2) no. of replicates per treatment	3	3	3	3
(3) no. of plants sampled per replicate	3[e]	3[e]	4[e]	6[e]
(4) no. of plants sampled per treatment (2) × (3)	9[f]	9[f]	12[f]	18[f]
(5) no. of plants sampled per stage (4) × 5[d]	45	45	60	90

[a]Middle tillering stage. [b]Panicle initiation stage. [c]Flowering stage. [d]5 treatments (T0–T4). [e]Number of plants taken to determine growth parameters (except dry weight). [f]Number of plants taken to analyze dry weight, 2AP, proline, and GABA.

with a DB-Wax (J&W Scientific, Folson, CA) fused silica capillary column (30 m × 0.32 mm i.d., film thickness = 0.5 μm). GC was coupled with a Saturn 2000 ion-trap mass spectrometer (Varian, Walnut Creek, CA, USA). Helium gas was the carrier gas at flow rate of 1 $mL \cdot min^{-1}$. The temperature of the GC oven was 50 °C (1 min), increased at 2 $°C \cdot min^{-1}$ to 85 °C and then to 245 at 30 $°C \cdot min^{-1}$, and held at 245 °C for 8 min. The ion trap temperature was 150 °C, and the manifold and transfer line temperatures were 45 and 250 °C respectively. For PCI acetonitrile was used as reagent gas.

2AP was quantified through the calibration curves established by area ratio between ions at m/z 70 and 72, from 2AP and 2AP-d_2 respectively. These ions were major daughter ions in PCI-MS–MS analysis of 2AP and 2AP-d_2 respectively.[19] Each sample was extracted in duplicate. The coefficient of variation of 2AP analysis was less than 10%. The results are expressed as $\mu g \cdot kg^{-1}$ of dry weight (DW) (mean ± SD). Under these conditions, the retention times of 2AP and 2AP-d_2 were 11.65 and 11.57 min, respectively.

Two standard curves were established separately for the leaves and the grains from a nonfragrant rice variety (Gladio × Fidji K2) involving solid-phase microextraction (SPME) technique. For leaves, ten different amounts of 2AP ranging from 0.006 to 3.757 μg in ethanol and a constant amount of 2AP-d_2 (0.195 μg) were introduced into 10 mL vials added with 0.5 g of leaves and 4 mL of sodium carbonate buffer (pH 9.2, 0.1 M). For rice grains (0.5 g) eight different amounts of 2AP (0.006–1.503 μg) were used together with a constant amount of 2AP-d_2 (0.195 μg) to establish the calibration curve. The experiment was done in duplicate. GC–PCI-MS–MS analyses were performed as above. Both calibration curves yielded a good linearity with a regression coefficient of 0.9961 and 0.9974 for leaves and grain matrix, respectively.

Determination of Proline and GABA Concentration. Proline and GABA extraction was performed as described previously.[7] 100 mg of leaves or grains ground under liquid nitrogen was added to 4 mL of sodium carbonate buffer (pH 9.0, 0.1 M) and 50 μL of 25 $\mu mol \cdot mL^{-1}$ carboxymethylcysteine (CMC) solution as an internal standard. The ensemble was agitated at room temperature for 1 h. Following filtration on 0.45 μm Millipore filter, 25 μL of the extract was mixed with 75 μL of sodium carbonate buffer (pH 9.0, 0.1 M) and 100 μL of 6 mM dabsyl (4-(dimethylamino)azobenzene-4′-sulfonyl chloride) solution in acetonitrile. After incubation for 10 min at 70 °C, the solution was added with 300 μL phosphate buffer (pH 7.0, 9 mM). Twenty microliters of the medium was analyzed by HPLC on a Dionex liquid chromatography system equipped with a UVD 340U diode array detector coupled to a HP ChemStation (Dionex, France). The column was a RP18 (250 mm × 4.6 mm, 5 μm particle size; Waters, Ireland). Mobile phase A consisted of 1% (v/v) 0.9 M NaH_2PO_4, 4% (v/v) dimethyl formamide and 0.1% triethylamine in water. The pH was adjusted to 7.5. Mobile phase B was acetonitrile–water (80:20, v/v). The elution was performed at 1 $mL \cdot min^{-1}$ with a linear gradient from 28% B at 0 min to 42% B at 30 min. The column was then rinsed with 100% B and then equilibrated with 28% B for at least 10 min. The retention times of proline and GABA were 24.8 and 44.8 min, respectively. Concentrations of proline and GABA were determined through the standard calibration curves. Each sample was analyzed in duplicate. The coefficients of variation of proline and GABA analysis were less than 10%. The levels of targeted compounds were expressed as $\mu g \cdot g^{-1}$ DW (mean ± SD).

Statistical Analysis. One-way analysis of variance (ANOVA) in mixed model using Dunnett's test for the comparisons of the means vs control was performed. Principal component analysis (PCA) was done using the correlation matrix of targeted compounds and yield components. EC was used in PCA as controlled variable. All statistical analysis was performed with XLSTAT software (Addinsoft, France).

RESULTS

Effect of Salinity Treatment on Plant Growth and Yield Components. Salinity treatment caused a significant reduction ($p < 0.05$) of plant height from 4 to 18% at the middle tillering (MT), panicle initiation (PI), and harvest stages except at the FLO stage compared to the control (T0). The reduction was the highest in the plants subjected to salt solution during the whole vegetative period (28 days), i.e., treatment T3.

Dry weight of the leaves (22.7 to 30.0%) and the grains (81.1 to 83.7%) was not significantly affected by salt treatment, suggesting that the plant did not need to sequester water to counter salt stress. Furthermore salt treatment did not induce significant differences in the number of tillers.

Salt treatment during the reproductive phase (T4) caused a significant decrease ($p < 0.01$) in the thousand grain weight (TGW) (26.15 g) compared to the TGW from the control (30.89 g) and other salinity treatments (28.66 to 31.08 g).

Table 3. 2AP Concentration ($\mu g \cdot kg^{-1}$) (DW) in the Leaves at Different Stages of Plant Growth in Relation to Salinity Treatments[a]

	growth stages		
treatments	MT	PI	FLO
T0	4444.60 ± 335.90	4902.10 ± 180.90	4729.80 ± 264.10
T1	5740.90 ± 424.40***	5433.50 ± 221.90	5717.80 ± 286.90***
T2	4276.60 ± 219.20	5261.51 ± 154.30	4531.70 ± 302.60
T3	5256.90 ± 126.90**	6168.80 ± 480.30***	5138.40 ± 294.60
T4	4843.55 ± 238.20	4839.40 ± 163.40	4906.30 ± 375.80

[a]Data are means ± SD (six replicates analyzed from 9 to 12 plants as shown in Table 2). Asterisks indicate significant differences vs control (**p < 0.01, ***p < 0.001). Note that ANOVA analysis was done independently for each stage.

Table 4. Proline and GABA Concentrations (DW) in the Leaves at Different Stages of Plant Growth in Relation to Salt Treatments[a]

	proline content ($\mu g \cdot g^{-1}$)			GABA content ($\mu g \cdot g^{-1}$)		
treatments	MT	PI	FLO	MT	PI	FLO
T0	96.3 ± 24.7	87.8 ± 12.5	87.4 ± 10.8	1002.3 ± 194.6	682.7 ± 84.8	855.9 ± 61.0
T1	125.9 ± 24.7	107.7 ± 7.8	94.3 ± 5.4	1011.7 ± 155.2	699.8 ± 72.0	736.2 ± 27.5*
T2	75.6 ± 3.9	119.9 ± 23.1**	92.6 ± 9.5	932.0 ± 129.5	1064.7 ± 71.0***	763.7 ± 62.2
T3	114.6 ± 22.2	132.4 ± 11.6***	106.6 ± 5.0**	1138.2 ± 110.1	968.4 ± 62.0***	836.6 ± 97.3
T4	98.6 ± 17.5	73.2 ± 12.1	90.6 ± 9.0	927.5 ± 70.7	696.5 ± 54.2	704.4 ± 49.3**

[a]Data are means ± SD (six replicates analyzed from 9 to 12 plants as shown in Table 2). Asterisks indicate significant differences vs control (*p < 0.05, **p < 0.01, ***p < 0.001). Note that ANOVA analysis was done independently for each stage.

Table 5. Correlation Coefficients from Regression Analyses between 2AP, Proline, and GABA Concentrations in the Leaves[a]

stages	$2AP_{MT}$	PRO_{MT}	$GABA_{MT}$	$2AP_{PI}$	PRO_{PI}	$GABA_{PI}$	$2AP_{FLO}$	PRO_{FLO}	$GABA_{FLO}$
$2AP_{MT}$	1	*0.955**	0.511	0.510	0.218	−0.304	*0.981***	0.500	−0.236
PRO_{MT}	*0.955**	1	0.617	0.462	0.122	−0.453	*0.945**	0.445	−0.017
$GABA_{MT}$	0.511	0.617	1	0.858	0.624	0.180	0.420	0.825	0.636
$2AP_{PI}$	0.510	0.462	0.858	1	*0.885**	0.556	0.393	*0.969***	0.351
PRO_{PI}	0.218	0.122	0.624	*0.885**	1	0.793	0.147	0.783	0.368
$GABA_{PI}$	−0.304	−0.453	0.180	0.556	0.793	1	−0.399	0.532	0.184
$2AP_{FLO}$	*0.981***	*0.945**	0.420	0.393	0.147	−0.399	1	0.350	−0.247
PRO_{FLO}	0.500	0.445	0.825	*0.969***	0.783	0.532	0.350	1	0.252
$GABA_{FLO}$	−0.236	−0.017	0.636	0.351	0.368	0.184	−0.247	0.252	1

[a]Significant correlations at *p < 0.05 and **p < 0.01.

Effect of Salinity Treatment on 2AP, Proline, and GABA Concentrations in the Leaves. 2AP, proline, and GABA concentrations in the leaves in response to salt treatments are shown in Tables 3 and 4. Average concentrations of three compounds in the leaves were in the range of 4276.6−6168.8 $\mu g \cdot kg^{-1}$ (DW) for 2AP, 73.2−132.4 $\mu g \cdot g^{-1}$ (DW) for proline, and 682.7−1138.2 $\mu g \cdot g^{-1}$ (DW) for GABA. The 2AP amount appeared to be 8- to 15-fold higher than those previously reported in the leaves from fragrant varieties, for example, 407.2 $\mu g \cdot kg^{-1}$ in Khao Dawk Mali 105 variety[20] and from 520 to 680 $\mu g \cdot kg^{-1}$ in Basmati 370 and Jasmine varieties.[13] This difference may be due to the genotype effect and/or to the quantification methodology for 2AP.

Significant increases (p < 0.01 and p < 0.001) in 2AP amount in the leaves were observed when salt treatment was initiated at the beginning of the vegetative phase (T1 and T3). On the contrary salt treatment at the reproductive phase was not favorable to stimulate 2AP synthesis in the leaves.

At the vegetative phase proline level was significantly increased (p < 0.01 and p < 0.001) in treatments T2 and T3 at the panicle initiation stage (PI), i.e., in the second vegetative phase compared to the control. In the reproductive phase there was only one significant change (increase) in proline level (treatment T3 at flowering stage).

GABA level increased also significantly (p < 0.001) in the second vegetative phase as in the case of proline. Conversely, its level decreased significantly (p < 0.05 and p < 0.01) at the reproductive stage (treatments T1 and T4).

Relationship between 2AP, Proline, and GABA Concentrations of the Leaves and Yield Components. Correlation coefficients between 2AP, proline, and GABA concentrations were determined on the leaves from vegetative and reproductive phases at the MT, PI, and FLO stages, noted as $2AP_{MT}$, $2AP_{PI}$, $2AP_{FLO}$, PRO_{MT}, PRO_{PI}, PRO_{FLO}, $GABA_{MT}$, $GABA_{PI}$, and $GABA_{FLO}$ (Table 5). Significant positive correlations were observed between proline and 2AP in some stages: between PRO_{MT} and $2AP_{MT}$, and PRO_{MT} and $2AP_{FLO}$; between PRO_{PI} and $2AP_{PI}$; between PRO_{FLO} and $2AP_{PI}$. On the contrary no correlation was found between GABA concentrations in any of the stages either with 2AP or with proline levels.

Principal component analysis (PCA) on variables from the leaves indicated that the first two components explained 71.53% of the total variation (Figure 3a). 2AP, proline, and GABA were positively correlated with PC1. Moreover there

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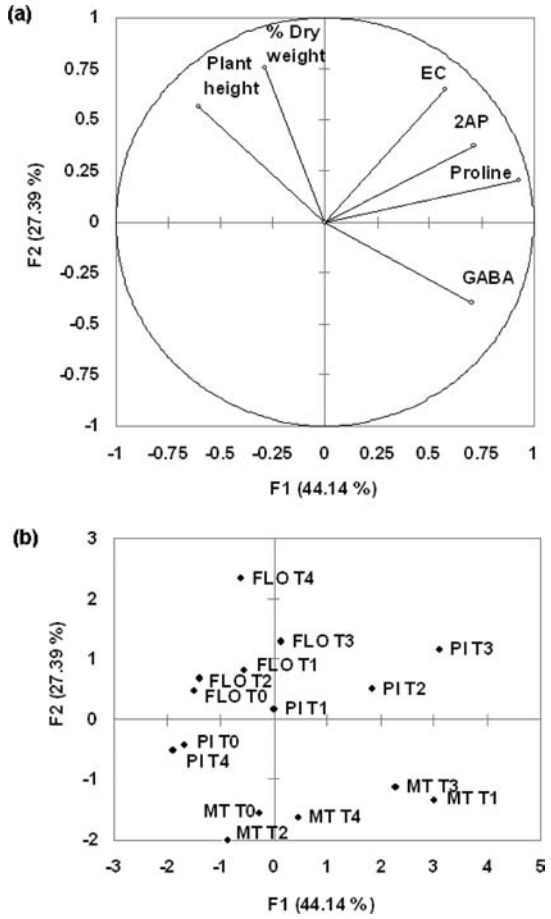

Figure 3. PCA plots of variables from the leaves (a) and position of salt treatment growth stages (b).

was a positive correlation with EC and 2AP level. On the contrary plant height and % dry weight were positively correlated on PC2. Here, PC2 does not seem to have a very strong and clear interpretation per se, since it does not materialize a clear variable bundle. However, planes 1 and 2 reveal the existence of two distinct variable bundles: one opposes dry weight and plant height to GABA, while the other correlates EC, 2AP, and proline.

Figure 3b shows PCA of the position of growth stages in relation to salinity treatments. MT_{T1}, MT_{T3}, PI_{T2}, and PI_{T3} have positive coordinates along the PC1 axis. Salt treatment during whole vegetative phase (PI_{T3}) and second vegetative phase (PI_{T2}) resulted in higher levels of proline and 2AP. FLO_{T4} had a positive value of PC2 while MT_{T0}, MT_{T2}, and MT_{T4} had negative values of PC2.

Effect of Salinity Treatment on 2AP, Proline, and GABA Concentrations in the Grains. The average concentrations of 2AP, proline, and GABA in the grains in response to different salt treatments varied from 592.2 to 997.8 $\mu g \cdot kg^{-1}$ (DW) for 2AP, 33.3 to 40.9 $\mu g \cdot g^{-1}$ (DW) for proline, and 26.9 to 74.6 $\mu g \cdot g^{-1}$ (DW) for GABA (Table 6). 2AP level was found to be lower than that in the grains from cv. Aychade cultivated in the fields under two different salinity conditions.[7] In the latter case 2AP content was between 715 and 1633 $\mu g \cdot kg^{-1}$ (DW). GABA content in the grains was close to that obtained in the field while proline content was higher (about by 2-fold) in our samples.[7]

Table 6. 2AP, Proline, and GABA Concentrations (DW) in the Mature Grains (Harvest Stage) Issued from Different Salt Treatments[a]

treatments	2AP ($\mu g \cdot kg^{-1}$)	proline ($\mu g \cdot g^{-1}$)	GABA ($\mu g \cdot g^{-1}$)
T0	592.2 ± 45.9	33.5 ± 1.3	26.9 ± 1.6
T1	763.6 ± 41.4**	35.5 ± 6.2	34.7 ± 5.2
T2	733.3 ± 15.6*	33.3 ± 4.7	30.8 ± 2.6
T3	997.8 ± 118.7***	40.9 ± 5.6	74.6 ± 13.7***
T4	858.9 ± 54.3***	40.5 ± 4.3	40.3 ± 0.8

[a]Data are means ± SD (six replicates analyzed from 18 plants as shown in Table 2). Asterisks indicate significant differences vs control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

There were no significant changes on proline level in the grains due to the salt treatment during plant growth (Table 6). The same occurred for GABA except for a dramatic increase in treatment T3. In contrast 2AP content increased significantly in the grains from all the salt-treated plants (T1−T4) compared to the control (T0). A highly significant increase ($p < 0.001$) was observed in the grains from treatments T3 and T4, i.e., plants treated with salt during whole vegetative and reproductive phases respectively.

Relationship between 2AP, Proline, and GABA Concentrations in the Grains and Yield Components. PCA on the variables from the grains produced a first component plane capturing 98.40% of the information: 75.12% and 23.28% by PC1 and PC2 respectively (Figure 4a). EC, proline, 2AP, and GABA were highly positively correlated with PC1, which thus materialized the clear bundle they made. Besides, TGW and % dry weight are farther astray from axes. The variable pattern thus appears to have a triangular shape. A good discrimination of the grains from different treatments was observed (Figure 4b). PC1 allowed scaling of treatments with respect to variables EC, proline, 2AP, and GABA and, in particular, allowed discrimination of T0 from the other treatments. Grains from the treatment T3 were positively correlated with PC1.

DISCUSSION

Our preliminary experiments in the greenhouse showed that cv. Aychade was not able to produce grains in high salinity conditions (EC = 5000 $\mu S \cdot cm^{-1}$). Sensitivity of this variety to salt stress was also observed in the field.[7] Since the objective of this work was to evaluate the effect of salinity on the grains as well, a salt solution yielding 3800 ± 400 $\mu S \cdot cm^{-1}$ was used in the experimental setup. This value was higher than the lowest EC value (2000 $\mu S \cdot cm^{-1}$) to induce salt stress in rice.[21] The experimental setup was satisfactory since the salt level applied at the vegetative phase and the reproducing phase with different durations allowed production of the grains. TGW was significantly decreased only in the treatment where salt application occurred during the reproductive phase. Similarly yield losses estimated through TGW evaluation were reported when rice plant was grown under salinity conditions during the reproductive phase.[22] However when Aychade cultivar was grown in the field, there was a significant negative correlation between TGW and EC not only at the reproductive phase but also at the vegetative phase.[7]

Salinity treatments in our experimental setup did not induce severe changes in the plant's height, dry weight of the leaves or panicle, number of panicles, or panicle length. It can be assumed that salt stress in our experimental conditions was

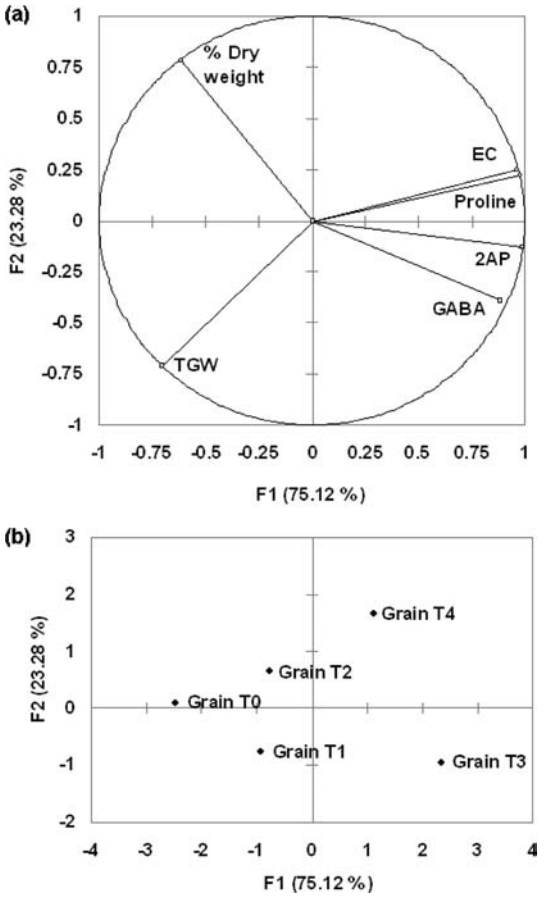

Figure 4. PCA plots of variables from the grains (a) and position of salt treatment growth stages (b).

moderate. The response of the plant to salt stress was evidenced by the increase in proline level at the vegetative phase in the leaves. Under salt stress conditions proline accumulation in rice leaves was already reported.[23−26] Similarly the increase in GABA level, one of the osmoprotectant compounds was observed in the vegetative phase. This may be explained by the fact that the rice plant had to synthesize more proline and GABA to counteract salt stress during the intense vegetative growth phase. No significant difference in GABA levels between seedlings from fragrant and nonfragrant rice varieties grown at 0 mM and 17 mM was observed.[14] The lower concentration of salt used in this work together with shorter duration of salt treatment (10 days) could explain no changes in GABA levels.

PCA showed that salt treatment applied during the whole vegetative phase gave the highest levels of proline and 2AP in the leaves. Both compounds were also positively correlated with EC, suggesting that salinity in the whole vegetative phase induces 2AP biosynthesis through the accumulation of proline. Positive correlations observed through correlation coefficients between 2AP and proline levels at vegetative phases support our assumption that proline could have participated in the biosynthesis of 2AP. In a previous work, however, salt treatment at the concentrations from 5 to 170 mM during growth of Jasmine and Basmati 370 cultivars at the vegetative stage did not result in the increase of 2AP amount in the leaves.[13] This difference may be due to the genetic trait or to the timing and duration of salt treatment. Indeed we observed the most significant increase in 2AP when salt treatment started at the beginning of the tillering stage and lasted during the whole vegetative phase. With regard to GABA level no correlation was found either with 2AP or with proline whatever salt treatment was applied.

This is the first time that we demonstrate significant increases in 2AP amount in the grains regardless the timing and duration of salt treatment. In particular the salt application during the whole vegetative and reproductive phases yielded the highest concentration of 2AP in the grains (998 and 859 $\mu g \cdot kg^{-1}$ DW respectively). However grain yield significantly decreased when salt treatment took place during the reproductive phase. A significant negative correlation was reported between grain yield and 2AP amount as well as between grain yield and mean EC during the entire crop season when Aychade and two other rice cultivars were grown in the field.[7] The authors suggested that increase of 2AP amount with salinity was partly due to 2AP concentration in smaller grain sizes. They deduced the formation of smaller grain sizes from the decrease of TGW. Such reduction in yield is one of the major constraints in rice cultivation. Our experimental setup clearly showed that it could be possible to produce grains with higher levels of 2AP under salinity without affecting yields in proviso that plants are exposed to salinity prior to the reproductive phase.

Proline level was not significantly affected in the grains by salinity although those grains contained a significantly higher level of 2AP compared to the control. A similar conclusion was reported in the experiments conducted in the field.[7] On the contrary we observed significant increases in 2AP and proline levels in the leaves under salinity treatments in both the vegetative and reproductive phases. Hence two possibilities could explain increases in 2AP in the grains. Either 2AP synthesized in the leaves could have been transported into the grains or proline was translocated from the leaves into the grains where 2AP synthesis occurred. The demonstration of such assumptions may have been undertaken through feeding of labeled targeted compounds into rice plant at different developmental stages.

In conclusion, overall data suggest that salinity management during rice cultivation could lead to better control of 2AP synthesis in rice without leading to a significant reduction in the grain yield. Further experiments should be undertaken using our experimental conditions with other fragrant rice to determine if there is genotype variability with regard to the relation between 2AP biosynthesis and grain productivity under salt stress.

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Notes

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ABBREVIATIONS USED

2AP, 2-acetyl-1-pyrroline; 2AP-d_2, 2-acetyl-1-d_2-pyrroline; BT, beginning tillering stage; GABA, γ-aminobutyric acid; DAS, days after sowing; DW, dry weight; EC, electrical conductivity; FLO, flowering stage; GC−PCI-MS−MS, gas chromatography

positive chemical ionization tandem mass spectrometry; MT, middle tillering stage; PCA, principal component analysis; PI, panicle initiation stage; RO, reverse osmosis; SIDA, stable isotope dilution assay; SPME, solid-phase microextraction; T, treatment; TGW, thousand grain weight

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