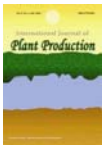

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Water relations, pigment stabilization, photosynthetic abilities and growth improvement in salt stressed rice plants treated with exogenous potassium nitrate application

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Abstract

Potassium is a major nutrient which may play an important role in many processes such as ion homeostasis in plant cells and osmotic adjustment of guard cells during stomatal opening and closing. Pathumthani 1 (PT1) rice has been reported as being a salt sensitive cultivar and has been selected as a model plant in this study to investigate the possibility of improving the osmotic potential, pigment stabilization, photosynthetic efficiency and growth characteristics of this plant under salinity stress by potassium nitrate (KNO_3) application. Sodium ion accumulation in root and leaves of KNO_3 (11.8 mM) treated plants declined as compared to the control plants. In control plants, however, due to decline in potassium ion content, the Na^+ / K^+ ratio increased significantly. A positive relation between Na^+ accumulation and osmotic potential was found. Osmotic potential (Ψ_s) in the root and leaf tissues of PT1 rice treated with 11.8 mM KNO_3 was maintained at low levels, which was accompanied with the stabilization of photosynthetic pigments, high photosynthetic performance and better growth characters under 200 mM NaCl. The photosynthetic ability in plants with KNO_3 application was positively related to plant dry weight. Exogenous KNO_3 application to rice crops may play a vital role as a short-gun technique for the improvement of salt tolerance.

Keywords: Chlorophyll fluorescence; Net photosynthetic rate; *Oryza sativa*; Osmotic potential; Potassium; Quantum yield; Salinity.

Introduction

Soil salinity is a serious abiotic stress, directly and indirectly affecting plant growth and development, leading to loss of productivity especially in glycophyte species (Hasegawa et al., 2000; Qadir et al., 2008). Sodium ion (Na^+) is a major contaminant of salt affected soil

and has been reported as being toxic to plants which is quickly absorbed and taken up by root cells (Tester and Davenport, 2003; Malagoli et al., 2008).

One of the most important salt defense mechanisms in higher plants, especially halophyte species is Na^+/K^+ homeostasis, which is defined as a primary defense response when plants are exposed to salt stress (Cuin et al., 2003; Munns and Tester, 2008). There are many documents reporting on this issue in terms of Na^+ / K^+ ratio, Na^+ and K^+ interactions, potassium transporter proteins and the potassium transport gene family (Schachtman and Liu, 1999; Rodríguez-Navarro, 2006; Amrutha et al., 2007; Chen et al., 2007; Gierth and Mäser, 2007; Britto and Kronzucker, 2008; Cuin et al., 2008; Alemán et al., 2009; Szczerba et al., 2009). The role of potassium in salt tolerance mechanisms has been investigated, including ion homeostasis, osmoregulation and antioxidant systems (Cakmak, 2005; Chen et al., 2007; Szczerba et al., 2009). There are several factors influencing Na^+/K^+ homeostasis, such as different genetic resources (Golldack et al., 2003; Kader et al., 2006; Huang et al., 2008), mutant lines (Wu et al., 1996; Zhu et al., 1998) and over-expression of potassium-related gene (s) (Rubio et al., 1999; Obata et al., 2007; Takahashi et al., 2007; Mangano et al., 2008). In addition, K^+ is known to function in osmotic adjustment in the guard cell controlling the stomatal movements and thus CO_2 assimilation in photosynthesis (Chartzoulakis et al., 2006; Degl'Innocenti et al., 2009).

Potassium application has been reported as being effective in salt tolerance mechanism of plants through Na^+/K^+ homeostasis (Chen et al., 2007; Cuin et al., 2008; Kader and Lindberg, 2008; Alemán et al., 2009), osmoregulation (Szczerba et al., 2009) and antioxidant systems (Cakmak, 2005). K^+ in plant tissues evidently decreases when plants are exposed to salt stress, especially rice genotypes (Basu et al., 2002; Castillo et al., 2007; Ahmad et al., 2007). Exogenous potassium in the culture medium is directly absorbed and taken up by the root tissues and translocated into the whole plant body (Akram et al., 2007; Akram et al., 2009; Kaya et al., 2007; Shaibur et al., 2008; Zheng et al., 2008). An alternative way to improve salt tolerance in plants is to increase the level of endogenous potassium by the application of potassium salts (Ahmad and Jabeen, 2005; Kaya et al., 2007; Zheng et al., 2008; Akram et al., 2009). Thus growth parameters including plant height and dry weight in salt stressed wheat pretreated with 16 mM KNO_3 were improved in both salt tolerant (DK961) and salt sensitive (JN17) cultivars (Zheng et al., 2008). Improved growth characters, in terms of shoot dry weight, root dry weight and fruit weight, were also found in salt stressed melons treated with 5 mM KNO_3 (Kaya et al., 2007). Leaf area and fruit yield of salt stressed *Lagenaria siceraria* were improved by 2.47 mM KNO_3 (Ahmad and Jabeen, 2005).

Rice (*Oryza sativa* L. spp. *indica*) is the main cereal crop in Asian counties. It plays a major role as a staple food, representing 50-80% of people's daily calorie intake (Khush, 2005). In previous reports, rice was found to be highly sensitive to salt stress in both vegetative and reproductive stages (Zeng et al., 2001; Moradi and Ismail, 2007), leading to crop yield losses of more than 50% when exposed to saline media with electrical conductivity of 6.65 dS m^{-1} (Zeng and Shannon, 2000). In Thailand, the Pathumthani 1 (PT1) cultivar is a long grain, aromatic rice with high cooking quality and a soft texture (Laohakunjit and Kerdchoechuen, 2007). It is widely cultivated in irrigated paddy fields and has been reported as being salt susceptible (Cha-um et al., 2007). The objective of this study was to investigate the ameliorative effects of potassium nitrate application on some

physiological responses such as water balance, ion homeostasis and photosynthetic parameters of salt-stressed rice plants.

Materials and Methods

Plant materials and treatments

Seeds of the salt-sensitive rice cultivar PT1 (*Oryza sativa* L. spp. *indica* cv. Pathumthani 1), provided by Pathumthani Rice Research Center, (Rice Research Institute, Department of Agriculture, Ministry of Agriculture and Cooperative, Thailand) were manually de-husked, surface-sterilized first in 5% Clorox® for 60 min, followed by 30% Clorox® for 30 min, and then rinsed three times with sterile distilled water. Surface-sterilized seeds were germinated on 0.25% Phytagel®-solidified MS media with 3% sucrose (photomixotrophic condition) in a 250 mL glass vessel. All media were adjusted to pH 5.7 and autoclaved. Rice seedlings were cultured *in vitro* under conditions of 25±2 °C ambient temperature, 60±5% relative humidity (RH) and 60±5 μ mol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) provided by fluorescent lamps with 16 h d^{-1} photoperiod. Seven-day-old seedlings were aseptically transferred to MS-liquid sugar-free media (photoautotrophic conditions) with 0, 9.4 (control), 11.8 and 24.1 mM KNO_3 using vermiculite as supporting material. The uncovered vessels containing photoautotrophic seedlings were transferred aseptically to a culture box chamber (Carry Box Model P-850, size 26×36×19 cm, Japan) with RH maintained at 65±5% by 1.5 L saturated NaCl solution (360 gL^{-1}). The number of air exchanges in the culture box chambers was increased to 5.1±0.3 h^{-1} by perforating the sides of the plastic chamber with 32 holes and placing gas-permeable microporous polypropylene film (0.22 μ m pore size) over each hole (Cha-um et al., 2004). The chamber containing the rice seedlings was acclimated for 14 days in a Plant Growth Incubator under a temperature shift of 28±2 °C/25±2 °C (light/dark), 500±100 μ mol mol^{-1} CO_2 concentration, 60±5% RH, 120±5 μ mol m^{-2} s^{-1} PPFD provided by fluorescent lamps with 12 h d^{-1} photoperiod. The culture media were adjusted to 200 mM NaCl (salt stress) for 4 days. Osmotic potential, photosynthetic pigments, photosynthetic performance and growth characters were analyzed.

Analytical methods and data collection

One hundred milligrams of whole plant materials were ground in liquid nitrogen. Sodium and potassium ions in plant materials were extracted by acidic methods (HNO_3 and $HClO_4$) and assayed according to Dionisio-Sese and Tobita (1998) using an Atomic Absorption Spectrophotometer (AA, Model M6, Thermo Elemental, MA, USA).

Osmolarity in the roots and leaves of rice seedlings was measured, according to Lanfermeijer et al. (1991). A hundred milligrams each of fresh root and leaf tissue were cut into small pieces, transferred to 1.5 mL micro tube then stirred with a glass rod. Twenty micro liters of extracted solution was dropped directly onto a disc-shaped filter paper in an osmometer chamber (Wescor, USA). The osmolarity was then measured.

Chlorophyll a (Chl_a), chlorophyll b (Chl_b) and total chlorophyll (TC) concentrations were determined following the method of Shabala et al. (1998) and the total carotenoids (C_{x+c}) concentration was determined following the method of Lichtenthaler (1987). One hundred milligrams of leaf material was collected from the second and third nodes of the shoot tip. The leaf samples were placed in a 25 mL glass vial, along with 10 mL 95.5% acetone, and blended using a homogenizer. The glass vials were sealed with parafilm to prevent evaporation and then stored at 4 °C for 48 h. The Chl_a and Chl_b concentrations were measured using a UV-visible spectrophotometer at 644 nm and 662 nm. The C_{x+c} concentration was also measured by spectrophotometer at 470 nm. A solution of 95.5% acetone was used as a blank.

The chlorophyll fluorescence emission from the adaxial surface of the third leaf from the shoot tip was monitored using a fluorescence monitoring system in the pulse amplitude modulation mode, as previously described by Loggini et al. (1999). A leaf, adapted to dark conditions for 30 min using leaf-clips, was initially exposed to the modulated measuring beam of far-red light (LED source with typical peak at wavelength 735 nm). Original (F_0) and maximum (F_m) fluorescence yields were measured under weak modulated red light (<0.5 μ mol m^{-2} s^{-1}) with 1.6 s pulses of saturating light (>6.8 μ mol m^{-2} s^{-1} PAR) and autocalculated using FMS software for Windows®. The variable fluorescence yield (F_v) was calculated by the equation of F_m-F_0. The ratio of variable to maximum fluorescence (F_v/F_m) was calculated as maximum quantum yield of PSII photochemistry. The photon yield of PSII (Φ_{PSII}) in the light was calculated by $\Phi_{PSII} = (F_m'-F)/F_m'$ after 45 s of illumination, when steady state was achieved.

Net photosynthetic rate (P_n) was calculated by comparing the CO_2 concentration inside the glass vessel containing the rice seedlings with that of outside. CO_2 concentrations inside and outside the glass vessel (C_{in} and C_{out}) at steady state were measured by Gas Chromatography (GC; Model GC-17A, Shimadzu Co. Ltd., Japan). The GC capillary column and detector were a GS-Q (J&W Scientific®, Germany) and a thermal conductivity detector (TCD), respectively. The detector and injector were set to a temperature of 250 °C. The temperature of the column was set at 30 °C for 1 min at initial state, then increased by 20 °C per min to 100 °C, and held for 1 min. The net photosynthetic rate of *in vitro* cultivated plantlets was calculated according to the method of Fujiwara et al. (1987), as follows:

$$[P_n] = K \times E \times V (C_{out}-C_{in}) / \text{Leaf area}$$

where K is a conversion factor converting the amount of CO_2 from volume to mole (40.5 mol m^{-3} at 28 °C), E is the number of air exchanges per hour (2.32 h^{-1}) and V is the volume of air in the vessel (0.0025 m^3).

Shoot fresh weight, root fresh weight, shoot dry weight, root dry weight and leaf area of rice seedlings were measured as described by Cha-um et al. (2006). Rice seedlings were dried in a hot-air oven (Memmert, Model 500, Germany) for 2 days, and then incubated in a desiccator before measurement of dry weight. The leaf area of rice seedlings was measured using a Leaf Area Meter DT-scan (Delta-Scan Version 2.03, Delta-T Devices, Ltd., UK).

Experimental design

The experiment was arranged as a Completely Randomized Design (CRD) with six replicates (*n*=6). The mean values obtained were compared by Least Significant Difference (LSD) and analyzed using SPSS software. The correlations between physiological and biochemical parameters were evaluated using Pearson's correlation coefficients.

Results and Discussion

Sodium ions (Na^+) in the salt stressed root and leaf tissues of rice seedlings without KNO_3 treatment were accumulated to 18.26 and 33.66 mg g^{-1}FW, whereas those in the 11.8 KNO_3 pretreatment were lowest at 3.64 and 15.70 mg g^{-1}FW, respectively (Table 1). In contrast, potassium ions in the root and leaf tissues were dropped to 2.96 and 4.80 mg g^{-1} FW when exposed to 200 mM NaCl, leading to enhanced Na^+ / K^+ ratio in the plant tissues (Table 1). Potassium content in root and leaf tissues of PT1 salt sensitive rice was unaltered, whereas it was enriched in HJ salt tolerant rice when exposed to salt stress (Siringam et al., 2009). It is possible that K^+ accumulation may play a key role in salt tolerance mechanisms in rice. The K^+ was enriched in root and leaf tissues of salt stressed seedlings pretreated with 11.8 mM KNO_3, controlling the low level of Na^+ in salt-stressed seedlings. In olive, Na^+ content in the leaf tissues of the exogenously applied plants with potassium salts is lower than those without K treatment when exposed to 100 mM NaCl, whereas K^+ was accumulated (Chartzoulakis et al., 2006). Similarly, the Na^+ in wild barley (*Hordeum maritimum* L.) treated with 3 mM K^+ subsequently exposed to 100 mM NaCl is lowest, while K^+ is highest (Degl'Innocenti et al., 2009). The Na^+ reduction and K^+ accumulation in the salt stressed plants pretreated with potassium is directly reduced the Na^+ / K^+ ratios, which are reported in wild barley (Degl'Innocenti et al., 2009), olive (Chartzoulakis et al., 2006), *Arabidopsis* (Kaddour et al., 2009), melon (Kaya et al., 2007), sunflower (Akram et al., 2009) and winter wheat (Zheng et al., 2008). In the present study, exogenous application of KNO_3 was adopted to improve salt tolerance of PT1 rice plants exposed to 200 mM NaCl through the increase of the internal potassium levels.

Table 1. Sodium (Na^+), potassium (K^+) ions and Na:K ratios in the root and leaf tissues of PT1 rice treated with 0, 9.4, 11.8 and 24.1 mM KNO_3 subsequently grown under 200 mM NaCl salt stress for 4 days.

KNO_3 (mM)	Root			Leaf		
	Na^+ (mg g^{-1}FW)	K^+ (mg g^{-1}FW)	Na:K	Na^+ (mg g^{-1}FW)	K^+ (mg g^{-1}FW)	Na:K
0	18.26^a	2.96^d	6.16^a	33.66^a	4.80^d	7.08^a
9.4	12.92^b	4.17^c	3.10^b	25.11^b	7.43^c	3.38^b
11.8	3.64^d	5.62^a	0.65^d	15.70^c	12.93^a	1.22^c
24.1	5.94^c	4.96^b	1.21^c	17.34^c	10.96^b	1.58^c
ANOVA	**	**	**	**	**	**

Different letters in each column show significant difference at P≤0.01 (**) by Least Significant Difference (LSD).

Na^+ accumulation in the salt-stressed root (Figure 1 A) and leaf tissues (Figure 1 B) was positively correlated to osmotic potential (Ψ_s), with $r^2 = 0.99$ for both tissues. Also, the Ψ_s in the leaf tissues was higher than in the root tissues when plants were exposed to 200 mM

NaCl (Figure 1). A major role of K in plant cells is osmoregulation, which is marked by the status of osmotic potential Ψ_s). The Ψ_s in both root and leaf tissues of salt stressed PT1 seedlings under the KNO_3 treatments was better than those without KNO_3 treatment. Improvement of the plant water relations including water content, water use efficiency, relative water content and water potential in K pretreated plants exposed to salt stress has been reported (Kaya et al., 2007; Zheng et al., 2008; Akram et al., 2009; Kaddour et al., 2009). The reduction of Ψ_s in the salt stressed leaves was positively correlated to total chlorophyll content ($r^2 = 0.96$) (Figure 2). Chlorophyll a (Chl_a), chlorophyll b (Chl_b), total chlorophyll (TC) and total carotenoids (C_{x+c}) concentrations in salt stressed leaves of PT1 rice seedlings were at 167.02, 53.72, 220.74 and 82.20 μg g^{-1} FW in 11.8 mM KNO_3 treated seedlings which were greater than those without KNO_3 by 2.49, 2.49, 2.49 and 2.54 times, respectively (Table 2). The chlorophyll content in salt stressed wheat pretreated with 16 mM KNO_3 is maintained (Zheng et al., 2008). Also, the photosynthetic pigments, Chl_a, Chl_b and TC in salt stressed melon plants pretreated with 5 mM KNO_3 are stabilized better than those without KNO_3 (Kaya et al., 2007). The Chl_a content in salt stressed leaves of PT1 rice was positively correlated with maximum quantum efficiency of PSII (F_v/F_m) with $r^2 = 0.89$ (Figure 3). The F_v/F_m, photon yield of PSII (Φ_{PSII}) and net photosynthetic rate (P_n) of rice seedlings exposed to NaCl stress (200 mM) were greatest under 11.8 mM KNO_3 pretreatment. The levels in plants pretreated with 9.4, 11.8 and 24.1 mM KNO_3 were higher than those in plants without KNO_3 by 1.77, 2.21 and 10.36 times, respectively (Table 3). Maximum quantum yield of PSII (F_v/F_m) in salt stressed sunflowers without K diminished to a greater degree than in plants treated with K, leading to high P_n (Akram et al., 2009). In the dark reaction of photosynthesis, K^+ in the guard cells affects CO_2 assimilation through controlling stomatal movements. Proper stomatal function in salt stressed leaves treated with exogenous potassium may be maintained by enriched K^+ in the cells, leading to high P_n (Chartzoulakis et al., 2006; Akram et al., 2009; Degl'Innocenti et al., 2009). This is in congruence with the present results that show high P_n in salt stressed PT1 pretreated with 11.8 mM KNO_3, as well as improved growth when compared to plants without pretreatment of KNO_3. A positive relationship between Φ_{PSII} and P_n was found ($r^2 = 0.99$) (Figure 4). In addition, physiological parameters, including Chl_a, Chl_b, TC, C_{x+c}, F_v/F_m, Φ_{PSII} and P_n, were positively correlated to each other to a highly significant ($P \leq 0.01$) level in statistical analysis (Table 4). Photosynthetic activity in rice pretreated with KNO_3 and subsequently exposed to salt stress was enhanced, leading to high growth rates ($r^2 = 0.73$) (Figure 5). Growth parameters, shoot fresh weight (SFW), root fresh weight (RFW), shoot dry weight (SDW), root dry weight (RDW) and leaf area (LA), were similarly affected in KNO_3 pretreated plants under salt stress. Growth parameters of salt stressed seedlings under 11.8 mM KNO_3 application were highest and significantly better than those without KNO_3 treatment (Table 5).

In conclusion, the exogenous application of 11.8 mM KNO_3 to PT1 rice seedlings produced optimal effects under 200 mM NaCl stress and led to the better ionic balance, pigment stabilization, reduced chlorophyll fluorescence emission and greater net photosynthetic rate, leading to improved growth performance. A short-gun technique, the exogenous application of KNO_3 to rice crops is an alternative procedure to be adopted in improving salt tolerance in rice crop grown on salt-affected land.

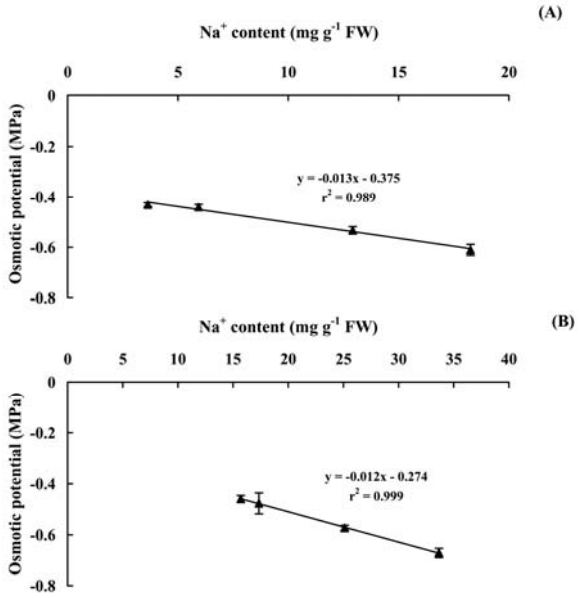

Figure 1. Relationship between sodium ions (Na^+) and osmotic potential (Ψ_s) in root (A) and leaf tissues (B) of PT1 rice treated with 0, 9.4, 11.8 and 24.1 mM KNO_3 subsequently exposed to 200 mM NaCl salt stress for 4 days. Error bars represent by ±SE.

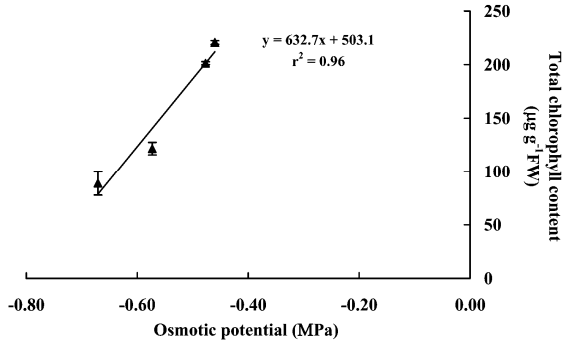

Figure 2. Relationship between osmotic potential (Ψ_s) in the leaf tissues and total chlorophyll contents in PT1 rice treated with 0, 9.4, 11.8 and 24.1 mM KNO_3 subsequently exposed to 200 mM NaCl salt stress for 4 days. Error bars represent by ±SE.

Table 2. Chlorophyll a (Chl_a), chlorophyll b (Chl_b), total chlorophyll (TC) and total carotenoids (C_{x+c}) concentrations in PT1 rice treated with 0, 9.4, 11.8 and 24.1 mM KNO_3 subsequently grown under 200 mM NaCl salt stress for 4 days.

KNO_3 (mM)	Chl_a (µg g^{-1} FW)	Chl_b (µg g^{-1} FW)	TC (µg g^{-1} FW)	C_{x+c} (µg g^{-1} FW)
0	67.07^c	21.62^d	88.69^b	32.34^c
9.4	90.69^b	30.92^c	121.61^b	43.87^b
11.8	167.02^a	53.72^a	220.74^a	82.20^a
24.1	155.98^a	45.42^b	201.40^a	73.59^a
ANOVA	**	**	**	**

Different letters in each column show significant difference at P≤0.01 (**) by Least Significant Difference (LSD).

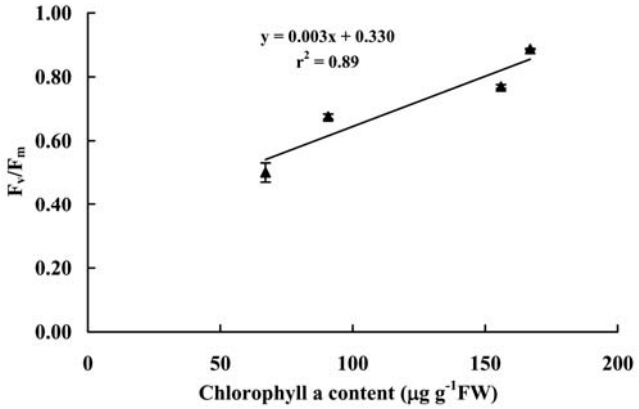

Figure 3. Relationship between chlorophyll a content and maximum quantum yield of PSII (F_v/F_m) in PT1 rice treated with 0, 9.4, 11.8 and 24.1 mM KNO_3 subsequently exposed to 200 mM NaCl salt stress for 4 days. Error bars represent by ±SE.

Table 3. Maximum quantum yield of PSII (F_v/F_m), photon yield of PSII (Φ_{PSII}) and net photosynthetic rate (P_n) in PT1 rice treated with 0, 9.4, 11.8 and 24.1 mM mgL^{-1} KNO_3 subsequently grown under 200 mM NaCl salt stress for 4 days.

KNO_3 (mM)	F_v/F_m	Φ_{PSII}	P_n (μmol CO_2 $m^{-2}s^{-1}$)
0	0.501^d	0.288^d	0.22^c
9.4	0.676^c	0.389^c	0.89^b
11.8	0.887^a	0.637^a	2.28^a
24.1	0.770^b	0.443^b	1.13^b
ANOVA	**	**	**

Different letters in each column show significant difference at P≤0.01 (**) by Least Significant Difference (LSD).

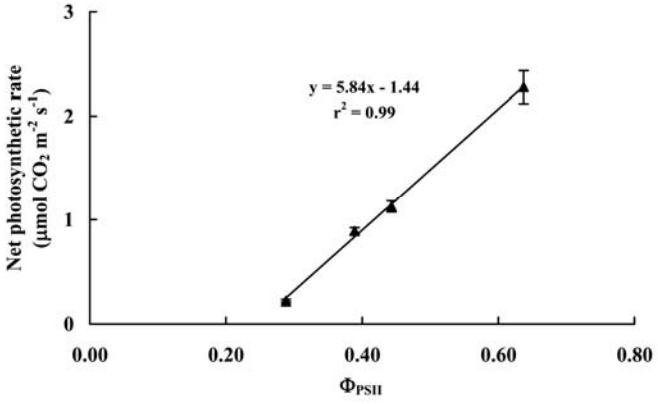

Figure 4. Relationship between photon yield of PSII (Φ_{PSII}) and net photosynthetic rate (P_n) in PT1 rice treated with 0, 9.4, 11.8 and 24.1 mM KNO_3 subsequently exposed to 200 mM NaCl salt stress for 4 days. Error bars represent by ±SE.

Table 4. Relationship between physiological and biochemical parameters of rice seedlings treated with 0, 9.4, 11.8 and 24.1 mM KNO_3 subsequently exposed to 200 mM NaCl for 4 days.

Parameters	Chl_a	Chl_b	TC	C_{x+c}	F_v/F_m	Φ_{PSII}	P_n
Chl_a	1	-	-	-	-	-	-
Chl_b	0.976**	1	-	-	-	-	-
TC	0.999**	0.986**	1	-	-	-	-
C_{x+c}	0.981**	0.960**	0.980**	1	-	-	-
F_v/F_m	0.879**	0.911**	0.890**	0.886**	1	-	-
Φ_{PSII}	0.843**	0.886**	0.857**	0.850**	0.928**	1	-
P_n	0.811**	0.851**	0.824**	0.820**	0.898**	0.958**	1

Significant levels at P≤0.01 is represented by ** using Pearson's correlation coefficients.

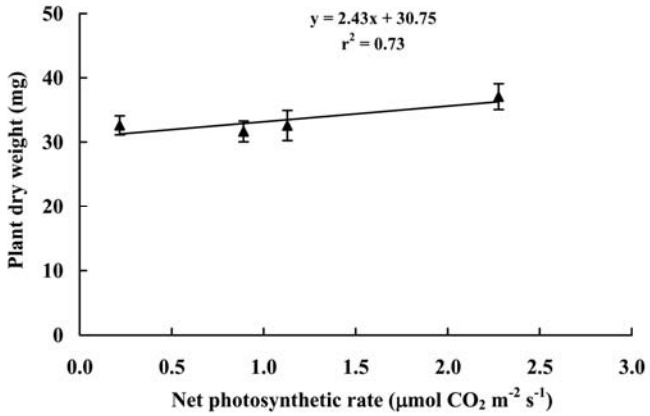

Figure 5. Relationship between net photosynthetic rate (P_n) and plant dry weight in PT1 rice treated with 0, 9.4, 11.8 and 24.1 mM KNO_3 subsequently exposed to 200 mM NaCl salt stress for 4 days. Error bars represent by ±SE.

Table 5. Shoot fresh weight (SFW), root fresh weight (RFW), shoot dry weight (SDW), root dry weight (RDW) and leaf area (LA) in PT1 rice treated with 0, 9.4, 11.8 and 24.1 mM KNO_3 subsequently grown under 200 mM NaCl salt stress for 4 days.

KNO_3 (mM)	SFW (mg)	RFW (mg)	SDW (mg)	RDW (mg)	LA (cm^2)
0	145.1^{b}	31.3^{b}	31.7^{b}	3.0^{b}	5.50^{d}
9.4	158.9^{b}	34.4^{ab}	32.6^{b}	3.4^{ab}	7.48^{c}
11.8	181.3^{a}	36.9^{a}	37.1^{a}	3.8^{a}	12.24^{a}
24.1	159.9^{b}	36.2^{a}	32.6^{b}	3.7^{a}	10.55^{b}
ANOVA	*	*	*	*	**

Different letters in each column show significant difference at P≤0.05 (*) or P≤0.01 (**) by Least Significant Difference (LSD).

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