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Effects of 5-aminolevulinic acid (ALA)-containing supernatants from selected *Rhodopseudomonas palustris* strains on rice growth under NaCl stress, with mediating effects on chlorophyll, photosynthetic electron transport and antioxidative enzymes

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ABSTRACT

Background: Rice is globally one of the most important food crops, and NaCl stress is a key factor reducing rice yield. Amelioration of NaCl stress was assessed by determining the growth of rice seedlings treated with culture supernatants containing 5-aminolevulinic acid (ALA) secreted by strains of *Rhodopseudomonas palustris* (TN114 and PP803) and compared to the effects of synthetic ALA (positive control) and no ALA content (negative control). *Results:* The relative root growth of rice seedlings was determined under NaCl stress (50 mM NaCl), after 21 d of pretreatment. Pretreatments with 1 μM commercial ALA and 10X diluted culture supernatant of strain TN114 (2.57 μM ALA) gave significantly better growth than 10X diluted PP803 supernatant (2.11 μM ALA). Rice growth measured by dry weight under NaCl stress ordered the pretreatments as: commercial ALA > TN114 > PP803 > negative control. NaCl stress strongly decreased total chlorophyll of the plants that correlated with non-photochemical quenching of fluorescence (NPQ). The salt stress also strongly increased hydrogen peroxide (H_2O_2) concentration in NaCl-stressed plants. The pretreatments were ordered by reduction in H_2O_2 content under NaCl stress as: commercial ALA > TN114 > PP803 > negative control. The ALA pretreatments incurred remarkable increases of total chlorophyll and antioxidative activities of catalase (CAT), ascorbate peroxide (APx), glutathione reductase (GR) and superoxide dismutase (SOD); under NaCl stress commercial ALA and TN114 had generally stronger effects than PP803.
Conclusions: The strain TN114 has potential as a plant growth stimulating bacterium that might enhance rice growth in saline paddy fields at a lower cost than commercial ALA.

1. Introduction

Rice (*Oryza sativa*) is worldwide one of the most important cereals, and Thailand is in the top ranks of rice exporting countries. Rice production is; however, negatively impacted by some environmental factors, in particular by salt stress and droughts. Salt stress or NaCl stress because NaCl is the main salt dissolved in saline water or soil and is one of the most serious environmental stresses on plants in general, and specifically on rice. Salt stress is known to adversely affect endogenous levels of phytohormones that influence a variety of processes in plants [1] such as reducing seed germination, ion uptake, stomatal opening, and photosynthetic rate [2]. In Sorghum, Netondo et al. [3] reported that maximum quantum yield of photosystem II (Fv/Fm), photochemical quenching coefficient (qP) and electron transport rate (ETR) significantly decreased, but non-photochemical quenching (NPQ) increased substantially under saline conditions. Sensitivity to salt stress in cereals might thus be associated with both reduction in PSII photochemical efficiency and enhanced NPQ to dissipate excess energy. Consequently, saline soil has detrimental effects on plant growth and yield. The reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH), are produced in normal aerobic metabolism, but their levels are increased under stress [4]. Changes in antioxidative enzyme activities; catalase (CAT), ascorbic peroxidase (APx), glutathione reductase (GR), and superoxide dismutase (SOD), involved in the detoxification of ROS,

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are often observed in plants under salt stress. Normally, the salt stress effects are obvious, especially during rice germination, as the percentage of successful germination rate is reduced [2]. The 5-aminolevulinic acid (ALA) has been reported to increase ascorbate–glutathione cycle activity and to increase the level of antioxidant enzymes such as SOD, CAT, and APx [5].

It has been repeatedly demonstrated that the problems caused by salinity can be counteracted by use of ALA [5,6]. ALA is a potential plant growth regulator in stress conditions, being an essential biosynthetic precursor of tetrapyrrole compounds such as heme, cytochromes and chlorophyll [7]. ALA is also known to regulate several key physiological processes associated with plant growth under saline regimes [7] including improved cell ultra-structure, leading to less ultra-structural damage in the root tip under stress conditions [8]. There have been a number of demonstrations that ALA at low concentrations can promote plant growth [6,9,10]. The exogenous application of ALA is very effective in minimizing the salt-induced adverse effects in various crops, *e.g.* spinach (*Spinacia oleracea*) [11], pakchoi (*Brassica campestris*) [12], potato (*Solanum tuberosum*) [13], date palm (*Phoenix dactylifera*) [14] and oilseed rape (*Brassica napus*) [6]. Unfortunately, commercial ALA is too expensive for many common agricultural applications. The use of microorganisms that directly produce ALA may be a lower cost option that is economically feasible in rice cultivation.

Among the ALA-producing microbes, only the phototrophic purple nonsulfur bacteria (PNSB) are widely distributed in paddy fields. Nunkaew et al. [15] obtained 210 PNSB isolates from 60 samples of paddy fields, cultured in a rice straw broth medium. The volatile fatty acids (VFAs), from anaerobic digestion of organic material in paddy fields, are a good carbon source for ALA production by PNSB [16]. This is in agreement with our previous work, where *Rhodopseudomonas palustris* strains TN114 and PP803 (isolated from paddy fields in southern Thailand) produced high amounts of ALA in rice straw broth under microaerobic light conditions. These ALA producing PNSB strains provide an opportunity to assess whether they can help solve the salinity stress problems of rice in grown in saline paddy fields. In addition of producing ALA PNSB also fix N_2 gas and thus they can be considered as one of the 'natural biofertilizers' [15]. Therefore, the goal of this study was to assess the effects on rice growth, under salt stress, of selected ALA-producing PNSB strains and systematically compare them to commercial ALA. Such results are likely to be of general interest to researchers interested in microbial plant growth promotion, assisting in understanding these effects. In addition, the effects of these treatments on antioxidative enzyme activities, photosynthetic electron transport and chlorophyll content of rice, under NaCl stress, were also investigated.

2. Materials and methods

2.1. PNSB used

The *R. palustris* strains used in this study, TN114 and PP803, were isolated from water and sediment samples collected from saline paddy fields in Phatthalung and Nakhon Si Thammarat provinces, Thailand, respectively. Both PNSB strains were selected on the basis of their ability to produce ALA in rice straw broth medium with saline condition, under microaerobic light conditions. Rice straw broth medium was deliberately chosen because it would be readily useable in rural Thailand by farmers to grow the PNSB.

2.2. Preparation of PNSB supernatant containing ALA

Glutamate–acetate (GA) broth medium was used because acetate is a major available carbon source in paddy fields; this substance is produced in one of the anaerobic decomposition steps of breakdown of organic matter. To prepare inoculum, one loopful of pure culture from a stab culture was inoculated into a screw cap test tube (150 × 15 mm: 20 ml) containing 18 ml GA medium, leaving a small space on top of the medium to achieve microaerobic conditions. The culture was incubated with tungsten light intensity of 3500 lx for 48 h. Growth in the culture broth was monitored by turbidity measurement, with a spectrophotometer at wavelength 660 nm, and for use as inoculums the culture broth was adjusted to an optical density (OD_{660}) of 0.5 by diluting with GA broth. To provide similar microaerobic-light conditions as those in a paddy field, for ALA production, a 2 ml inoculums was added into a screw cap test tube (150 × 15 mm: 20 ml) containing 18 ml GA with 0.25% NaCl (electrical conductivity, approximately 4 mS/cm; 43 mM). This is an average salt concentration in the paddy fields of southern Thailand [15]. The PNSB cultures grew well in media containing 0.25% NaCl and so would be suitable for field use in saline soils. All culture tubes were incubated in a shaking water bath (30 rpm) at 30°C for 72 h, for maximal ALA production, based on our preliminary work. After that the culture broth was centrifuged at 4032 g for 15 m to remove PNSB cells, and the culture supernatant was collected for ALA analysis and for testing its effects on saline stressed rice.

The amounts of ALA in the culture supernatants were determined using HPLC with a RF-10AXL fluorescence detector, following the method described by Tangprasittipap et al. [17]. Briefly, 50 μl of culture supernatant was mixed with 3.5 ml acetylacetone/ethanol/water (15:10:75, v:v:v) containing 0.4% NaCl and 450 μl aqueous formalin (8.5% v/v), and left at 100°C for 30 m. The HPLC conditions were as follows: Inertsil ODS-3 column (5 μm, 250 × 150 mm) (GL Science Inc., Tokyo, Japan) at 40°C with methanol mixed into 2.5% (v/v) acetic acid in the ratio of 60:40 (v/v) as the mobile phase with flow rate 0.2 ml/min. The eluted sample was monitored at excitation and emission wavelengths 363 and 473 nm. The extracellular ALA concentration was calculated from the peak area, using 99.9% ALA-HCl as an authentic standard.

2.3. Pretreatment with ALA-containing PNSB supernatant

Culture supernatants of PNSB collected after 72 h incubation, prepared as previously described, were used in ALA-containing treatments of experiments designed to assess relief of the effects of salt stress. Seeds were grown either with no supplement (normal control conditions) or with 50 mM NaCl (salt stress conditions). There were 8 experimental treatment groups: negative controls (distilled water ± NaCl), positive controls (1 μM ALA ± NaCl), and 10X diluted culture supernatants of either strain TN114 or PP803 without and with salt stress (TN114 or PP803 ± NaCl) (Table 1). The 10 fold dilution of PNSB culture supernatants was chosen based on preliminary work, where this dilution was found to be best for stimulating rice growth under salt stress. Each treatment group consisted of 9 seedlings. Rice seeds (*O. sativa* L.) were sterilized by 5% sodium hypochlorite for 3 min. After the seeds were soaked in distilled water at 30°C for 2 d and sown in plastic pots containing commercial compost mixture moistened sufficiently with distilled water. The plants were grown in a growth chamber at 30/25°C (day/night) with 12 h of light daily, for 24 d at which time the 1.5–2.0 leaf stage was reached [5]. Distilled water was added to the soil daily as required.

Before pretreatments, the seedlings were removed and their roots were carefully washed to remove soil. For pretreatment, they were then immersed in commercial ALA (1 μM), or the 10X-diluted PNSB culture supernatant (with 2.57 μM ALA for TN114 and 2.11 μM for PP803), or distilled water, for 12 h with continuous light. After that, the rice seedlings were transplanted into Kasugai nutrient solution [18] in plastic pots and placed in the growth chamber. Five days later, stress conditions for appropriate groups were created by adding NaCl to the nutrient solution (final salt concentration 50 mM). The nutrient solutions were renewed every 4 d, with or without salt stress depending on the treatment group. After 21 d of pretreatment, the fresh and standard dry weights of the whole plants were measured. The root length of each rice plant was measured, and the relative root growth (RRG)

Table 1
Relative root growth (RRG), fresh and dry weights of seedlings without and with salt stress (50 mM NaCl) after 21 d of pretreatments. RRG values use the negative control with the same status as the baseline referred to.

Experimental set	Relative root growth (%)	Fresh weight (mg/plant)	Dry weight (mg/plant)
1. Negative control: Distilled water (DW)	–	241.12 ± 6.58^{b}	43.50 ± 3.30^{c}
2. Negative control: DW + 50 mM NaCl	–	128.50 ± 2.48^{d}	21.15 ± 1.22^{d}
3. Positive control: 1 μM commercial ALA	166.11 ± 8.30^{bc}	270.90 ± 3.43^{a}	75.74 ± 1.75^{a}
4. Positive control: 1 μM ALA + 50 mM NaCl	178.42 ± 6.41^{a}	270.37 ± 2.87^{a}	76.23 ± 1.95^{a}
5. Strain TN114: 2.67 μM ALA	157.87 ± 5.17^{c}	283.72 ± 3.05^{a}	77.54 ± 1.47^{a}
6. TN114 (2.67 μM ALA) + 50 mM NaCl	172.38 ± 7.54^{ab}	221.43 ± 4.97^{c}	46.45 ± 2.89^{b}
7. Strain PP803: 2.11 μM ALA	137.14 ± 5.14^{d}	223.30 ± 5.03^{bc}	43.97 ± 2.45^{c}
8. PP803 (2.11 μM ALA) + 50 mM NaCl	157.36 ± 6.62^{c}	236.50 ± 2.86^{bc}	41.95 ± 1.32^{c}

Each value is the mean of nine replicates, given with standard deviation. Different superscripts in the same column indicate significant differences ($P < 0.05$).

calculated by comparison to control treatments with the non-saline or saline condition as appropriate.

2.4. Chlorophyll content in rice seedlings

The chlorophyll content was determined according to Chappelle et al. [19]. The shoot parts from the 9 rice seedlings of each treatment group were excised and soaked in DMSO (100 ml/g FW: fresh weight) in a glass vial. The vial was sealed tightly and incubated at 30°C for 48 h in darkness. The concentrations of the extracted pigments (chlorophyll *a*, chlorophyll *b*) were calculated from absorbance values at 664 and 648 nm, respectively using the formulae quoted by Chappelle et al. [19]. This determination was done in three replicates.

2.5. Chlorophyll fluorescence measurements

Fluorescence yield determinants were measured on the youngest leaf from each experiment as described by Dionisio-Sese and Tobita [20]. Chlorophyll fluorescence in dark- and light-adapted leaves was excited and measured using a portable PAM (Pulse Amplitude Modulation) fluorometer (Junior-PAM, Gademann Instruments, Wurzburg, Germany) [21]. Minimal fluorescence (F_0) was measured after a 20 m dark acclimation to empty photosystem II, while maximal fluorescence (Fm) was measured after the leaf received a flash of actinic saturating light (2000 μmol (quanta)/m^2/s) for 0.8 s. Data was analyzed using standard Walz Software (Walz, Wurzburg, Germany) using the equations of Genty et al. [22]. F_0 and Fm was calculated maximal photochemical quantum yield of PSII or Fv/Fm = Fm − F_0 / Fm. Actual quantum yield (PSII) was measured on the youngest fully expanded leaves that were illuminated for 2 min in the growth chamber with actinic light after dark adaptation and was calculated as ΦPSII = (Fm′ − Ft) / Fm′, where F′m and Ft refer to maximum and steady-state fluorescence in the light, respectively. ETR was calculated as ETR = ΦPSII × PPFD × 0.5 × 0.84, where Photosynthetic Photon Flux Density (PPFD) is the incident on the leaf using the standard settings for the Junior PAM; the factor 0.5 is based on the assumption that photons are absorbed equally by both two photosystems and the factor 0.84 is the standard assumed leaf absorbance used by the Walz software [23]. Non-photochemical quenching of fluorescence (NPQ) which is proportional to the rate of heat dissipation of PSII [22] was calculated as NPQ = Fm / Fm′ − 1.

2.6. H_2O_2 content in rice seedlings

H_2O_2 levels were assayed by using 500 mg of leaf tissue from the 9 rice seedlings of each group, and the tissue was homogenized in an ice bath with 5 ml 0.1% (w/v) TCA (trichloroacetic acid). The homogenate was centrifuged at 12,000 g for 15 m, and then 0.5 ml of the supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M KI (potassium iodide). The absorbance was then measured at 390 nm, and the content of H_2O_2 was determined from a standard curve [5]. The assay was carried out in triplicate.

2.7. Activities of antioxidative enzymes in rice seedlings

500 mg excised leaves, from the 9 rice seedlings in each group, was homogenized in 5 ml of 25 mM potassium phosphate buffer (pH 7.8). To obtain the extract, the homogenate was centrifuged at 15,000 g for 20 m at 4°C [5], and the supernatant was filtered through one layer of Millex® (Merck Millipore, Cork, Ireland) prior to use in the triplicated enzyme assays.

SOD activity was assayed at 30°C in a 1 ml reaction mixture comprised of 0.1 ml of 500 mM potassium phosphate buffer (pH 7.8, containing 0.1 mM EDTA), 0.1 ml of 0.1 mM cytochrome *c* (from horse heart), 0.1 ml of 1 mM xanthine dissolved in 10 mM NaOH, 0.02 ml xanthine oxidase, 0.66 ml distilled water and 0.02 ml enzyme extract. SOD activity was measured as the reduction of cytochrome *c* using a standard spectrophotometer at 550 nm [24].

CAT activity was assayed at 30°C in a 1 ml reaction mixture consisting of 0.95 ml of 50 mM potassium phosphate buffer (pH 7.0, containing 10 mM H_2O_2) and 0.05 ml enzyme extract. Activity was determined spectrophotometrically at 240 nm by measuring the decomposition of H_2O_2 [5]. A range of H_2O_2 solutions was made up to construct a standard calibration curve, and the CAT activities of extract samples were calculated from the standard curve.

APx activity was assayed at 30°C in a 1 ml reaction mixture containing 0.25 ml of 100 mM potassium phosphate buffer (pH 7.0), 0.25 ml of 1 mM ascorbic acid, 0.25 ml of 0.4 mM EDTA, 0.01 ml of 10 mM H_2O_2, 0.19 ml distilled water and 0.05 ml enzyme extract. Activity was determined at 290 nm, measuring the decrease of ascorbic acid. Ascorbic acid was used to produce a standard calibration curve, and the APx activities of extracts were calculated from the standard curve [24].

GR activity was assayed at 30°C in a 1 ml reaction mixture containing 0.25 ml of 100 mM potassium phosphate buffer (pH 7.8), 0.05 ml of 10 mM oxidized glutathione (GSSG), 0.12 ml of 1 mM NADPH, 0.48 ml distilled water and 0.1 ml enzyme extract. Activity was determined at 340 nm, measuring the decrease of NADPH absorbance [24]. NADPH solutions were used to create a standard calibration curve for calculating the GR activities of extract samples.

2.8. Statistical analysis

All experiments were carried out in triplicates unless otherwise stated. Data are presented as means with standard deviations, from the three determinations. One way ANOVA was used to analyze statistical differences, considered significant at a *P*-value < 0.05, and mean comparisons were performed by Duncan's multiple range test.

3. Results

3.1. Effect of PNSB supernatant pretreatment on rice growth under NaCl stress

The growth of rice seedlings with/without salt stress, based on RRG as well as fresh and dry weights of whole plants, after 21 d of pretreatments

(commercial ALA: positive control, ALA in PNSB supernatant, or distilled water as negative control), is presented in Table 1. Under normal conditions (without 50 mM NaCl) the dry weight of whole rice seedlings was significantly different ($P < 0.05$) between the treatment groups as follows: commercial ALA = TN114 > PP803 = distilled water. Under NaCl stress the dry weight of the whole plants was significantly ($P < 0.05$) reduced in sets of distilled water (negative control) and 10X diluted TN114 supernatant. The highest dry weight was found in the sets treated with commercial ALA solutions (positive control) with and without salt, and the 10X diluted TN114 supernatant without NaCl addition (75.74 ± 1.75 to 77.54 ± 1.47 mg/seedling). In contrast, the lowest dry weight of rice seedlings (21.15 ± 1.22 mg/seedling) was found in the negative control group, where plants had been immersed in distilled water with 50 mM NaCl. The dry weights of rice seedlings in the other treatments were in the range 41.95 ± 1.32 to 46.45 ± 2.89 mg/seedling. Trends in the fresh weights were similar to those in the dry weights. This indicated that the NaCl stress had no dramatic effects on the water contents of the rice plants. The root lengths of rice seedlings were measured and used to calculate the RRG. Under NaCl stress conditions, the 178.42 ± 6.41% RRG for treatment with commercial ALA was significantly higher than for treatments without NaCl stress (137.14 ± 5.14 to 166.11 ± 8.30). With or without NaCl stress, the commercial ALA and the 10X diluted TN114 supernatant had no significant difference in % RRG. However, the 10X diluted PP803 supernatants had the lowest RRG in both conditions (137.14 ± 5.14% for normal and 157.36 ± 6.62% for NaCl stress conditions).

Fig. 1 shows physical characteristics of rice seedlings under NaCl stress, after 21 d of pretreatment. The maximum root length was obtained without NaCl stress, with commercial ALA and 10X diluted TN114 supernatant (average length: 4.32 ± 1.86 cm) treatments, and the minimum (average length: 1.57 ± 1.83 cm) in the negative control set under salt stress (50 mM NaCl). In the case of the negative control under NaCl stress, the whole plant had a brown color and noticeable wilt, along with the shortest roots. In contrast, healthy control plants were green in color and long roots were observed in the negative control grown under normal conditions, and under both NaCl-treated conditions when treated with commercial ALA or TN114.

3.2. Chlorophyll content in rice seedlings under NaCl stress

The total chlorophyll content in rice seedlings, measured at day 21 after pretreatment, was significantly higher in normal conditions than under salt stress for any pretreatment type, including controls (Table 2). Under the normal conditions, the commercial ALA gave the best total chlorophyll production (roughly 0.64 mg/g FW), followed by the treatments with 10X diluted culture supernatants (TN114 or PP803), and the total chlorophyll production was lowest in the negative control set (0.49 mg/g FW). It was of interest that, under salt stress, the total chlorophyll was not significantly different between the commercial ALA and the 10X diluted culture supernatants (TN114 or PP803) (roughly 0.40 mg/g FW). Again, the lowest total chlorophyll production (roughly 0.07 mg/g FW) was observed in the negative control set grown under salt stress.

Fig. 1. The physical characteristics of rice seedlings without and with salt stress (50 mM NaCl) after 21 d of pretreatments. The treatment groups are indicated by code numbers, matching those in Table 1.

3.3. Chlorophyll fluorescence during seedling stages

During the seedling stage, measurements of chlorophyll PAM fluorescence were conducted 21 d after salinization. Maximal photochemical quantum yield of PSII (Fv/Fm) was significantly higher in normal conditions than under salt stress in negative and positive controls, while treatments with 10X diluted culture supernatants showed slight increases in value (Fig. 2a). In the NaCl stress condition, no significant differences between ALA treatment and both 10X diluted culture supernatants were observed but the negative control showed that Fm/Fv decreased significantly (Fig. 2a). Moreover, actual quantum yield of photosynthesis (ΦPSII) and ETR were found to exhibit the same trend as shown in plots of Fm/Fv (Fig. 2b, c). An opposite trend was observed with NPQ of fluorescence which increased substantially with salt addition in the negative control. NPQ values in salinity in ALA treatment and both 10X diluted culture supernatants were similar to the normal condition (Fig. 2d). Thus, ALA or 10X diluted culture supernatants were protecting the plants from increase in NPQ in the presence of salinity stress.

3.4. Hydrogen peroxide content in rice seedlings under NaCl stress

The H_2O_2 content was determined to confirm the effectiveness of pretreatment by commercial ALA and both 10X diluted PNSB supernatants in scavenging H_2O_2 (Table 2). Under normal conditions, all sets had a low content of H_2O_2 in the range 0.10–0.12 μmol/g FW. In contrast, plants under NaCl stress, the H_2O_2 content increased remarkably in all sets and the negative controls had the highest H_2O_2 level (0.55 μmol/g FW) followed by treatments with PP803, TN114, and the positive control group. It should be noted that, the H_2O_2 contents differed significantly for each treatment, between the cases with and without salt stress.

3.5. Antioxidative enzyme activities in rice seedlings under NaCl stress

Activity of SOD in rice seedlings at day 21 after pretreatment with both 10X diluted PNSB supernatants, with and without salt stress, is presented in Fig. 3a. This enzyme binds O_2^- and converts it to superoxide H_2O_2 that reacts with cytochrome *c* which reduces it. The reduction of cytochrome *c* is in this way related to SOD activity [24]. The SOD activity in salt stress was higher than in normal conditions, for the positive controls and treatments with TN114 and PP803 supernatants, although these differences were not significant. In contrast, the opposite result was found for the negative controls: the SOD activity was significantly higher in the normal conditions. The SOD activity in the positive controls and TN114 supernatant treatment was 472.22 ± 48.11 unit/g FW under normal conditions, whereas in salt stress both sets had the highest activity (500.00 ± 0.01 unit/g FW). The lowest SOD activity (74.42 ± 16.06 unit/g FW) was observed in the negative controls under salt stress but with no ALA or diluted culture supernatant treatment. The presence of ALA or 10X diluted culture supernatants prevented the inhibition of SOD activity induced by increased salinity.

CAT activities in rice seedlings at day 21 after pretreatment with 10X diluted PNSB supernatants (TN114 or PP803), under normal and salt stress conditions, are presented in Fig. 3b. In normal conditions without

Table 2
Total chlorophyll and H_2O_2 contents of rice seedlings without and with salt stress (50 mM NaCl) after 21 d of pretreatments.

Experimental set	Total chlorophyll (mg/g FW)	H_2O_2 (μmol/g FW)
1. Negative control: Distilled water (DW)	0.489 ± 0.018^{c}	0.107 ± 0.001^{f}
2. Negative control: DW + 50 mM NaCl	0.068 ± 0.013^{e}	0.551 ± 0.006^{a}
3. Positive control: 1 μM commercial ALA	0.637 ± 0.003^{a}	0.095 ± 0.001^{g}
4. Positive control: 1 μM ALA + 50 mM NaCl	0.391 ± 0.006^{d}	0.315 ± 0.012^{d}
5. Strain TN114: 2.67 μM ALA	0.554 ± 0.006^{b}	0.102 ± 0.001^{fg}
6. TN114 (2.67 μM ALA) + 50 mM NaCl	0.410 ± 0.004^{d}	0.338 ± 0.004^{c}
7. Strain PP803: 2.11 μM ALA	0.568 ± 0.004^{b}	0.122 ± 0.001^{e}
8. PP803 (2.11 μM ALA) + 50 mM NaCl	0.405 ± 0.006^{d}	0.381 ± 0.008^{b}

Each value is the mean of nine replicates, given with standard deviation. Different superscripts in the same column indicate significant differences ($P < 0.05$). FW = fresh weight.

addition of NaCl, the positive controls with addition of 1 μM commercial ALA had the highest CAT activity in rice seedlings: H_2O_2 was degraded at the rate of 1.15 ± 0.11 mmol/g FW/min. The other treatments in the rank order are follows: TN114 supernatant, negative controls, and PP803 supernatant; however, the differences between these were not significant. On the other hand, the CAT activity under NaCl stress was significantly lower than in normal conditions; for the positive controls, TN114, and PP803 treatments it was at the same level, and significantly lower for the negative controls. The lowest activity in the negative control was as little as 0.06 ± 0.03 mmol/g FW/min in NaCl stress, while the other treatment groups had the range 0.63–0.70 mmol/g FW/min. Thus, CAT activity is high in untreated plants whether or not ALA is present.

Fig. 3c shows APx activities in rice seedlings at day 21 after pretreatments with both 10X diluted PNSB supernatants, under normal and salt stress conditions. Ascorbic acid (AsA) as a substrate was decomposed in the reaction used for quantitative analysis. The pattern of APx activity was similar for commercial ALA and PNSB supernatants, in that the activity in normal condition was lower than under NaCl stress; these differences were not significant though. In contrast, the opposite pattern was found in the negative controls: the APx activity in normal conditions was significantly higher than under NaCl stress. For the commercial ALA treatment group, under normal conditions AsA was decomposed at rate 6.33 ± 0.41 μmol/g FW/min, while under salt stress the rate was 7.10 ± 0.25 μmol/g FW/min.

Results of GR activities in rice seedlings at day 21 after pretreatment with both 10X diluted PNSB supernatants, under both tested conditions are shown in Fig. 3d. For all treatment types, the GR activity in normal conditions was significantly higher than in NaCl stress. NADPH as an indicator was oxidized at the rate of 0.612 ± 0.01 μmol/g FW/min in the commercial ALA set under normal condition. In NaCl stress conditions, the highest GR activity was observed in sets treated with commercial ALA and TN114, followed by PP803, and the negative control had the lowest rate of 0.185 ± 0.01 μmol/g FW/min.

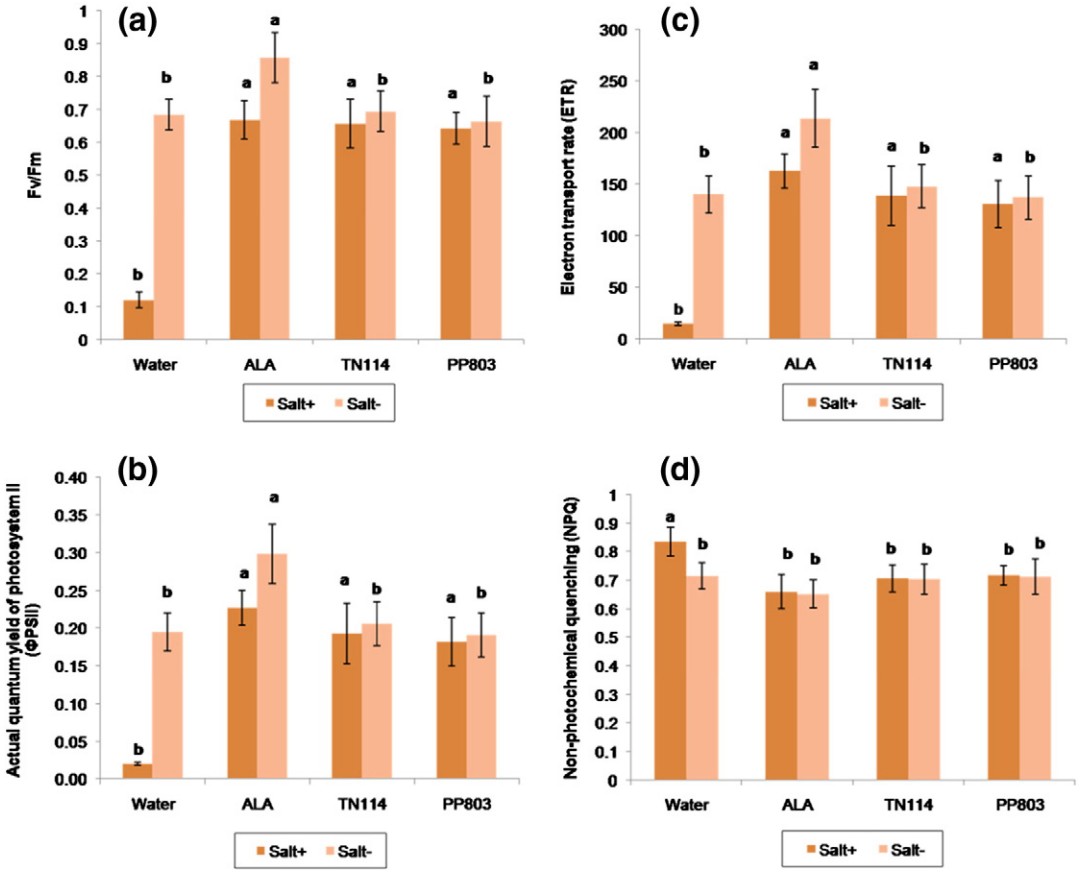

Fig. 2. The chlorophyll fluorescence parameters: maximum quantum yield, Fv/Fm (a); actual quantum yield, ΦPSII (b); electron transport rate, ETR (c); and non-photochemical quenching, NPQ (d) of rice seedlings without and with salt stress (50 mM NaCl) after 21 d of pretreatments. The treatment types were ALA, the culture supernatant of *R. palustris* strains (TN114 or PP803), and distilled water as negative control. Each value represents mean ± SD (n = 9). Values with different lowercase letters indicate significant differences at $P < 0.05$.

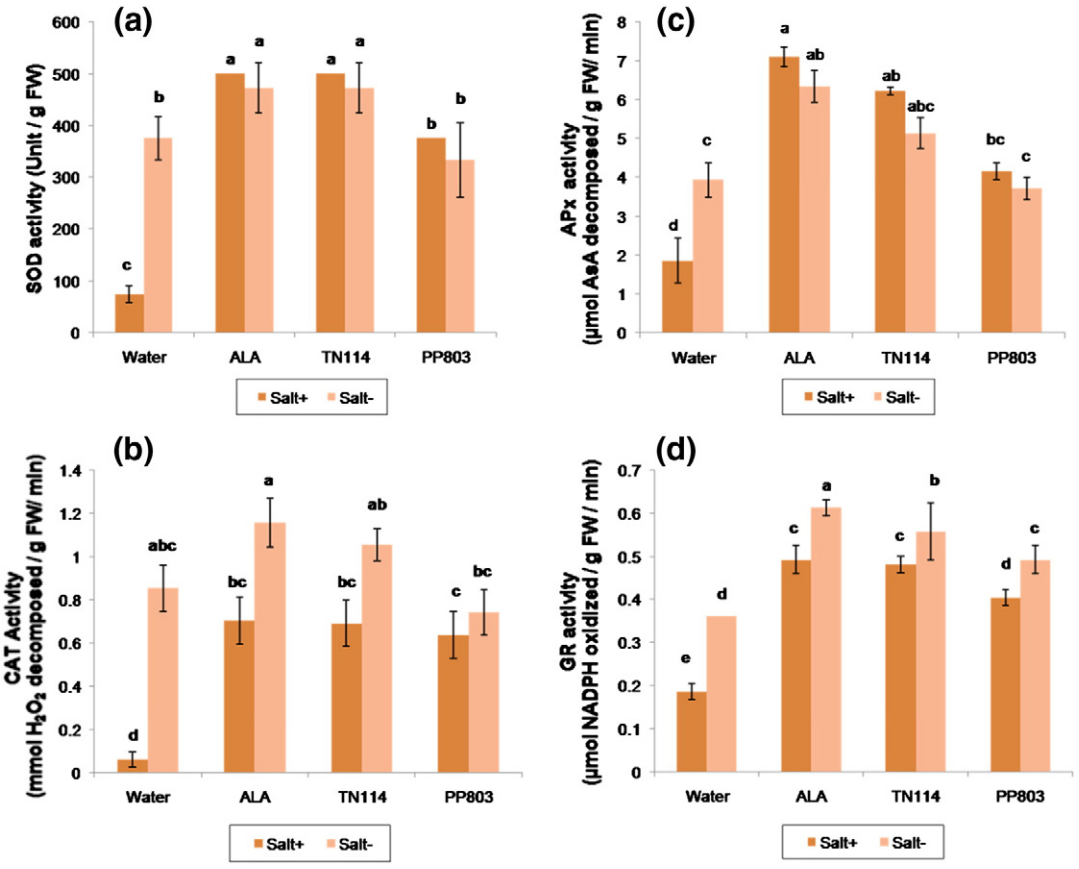

Fig. 3. Activities of antioxidative enzymes; superoxide dismutase (SOD) (a); catalase (CAT) (b); ascorbate peroxide (APx) (c); and glutathione reductase (GR) (d) of rice seedlings without and with salt stress (50 mM NaCl) after 21 d of pretreatments. The treatment types were ALA, the culture supernatant of *R. palustris* strains (TN114 or PP803), and distilled water as a negative control. Each value represents mean ± SD (n = 9). Values with different lowercase letters indicate significant differences at $P < 0.05$.

4. Discussion

The salinity of soil and water is most commonly caused by high Na^+ and Cl^- concentrations. This salinity reduces the initial growth and productivity of plants, by generation of ROS such as $^1O_2$, O_2^-, H_2O_2, and •OH. The ROS are produced in shoot cultures of plants, including rice in salt solution [25]. Zhen et al. [9] reported that ALA affected antioxidant enzyme activities in plants; thereby plant cells were protected from the negative effects of salinity. In this study we have confirmed that the pretreatment with ALA, contained in each of the 10X diluted PNSB supernatants could ameliorate rice seedling growth in 50 mM NaCl: the % RRG was higher than in those treatments grown without 50 mM NaCl (Table 1). This means that the culture supernatants restored the growth of rice seedlings under NaCl stress to levels comparable to those control plants not exposed to NaCl stress. According to percentage RRG, there was no significant difference of rice growth amelioration between commercial ALA and the culture supernatant from strain TN114, under both conditions tested (Table 1 and Fig. 1). In contrast, NaCl stress strongly affected the rice seedling growth in the negative control set. In Fig. 1 the negative control with 50 mM NaCl (Plant 2) had obvious salt stress symptoms such as wither, but these symptoms were not apparent in both 10X diluted PNSB supernatant treatments in salt condition (plants 4, 6, 8). However, regarding the fresh and dry weights, the rice treated with commercial ALA grew better than the plants treated with PNSB supernatants (Table 1). This could indicate that some bioactive compounds other than ALA are present in the PNSB supernatants that counteract the effects of the ALA. However, it should be pointed out that although statistically significant, the difference was small compared to the very negative effects of NaCl treatment with no benefit of added ALA or PNSB supernatants.

The treatments with 10X diluted culture supernatants had higher amounts of ALA than the treatments with commercial ALA, because they had been tested for the best dilution to stimulate rice growth under NaCl stress (data not shown). The ALA in culture supernatants is mixed with other compounds that may reduce its activity as previously described. However, the results indicate that both PNSB supernatants, particularly TN114, had enough ALA to restore rice seedling growth in saline paddy fields, with the dilution used. The use of supernatants containing ALA would be a cheaper alternative to farmers than commercial ALA. PNSB can easily be grown in a shallow pond [26]. This is in accordance with our previous work that stimulated the growth of PNSB by adding 0.13% fermented pineapple extract into rubber sheet wastewater [27]. In addition of producing ALA, *R. palustris* strains are typically able to fix N_2 [28] and so are an effective biofertilizer. Our findings show that it not only provides N-fertilizer , but also gives extracellular secretion of ALA that has positive effects to reduce NaCl stress.

In Table 2, it can be seen that with no ALA treatment NaCl stress strongly decreased the total chlorophyll content of rice seedlings. Under NaCl stress conditions, there was a big increase of total chlorophyll in the sets pretreated with commercial ALA and PNSB supernatants, compared to the negative control. The increase in the total chlorophyll content in sets of positive controls and treatments (TN114 or PP803) is usually thought of as being primarily due to ALA being a precursor for chlorophyll synthesis [7]. This is supported by Santos [29], who reported that the decrease in the chlorophyll content in NaCl-stressed sunflower (*Helianthus annuus* L. cv. SH222) leaves was mainly because of the decrease in ALA synthesis. The results also indicate that both culture supernatants were effective in eliminating salt stress effects, as there was no significant difference in the amount of total chlorophyll between

the commercial ALA and the PNSB supernatant treatments. Exogenously applied ALA can help accumulate chlorophyll, leading to a higher rate of photosynthesis and plant growth.

It can also be seen from Fig. 2 that the ALA in both of the 10X diluted PNSB supernatants was enough to enhance the chlorophyll fluorescence of rice seedlings and also significantly increase the photosynthetic ETR value over the negative control set. In rice cultivar (*O. sativa*), Moradi and Ismail [30] reported that Fv/Fm and ETR significantly decreased, but NPQ increased substantially under saline conditions. However, for this study, in contrast, Fv/Fm, ΦPSII, and ETR were elevated and NPQ was increased under saline conditions when compared between positive and negative controls. The promotion of ALA experiment on ΦPSII and ETR are beneficial to photosynthesis. Sun et al. [31] found that ALA treatment permits more energy dissipation in antenna light harvesting proteins, which protects the photosynthetic apparatus under stress conditions. Sun et al. [31] also found that ALA improved the ETR leading to more rapid production of ATP and NADPH, which are necessary for CO_2 fixation. More photosynthetic accumulation in leaves is not only the base for stress resistance but also provides the organic carbon necessary for plant growth. Moreover, no significant differences in Fv/Fm, ΦPSII, ETR and NPQ were observed in commercial ALA and both 10X diluted PNSB supernatants under saline conditions which agreed with their relative amounts of chlorophyll content under NaCl stress. Therefore, this study demonstrated that these two PNSB strains have the potential as plant growth stimulating bacteria for ameliorating the growth of rice in saline soil paddy fields and we have shown that this effect is upon both the chlorophyll status of the leaves and their photosynthetic ETR.

Averina et al. [32] reported that ROS, such as H_2O_2, can be generated in a plant cell at low NaCl levels when cultivated in normal conditions and this is confirmed by the results in Table 2. High H_2O_2 levels are consistent with photosystems being under severe stress because under stress H_2O_2 is formed by H_2O acting as an electron acceptor. Hence, we found high levels in the NaCl stressed plants where no ALA or 10X diluted PNSB supernatants were added. The results also showed no effect on H_2O_2 levels in unstressed condition, by pretreatments with commercial ALA or the 10X diluted PNSB supernatants. In contrast, both ALA and the 10X diluted PNSB supernatants strongly decreased H_2O_2 levels in salt stressed rice seedlings. Reduction in H_2O_2 content might be caused by well-organized functioning of the following enzymes; APx, CAT, GR and SOD, which were stimulated by ALA content in those treatment sets.

Generally, the production of antioxidative enzymes, such as CAT, APx, GR, and SOD, is a protective mechanism of plants, used against the damaging effects of ROS [25]. Hence, plants producing higher levels of antioxidative enzymes show greater resistance to oxidative damage from ROS [33]. SOD is a ubiquitous enzyme in aerobic organisms, used as a forefront defense mechanism against ROS in the antioxidative system, and catalyzes the conversion of O_2^- into O_2 and H_2O_2 [25]. There are several reports that plants treated with a low concentration of ALA, before cultivation in stress conditions, have significantly increased SOD activity [11,34]. This is in accordance with the current study, where we found significantly enhanced SOD activity of rice seedlings in NaCl stress, for the immersion pretreatments with commercial ALA solution or the 10X diluted PNSB supernatants compared to the negative control (Fig. 3a). However, there were no significant differences in SOD activity between normal and stress conditions, for all the treatments. The SOD activity under both conditions was similar between the TN114 supernatant and the 1 μM commercial ALA treatments as there were no significant differences.

This study found that after 21 d of pretreatment there was a significantly increased CAT activity in rice seedlings under NaCl stress for the 1 μM commercial ALA or the 10X diluted PNSB supernatants when compared with a negative control (Fig. 3b). This means that commercial ALA and the 10X diluted PNSB supernatants containing ALA acted to decompose H_2O_2 in NaCl-stress conditions, as indicated by Table 2. On the other hand, the CAT activity in normal conditions was significantly higher than under NaCl stress, for all treatment types. Plants in general possess a small family of CAT genes; in rice the genes are *CatA*, *CatB*, and *CatC* [35]. Among these, only *CatB* was inhibited in rice by salt stress, while salinity did not alter the phasing of *CatA* and *CatC* expressions [36]. Hence, without the inhibition of *CatB* expression the CAT activity in rice seedlings under normal conditions is higher than under salinity stress conditions.

The CAT and APx activities play an important role in scavenging H_2O_2 to form H_2O and O_2, in protecting a plant against oxidative stress [37]; particularly photooxidative stress where H_2O_2 is formed by Mehler reactions in the chloroplasts [38]. In pretreatment sets, the APx activity in rice seedlings under NaCl stress was higher than that found in normal conditions (Fig. 3c). The role of this enzyme may be related to heme synthesis as it is a heme-containing protein [39], and one family of CAT uses heme as a cofactor [40]. This is in agreement with Wongkanthakorn et al. [5], who reported that activities of CAT and APx in rice under salt stress increased when the rice was pretreated with ALA in a range of 0.1–1.0 μM. The high level of this enzyme indicated that ALA (either commercial or in PNSB supernatants) improved the salt tolerance of rice. Interestingly, there were no significant differences in the CAT and APx activities between treatments with culture supernatant from strain TN114 and commercial ALA. This suggests that ALA produced by the selected PNSB had an equal potential to enhance the salt tolerance of rice seedlings as the commercial ALA.

The APx and GR activities are interrelated because APx uses AsA as a reducing substrate for converting H_2O_2 to form H_2O and O_2, as previously described; thereby the oxidized ascorbate is recycled using glutathione as an electron donor [33]. GR then converts oxidized glutathione (GSSG) to its reduced form (GSH) and so GR is an important enzyme in the ROS protective mechanism of the respiratory system [25]. The ratio of $NADP^+$/NADPH increases along with increasing GR activity to ensure the availability of $NADP^+$ for trapping electrons from the photosynthetic electron transport chain [41] resulting in less leakage of electrons to O_2 for the generation of O_2^- [42]. Immersion of *Gingko biloba* seedlings in an ALA solution enhanced APx and dehydroascorbate reductase (DHAR) activities: the numbers of AsA and GSSG increased [43]. In contrast, dehydroascorbate (DHA) decreased in the system leading to an increase the ratio of AsA/DHA. This suggests that the relation between APx and GR activities is affected by ALA, in agreement with the negative correlation between them under NaCl stress in the current study (Fig. 3c, d).

5. Concluding remarks

The ALA containing culture supernatant of *R. palustris* TN114, at 10X dilution, was comparable to commercial ALA in ameliorating the rice seedling growth under NaCl stress. Pretreatment with it increased total chlorophyll content and more crucially the electron transport rate of leaves and the activities of antioxidative enzyme (CAT, APx, GR and SOD) that scavenge ROS and decrease H_2O_2, and these effects would have contributed to the growth effects. These laboratory results suggest that TN114 strain has great potential as a plant growth stimulating bacterium, for enhancing rice growth in a saline soil environment at a low cost and easy to grow in rural Thailand. Our findings seem to warrant the pursuit of agronomic field studies.

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References

[1] Perveen S, Shahbaz M, Ashraf M. Regulation in gas exchange and quantum yield of photosystem II (PSII) in salt-stressed and non-stressed wheat plants raised from seed treated with triacontanol. Pak J Bot 2010;42:3073–81.

[2] Iqbal M, Ashraf M. Changes in hormonal balance: A possible mechanism of pre-sowing chilling-induced salt tolerance in spring wheat. J Agron Crop Sci 2010;196:440–54. http://dx.doi.org/10.1111/j.1439-037X.2010.00434.x.

[3] Netondo GW, Onyango JC, Beck E. Sorghum and salinity: II. Gas exchange and chlorophyll fluorescence of sorghum under salt stress. Crop Sci 2004;44:806–11.

[4] Ashraf M, Harris PJC. Potential biochemical indicators of salinity tolerance in plants. Plant Sci 2004;66:3–16. http://dx.doi.org/10.1016/j.plantsci.2003.10.024.

[5] Wongkanthakorn N, Sunohara Y, Mutsumoto H. Mechanism of growth amelioration of NaCl-stressed rice (*Oryza sativa* L.) by δ-aminolevulinic acid. J Pestic Sci 2009;34:89–95. http://dx.doi.org/10.1584/jpestics.G08-43.

[6] Naeem MS, Rasheed M, Liu D, Jin ZL, Ming DF, Yoneyama K, et al. 5-Aminolevulinic acid ameliorates salinity-induced metabolic, water-related and biochemical changes in *Brassica napus* L. Acta Physiol Plant 2011;33:517–28. http://dx.doi.org/10.1007/s11738-010-0575-x.

[7] Hotta Y, Tanaka T, Takaoka H, Takeuchi Y, Konnai M. Promotive effects of 5-aminolevulinic acid on the yield of several crops. Plant Growth Regul 1997;22:109–14. http://dx.doi.org/10.1023/A:1005883930727.

[8] Ali B, Tao Q, Zhou Y, Gill RA, Ali S, Rafiq MT, et al. 5-Aminolevolinic acid mitigates the cadmium-induced changes in *Brassica napus* as revealed by the biochemical and ultra-structural evaluation of roots. Ecotoxicol Environ Saf 2013;92:271–80. http://dx.doi.org/10.1016/j.ecoenv.2013.02.006.

[9] Zhen A, Bie ZL, Huang Y, Liu ZX, Fan ML. Effects of 5-aminolevulinic acid on the H_2O_2-content and antioxidative enzyme gene expression in NaCl-treated cucumber seedlings. Biol Plant 2012;56:566–70. http://dx.doi.org/10.1007/s10535-012-0118-y.

[10] Ali B, Huang CR, Qi ZY, Ali S, Daud MK, Geng XX, et al. 5-Aminolevulinic acid ameliorates cadmium-induced morphological, biochemical, and ultrastructural changes in seedlings of oilseed rape. Environ Sci Pollut Res Int 2013;20:7256–67. http://dx.doi.org/10.1007/s11356-013-1735-5.

[11] Nishihara E, Kondo K, Parvez MM, Takahashi K, Watanabe K, Tanaka K. Role of 5-aminolevulinic acid (ALA) on active oxygen-scavenging system in NaCl-treated spinach (*Spinacia oleracea*). J Plant Physiol 2003;160:1085–91. http://dx.doi.org/10.1078/0176-1617-00991.

[12] Wang JJ, Jiang WB, Liu H, Liu WQ, Kang L, Hou XL. Promotion by 5-aminolevulinic acid of germination of pakchoi (*Brassica campestris* ssp. Chinensis var. communis Tsen et Lee) seeds under salt stress. J Integr Plant Biol 2005;47:1084–91. http://dx.doi.org/10.1111/j.1744-7909.2005.00150.x.

[13] Zhang ZJ, Li HZ, Zhou WJ, Takeuchi Y, Yoneyama K. Effect of 5-aminolevulinic acid on development and salt tolerance of potato (*Solanum tuberosum* L.) microtubers *in vitro*. Plant Growth Regul 2006;49:27–34. http://dx.doi.org/10.1007/s10725-006-0011-9.

[14] Youssef T, Awad MA. Mechanisms of enhancing photosynthetic gas exchange in date palm seedlings (*Phoenix dactylifera* L.) under salinity stress by a 5-aminolevulinic acid–based fertilizer. J Plant Growth Regul 2008;27:1–9. http://dx.doi.org/10.1007/s00344-007-9025-4.

[15] Nunkaew T, Kantachote D, Nitoda T, Kanzaki H. The use of rice straw broth as an appropriate medium to isolate purple nonsulfur bacteria from paddy fields. Electron J Biotechnol 2012;15. http://dx.doi.org/10.2225/vol15-issue6-fulltext-8.

[16] Tanaka T, Sasaki K, Noparatanaraporn N, Nishio N. Utilization of volatile fatty acids from the anaerobic digestion liquor of sewage sludge for 5-aminolevulinic acid production by photosynthetic bacteria. World J Microbiol Biotechnol 1994;10:677–80. http://dx.doi.org/10.1007/BF00327958.

[17] Tangprasittipap A, Prasertsan P, Choorit W, Sasaki K. Biosynthesis of intracellular 5-aminolevulinic acid by a newly identified halotolerant *Rhodobacter sphaeroides*. Biotechnol Lett 2007;29:773–7. http://dx.doi.org/10.1007/s10529-006-9303-4.

[18] Nagaya Y, Matsuoka T, Kobayashi T, Taniyama T. Development of automatic fog culture system for year-round rice production in greenhouse. Jpn Agric Res Q 1998;32:249–56.

[19] Chappelle EW, Kim MS, Mcmurtrey III JE. Ratio analysis of reflectance spectra (RARS): an algorithm for the remote estimation of the concentrations of chlorophyll A, chlorophyll B and carotenoides in soybean leaves. Remote Sens Environ 1992;39:239–47. http://dx.doi.org/10.1016/0034-4257(92)90089-3.

[20] Dionisio-Sese ML, Tobita S. Effects of salinity on sodium content and photosynthetic responses of rice seedlings differing in salt tolerance. J Plant Physiol 2000;157:54–8. http://dx.doi.org/10.1016/S0176-1617(00)80135-2.

[21] Schreiber U, Bilger W, Neubauer C. Chlorophyll fluorescence as a non-intrusive indicator for rapid assessment of *in vivo* photosynthesis. In: Schulze ED, Caldwell MM, editors. Ecophysiology of photosynthesis: ecological studies. Berlin: Springer; 1995. p. 49–70. http://dx.doi.org/10.1007/978-3-642-79354-7_3.

[22] Genty B, Briantais JM, Baker NR. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochim Biophys Acta 1989;990:87–92. http://dx.doi.org/10.1016/S0304-4165(89)80016-9.

[23] Ritchie RJ, Bunthawin S. The use of pulse amplitude modulation (PAM) fluorometry to measure photosynthesis in a cam orchid, *Dendrobium* spp. (cv. Viravuth Pink). Int J Plant Sci 2010;171:575–85. http://dx.doi.org/10.1086/653131.

[24] Asada K. Chloroplasts: formation of active oxygen and its scavenging. Methods Enzymol 1984;105:422–9. http://dx.doi.org/10.1016/S0076-6879(84)05059-X.

[25] Apel K, Hirt H. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol 2004;55:373–99. http://dx.doi.org/10.1146/annurev.arplant.55.031903.141701.

[26] Kim MK, Choi KM, Yin CR, Lee KY, Im WT, Lim JH, et al. Odorous swine wastewater treatment by purple non-sulfur bacteria, *Rhodopseudomonas palustris*, isolated from eutrophicated ponds. Biotechnol Lett 2004;26:819–22. http://dx.doi.org/10.1023/B:BILE.0000025884.50198.67.

[27] Kantachote D, Kornochalert N, Chaiprapat S. The use of the purple non sulphur bacterium isolate P1 and fermented pineapple extract to treat latex rubber sheet wastewater for possible use as irrigation water. Afr J Microbiol Res 2010;4:2604–16.

[28] Larimer FW, Chain P, Hauser L, Lamerdin J, Malfatti S, Do L, et al. Complete genome sequence of the metabolically versatile photosynthetic bacterium *Rhodopseudomonas palustris*. Nat Biotechnol 2004;22:55–61. http://dx.doi.org/10.1038/nbt923.

[29] Santos CV. Regulation of chlorophyll biosynthesis and degradation by salt stress in sunflower leaves. Sci Hortic 2004;103:93–9. http://dx.doi.org/10.1016/j.scienta.2004.04.009.

[30] Moradi F, Ismail AM. Responses of photosynthesis, chlorophyll fluorescence and ros-scavenging systems to salt stress during seedling and reproductive stages in rice. Ann Bot 2007;99:1161–73. http://dx.doi.org/10.1093/aob/mcm052.

[31] Sun YP, Zhang ZP, Wang LJ. Promotion of 5-aminolevulinic acid treatment on leaf photosynthesis is related with increase of antioxidant enzyme activity in watermelon seedlings grown under shade condition. Photosynthetica 2009;47:347–54. http://dx.doi.org/10.1007/s11099-009-0055-y.

[32] Averina NG, Gritskevich ER, Vershilovskaya IV, Usatov AV, Yaronskaya EB. Mechanisms of salt stress tolerance development in barley plants under the influence of 5-aminolevulinic acid. Russ J Plant Physiol 2010;57:849–56. http://dx.doi.org/10.1134/S1021443710060075.

[33] Azevedo-Neto AD, Prisco JT, Enéas-Filho J, De Abreu CEB, Gomes-Filho E. Effect of salt stress on antioxidative enzymes and lipid peroxidation in leaves and roots of salt-tolerant and salt-sensitive maize genotypes. Environ Exp Bot 2006;56:87–94. http://dx.doi.org/10.1016/j.envexpbot.2005.01.008.

[34] Zhang WF, Zhang F, Raziuddin R, Gong HJ, Yang ZM, Lu L, et al. Effects of 5-aminolevulinic acid on oilseed rape seedling growth under herbicide toxicity stress. J Plant Growth Regul 2008;27:159–69. http://dx.doi.org/10.1007/s00344-008-9042-y.

[35] Iwamoto M, Maekawa M, Saito A, Higo H, Higo K. Evolutionary relationship of plant catalase genes inferred from exon–intron structures: isozyme divergence after the separation of monocots and dicots. Theor Appl Genet 1998;97:9–19. http://dx.doi.org/10.1007/s001220050861.

[36] Menezes-Benavente L, Teixeira FK, Kamei CLA, Margis-Pinheiro M. Salt stress induces altered expression of genes encoding antioxidant enzymes in seedlings of *Brazilian indica* rice (*Oryza sativa* L.). Plant Sci 2004;166:323–31. http://dx.doi.org/10.1016/j.plantsci.2003.10.001.

[37] Wei-Feng X, Wei-Ming S, Ueda A, Takabe T. Mechanisms of salt tolerance in transgenic *Arabidopsis thaliana* carrying a peroxisomal ascorbate peroxidase gene from barley. Pedosphere 2008;18:486–95. http://dx.doi.org/10.1016/S1002-0160(08)60039-9.

[38] Murata N, Allakhverdiev SI, Nishiyama Y. The mechanism of photoinhibition *in vivo*: re-evaluation of the roles of catalase, α-tocopherol, non-photochemical quenching, and electron transport. Biochim Biophys Acta 1817;2012:1127–33. http://dx.doi.org/10.1016/j.bbabio.2012.02.020.

[39] Wada K, Tada T, Nakamura Y, Ishikawa T, Yabuta Y, Yoshimura K, et al. Crystal structure of chloroplastic ascorbate peroxidase from tobacco plants and structural insights into its instability. J Biochem 2003;134:239–44. http://dx.doi.org/10.1093/jb/mvg136.

[40] Hofrichter M, Ullrich R, Pecyna MJ, Liers C, Lundell T. New and classic families of secreted fungal heme peroxidases. Appl Microbiol Biotechnol 2010;87:871–97. http://dx.doi.org/10.1007/s00253-010-26330.

[41] Hamurcu M, Sekmen AH, Turkan IS, Gezgin S, Demiral T, Bell RW. Induced anti-oxidant activity in soybean alleviates oxidative stress under moderate boron toxicity. Plant Growth Regul 2013;70:217–26. http://dx.doi.org/10.1007/s10725-013-9793-8.

[42] Verma S, Mishra SN. Putrescine alleviation of growth in salt stressed *Brassica juncea* by inducing antioxidative defense system. J Plant Physiol 2005;162:669–77. http://dx.doi.org/10.1016/j.jplph.2004.08.008.

[43] Xu F, Chang J, Cheng SY, Zhu J, Li LL, Wang Y, et al. Promotive effect of 5-aminolevulinic acid on the antioxidant system in *Ginkgo biloba* leaves. Afr J Microbiol Res 2009;8:3769–76.