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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)