Sodium Lignosulfonate Improves Shoot Growth of Oryza Sativa via Enhancement of Photosynthetic Activity and Reduction of Stress Response

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Abstract

Lignosulfonate (LS) is a by-product obtained during sulfite pulping process and is commonly used as a growth enhancer in plant growth. However, the underlying growth promoting mechanism of LS on shoot growth remains largely unknown. Hence, this study was undertaken to determine the potential application of eco-friendly LS chelated ion complex (NaLS and CaLS) to enhance recalcitrant indica rice MR219 shoot growth and to elucidate its underlying growth promoting mechanisms. The NaLS was shown to be a better shoot growth enhancer as compared to CaLS, with optimum concentration of 300 mg/L. Subsequent comparative proteomic analysis revealed an increase of photosynthesis-related proteins and stress regulator proteins abundance in NaLS-treated rice as compared to MSO (control). Consistently, biochemical analyses showed a significant increase of rubisco activity, total chlorophyll, total sugar and total protein contents in NaLS-treated rice, implying NaLS role in empowering photosynthesis activities that led to plant growth enhancement. In addition, low level of peroxidase activity, malondialdehyde content and phenylalanine ammonia lyase activity were also observed in NaLS-treated rice. These results suggest that NaLS plays a role in modulating cellular homeostasis to provide a conducive cellular environment for plant growth. Taken together, NaLS improved shoot growth of recalcitrant MR219 rice by upregulation of photosynthetic activities and reduction of cellular stress leading to better plant growth.

Introduction

Rice is an important food crop in the world, and it is a staple food for more than half the global population. However, current rice cultivation that relies heavily on chemical fertilizers has caused sustainability food production to be at stake due to deterioration of soil health and pollutions, which lead to loss of fertile land. Approximately, one ton of rough rice production requires 20 kg of mineral nitrogen, 11 kg phosphorus oxide and 30 kg potassium oxide. Hence, incorporation or replacement of current rice fertilization regime with environmental-friendly growth enhancer would be a promising alternative to sustain global rice production.

Lignosulfonate (LS) is a by-product obtained during sulfite pulping process. LS comprises of hydrophilic sulfonic groups and electroactive methoxyphenol, forming phenylpropane segments. Similar to humic substances, LS contains high amount of carboxylic and phenol groups bound to aromatic rings, which enables chelation, buffering and cation exchange flexibilities. In general, LS can be found chelated with different cations, such as calcium (Ca), natrium (Na), zinc (Zn), potassium (K) and ferum (Fe), forming ion-chelated LS complex. The ability of LS to chelate different micronutrient ions enables it to be used as a plant fertilizer. In addition, LS chelator is more cost saving and eco-friendly as compared to other synthetic chelates, making it a good soil conditioners and plant stimulants in agriculture.

Aside from agriculture application, several ion-chelated LS have also been used to enhance in vitro shoot and root growth of several plant species. For instance, the incorporation of FeLS and CaLS in medium successfully enhanced growth performance of holly, ginseng and poplar. It was hypothesized
that these ion-chelated LS are able to modulate the endogenous auxin and act as an auxin protector in order to prevent auxin degradation, which in turns resulted in vigorous root growth of the plants. In addition, study by Docquier et al. suggested that incorporation of CaLS in the medium was able to enhance the nutrient ion availability to the plant, leading to better nutrient ion acquisition and growth performance of orchid, poplar and California redwood. Recent study by Wan Abdullah et al. also demonstrated that incorporation of CaLS improved the shoot proliferation rate of *in vitro* vanilla plant. The increased in vanilla shoot proliferation rate was associated with enhanced chlorophyll content, that contributed to the better photosynthetic activity for plant growth. Similarly, other humic substances like lignosulfonate-humate a, lignosulfonate-humate b and leonardite humic acid, have demonstrated to improve photosynthetic metabolism and stimulation of photosynthetic enzymes in maize.

To date, incorporation of LS-ion complex in fertilizer regime and tissue culture medium have been demonstrated to enhance growth of several plant species. However, its efficiency as a growth enhancer in plant is highly dependent on the types of chelated ion and plant species. Besides, the effects of ion-chelated LS complex on rice has not been reported. Therefore, this study was undertaken to evaluate the effect of ion-chelated LS complexes namely CaLS and NaLS on shoot growth of recalcitrant *indica* rice cultivar. Moreover, the underlying growth promoting mechanisms induce by the NaLS was determined through biochemical analysis and proteome profiling. Ultimately, deeper understanding on the effects and underlying growth promoting mechanism of LS-chelated ion complex would maximize its usage efficiency in agricultural sector, specifically in rice cultivation.

**Results**

**Physiological responses of MR219 rice shoot towards CaLS and NaLS treatments**

Utilization of ion chelated LS required details optimization in order to provide beneficial effects to the plant. In this study, different concentrations of NaLS and CaLS (0, 100, 200, 300 and 400 mg/L) were supplemented into MS medium in order to determine its growth promoting effects during shoot growth of MR219 rice. Shoot apex was selected in this study as it contained shoot apical meristem cells that contributed to building block of specialized tissues in the aerial parts of the plant. In general, high concentration of NaLS (200 mg/L ≥) was found to enhance shoot elongation of the MR219 rice. The optimum concentration of NaLS was recorded at 300 mg/L, with significance increment of shoot growth (28.15%) as compared to control plant (Fig. 1a). On the other hand, no significant changes were recorded on all concentrations tested for CaLS (Fig. 1a). To understand the underlying mechanisms of NaLS in stimulating the shoot growth of rice, comparative proteome profiling and biochemical assays were performed on control plantlet (MSO) and 300 mg/L NaLS plantlet (NaLS-treated rice).

**Comparative proteome analysis reveals an increased in photosynthesis-related proteins in NaLS-treated rice**
Comparative proteome analysis was carried out to elucidate the changes in MR219 rice proteome in response to NaLS in plant growth. The proteome analysis was performed using Perseus Software v1.6.0.7 (Max Planck Institute of Biochemistry) to execute the Pearson correlation and Principal Component Analysis (PCA). Pearson correlation reveals strong positive correlation (> 0.7) between the control and NaLS-treated rice (Supplementary Figure S1), which indicates that both control and NaLS-treated rice used were derived from the same organism with minimal contamination in the samples. In addition, the score plot of PCA shows grouping pattern of sample, wherein the control samples and NaLS-treated rice samples are totally separated because of significant change in proteome profile detected between treatment groups (Supplementary Figure S1). Score plot of PCA shows two principal components with PC1 and PC2 indicate 56.2% and 19.2%, respectively, thus making the total variance for the PCA is 75.4%. Meanwhile, the loading plot of PCA (Supplementary Figure S1) describes the relationship among all proteins identified. These proteins identified in loading plot correlates to the samples as shown in score plot of PCA (Supplementary Figure S1).

Based on comparative proteome analysis, a total of 41 and 60 unique proteins were successfully identified in the control and NaLS-treated rice, respectively; wherein, a total of 371 proteins were shared between the two samples (Fig. 2a). Through volcanic plot analysis, a total of 56 differentially expressed proteins were identified in response to NaLS. Among these proteins, a total of 15 proteins were upregulated, while a total of 41 proteins were downregulated in NaLS-treated rice as compared to the control plantlet (Fig. 2a – c). In response towards NaLS, proteins with the greatest increase in abundance were photosystem II (PSII) CP43 reaction center protein, photosystem I (PSI) iron-sulfur center, PSII CP47 reaction center protein and PSII protein D1. Meanwhile, proteins with greatest decrease in abundance were chitinase 5, chitinase 1, ATP synthase epsilon chain, thioredoxin reductase NTRB (Table 1). Two upregulated proteins [PSI iron-sulfur center (PSAC), PSII CP47 reaction center protein (PSBC)] and two downregulated proteins [chitinase 5 (CHT5) and chitinase 1 (CHT1)] were selected for gene expression analysis. Gene expression of selected proteins were observed to be in a similar trend with the proteome profile (Supplementary Figure S2). The list of primers used in RT-qPCR can be found in Supplementary Table S2.
Table 1
Top 15 up- and down-regulated proteins of significant difference in NaLS-treated rice.

| No | Proteins                                                                 | Uniprot Accession No. | General Function          | Fold Change |
|----|---------------------------------------------------------------------------|------------------------|---------------------------|-------------|
|    | **Upregulated Proteins**                                                 |                        |                           |             |
| 1  | Photosystem II CP43 protein                                              | P0C367                 | Photosynthesis            | 1.1811      |
| 2  | Photosystem I iron-sulfur center                                         | P0C359                 | Photosynthesis            | 1.07513     |
| 3  | Photosystem II CP47 protein                                              | P0C362                 | Photosynthesis            | 0.962446    |
| 4  | Photosystem II protein D1                                                | P0C433                 | Photosynthesis            | 0.937846    |
| 5  | Clathrin heavy chain 2                                                   | Q2QYW2                 | N/A                       | 0.927112    |
| 6  | Chlorophyll a/b binding protein, chloroplastic                           | A2XJ35                 | Photosynthesis            | 0.922371    |
| 7  | Photosystem II D2 protein                                                | P0C436                 | Photosynthesis            | 0.874711    |
| 8  | Ribulose bisphosphate carboxylase small chain A, chloroplastic          | P18566                 | Photosynthesis            | 0.66129     |
| 9  | Chaperone protein ClpC2, chloroplastic                                   | Q2QVG9                 | Translational Modification| 0.634115    |
| 10 | Probable aldo-keto reductase 3                                           | A2XRZ6                 | N/A                       | 0.585803    |
| 11 | Elongation factor 1-delta 2                                              | Q40682                 | Protein Biosynthesis      | 0.584471    |
| 12 | Elongation factor 1-alpha                                                | O64937                 | Protein Biosynthesis      | 0.524749    |
| 13 | Chaperone protein ClpC1, chloroplastic                                   | Q7F9I1                 | Translational Modification| 0.504763    |
| 14 | Glyceraldehyde-3-phosphate dehydrogenase 3, cytosolic                   | Q6K5G8                 | Carbohydrate Metabolism   | 0.440004    |
| 15 | Fructose-bisphosphate aldolase, chloroplastic                            | Q40677                 | Carbohydrate Metabolism   | 0.322455    |
|    | **Downregulated Proteins**                                               |                        |                           |             |
| 16 | Chitinase 5                                                               | Q7Y1Z0                 | N/A                       | -1.50325    |
| 17 | Chitinase 1                                                               | Q42993                 | N/A                       | -1.48961    |
| 18 | ATP synthase epsilon chain, chloroplastic                                | P0C2Z1                 | N/A                       | -1.34773    |

N/A, not available
| No | Proteins                                                                 | Uniprot Accession No. | General Function               | Fold Change |
|----|--------------------------------------------------------------------------|-----------------------|--------------------------------|-------------|
| 19 | Thioredoxin reductase NTRB                                              | Q6ZFU6               | Amino Acid Biosynthesis        | -0.93522    |
| 20 | Acyl transferase 9                                                       | Q9LGQ6               | N/A                            | -0.904535   |
| 21 | Probable UDP-arabinopyranose mutase 2                                   | Q7FAY6               | Amino Acid Biosynthesis        | -0.885329   |
| 22 | Ribose-phosphate pyrophosphokinase 4                                    | Q6ZFT5               | Amino Acid Biosynthesis        | -0.873207   |
| 23 | Pyruvate kinase 1, cytosolic                                            | B8BJ39               | Glycolysis                     | -0.842546   |
| 24 | 40S ribosomal protein S7                                                | Q8LJU5               | Translational Modification     | -0.808968   |
| 25 | Glucosidase 2 subunit beta                                               | A2WNF5               | Signalling and Cellular Process| -0.803812   |
| 26 | Cytochrome c                                                             | A2Y4S9               | N/A                            | -0.750532   |
| 27 | Proteasome subunit alpha type-3                                          | Q9LSU0               | Protein folding                | -0.731038   |
| 28 | Probable NADPH:quinone oxido-reductase 1                                 | Q941Z0               | N/A                            | -0.723101   |
| 29 | Glutaredoxin-C8                                                          | Q0DAE4               | Protein folding                | -0.69478    |
| 30 | Glutamine synthetase cytosolic isozyme 1–1                               | P14656               | Nitrogen Metabolism            | -0.640245   |

N/A, not available

The proteins identified were subjected to gene ontology (GO) analysis, in which they were classified into three categories, namely, biological processes, cellular components and molecular functions (Fig. 2d – f). From GO analysis (Fig. 2d – f) of biological processes, majority of the proteins were categorized under metabolic process (32.50%), followed by cellular process (34.58%), response to stimulus (8.33%) and biological regulation (7.92%). Meanwhile, when categorized under cellular components, majority of the proteins were involved in chloroplast (30.77%), cytoplasm (23.08%), nucleus (14.42%) and mitochondria (8.65%). In molecular functions, majority of the proteins identified were involved in binding (45.27%) and catalytic activity (40.54%). The changes in protein abundance in each category are shown in Fig. 2g. Subsequently, the identified proteins were subjected to KEGG pathway analysis in order to determine the overall effects of NaLS on the proteome during shoot growth (Fig. 2h). Based on KEGG pathway analysis, proteins involved in secondary metabolite biosynthesis were found to be the most affected pathway in response towards NaLS, followed by photosynthesis, carbohydrate metabolism, and lastly, amino acid biosynthesis pathway (Fig. 2h). A detailed categorization of the proteome profile can be found in Supplementary Table S2.
NaLS enhances protein synthesis and photosynthetic activities during shoot growth

In addition to proteome analysis, biochemical assessments were performed to further elucidate the possible roles of NaLS during shoot growth. Our results in Fig. 3a recorded 3.54 mg/g fresh weight (FW) of total chlorophyll content in NaLS-treated rice as compared to the control (2.47 mg/g FW). The accumulation of chlorophyll content was coherent with the significant increase in rubisco activity, whereby significant increase in rubisco activity (1.39 µmol CO$_2$/mg protein) was detected in NaLS-treated rice as compared to the control (0.54 µmol CO$_2$/mg protein; Fig. 3b). Besides, significant increment of sugar content was also recorded in NaLS-treated rice (1.50 mg/g FW) as compared to the control (2.11 mg/g FW; Fig. 3c). Likewise, significant increment in protein contents were recorded in NaLS-treated rice (7.82 mg/g FW) as compared to the control (4.12 mg/g FW).

Regulation of cellular homeostasis and stress response in NaLS-treated rice

To investigate the possible involvement of secondary metabolites and stress response in NaLS-treated rice, peroxidase activity, malondialdehyde (MDA) content and phenyl ammonia lyase (PAL) activity were determined. The NaLS-treated rice recorded a significant decrease in peroxidase activity (0.08 µmol/min mg protein) as compared to control (0.13 µmol/min mg protein; Fig. 4a). Similar trend was observed in MDA content, in which a significant decrease was recorded in NaLS-treated rice (3.38 nmole/mg FW) when compared to the control (3.94 nmole/mg FW; Fig. 4b). As for PAL activity, a significant drop was also observed in NaLS-treated rice (16.84 nM/mg protein) as compared with the control (10.65 nM/mg protein; Fig. 4c).

Discussion

Previous study has shown that beneficial effects of LS ion chelated complex is very much dependent on the type of tissues, plant species and type of chelated ions used. For instance, Docquier et al.\textsuperscript{10} reported the usage of CaLS resulted in superior growth promoting effects compared to FeLS and KLS in both Phalaenopsis and Sequoia sempervirens. Therefore, proper optimization on type of ion chelated and concentration used are prerequisite prior to its application in agriculture. In this study, NaLS was shown to be a better additive to improve the shoot growth of MR219 rice as compared to CaLS. Although many studies have regarded that sodium ions (Na$^+$) as non-essential element in terrestrial plant, their benefits in stimulating plant growth should not be ignored. In optimum concentration, Na$^+$ could aids in regulating leaf turgor pressure and chlorophyll concentrations resulting in an enhancement of overall plant photosynthetic activity, which is essential for plant growth and development\textsuperscript{15}. On top of that, Na$^+$ could also be used as a substitute for K$^+$, reducing K$^+$ requirements for plant growth\textsuperscript{16}. For instance, the germination rate, total dry weight and nutrient absorption of cotton plants were improved by Na supplementation\textsuperscript{17}. Thus, application of NaLS increased the bioavailability of Na$^+$ in the medium and enhanced uptake of Na$^+$ in the plant, while reducing dependability towards K$^+$ for plant growth.
Based on our proteomic data, increased of proteins abundance related to photosynthesis (PSII CP47, CP43, D1 and D2 proteins) was observed suggesting the participation of NaLS in governing photosynthetic rate. These proteins play an important role in modulation of photosynthetic activities in plant, as photosynthesis involves in production of energy and sugar required for plant growth. Moreover, previous study reported that moderate concentration of sodium chloride (up to 50 mM) allowed optimum Na\(^+\) accumulation on the leaves of *Zygiphyllum xanthoxyllum* which led to stimulation of photosynthesis activity and significantly increased relative growth rate\(^{15}\). Hence, NaLS may help in enhancing the photosynthetic rate to improve the rice growth as observed in this study.

Photosynthetic process is governed by a complex protein phosphorylation/dephosphorylation cascade\(^{18}\). The process is initiated by light-harvesting complexes, which absorb light and transfer excitation energy to the reaction centers of PSII and PSI. Subsequently, linear electron flow from PSII was initiated through a series of electron carrier intermediates producing and eventually reduced NADP to NADPH\(^{19}\). In present study, comparative proteome analysis reveals upregulation of photosynthesis-related proteins in response to NaLS (Table 1). The PSII is a multiprotein-pigment complex, composed of major protein subunits such as reaction center subunits (D1 and D2 proteins) and PSII internal antenna subunits (CP43 and CP47 proteins). According to Table 1, upregulation of PSII major protein subunits (PSII CP47, CP43, D1 and D2 proteins) were observed in NaLS-treated rice, suggesting an enhancement of PSII biogenesis. The PSII is one of the key components involved in initiation of linear electron flow of photosynthesis generating reducing power required for CO\(_2\) fixation to produce NADPH during photosynthesis\(^{20}\). Lacking in one of the major photosystem protein subunits may result in reduction of photosynthetic capacity and cellular metabolism. For instance, degradation of reaction center subunits (D1 and D2 proteins) was demonstrated to reduce photosynthetic performance in plants, which eventually reduced plant growth\(^{20,21}\).

In addition to proteome analysis, biochemical assessments were conducted to provide a further understanding on the possible roles of NaLS on shoot growth. Consistent with the upregulation of photosynthesis-related proteins in proteome profiling, the total chlorophyll content was significantly enhanced in response to NaLS treatment (Fig. 3a). The positive relationship between chlorophyll content and photosynthetic activity in plant has been widely observed\(^{22}\). In plants, chlorophyll is responsible for light-harvesting in photosynthesis, resulting in the excitation of electrons that are used to drive the linear electron flow in photosynthesis. Photosynthetic products produced will then be used for sugar production via Calvin cycle. In Calvin cycle, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) plays an important role, whereby Rubisco catalyzes the assimilation of CO\(_2\) with ribulose-1,5-bisphosphate (RuBP) producing 3-phosphoglycerate (PGA). The PGA produced will be integrated into the Calvin cycle producing sugars\(^{23}\). In present study, the upregulation of Rubisco protein (Table 1) and enhanced Rubisco activity (Fig. 3b) were observed in NaLS-treated rice. Therefore, the increased in chlorophyll content accompanied with enhanced Rubisco activity support the role of NaLS in improving rice growth via enhanced photosynthetic activity.
Photosynthetic activity was known to influence sugar accumulation in plant. The data obtained in Fig. 3c shows that NaLS-treated rice had recorded significant amount of sugar content compared to the control. These observations were consistent with previous studies, in which enhanced photosynthetic activity were accompanied with enhanced sugar production\textsuperscript{24,25}. On top of that, proteomic analysis revealed an upregulation of carbohydrate metabolism-related proteins [glyceraldehyde-3-phosphate dehydrogenase 3 (GAPDH) and fructose-bisphosphate aldolase (FBA); Table 1] in NaLS-treated rice. These two proteins were known to play a role in glycolysis pathway catalyzing the breakdown of glucose into pyruvate and energy, which will then be used for plant growth\textsuperscript{26}. Likewise, NaLS-treated rice recorded a significant increase protein accumulation (Fig. 3d). The increased in protein content is consistent with the enhanced shoot growth and photosynthetic activity. Besides, studies have found positive correlation between protein content with nitrogen supply in plant\textsuperscript{27,28}. Supporting this, it was demonstrated that deficiency in nitrogen supply in plant often reduces the growth of leaves by half. Nitrogen content is utilized as a source of building blocks for amino acid, which are prerequisite for protein biosynthesis in plant\textsuperscript{27}. Besides, high protein content is crucial during plant growth and development as well. For instance, protein is involved as structural protein, transporter protein, and metabolic reaction in the cells\textsuperscript{28}. These activities are important for proper functioning of the plant cells which will provide energy and food source to the plant and subsequently lead to improve plant growth.

Aside from photosynthetic process, NaLS was observed to be involved in modulation of reactive oxygen species (ROS) and stress in plant (Fig. 4 & Supplementary Table S1). During photosynthetic process, ROS were constantly generated. While ROS play an important role in plant signaling, excessive accumulation of ROS may cause oxidative damage towards plant cells, which leads metabolic homeostasis impairment. For instance, excessive ROS production caused by absorption of excessive sunlight by the light-harvesting complex resulted in photoinhibition of PSI\textsuperscript{29}. Hence, it is vital to maintain cellular homeostasis in the plant during enhancement of photosynthetic activity. In present study, increased in protein abundance of peptide methionine sulfoxide reductase A4 (PMSRA4) and delta-1-pyrroline-5-carboxylate synthase 1 (P5CS1) that were found in NaLS-treated rice could act as stress regulator (Supplementary Table S1). Involvement of these proteins in regulating stress response in plant have been elucidated previously. As suggested by Romero et al.\textsuperscript{30}, increased of PMSR4 activity could enhance tolerance level towards oxidative stresses. Similarly, P5CSI was shown to be vital as a stress regulator upon abiotic stress treatment.

Additionally, application of NaLS recorded a significant decrease in peroxidase activity (Fig. 4a). Low levels of peroxidase activity indicate low levels of ROS in plant cell. In line with the decrease in peroxidase activity, NaLS recorded a significant decrease in malondialdehyde (MDA) content (Fig. 4b). MDA is a major product of lipid peroxidation induced mainly by ROS and it reflects lipid peroxidation in plant cells in response towards stress\textsuperscript{31}. Significant increase in MDA levels implies severe oxidative damage towards cell membrane. Studies have found that lipid peroxidation is a common phenomenon that occurs in plant cells when subjected to stress, and MDA are often used as a marker to determine the physiological status of the plant during plant growth\textsuperscript{32}. On top of that, our study recorded a significant
decrease of phenylalanine ammonia lyase (PAL) activity in NaLS-treated rice (Fig. 4c). The PAL is one of the rate-limiting enzymes, known to play a key role in secondary metabolite biosynthesis pathway. Secondary metabolites biosynthesis involves production of phenolic and flavonoid compounds which plays an important role in regulation of cellular homeostasis in plants in response towards stress\textsuperscript{33,34}.

In general, regulation of cellular homeostasis is vital for enhancement of plant growth and development. During active acclimatization and adaptation process in response to stress environment, plant requires a bulk amount of energy for ATP biosynthesis and metabolism\textsuperscript{35}. This could be seen during salt stress treatment of \textit{Hordeum marinum}, whereby mitochondrial ATP synthase precursor and soluble inorganic pyrophosphatase 1 were upregulated to catalyze more energy to the plant indicating the urgent need for energy splurge in the form of ATP to establish homeostasis environment in the plant\textsuperscript{36}. Therefore, the increase in abundance of stress regulator proteins, decrease in peroxidase activity, MDA content and PAL activity imply that incorporation of NaLS may play a role in reducing stress in plant cell. This allows the plant to be grown in a conducive environment, whereby energy produced by the plant could be used efficiently to promote plant growth and development rather than directed it for plant defense mechanism to maintain cellular homeostasis.

**Conclusions**

To date, the growth promoting mechanisms of LS on plant remain largely fragmented. Our study demonstrated that the incorporation of 300 mg/L of NaLS in MS medium effectively enhanced shoot growth of MR219 rice. Improvement of shoot growth was due to the stimulation of photosynthetic activities in the MR219 rice, which could be evidenced by increased of PSI- and PSII- related proteins in proteome profiling as well as enhanced chlorophyll content and Rubisco activity in the plant (Fig. 5). In addition, it was found that NaLS successfully regulates cellular homeostasis in the plants, which was demonstrated by enhanced stress regulator proteins, reduced peroxidase activity, MDA content and PAL activity (Fig. 5). Hence, NaLS improved shoot growth of MR219 rice via enhanced photosynthetic activities and ROS stabilization.

**Methods**

**Plant materials**

The seeds of the recalcitrant Malaysian rice cultivar MR 219 used in this research were obtained from Malaysian Agricultural Research and Development Institute (MARDI), Seberang Prai, Penang.

**Lignosulfonates preparation**

Analytical grade NaLS (471038; Sigma-Aldrich, USA) and CaLS (471054; Sigma-Aldrich, USA) were prepared in a stock solutions of 50 mg/mL. All the stock solutions were filtered sterilized using 0.22 μm cellulose acetate membrane before being kept at 4 °C.
Seeds sterilization and LS treatment

Surface sterilization were performed on mature seeds according to previously described protocol\textsuperscript{37} with slight modifications. Firstly, the seeds were de-husked and sterilized with 70% (v/v) ethanol for 1 min, followed by 50% (v/v) Clorox for 30 min. The seeds were rinsed with sterile distilled water before being dried on filter paper. The sterilized seeds were then transferred into our previously established callus induction medium containing Gamborg's B5 basal medium\textsuperscript{38} supplemented with 10 g/L maltose, 0.1 g/L L-glutamine, 0.1 g/L L-asparagine, 0.1 g/L L-arginine, 10 mg/L NAA and 1 mg/L 2,4-D, pH 5.8\textsuperscript{11}. The seeds were then incubated under a photoperiod of 16 h light and 8 h darkness at 25 ± 2 ºC. After one week, approximately 1 cm of the shoot apices were removed from the seed and cultured in shoot growth medium containing Murashige and Skoog medium (MS)\textsuperscript{39} with 30 g/L sucrose, 3 mg/L kinetin and 0.5 mg/L NAA supplemented with CaLS or NaLS at different concentrations (100, 200, 300, and 400 mg/L). The shoot apices were incubated under a photoperiod of 16 h light and 8 h darkness at 25 ± 2 ºC for three weeks.

Protein extraction and protein digestion

In proteomic analysis, plant samples were ground into fine powder using liquid nitrogen. The samples were then mixed with 500 μL protein extraction buffer containing 50 mM of ammonium bicarbonate and 10 mM phenylmethylsulfonyl fluoride (PMSF). The mixture was then vortexed, sonicated and centrifuged according to Yang et al.\textsuperscript{40} and solubilized protein was collected. Desalting was carried out on total soluble protein obtained using acetone precipitation method\textsuperscript{41}. Subsequently, the proteins content in the plant sample was determined at 595 nm through Bradford assay\textsuperscript{42}. The proteins sample (100 μg) was digested with Trypsin Gold (Promega, USA) at a ratio of 1:200 parts of protein, according to previous study\textsuperscript{40}.

Peptide separation and protein identification

Nano liquid chromatography tandem-mass spectrometry (nanoLC-MS/MS; Thermo Scientific, USA) analysis was performed according to Yang et al.\textsuperscript{40}. Briefly, aliquot of 2 μL digested sample was injected into EASY-Spray Column Acclaim PepMapTM C18 100 (A0, 2μm particle size, 50μm id x 15cm) at 35 ºC. The sample elution process was performed similarly as described by Yang et al.\textsuperscript{40}. The eluents from the LC were directly introduced into a mass spectrometer (Orbitrap Fusion – Thermo Fisher Scientific, US). The instrument was operated in the data dependent acquisition. Full scan spectra were collected (OTMS1) using parameters defined by previous study\textsuperscript{40}. Only precursors with an assigned monoisotopic m/z and a charge state up to 4 were further analyzed for MS2. All precursors were filtered using a 20 sec dynamic exclusion window and intensity threshold of 5,000. The MS2 spectra were analyses (ITMS2) following parameters reported by Yang et al.\textsuperscript{40}. Precursors were fragmented by CID and HCD at normalized collision energy of 30% and 28%, respectively.
Subsequently, raw data was analyzed using Thermo Scientific™ Proteome Discoverer™ Software 2.1 and SEQUEST HT was used as the database searching algorithm. The intensities of each MS ion were measured according to the accurate mass and time tag strategy\textsuperscript{43}. Identification of the proteins was performed based on the searched against the UniprotKB database restricted to \textit{O. sativa} (2020_01: 48,904 sequences) with a 1\% strict FDR and 5\% relax FDR criteria using Percolator®. Search parameters were set up according to previous study\textsuperscript{40}.

**Protein quantification and data analysis**

The experiment was performed in triplicates with three biological replicates for MSO and NaLS-treated samples. The protein files (txt.format) obtained from Proteome Discoverer™ were uploaded to Perseus for comparative proteome analysis between MSO and NaLS-treated samples. Data processing such as PCA and Pearson correlation was performed in Perseus according to Ramdas et al.\textsuperscript{43}. Significant differences of protein abundance were determined based on the Student’s t test ($p < 0.05$). The $p$-values were also adjusted for multiple-testing using the permutation-based false discovery rate, with a randomization number of 250. Proteins were considered to be significantly differentially expressed between treatment groups with adjusted $p$-values of $<0.05$.

**Total photosynthetic pigments content**

One hundred milligram of sample was ground into fine powder with the presence of liquid nitrogen. The ground powder was mixed with 2 mL of 80\% (v/v) of acetone for 1 min in darkness. The homogenate was then centrifuged at 400 x g for 5 mins. The absorbance of the samples was then recorded at 470, 647 and 663 nm. The concentration of photosynthetic pigments (chlorophyll a, chlorophyll b, total chlorophyll and carotenoids) were calculated according to calculations described in Lichtenthaler et al.\textsuperscript{44} and expressed in mg/g fresh weight (FW).

**Rubisco activity**

Rubisco activity was measured spectrophotometrically according to Usuda\textsuperscript{45} with slight modifications. In brief, 1 g of leaf sample was homogenized to fine powder with presence of liquid nitrogen. Then, the powder was mixed with ice-cold extraction buffer containing 0.25 M Tris-HCl (pH 7.8), 0.05 M MgCl$_2$, 0.0025 M EDTA and 37.5 mg of DTT. Centrifugation of 10,000 x g for 10 minutes at 4°C was performed and the supernatant was collected as crude enzyme. Approximately 40 $\mu$L of crude enzyme was mixed with 960 $\mu$L reaction buffer containing 100 mM Tris-HCl (pH8), 40 mM NaHCO$_3$, 10 mM MgCl$_2$, 0.2 mM NADH, 4 mM ATP, 0.2 mM EDTA, 5 mM DTT, 1 U of glyceraldehyde-3-phosphodehydrogenase, 1 U of 3-phosphoglycerate kinase, and 0.2 mM ribulose 1,5-bisphosphate (RuBP). The absorbance of enzyme activities was recorded at 340 nm and expressed in $\mu$mol CO$_2$ mg$^{-1}$ protein.

**Total soluble sugar content**
Total soluble sugar content was estimated according to Dubois et al.\textsuperscript{46} with slight modifications. In brief, 0.1 g of sample was ground into powder in liquid nitrogen and extracted twice in 2 mL of 90\% (v/v) ethanol at 60°C for one hour. After each extraction, samples were centrifuged at 400 x g for 5 mins. One mL of the supernatant was mixed with 1 mL of 5\% (v/v) phenol together with 5 mL of concentrated sulphuric acid. The mixture was allowed to cool before the readings was taken at 495nm spectrophotometrically. The soluble sugar content was determined using glucose as a standard and expressed in mg/g FW.

**Total protein content**

Two hundred fifty milligram of plant sample were ground into powder with liquid nitrogen. Ice cold extraction buffer containing 1.8 mL of 50 mM ammonium bicarbonate (ABC) and 0.2 mL of 100 mM PMSF was added to the powdered sample. The homogenate was centrifuged at 10,000 x g for 30 mins and supernatant was collected as crude enzyme. All steps in enzyme extraction was performed at 4°C. Bradford assay was performed to determine the protein concentration at 595 nm\textsuperscript{42}. Total protein content in the sample was then determined by using bovine serum albumin as standard.

**Peroxidase activity**

The crude enzyme obtained during protein extraction was used in determination of peroxidase activity. One hundred microliters of crude enzyme were added with 950 µL of distilled water, 750 µL of 100 mM potassium phosphate buffer (pH 6.8), 600 µL of 100 mM pyrogallol and 600 µL of 100 mM H\textsubscript{2}O\textsubscript{2}. Peroxidase activity was measured at 420 nm between the second and fifth minute after addition of crude enzyme into the substrate. The peroxidase activity was expressed as micromoles of purpurogallin formed per minute per milligram of proteins\textsuperscript{47}

**Malondialdehyde (MDA) content**

MDA content was measured according to Luo et al.\textsuperscript{48} with slight modifications. Approximate 0.2 g of frozen powdered samples were dissolved in 10 mL of 10\% (w/v) trichloroacetic acid (TCA). The homogenate was then centrifuged at 12,000 x g for 10 mins. Subsequently, 2 mL of supernatant was mixed with 2 mL of 10\% (w/v) TCA containing 0.6\% (w/v) of thiobarbituric acid (TBA) and incubated at 100°C for 20 mins. Then, the homogenate was allowed to cool on ice followed by centrifugation at 12,000 x g for 10 mins. The supernatant was then measured at 532, 600 and 450 nm. The MDA content was calculated using the following formula; MDA content (uM) = 6.45 (OD\textsubscript{532} − OD\textsubscript{600}) − 0.56 (OD\textsubscript{450}).

**Phenylalanine ammonia-lyases (PAL) activity**

PAL activity was measured according to Schmidt et al.\textsuperscript{49} with slight modifications. The crude enzyme obtained during protein extraction in total protein content determination was used to determine PAL activity in the sample. One hundred microliter of crude enzyme obtained were mixed with 1.15 mL containing 0.1 M sodium borate buffer (SBB; pH 8.8) and 10 mM L-phenylalanine. The mixture was then
incubated at 37°C for 1 hour following which 250 µL of 5 N HCl was added to stop the reaction. PAL activity was then measured at 290 nm and the result was expressed in nanomoles of trans-cinnamic acid formed per milligram of proteins.

**Real-time reverse transcription polymerase chain reaction (RT-qPCR) analysis**

The RNA was isolated via RNeasy Plant Mini Kit (Qiagen, Germany) following the protocol described in Lai. First strand cDNA was converted from 1 µg of isolated total RNA using QuantiNova Reverse Transcription Kit (Qiagen, Germany). Expression profile were assessed via RT-qPCR analysis. Real-time PCR was performed with Bio-Rad CFX96 system (Bio-Rad, US) with QuantiNova SYBR Green PCR (Qiagen, Germany) following the protocol as described in Lai et al. The real-time PCR reaction conditions used were as follows: 95 ºC for 30 s followed by 40 cycles of 95 ºC for 5 s and 60 ºC for 5 s. Three technical replicates on three biological replicates were performed on each sample. The data were analyzed using Bio-rad CFX Manager 3.1 software. The relative expression levels \(2^{-ΔΔCT}\) were calculated according to Livak's method. The reference genes used in this study were rice cyclophilin \((OsCYC)\) and ubiquitin 5 \((OsUBQ5)\).

**Statistical analysis**

All data presented were the average ± standard deviation (SD) of three biological replicates. The Student's t test was applied in evaluating the level of significant differences at \(p < 0.05\) between the different treatments using the SPSS v.20 software (IBM Corp., Armonk, USA).

**Declarations**

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**Author contributions**

KSL conceived and designed the experiments; ADXK, WMANWA, LYL and CNT performed the experiments; NPT, JOA, MHY and KSL contributed materials/reagents/analysis tools/funding acquisition; ADXK, WMAZWA, CNT wrote the manuscript. All authors have read, contributed and approved the manuscript.

**Additional Information**

The author(s) declare no competing interests.

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**Figures**
Figure 1

Shoot growth in response to NaLS and CaLS treatments. (a) Shoot height after three weeks culture period in MSO, NaLS- and CaLS-supplemented medium. Shoot growth after three weeks incubation on (b) control and (c) 300 mg/L CaLS- and (d) 300 mg/L NaLS-supplemented medium. Data shows mean of three biological replicates, n=10, with error bars represent standard deviation. Asterisk represent the
significantly difference between treatments at \( p < 0.05 \) when compared to control. Scale bars represent 1 cm.