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Research Article

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Posted Date: February 24th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-212038/v1

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Version of Record: A version of this preprint was published at Environmental Science and Pollution Research on September 13th, 2021. See the published version at https://doi.org/10.1007/s11356-021-
Seed priming with Brassinosteroids alleviates Aluminum toxicity in rice via improving antioxidant defense system and suppressing aluminum uptake

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Abstract

Brassinosteroids (BRs) are growth-promoting hormones that exhibit high biological activities inside numerous plant species. BRs play a protective role in plants against various stresses. In the present study, seed priming of 24-epibrassinolide (0.01µM) was used to demonstrate the mitigation effect of Aluminum (400µM) in rice plants. BRs application was found effective in control beside plants under aluminum (Al) toxicity. It enhanced seed germination energy, germination percentage as well as root length, shoots length, fresh and dry weight as well under the stressed condition as well as inside control (primed with BRs). Although, Aluminum toxicity induced reduction in seed growth parameters, also in photosynthetic pigments, increased MDA content, and H$_2$O$_2$ production by enhancing the activities of antioxidative enzymes such as ascorbate peroxidase, catalase, and peroxidase in both roots and shoots. These changes were noticed higher inside tolerant variety (YLY-689) as compared to sensitive cultivar (CY-927). Interestingly, 24-EBL coped the stress through decreasing MDA content as well as H$_2$O$_2$ production by more stimulating antioxidant activities to modulate the stress condition under Al stress. Gene expression analysis of $SOD$-$Cu$-$Zn$, $SOD$-$Fe$, $CAT_a$, $CAT_b$, $APX02$, and $APX08$ also supported the data related to antioxidant activities. These findings led us toward the conclusion that more induction of antioxidant activities with effective response toward other seed growth parameters with low uptake of Aluminum under 400µM Aluminum stress, 24-EBL was responsible for the mitigation of aluminum stress in rice seedlings.

Keywords: Aluminum, Brassinosteroids, Rice, antioxidant enzyme activity, heavy metals, phytohormones

1. Introduction

Rice is considered a more edible crop in most countries of Asia and its overall production is estimated 90% world widely (Dawe et al. 2010). China is one of the leading rice-producing countries. In southern China,
Rice is the main source to meet the hunger of underprivileged people who fulfill their nutrients from rice uptake (Huang et al. 2013). Lately, soil pollution concern is arising due to industrialization and heavy metal contamination such as cadmium (Cd), mercury (Moghaddasi et al.) and lead (Pb) in soil, water and air (Fu & Kane 2008) is the biggest issue inside all over the world. Aluminum is one of the most growths inhibiting factor inside acidic soils. Almost 30-50% of soil is polluted with aluminum word widely and 21% inside China as well (Agha et al. 2018). It directly causes influences root length as well as affects membrane lipids and other peroxidation like Fe (Mannon et al. 2004). It increases the peroxidation of lipids and enhances the action of antioxidant enzymes such as catalase, peroxidase, superoxide dismutase, and glutathione reductase (Liu et al. 2003) that lead to the plant stress stage. It is investigated that Al toxicity is also observed in shoots which are observed as an upshot of root system damage (Vitorello et al. 2005). There are some nastiest effects caused by Al toxicity in plants such as water relation, reduces stomatal opening, reductions of photosynthetic activity besides it; reasons chlorosis as well as necrosis of leaves. Nevertheless, it surges the level of proline (Nandi & Neogy 2002) which performs itself as an osmoprotectant, membrane stabilizer as well as ROS vulture (Apel & Hirt 2004).

Brassinosteroids (BRs) are polyhydroxy steroidal phytohormones that have great capability to ardently demonstrate the plant growth-promoting effect (Latha & Vidya Vardhini 2018). It was firstly discovered in the rape plant, Brassica napus pollen based on its capability to stimulate growth (Leaska 1970). Afterward, 70 various types of BRs steroidal growth-promoting hormones were identified from virtually all plants like gymnosperms, monocots, dicots, pteridophytes, and alga from various parts of plants such as pollen, flower buds, fruits, seeds, vascular cambium, leaves, shoots, and roots (Bajguz & Hayat 2009, Haubrick & Assmann 2006, Piotrowska-Niczyporuk & Bajguz 2014). Wide research on BRs indicates that it plays an important role to mitigate various plant stresses including biotic and abiotic stresses (Wu et al. 2014).
24-Epibrassinolide is the most biologically active BR compound that is involved in developmental processes, cell division, elongation, gene expression, and vascular differentiation, etc. (Bergonci et al. 2014). It improves the plant growth by enhancing the chlorophyll contents which have a crucial role to increase photosynthetic capability, improves antioxidant system capacity, surges enzymatic activity, and up-regulates stress response genes [superoxide (SOD), peroxide (POD), catalase (CAT), glutathione reeducates (GR) and ascorbate peroxide (APX)] (Liu et al. 2016, Yuan et al. 2012). Various mutants of BRs showed many kinds of growth flaws such as dwarfism, deep green leaves, late flowering, and male sterility (Chakraborty et al. 2015, Hou et al. 2017) in ideal plants Arabidopsis and Brassica (Clouse et al. 1996, Russinova et al. 2004). These investigations specify that BR has an optimistic response toward numerous types of stresses as well as it stimulates various physiological and molecular mechanisms to improve plant growth by inducing stress tolerance.

The current study is aimed to deliberate the role of Brassinosteroids in lessening aluminum stress in rice plants as well as to drill the association of antioxidant system and capability of brassinosteroids to produce resistance against aluminum stress in rice plants. In this study, the application of brassinosteroids (EBL) to alleviate the aluminum stress in rice plants is scrutinized.

2. Materials and methods

2.1. Brassinosteroids (BRs) preparation

24-Epibrassinolide (EBL) was obtained from the Institute of crop science, Zhejiang University, China. The BR was liquefied in an adequate quantity of ethanol and a stock solution of $10^{-5} \text{ M}$ was prepared by adding ddH$_2$O comprising 0.05% Tween-20 as a surfactant.

2.2. Plant materials and growth conditions
The seeds of two cultivars of *Oryza sativa, L.* (cvs. CY-927 and YLY-689) were obtained from the Center of Seed Science, College of Agriculture and Biotechnology, Zhejiang University, China. Seeds were surface sterilized by using 0.5% NaClO solution for 15 minutes and then washed several times through tap water followed by washing with sterilized distilled water thrice to eradicate the smidgens of the disinfectant. Priming of sterilized seeds was done at 15 °C in darkness for 24 hrs with 0.01µM BRs. Then, seeds were dried back to their original moisture contents at room temperature. The unprimed dry seeds were used as control (CK). After priming, seed germination tests were carried out. Fifty seeds were used for each treatment positioned in a plastic germination box (12 cm × 18 cm) as well as each treatment was replicated three times. Then, incubation of seeds was carried out in a germination chamber at 25 °C under an interchanging cycle of 8 hr lighting and 16 hr darkness for 14 days (Zeng et al. 2006). The incubated seeds were treated with 400µM concentration of Aluminum with nutrient media solution. The composition of nutrient solution was 0.5µM MKNO₃, 0.5 µM MCa(NO₃)₂, 0.5µM MgSO₄, 2.5µM MKH₂PO₄, 2.5 µM NH₄Cl, 100µM Fe–K–EDTA, 30µM MH3BO₃, 5µM MnSO₄, 1µM CuSO₄, 1µM ZnSO₄ and 30µM H₃BO₃, 5µM MnSO₄, 1µM CuSO₄, 1µM ZnSO₄ and 1µM (NH₄)₆Mo₇O₂₄ per liter. The pH of the nutrient solution was adjusted to 5.0 with HCl and NaOH. The aluminum concentration was based on findings from a primary experiment which was conducted on the bases of various concentrations of Aluminum i.e. 0, 100, 200, 300, 400, 500, 600, 700, and 800µM. The aluminum concentrations (100-300) µM exhibited slight damage to plant growth. Although, aluminum concentration 400µM was exhibited substantial damage to plant growth. However, concentrations greater than 500µM were excessively toxic for the growth of the plant.

2.3. Measurement of physiological parameters
Counting of germinated seeds was carried out daily for 14 days. Total germinated seeds were counted on the 5th day of germination and were considered as germination energy. Then, the germination percentage was calculated on day 14th. Germination Index, Mean Germination Time, as well as Vigour Index, was carried out by following formulas (Hu et al. 2005):

\[
GI = \sum \frac{G_t}{T_t}
\]  
\[
MGT = \frac{\sum (G_t \times T_t)}{\sum G_t}
\]

\[
VI = \text{Germination} \times [\text{Shoot length (Clouse et al.)} + \text{Root length (Clouse et al.)}]
\]

\(G_t\) is the total calculated number of germinated seeds on day \(t\), and \(T_t\) is the time conforming to \(G_t\) in days (Hu et al. 2005).

2.4. Experimental design and treatment pattern

The two-week-old seedlings (primed with water as well as primed with 0.01 µM 24-epibrassinolide (EBL)) were treated with a 400 µM concentration of aluminum. The way of the experimental pattern was wholly randomized design (CRD) in addition to the position of the pots, inside the growth chamber was altered every day. The plants were sampled at 21 days to make the various observations.

2.5. Plant growth investigation

The plants were detached and immersed in a bucket, occupied with water, to confiscate the smidgens of the disinfectant, confirming the security of roots. The plants were impaired and the lengths of roots and shoots were measured, followed by their later weighing to record their fresh mass. The roots in addition to shoots were formerly dried out in an oven, run at 80 °C for 24 hr, and assessed to record their dry mass.
2.6. Measurement of photosynthetic pigments

Investigation of photosynthetic pigments such as chlorophyll-a, b, and total chlorophyll was determined by following the methodology of Hartmut et al. In short, Fresh leaf tissues (0.2 g) were standardized in 3 mL ethanol (95%, v/v). The homogenate was centrifuged at 5000 g for 10 min and then, the supernatant was extracted. 9 mL ethanol (95%, v/v) was further supplemented with 1 mL aliquot of the supernatant. Afterward, the mixture was determined by observing the absorbance at the wavelengths 665, 649, and 470 nm through an exhausting spectrophotometer (LICHTENTHALER & Wellburn 1983). The following equations were utilized for the cunning of pigment amounts:

\[
\text{Chlorophyll a (Ca)} = 13.95 \text{A}_{665} - 6.88 \text{A}_{649} \tag{4}
\]

\[
\text{Chlorophyll b (Cb)} = 24.96 \text{A}_{649} - 7.32 \text{A}_{665} \tag{5}
\]

\[
\text{Total chlorophyll content} = \text{Ca +Cb} \tag{6}
\]

The quantities of pigments were premeditated as milligrams per liter of plant excerpt.

2.7. Measurement of metal contents in plant tissues

Aluminum scrutiny was performed on dried roots and shoots. Dry plant samples (0.2 g) for each treatment, were assimilated by using 5 mL concentrated HNO\(_3\) and HClO\(_4\) (5:1, v/v) on a hot plate at 70ºC for almost 5 hr. The digested sample was diluted with 2% HNO\(_3\) to a final volume of 10 ml and sifted. The filtrate was used for the analysis of Al and the microelements Fe\(^{2+}\), Zn\(^{2+}\), Mn\(^{2+}\), and macro elements calcium (Ca\(^{2+}\)), potassium (K\(^+\)), and magnesium (Mg\(^{2+}\)) with an atomic absorption spectrometer (iCAT-6000-6300, Thermo Scientific, USA) (Khan et al. 2013).

2.8. Measurement of MDA contents and H\(_2\)O\(_2\) measurements
MDA concentration was investigated as 2-thiobarbituric acid (TBA) volatile metabolites. Approximately 1.5 mL excerpt was homogenized in 2.5 mL of 5% TBA prepared in 5% trichloroacetic acid (TCA). The mixture was intense at 95 °C for 15 min, besides then hastily chilled on ice. Afterward, centrifugation at 5,000 g for 10 min was carried out; the absorbance of the supernatant was calculated at 532 nm. Amendment of nonspecific turbidity was through by subtracting the absorbance value measured at 600 nm. The concentration of MDA was intended in terms of (nmol mg$^{-1}$ protein). To measure the concentration of hydrogen peroxide ($H_2O_2$), shadowed the protocol designated by (Velikova et al. 2000).

**2.9. Antioxidant enzyme activity assay**

Fresh samples (0.5 g) of both shoots and roots were homogenized in 8 ml of 50mM potassium phosphate buffer (pH 7.0 containing 1mM EDTA$_2$ as well as 0.5% PVP W/V) on ice. Then and there, centrifugation of the homogenate was done for 20 minutes at 12000rpm at 4°C. Supernatants were unruffled in discrete tubes and stored at 80°C. The method of Giannopolitis & Ries, (1977) was followed to examine the activity of superoxide dismutase (SOD). Peroxidase (POD) activity was assessed as designated by (Dobson & Wilson 1992) exhausting the elimination coefficient 25.5 mM$^{-1}$ cm$^{-1}$. Catalase (CAT) activity was inspected conferring to (Aebi, 1984) by the extermination constant of 39.4 mM$^{-1}$ cm$^{-1}$, whereas for the fortitude of ascorbate peroxidase (APX) activity scrutiny was done by the method of (Nakano & Asada 1981).

**2.10. RNA extraction and gene expression analysis**

Antioxidant gene expression was scrutinized by quantitative real-time PCR (qRT-PCR). Total RNA was extracted grounded on the Trizol reagent as described by Gunia, Barnes, & Sah, (2014). For cDNA synthesis, reverse transcription from 1 μg of total RNA through PrimeScript™ RT reagent kit was utilized as well as
q-PCR was performed according to (Zhang et al. 2016). OsActin was used as an inner standard. Primers used for qPCR are specified in Table S1.

**2.11. Statistical Analysis**

One-way analysis of variance treatments with the least significant differences (LSD) was applied as a posthoc test at a 95% assurance interlude amongst numerous data sets, using SPSS v16.0 (SPSS, Inc., Chicago, IL, USA). Three samples for one treatment were conducted from three different pots. Variance (Oberdörster et al.) analysis was done through Duncan's multiple range tests amongst the treatment’s mean to conclude the significant difference at p < 0.05 and 0.01 levels between mean values. Principle component analysis (PCA) and Agglomerative hierarchical clustering (AHC) was accomplished to scrutinize the classification of two different cultivars of rice grounded on their vulnerability toward Al by using XLSTAT.

**3. Results**

**3.1. Supplement of BRs significantly enhanced seed vigor and plant growth**

The current study has demonstrated that germination energy, percentage, vigor index, and germination index were significantly declined under 400µM Al toxicity in both rice cultivars as compared to control (Table 1). More reduction was observed in cultivar CY-927 as compared to cultivar YLY-689. As concerned, priming with 0.01µM concentration of BRs resulted in the resistance against 400µM Al stress as well as it significantly enhanced the germination energy, percentage, vigor index, and germination index under toxicity as compared to the unprimed seeds. The latent study demonstrated that mean germination time is reduced with priming of 0.01µM BRs under toxicity of 400µM Al in both cultivars (Table 1).

Disclosure of Al has shown the phenotypically changes in the shoot as well as root length in both cultivars (Fig. 1, Fig. 2). It was observed that there was a significant difference between shoot length, root length, fresh
weight, and dry weight at 400µM Al exposure in both cultivars. More reduction is observed in cultivar CY-927 as compared to cultivar YLY-689 and significant improvement in shoot length, root length, fresh weight, and dry weight under 400µM Al exposure were noticed with seed priming with 0.01µM BRs (Table 2).

3.2. Seed Priming with BRs significantly increased photosynthetic pigments

The latent study represented that alone treatment of Al caused a significant reduction in Chl a, b and total chlorophyll contents as compared to control. (Fig. 3). This reduction inside photosynthetic pigments was observed in both cultivars under Al stress. The decrease was more noticeable in CY-927 as compared to YLY-689. Seed priming with 0.01µM BRs increased photosynthetic pigments such as Chl a, b, and total chlorophyll contents as compared to control inside both treatments alone as well as under 400µM Al stresses in both cultivars. Plants treated with BRs alone exhibited more photosynthetic pigment than control in both cultivars (Fig. 3).

3.3. Supplement of BRs reduced Al accumulation under Al stress

Roots are the main part that interacts first with heavy metals and the main source of uptake for a nutrient solution as well as heavy metals. A recent study revealed that Al uptake was more in those plants which were primed by water as compared to the plants primed with 0.01µM BRs under Al toxicity. In roots, Al accumulation was observed higher as compared to shoots (Table 3-4). More Al accumulation was pragmatic in cultivar YLY-689 as compared to CY-927. More interestingly, it was observed that K, Ca, Fe and Mn contents decreased by exposure of Al toxicity in both roots and shoots whereas Zn was increased. The same trend was noticed in both cultivars (Table 3-4).

3.4. Supplement of BRs ameliorated Al generated oxidative stress
The presence of Al caused an enhancement in MDA contents in both cultivars as compared to control and also increased the production of H$_2$O$_2$. This increase was observed more significant in CY-927 as compared to YLY-689 cultivar. The application of BRs seed priming reduced the MDA contents as well as H$_2$O$_2$ production significantly inside both cultivars (Fig. 4). The MDA contents were observed higher in shoots as compared to roots under alone treatment of Al was noticed 64.7% in CY-927 and 55.4% in YLY-689. Whereas it was observed 56% and 42% in roots respectively. Moreover, 44.5%, 45% in shoots, and 26%, 39.7% decreased were recorded in roots of both cultivars under primed seeds with 0.01µM BRs respectively.

### 3.5. Determination of Antioxidant enzyme activities

Under alone treatment of Al, stimulated behavior of antioxidant activity was noticed. A recent investigation showed that under 400µM Al concentration; SOD, CAT, POD as well as APX was enhanced more in stressed plants as compared to the control and this effect was observed higher in YLY-689 than CY-927 (Fig. 5). These activities were observed higher in roots as compared to shoots. SOD activity under alone treatment of Al was noticed at 22.5% in CY-927 and 43.6% in shoots of YLY-689 cultivar. Whereas it was observed 47.7% and 58.2% in roots respectively. Interestingly, 44.6%, 46.3% in shoots, and 57.4%, 58.2% increment were recorded in roots of both cultivars under primed seeds with 0.01µM BRs respectively. For CAT activity, under separate treatment of Al was noticed 45% in CY-927 and 59% in YLY-689. Whereas it was observed 39% and 45% in roots respectively. Stimulatingly, 51%, 61% in shoots, and 46%, 58% increment were recorded in roots of both cultivars under primed seeds with 0.01µM BRs respectively. In the presence of Al alone, POD and APX were also noticed higher but with BRs priming this effect was observed further greater however POD activity was not
more enhanced. Under Al treatment, 14.8% in CY-927, 37.4% in YLY-689 POD was recorded in shoots as well as 30.2% and 46.8% were observed in roots respectively (Fig. 5).

3.6. Determination of gene expression analysis

A significant difference was noted in the expression of APX02 in both roots and shoots as compared to control inside both cultivars. Transcriptional level of APX02 was higher under the stress of 400µM Al as well as with the treatment of 0.01 µM BRs under 400µM toxicity. Expression of APX02 was higher in the YLY-689 cultivar as compared to the CY-927 rice cultivar (p < 0.01). Interestingly, the transcriptional level of APX02 in plants that were treated with seed priming of 0.01 µM BRs were higher than stressed plants and data was supporting the results of APX activity (Fig. 5). Correspondingly, the transcriptional level of APX08 was also prominent in both roots and shoots inside both cultivars as compared to control but inside roots, the expression level of APX08 was observed higher in YLY-689 as compared to CY-927 cultivar (Fig. 6a). Additionally, gene expression of CATa and CATb in both cultivars is observed higher in both roots and shoots as well as compared to the gene expression level of the control condition. It was investigated that increase was more pronounced inside YLY-689 as compared to CY-927 cultivars. As a result, under µM Al stress, the gene expression level of CATa and CATb was higher in both roots and shoots in both cultivars as compared to the control (Fig. 6b).

Significant up-regulation of SOD Cu-Zn and SOD-Fe2 gene expression was observed in both cultivars under stressed conditions but it was noticed higher in YLY-689. SOD Cu-Zn and SOD-Fe2 gene transcriptional level was observed higher in roots as compared to shoots. SOD Cu-Zn gene transcriptional level was more clearly up-regulated in roots as compared to control condition irrespective of 400µM Al stress (Fig. 6c). Interestingly, seeds primed with 0.01µM BRs showed higher expression of SOD Cu-Zn and SOD-Fe2 genes as compared to seeds primed with water. It may be happened to modulate the stress condition inside both cultivars by...
up-regulating specific genes expression momentarily irrespective of 400µM Al toxicity concentration (Fig. 6c).

Our data also supports the resultant behavior of antioxidant activities (Fig. 5). It clearly showed that 0.01µM BRs have a significant role to cope with the stress condition as compared to control plants by modulating and regulating the transcriptional expression level of specific genes.

3.7. Determination of cluster and correlation analysis between observations

Based on physiological traits of two different rice varieties; biplot graphs of Principle component analysis were constructed to investigate the sensitive and tolerant groups through F1 and F2 of numerous parameters under distinct treatments for example control primed with water (CY927-H2O, YLY689-H2O), control primed with BRs (CY927-BRs, YLY689-BRs), seed primed with BRs, and treatment under Al stress (CY927-BRs+Al, YLY689-BRs+Al), seed primed with H2O under Al stress (CY92-Al+ H2O, YLY689-AI+ H2O) (Fig7A-C).

MDA, MGT, and H2O2 were grouped and represented a positive correlation between each other. Although, MGT, H2O2, and MDA showed a significantly negative correlation with V.I, F/W, D/W, S.L, R.L, G.E, and G.P but simultaneously exhibited a negative correlation with SOD, POD, CAT, and APX as well. A similar trend was noticed in both varieties (Fig. 7A, and B). PCA analysis of both cultivars (CY927 and YLY689) demonstrated that YLY689 is a tolerant genotype to Al and CY927 as a sensitive genotype. It demonstrated the maximum contribution of F1 (84.92) followed by F2 (10.20), with total contribution of 95.12% in CY927 and for YLY689 the maximum contribution of F1 (86.39) followed by F2 (10.69), with total contribution of 97.09% is noticed. ACH outcomes also confirmed the same response of both varieties under distinct treatments (Fig. 7C). It represented the close correlation between both cultivars (CY927 and YLY689) primed with BRs as well as primed with water than cultivars primed with water under Al stress. Cultivars primed with BRs under Al...
Discussion

Aluminum is more 3rd abundant element in the earth's crust which is present in high quantity but slightly available in a soluble form which causes severe damage to the biological systems (da Silva Leite 2012). At pH less than 5.5, Al is present in an available form which can cause toxicity in plants especially inhibit the roots of plants; (Barcelo & Poschenrieder 2002) reduce shoots length, fresh weight, and dry weight (Table 2) because it has direct exposure to roots and cause inhibition of cell elongation at an early stage and later on cause damage in plants growth and development as well (Čiamporová 2002, Silambarasan et al. 2019). Al also causes nutritional imbalance inside plant because the intrusion in water retention besides membrane permeability (Olivares et al. 2009) such as in recent study; K, Ca, Fe and Mn contents decreased by exposure of Al in both roots and shoots (Table 3-4). Saliva et al., (2010) reported that K, Ca, Fe and Mn contents were reduced by the toxicity generated by Al in rice plants as its effect was more stated in sensitive variety as compared to tolerant. In tomato and maize, Al caused inhibitory effects on K, Ca, Fe, and Mn contents in both roots and shoots (Castro et al. 2010, Simon et al. 1994). As a consequence of it, photosynthetic pigments and associated processes are also affected (Fig. 3) resultant in a caused reduction in plant growth by Al exposure (Table 2). Nevertheless, seeds treated with brassinosteroids (24 EBL) priming showed better response toward Al stress resistance as compared to seeds primed with water (Table 1-2) and enhanced the photosynthetic pigments inside both sensitive, as well as tolerant variety under Al toxicity (Fig. 3) due to the brassinosteroids, stress resistance behavior in amendments and manipulations in plasma membrane under stressed environment (Hamada & Tsuruo 1986) as well as with stimulating the antioxidant enzyme activity (Fig. 5) Moreover, Brassinosteroids act as a proton pump which can enhance water uptake (Mei et al. 2005), regulate suppression and up-regulation
of genes, protein synthesis (Brand et al. 2003) besides nucleic acid stimulation as well as improvement in antioxidant enzyme activity (Khripach et al. 2003). All of these modified effects of brassinosteroids play an important role to stimulate plant growth under stressed conditions. It was reported that Al reduces the stress effect on plants and improves plant growth as well as photosynthetic pigments and water uptake (Abdullahi et al. 2003, Rajewska et al. 2016). Likewise, It inhibited toxicity caused by cadmium in cowpea plants (Santos et al. 2018), reduced salinity stress (Guinney et al. 2015, O’ zdemir &Edwards 2004), Chromium stress in Brassica Juncea L. (Arora et al. 2010), temperature stress (Di Angelantonio et al. 2016, Finlayson &Van der Valk 1995), drought (FILOVÁ 2014, Talaat &Shawky 2016, Talaat et al. 2015) and heavy metal stresses (Li et al. 2016, Singhal et al. 2015, Swamy et al. 2014) as well by improving antioxidant system.

The results obtained from present studies that antioxidant enzyme activities such as catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD) which acts as a defense system during plant stress periods are increased by aluminum stress (Fig. 5) because of interference inside ROS activity (Jones et al. 2006, Yamamoto et al. 2003b). In a likewise recent study, SOD, CAT, and APX have increased in both sensitive as well as tolerant varieties in maize after exposure to Al treatment alone (Liu et al. 2008, Yamamoto et al. 2003a). In tobacco plants, SOD, POD, APX, and CAT activity was increased after Al disclosure (Ghanati et al. 2005). Interestingly, in latent investigation brassinosteroids stimulated more antioxidant activities in both pensive and resistant cultivars (Fig. 5). The role of antioxidant activities i.e. SOD, POD, and CAT, etc. is very important and it is enhanced by BRs after Al exposure to reduce the toxicity induced by Al (Padmanabhan et al. 2010). It's found consistent in various studies that BRs increase the antioxidant enzymatic activity in stress conditions to mitigate various stresses and to (Arora et al. 2008, Baiguz &Hayat 2009) regulate plant's normal behavior (Ali et al. 2008, Sharma et al. 2007). Al stress causes damage inside the membrane as a result hydrolytic enzyme activity reduces and causes enhancement of ROS activity (Fig. 4). It is strongly believed that an increase in ROS
activity causes severe damage inside cellular structure as well as to macromolecules (Halliwell 1999). It is noted
that the application of BRs reduces H$_2$O$_2$ production as well as MDA content under a stress environment (Fig.
4) to protect the plant's membrane from oxidative damage. It lessens the rate of superoxide radicles and as a
result enhances the antioxidant activity in plants (Singh et al. 2008).

Gene expression study by checking the transcriptional level of plants under heavy metal stress provides a more
precise approximation of antioxidant genes toward the behavior of antioxidant enzyme activities. Hence, we
investigated multiple genes related to antioxidant activities to estimate both enzymatic as well as transcriptional
responses of both rice cultivars under stress conditions as compared to control. Transcriptional levels of APX02
and APX08 were higher in our study. This expression has gone toward up-regulation because of NaCl treatment
(Matsumoto et al. 2001). Likewise, APX transcripts influence was up-regulated through augmented levels of
H$_2$O$_2$ in tobacco chloroplasts as consequences of Cu-Zn-superoxide dismutase overexpression (Gupta et al.
1993). Moreover, significant up-regulation of catalase genes (CATa and CATb) was also observed in both roots
and shoots inside both cultivars. There is no significant change in catalase gene expression of leaves were
investigated in Arabidopsis thaliana (Mol et al. 2008). It may have occurred because of the presence of multiple
allo- or isozymes. In contrast, Al causes an increase of catalase gene expression because of the breakdown of
proteins which leads to up-regulation of the transcriptional level. In the present investigation, it is also examined
that gene expression SOD-Cu-Zn and SOD-Fe$_2$ were higher under Aluminium stress which indicated that
oxidative damage inside various cellular compartments were induced by Al toxicity. The pattern of gene
regulation was different because its expression was more up-regulated in roots as compared to shoots in both
cultivars as compared to control. It may happen because roots are the plant's primary interaction point which
first interacts with toxicity caused by Al stress. These interpretations reinforced the opinion of Smeets et al. who
specified that the fundamental mechanism of oxidative stress was dissimilar in the roots as compared to leaves.
Furthermore, the generation of superoxide besides the lipoxygenase activity is the foremost reason for oxidative stress inside roots, although inside the leaves H$_2$O$_2$ was appeared to be an imperative contestant. However, H$_2$O$_2$ was formed close by as a product of augmented Cd content of the leaves, or maybe it attained as an indication from roots, rests to be illuminated (Mol et al. 2008).

Principle component analysis (PCA) is a multivariate method which frequently used to categorize the values based on various biological statuses, quality as well as origins. To identify and categorize the large data set into a small number of vigorously correlated variables PCA is utilized (Shan et al. 2013). ACH disclosed the interaction between different genotypes of rice-based on distinct treatments (Fig 7C). On physiological traits based, various treatments were utilized to distinguish the sensitive and tolerant genotype and to represent the correlation between various traits by using the amalgamation of both PCA and ACH (Fig. 5). V.I, F/W, D/W, S.L, R.L, G.E, and G.P were showed a group and significantly positive correlation between each other but instantaneously negative relation with MGT, H2O2, and MDA. This investigation contributes to cognize the response mechanism of both cultivars (CY927, YLY689) under Al stress as well as seed priming with BRs under Al toxicity in both varieties on morphophysiological, biochemical, and molecular bases.

**Conclusion**

The latest study investigated the clear phytotoxic effect of aluminum (Al) on the physiological, antioxidant system as well as on the molecular mechanism of rice seedling. Data exposed that seed priming with brassinosteroids (BRs) mitigated the venomous effect of aluminum to *Oryza sativa* seeds besides enhanced both germination as well as early seedling growth under toxicity of Al. Antioxidant system (SOD, POD, CAT, and APX) was increased by the response of Al stress in rice plants which was remarkably further persuaded by BRs. Furthermore, a transcriptional study of antioxidant genes also confirmed the same pattern in both cultivars. Consequently, it may be suggested that the modified level of the antioxidant system, however in part, was the
reason for the improvement of resistance level against Al in rice seedlings. A recent study illustrated that YLY-689 proved as a tolerant variety as compared to CY-927 against Al toxicity. Furthermore, the application of BRs increased the degree of resistance by enhancing plant growth, photosynthetic pigments, and other associated processes under aluminum stress inside rice seedlings.

Author contribution

FB, HJ, and GY involved in conceptualization, GY and FB design experiment. FB, AJ, LZ performed experiment and writing manuscript. HC, LJ and CM writing and editing assistance the manuscript. HJ and ZX perform statistical analysis. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

Funding

The authors did not receive support from any organization for the submitted work.

Data availability statement

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Table 1. Seed priming effect with 0.01µM BRs on germination energy, germination percentage, germination index, mean germination time as well as vigour index of two rice cultivars under 400µM Al toxicity.

| Varieties Name | G.E       | G.P       | G.I       | MGT       | V.I       |
|----------------|-----------|-----------|-----------|-----------|-----------|
| CY-927-H₂O    | 88.00a±2.00 | 94.67b±1.15 | 20.13b±1.17 | 2.91b±0.17 | 0.88b±0.17 |
| CY-927-Al     | 46.00d±2.00 | 60.67d±1.15 | 10.87c±0.50  | 3.81a±0.08  | 0.15c±0.07  |
| CY927-BRs     | 90.67a±3.06 | 100.00a±0.00 | 31.62a±0.92  | 2.17c±0.07  | 1.47a±0.16  |
| CY927-BRs+Al  | 69.33c±3.06 | 85.33c±5.03  | 21.49b±1.82  | 2.91b±0.20  | 0.64b±0.06  |
| YLY689- H₂O   | 90.00b±2.00 | 99.33a±1.15 | 23.72b±0.43  | 2.59bc±0.26 | 1.26b±0.15  |
| YLY-689-Al    | 69.33c±1.15 | 72.67c±2.31  | 16.53c±0.58  | 2.88a±0.37  | 0.37d±0.19  |
| YLY689-BRs    | 98.00a±2.00 | 100.00a±0.00 | 32.44a±1.12  | 1.93c±0.14  | 2.06a±0.03  |
| YLY689-BRs+Al | 80.67b±3.06 | 88.00b±2.00  | 23.57b±0.56  | 2.63b±0.08  | 0.74c±0.03  |

Each value is demonstrating the mean of three repeats of every treatment. The similar letters inside a column specify that there was no significant difference at a 95% probability level at the \( p < 0.05 \) level, correspondingly.

Table 2. Seed priming effect with 0.01µM BRs on shoot length, root length, fresh weight and dry weight of two rice cultivars under 400µM Al toxicity.

| Varieties Name | S.L       | R.L       | F/W       | D/W       |
|----------------|-----------|-----------|-----------|-----------|
| CY-927-H₂O    | 14.26b±0.12 | 13.85b±0.37 | 0.41b±0.01 | 0.04a±0.01 |
| CY-927-Al     | 5.52c±0.23  | 6.85d±0.15  | 0.16d±0.01 | 0.01c±0.01 |
| CY927-BRs     | 16.40a±0.08 | 15.29a±0.44 | 0.53a±0.01 | 0.05a±0.00 |
| CY927-BRs+Al  | 8.38b±0.72  | 8.93c±0.46  | 0.29c±0.01 | 0.03b±0.00 |
| YLY689-H₂O    | 15.26a±0.35 | 18.96b±0.47 | 0.48b±0.01 | 0.06a±0.00 |
| YLY-689-Al    | 8.56d±0.15  | 9.19d±0.32  | 0.18d±0.01 | 0.02c±0.01 |
| YLY689-BRs    | 18.23a±0.11 | 19.54a±0.28 | 0.59a±0.01 | 0.06a±0.01 |
| YLY689-BRs+Al | 11.43c±0.22 | 11.48c±0.18 | 0.31c±0.01 | 0.04b±0.00 |
Each value is demonstrating the mean of three repeats of every treatment. The similar letters inside a column specify that there was no significant difference at a 95% probability level at the \( p < 0.05 \) level, correspondingly.

### Table 3. Seed priming effect with 0.01µM BRs on Al uptake and accumulation in shoots of two rice cultivars under 400µM Al toxicity

| Treatment      | Mg (mg/g) | Al (mg/g) | K (mg/l) | Ca (mg/l) | Mn (mg/l) | Fe (mg/l) | Zn (mg/l) |
|----------------|-----------|-----------|----------|-----------|-----------|-----------|-----------|
| CY927-H2O      | 2.32c±0.13 | -         | 31.72d±1.06 | 1.59ab±1.04 | 0.27ab±0.10 | 0.13b±0.10 | 0.05b±0.02 |
| CY927-BRs      | 2.37b±0.74 | -         | 32.43b±2.06 | 2.80a±0.99  | 0.43a±0.19  | 0.20a±0.19 | 0.05b±0.02 |
| CY927-Al       | 2.45a±0.46 | 0.61a±0.06 | 37.48a±1.78 | 1.18ab±0.24 | 0.09b±0.01  | 0.08b±0.01 | 0.07a±0.01 |
| CY927-BRs+Al   | 2.31d±0.17 | 0.43b±0.07 | 32.08c±1.10 | 0.80b±0.09  | 0.12b±0.02  | 0.11b±0.02 | 0.06b±0.00 |
| YLY689-H2O     | 1.77c±0.78 | -         | 26.36c±1.99 | 0.71b±0.48  | 0.15a±0.48  | 0.07a±0.04 | 0.02c±0.01 |
| YLY689-BRs     | 2.20b±0.65 | -         | 32.09bc±1.93 | 0.75ab±0.21 | 0.14a±0.23  | 0.09a±0.02 | 0.03c±0.00 |
| YLY689-Al      | 2.24ab±0.21 | 0.82a±0.34 | 40.43b±2.21 | 0.80ab±0.23 | 0.10b±0.21  | 0.07a±0.01 | 0.05a±0.01 |
| YLY689-BRs+Al  | 3.04a±0.30 | 0.24b±0.26 | 49.60a±0.52 | 1.29a±0.27  | 0.13a±0.27  | 0.10a±0.01 | 0.04b±0.00 |

Each value is demonstrating the mean of three repeats of every treatment. Same letters are representing no significant differentiation at 95% probability level (\( p<0.05 \)).

### Table 4. Seed priming effect with 0.01µM BRs on Al uptake and accumulation in roots of two rice cultivars under 400µM Al toxicity.

| Treatment      | Mg (mg/g) | Al (mg/g) | K (mg/l) | Ca (mg/l) | Mn (mg/l) | Fe (mg/l) | Zn (mg/l) |
|----------------|-----------|-----------|----------|-----------|-----------|-----------|-----------|
| CY927-H2O      | 2.34a±0.17 | -         | 19.69a±1.42 | 0.88a±0.34 | 0.08a±0.01 | 6.16a±0.30 | 0.08b±0.03 |
| CY927-BRs      | 1.52a±0.53 | -         | 14.21a±2.73 | 0.51b±0.33 | 0.07b±0.02 | 4.19ab±1.06 | 0.12a±0.05 |
| CY927-Al       | 1.14d±0.49 | 5.02a±2.34 | 9.81b±0.29 | 0.21bc±0.05 | 0.03b±0.01 | 0.52b±0.23 | 0.14a±0.05 |
| CY927-BRs+Al   | 1.19b±0.42 | 3.92b±1.23 | 12.60ab±1.10 | 0.11c±0.04 | 0.04b±0.02 | 0.46b±0.17 | 0.07b±0.01 |
| Treatment             | Chlorophyll a | Chlorophyll b | Chlorophyll a+b | MDA | H_{2}O_{2} | SOD | CAT | APX | POD |
|-----------------------|---------------|---------------|-----------------|-----|-----------|-----|-----|-----|-----|
| YLY689-H_{2}O         | 1.77b±0.50    | -             | 13.48b±1.29     | 1.31b±1.25 | 0.07a±1.25 | 4.03a±0.94 | 0.02c±0.01 |
| YLY689-BRs            | 2.13a±0.43    | -             | 17.46a±2.10     | 0.76b±0.17 | 0.05ab±0.17 | 3.63a±1.95 | 0.03c±0.00 |
| YLY689-Al             | 1.28cd±0.34   | 8.78a±0.25    | 13.47b±2.94     | 0.30b±0.17 | 0.04ab±0.17 | 1.53b±0.17 | 0.04b±0.00 |
| YLY689-BRs+Al         | 1.32c±0.76    | 7.50ab±2.45   | 11.02bc±1.74    | 1.50a±0.43 | 0.03ab±0.43 | 0.71b±0.24 | 0.05a±0.01 |

Each value is demonstrating the mean of three repeats of every treatment. Same letters are representing no significant differentiation at 95% probability level (p<0.05).

**Figure legends**

- **Fig. 1.** Physiological effect of Al toxicity on rice cultivar CY-927 and mitigation effect by 0.01μM BRs under 400μM Al stress.
- **Fig. 2.** Physiological effect of Al toxicity on rice cultivar YLY-689 and mitigation effect by 0.01μM BRs under 400μM Al stress.
- **Fig. 3.** Seed priming effect with 0.01μM BRs on (A) Chlorophyll a, (B) Chlorophyll b (C) Chlorophyll a+b in leaves of two different cultivars of *Oryza sativa* under 400μM Al concentration.
- **Fig. 4.** Seed priming effect with 0.01μM BRs on MDA contents and H_{2}O_{2} production in shoots and roots of two rice cultivars under 400μM Al toxicity.
- **Fig. 5.** Seed priming effect with 0.01μM BRs on SOD, CAT, APX and POD contents in both shoots and roots of two rice cultivars under 400μM Al toxicity.
- **Fig. 6a.** Effect of seed priming 0.01μM BRs on gene expression of (A) APX02, (B) APX08 in shoots and roots of both cultivars of rice under toxicity of 400μM Al.
- **Fig. 6b.** Effect of seed priming 0.01μM BRs on gene expression of (C) CATa, (D) CATb in shoots and roots of both cultivars of rice under toxicity of 400μM Al.
Fig. 6c. Effect of seed priming 0.01µM BRs on gene expression of (E) SOD Cu-Zn, (F) SOD-Fe, in shoots and roots of both cultivars of rice under toxicity of 400µM Al.

Fig. 7. Bioplot of principle component of 1 and 2 of the PCA extracted from results obtained from physiological data of two different rice cultivars (CY927, YLY689) under various treatments such as control primed with water (CY927-H₂O, YLY689-H₂O), control primed with BRs (CY927-BRs, YLY689-BRs), seed primed with BRs, and treatment under Al stress (CY927-BRs+Al, YLY689-BRs+Al), seed primed with H₂O under Al stress(CY92-Al+ H₂O, YLY689- Al+ H₂O). Sharp angle represented positive, obtuse angle showed a negative correlation, as well as a right angle, demonstrated a correlation between parameters. (A) Physiological parameters of rice variety CY927 illustration through Pearson's correlation coefficients under different treatments. (I) contains POD, CAT, APX, and SOD, (II) Showed G.I, F/W, D/W, G.E, G.P, V.I, and S.L, (III) Illustrated MDA, MGT, and H₂O₂; while (IV) represented R.L. (B) Physiological parameters of rice variety YLY689 representation via Pearson's correlation coefficients under different treatments. Distance between each circle represented the strength of correlation. (I) contains POD, CAT, APX, and SOD, (II) Showed G.I, F/W, D/W, G.E, G.P, and V.I, (III) Illustrated MDA, MGT, and H₂O₂; while (IV) represented R.L and S.L (C) Dendrogram of two different rice cultivars under various treatments obtained through Agglomerative hierarchical clustering using ward’s method on basis of physiological traits.
Fig. 1. Physiological effect of Al toxicity on rice cultivar CY-927 and mitigation effect by 0.01µM BRs under 400µM Al stress

Fig. 2. Physiological effect of Al toxicity on rice cultivar YLY-689 and mitigation effect by 0.01µM BRs under 400µM Al stress

Fig. 3. Seed priming effect with 0.01µM BRs on (A) Chlorophyll a, (B) Chlorophyll b (C) Chlorophyll a+b in leaves of two different cultivars of *Oryza sativa* under 400µM Al concentration
Fig. 4. Seed priming effect with 0.01µM BRs on MDA contents and H$_2$O$_2$ production in shoots and roots of two rice cultivars under 400µM Al toxicity.
Fig. 5. Seed priming effect with 0.01µM BRs on SOD, CAT, APX and POD contents in both shoots and roots of two rice cultivars under 400µM Al toxicity.
Fig. 6a. Effect of seed priming 0.01µM BRs on gene expression of (A) APX02, (B) APX08 in shoots and roots of both cultivars of rice under toxicity of 400µM Al.
Fig. 6b. Effect of seed priming 0.01µM BRs on gene expression of (C) $CAT_a$, (D) $CAT_b$ in shoots and roots of both cultivars of rice under toxicity of 400µM Al.
Fig. 6c. Effect of seed priming 0.01µM BRs on gene expression of (E) SOD Cu-Zn, (F) SOD-Fe₂ in shoots and roots of both cultivars of rice under toxicity of 400µM Al.
Fig. 7. Biplot of principle component of 1 and 2 of the PCA extracted from results obtained from physiological data of two different rice cultivars (CY927, YLY689) under various treatments such as control primed with water (CY927-H₂O, YLY689-H₂O), control primed with BRs (CY927-BRs, YLY689-BRs), seed primed with BRs, and treatment under Al stress (CY927-BRs+Al, YLY689-BRs+Al), seed primed with H₂O.
under Al stress (CY92-Al+ H₂O, YLY689- Al+ H₂O). Sharp angle represented positive, obtuse angle showed a negative correlation, as well as a right angle, demonstrated a correlation between parameters. (A) Physiological parameters of rice variety CY927 illustration through Pearson's correlation coefficients under different treatments. (I) contains POD, CAT, APX, and SOD, (II) Showed G.I, F/W, D/W, G.E, G.P, V.I, and S.L, (III) Illustrated MDA, MGT, and H₂O₂; while (IV) represented R.L. (B) Physiological parameters of rice variety YLY689 representation via Pearson's correlation coefficients under different treatments. Distance between each circle represented the strength of correlation. (I) contains POD, CAT, APX, and SOD, (II) Showed G.I, F/W, D/W, G.E, G.P, and V.I, (III) Illustrated MDA, MGT, and H₂O₂; while (IV) represented R.L and S.L (C) Dendrogram of two different rice cultivars under various treatments obtained through Agglomerative hierarchical clustering using ward’s method on basis of physiological traits.
Figures

Figure 1

Physiological effect of Al toxicity on rice cultivar CY-927 and mitigation effect by 0.01µM BRs under 400µM Al stress

Figure 2
Physiological effect of Al toxicity on rice cultivar YLY-689 and mitigation effect by 0.01µM BRs under 400µM Al stress

Figure 3

Seed priming effect with 0.01µM BRs on (A) Chlorophyll a, (B) Chlorophyll b (C) Chlorophyll a+b in leaves of two different cultivars of Oryza sativa under 400µM Al concentration
Figure 4

Seed priming effect with 0.01µM BRs on MDA contents and H2O2 production in shoots and roots of two rice cultivars under 400µM Al toxicity.
Figure 5

Seed priming effect with 0.01µM BRs on SOD, CAT, APX and POD contents in both shoots and roots of two rice cultivars under 400µM Al toxicity.
Figure 6

Fig. 6a. Effect of seed priming 0.01µM BRs on gene expression of (A) APX02, (B) APX08 in shoots and roots of both cultivars of rice under toxicity of 400µM Al. Fig. 6b. Effect of seed priming 0.01µM BRs on gene expression of (C) CATa, (D) CATb in shoots and roots of both cultivars of rice under toxicity of 400µM Al. Fig. 6c. Effect of seed priming 0.01µM BRs on gene expression of (E) SOD Cu-Zn, (F) SOD-Fe2 in shoots and roots of both cultivars of rice under toxicity of 400µM Al.
Figure 7

Biplot of principle component of 1 and 2 of the PCA extracted from results obtained from physiological data of two different rice cultivars (CY927, YLY689) under various treatments such as control primed with water (CY927-H2O, YLY689-H2O), control primed with BRs (CY927-BRs, YLY689-BRs), seed primed with BRs, and treatment under Al stress (CY927-BRs+Al, YLY689-BRs+Al), seed primed with H2O under Al stress (CY92-Al+ H2O, YLY689- Al+ H2O). Sharp angle represented positive, obtuse angle showed a negative correlation, as well as a right angle, demonstrated a correlation between parameters. (A) Physiological parameters of rice variety CY927 illustration through Pearson’s correlation coefficients.
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