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SHORT RESEARCH AND DISCUSSION ARTICLE

# 24-Epibrassinolide pre-treatment reduces alkaline-induced oxidative stress in red rice seedlings

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## Abstract

Soil alkalinity caused by salts, such as sodium bicarbonate ($NaHCO_3$), and the frequently associated waterlogging problems are pervasive in agriculture and have a deleterious impact on crop production. However, various plant growth regulators, including brassinosteroids, are considered to be important against different abiotic stresses experienced by plants due to drought, salinity, and heavy metal stress. We investigated the putative role of 24-epibrassinolide (EBL), an active brassinosteroid, on red rice plants experiencing alkaline stress. Seedlings were pre-treated with 0.01 μM EBL for 30 min and later, exposed to $NaHCO_3$ (25 mM) and were sampled, 5 days after treatments. Results showed that the pre-treatment of seedlings with EBL under non-stress conditions could promote rice plant growth. Growth parameters including dry weight (DW), root and coleoptile lengths were reduced under alkaline stress, whereas EBL application reduced the level of inhibition, as compared with $NaHCO_3$ treatment. Enhanced levels of malondialdehyde content, hydrogen peroxide, and superoxide radicals were significantly diminished by EBL pre-treatment. Moreover, pre-treatment of EBL to alkaline-treated rice seedlings largely stimulated the enzymatic activities of ascorbate peroxidase, catalase, and superoxide dismutase. Thus, the results suggest that pre-application of EBL significantly ameliorates alkaline stress in rice.

**Keywords** Sodium bicarbonate · Lipid peroxidation · Superoxide anion · Antioxidant enzymes · Stress amelioration

## Introduction

Agricultural crop productivity is constrained by saline and alkaline stresses that seriously affect more than 800 million hectares of land globally (Wei et al. 2015). Principally, the alkalinity problem in arid and semi-arid environments is worse than salinity. In this context, soil alkalinity is observed to be a major abiotic factor leading to an impairment of life processes and perturbing plant growth and yield, particularly in crops including rice, and in acute conditions, may even lead to plant death (Munns and Tester 2008). Previous published works have delineated the role of alkaline salts such as sodium bicarbonate ($NaHCO_3$) and sodium carbonate ($Na_2CO_3$) in causing alkaline stress (Shi and Sheng 2005; Shi and Wang 2005; Guo et al. 2010; Abdel Latef and Tran 2016). Excessive alkaline stress is characterized by an unusually high pH (ranging from 8.5 to 11) that induces a range of negative effects in plants, including ionic stress caused by high accumulation of sodium ions ($Na^+$), and osmotic stress due to water deficit accompanied with an excessive accumulation of free radicals resulting in oxidative stress (Cha-um et al. 2010; Wei et al. 2015; Mir et al. 2018). High pH also results in reduced activity of the root system, decline in photosynthesis, and disruption of membrane integrity (Mir et al. 2018). However, to overcome the stress caused by alkaline salts, plants have well-developed antioxidant defense systems that protect the plant cells against reactive oxygen species (ROS)-induced toxicity (Huang et al. 2014; Wu et al. 2017; Mir et al. 2018).

Of late, attempts have been made to utilize various bioactive molecules such as jasmonic acid (Mir et al. 2018) and nitric oxide (Gao et al. 2012) in alleviating the stress caused by alkaline salts. However, in this context, exogenously applied brassinosteroids (BRs) can also be regarded as a promising alternative as they independently or in conjunction with other

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hormones, play critical roles in plant physiology such as inducing stem elongation, root growth, xylem differentiation, regulating specific gene expression, and photosynthesis (Planas-Riverolo et al. 2019; Peres et al. 2019). BRs bind to a plasma membrane-localized receptor, BRI1 (brassinosteroid insensitive 1) and a co-receptor BAK1 (BRI1-associated receptor kinase 1). It initiates a cascade of phosphorylation events and activates the kinase, brassinosteroid-signaling kinase 1 (BSK1). BSK1 in turn phosphorylates the BRI1-suppressor 1 (BSU1) phosphatase, which then dephosphorylates and inactivates the kinase, brassinosteroid insensitive 2 (BIN2). Inactivation of BIN2 dephosphorylates BZR1 (brassinazole resistant 1) and BES1/BZR2 (BRI1-EMS-suppressor 1) and these unphosphorylated transcription factors accumulate in the nucleus and bind to the target BR-responsive genes that regulate cell growth and development and plant stress tolerance (Planas-Riverolo et al. 2019; Peres et al. 2019). 24-Epibrassinolide (EBL), an active plant growth regulator, has been previously shown to alleviate the harmful effects of various abiotic stressors including drought, salinity, and heavy metal poisoning (Wu et al. 2017; Planas-Riverolo et al. 2019). Previous studies have demonstrated the potential effect of EBL in ameliorating the saline stress-induced toxicity in perennial ryegrass (Sun et al. 2015; Wu et al. 2017), tomato (Ogweno et al. 2008; Yilmaz-Gokdogan and Burun 2017), and rice (Zdemir et al. 2004). Although various studies have been conducted on understanding EBL-mediated salinity stress tolerance in plants, the knowledge of biochemical, molecular, and physiological mechanisms activated in response to the damage caused by alkaline stress in rice, a staple food crop with great economic value, continues to be elusive.

Rice is a staple food crop grown worldwide, especially in Asia, which is capable of growing well in standing water in alkaline soil (Wei et al. 2015). Nevertheless, it is also considered as a sensitive crop to saline (Munns and Tester 2008) and alkaline soils as the growth and development of plants get significantly reduced under alkalinity (Wang et al. 2011; Wei et al. 2015). Hence, the development of alkali-resistant rice plants is critical for improving plant growth and ensuring sustainable rice production in saline and alkaline-stressed paddy fields. Traditionally used colored rice varieties are highly valued as they serve unique nutritional and medicinal qualities. These pigmented rice grains are rich sources of antioxidants and polyphenols and contain more micronutrients as compared with other rice varieties (Bhat and Riar 2015). Recently, colored rice varieties are gaining attention because of the presence of anthocyanins that are known to fight against various biotic and abiotic stresses including salinity and provide protection from oxidative damage by free radicals (Chunthaburee et al. 2016). Moreover, many of these rare varieties, such as red rice cultivar “Bao dhaan” cultivated in Assam, have been reported to be drought resistant (Anonymous 2018). However, no work in the past has been conducted with colored rice varieties vis-à-vis their tolerance and adaptation to alkalinity stress. Therefore, the present study was conducted with an aim to observe the effect of exogenous EBL pre-treatment on the plant growth and various biochemical adaptive responses involved in the amelioration of alkaline stress in red rice seedlings.

## Materials and methods

### Plant material and growth treatment

Seeds of red rice cultivar (“Karad”) were obtained from the local market in district Chamba, Himachal Pradesh, India, and used for the study. For surface sterilization, these were dipped in sodium hypochlorite (0.1%, *w*/*v*) solution for 20 min and thoroughly washed five times with deionized water. After soaking the seeds overnight in distilled water, they were germinated in plastic trays (33 cm × 22 cm × 8 cm), layered with moist cotton and a single sheet of filter paper. These were kept in a plant growth chamber maintained at 30 ± 2 °C room temperature, 75 ± 1% relative humidity, 12 h photoperiod and provided with an irradiance of ~ 240 μmol $m^{-2}$ $s^{-1}$. After 3–4 days of growth, seedlings were grown hydroponically for 24 h on a nylon mesh, with their roots dipped in 500 mL beakers filled with distilled water for acclimatization. For the pre-treatment of seedlings, 0.01 μM EBL (hereafter, EBL only) was prepared after initially dissolving in 100 μL of absolute ethanol. Seedlings were then primed with deionized water (for control) or dipped in EBL for 30 min. The selected concentration of EBL is non-toxic for plant growth and based on the earlier studies (Sun et al. 2015; Dong et al. 2017; Wu et al. 2017). A total of four treatments were prepared: (i) distilled water alone (control), (ii) distilled water + 0.01 μM EBL (hereafter, EBL), (iii) 25 mM $NaHCO_3$ (hereafter, $NaHCO_3$), (iv) 25 mM $NaHCO_3$ + 0.01 μM EBL (hereafter, $NaHCO_3$+EBL). The concentration of $NaHCO_3$ used in the present study is based on earlier findings that 20 mM of $NaHCO_3$ inhibited the root length in grasses by 67–91% (Lee and Woolhouse 1969) and caused a reduction of 38% in plant height in maize (Al-Mansouri and Alhendawi 2014). After exposing the primed and non-primed seedlings to distilled water or $NaHCO_3$ solution, they were kept in growth chamber at conditions described above. On the 6th day, seedlings were harvested and the root and coleoptile were excised and further biochemical tests were performed after storing the material at − 20 °C. Root and coleoptile lengths were also recorded by measurement with a centimeter ruler.

### Determination of superoxide anion ($O_2^{\bullet-}$) generation and hydrogen peroxide ($H_2O_2$) content

$H_2O_2$ content was quantified spectrophotometrically at 390 nm based on the oxidation of potassium iodide (KI), as described by Singh et al. (2008). The method involved homogenization of 50 mg root tissue in 5 mL of 0.1%, *w/v*, trichloroacetic acid (TCA). After centrifuging at 10,000 rpm for 25 min at 4 °C, a 0.5 mL aliquot was added to the solution containing 0.5 mL phosphate ($PO_4^{3-}$) buffer (pH = 7.0) and 1 mL KI (1 M). $H_2O_2$ content was calculated in terms of nM $g^{-1}$ DW (dry weight).

The generation of $O_2^{\cdot-}$ radical was measured by extracting 80 mg of root tissue using a $PO_4^{3-}$ buffer (8 ml; 100 mM; pH = 7.0) and centrifuging as previously described. Two hundred microliters of root extract was mixed with 1.8 mL solution containing adrenaline (1 mM) and $PO_4^{3-}$ buffer (75 mM; pH = 7.4). The difference in absorbance values at 480 nm was detected after 0 and 5 min and $O_2^{\cdot-}$ contents were expressed in μM $g^{-1}$ DW (Misra and Fridovich 1972).

### Measurement of malondialdehyde content

The amount of malondialdehyde (MDA) was calculated as a measure of lipid peroxidation as per Singh et al. (2008). One milliliters of supernatant obtained from homogenized root tissue in TCA (as above for $H_2O_2$ content) was treated with a solution prepared by mixing 4 mL of 0.5% thiobarbituric acid in 20% TCA and kept for 30 min in an oven set at 95 °C. The solution was subsequently ice-cooled and centrifuged again. The difference in the amount of absorbance was recorded at dual wavelengths (532 nm and 600 nm) for calculating the MDA content in terms of nM $g^{-1}$ DW.

### Measurement of antioxidant enzyme activity

Root sample (80 mg) was ground in liquid nitrogen and after extracting in a $PO_4^{3-}$ buffer (8 mL; 100 mM; pH = 7.0), the extract was subjected to centrifugation at 10,000×*g* at 4 °C rotor temperature for 30 min. Thereafter, an appropriate amount of supernatant was taken and used for determining the antioxidant enzyme activity.

For the estimation of catalase (CAT) activity, 0.05 mL extract was mixed in 1.8 mL solution containing $H_2O_2$ (10 mM) and $PO_4^{3-}$ buffer (25 mM; pH = 7.0) (Singh et al. 2008). The enzymatic activity was assayed spectrophotometrically by observing the disappearance rate of $H_2O_2$ at 240 nm for a period of 1 min and the unit was recorded as enzyme units (EU) per milligram tissue. The enzymatic activity of ascorbate peroxidase (APX) was measured by the consumption of ascorbic acid at a wavelength of 290 nm after a period of 1 min and expressing the unit as EU per milligram tissue (Kaur et al. 2015). The quantification of extent of reduction in the appearance of nitroblue tetrazolium chloride (NBT) at 560 nm was evaluated as a measure of superoxide dismutase (SOD) activity and recorded as EU per milliliter (Singh et al. 2008).

### Experimental design and statistical analysis

Treatments were completely assigned at random for the hydroponically designed experiment arranged as 2 × 2 factorial, with three replicates per treatment ($n = 3$). Data were analyzed separately for each of the parameters mentioned above by one-way analysis of variance with alkaline salt concentration kept as a fixed factor. A post hoc Tukey's test at 5% level of significance was used to determine the significant differences among treatments, as analyzed by SPSS software.

## Results

### Pre-treatment with EBL improves the growth of rice seedlings

Rice seedlings immersed in $NaHCO_3$ solution showed a significant decline in the root and coleoptile length by 25.3% and 27%, respectively, in comparison with the control (0 mM $NaHCO_3$+0 mM EBL), noticed after 5 days (Table 1). However, EBL pre-application alleviated the inhibitory effects of alkaline stress and thus, significantly ($p \leq 0.05$) enhanced the root and coleoptile length of the seedlings by ~ 57% and 48% relative to the $NaHCO_3$ treatment, respectively (Table 1).

### EBL declines $H_2O_2$ content and $O_2^{\cdot-}$ generation rate

The production of $H_2O_2$ was enhanced by 31.6% in $NaHCO_3$-treated rice seedlings over the untreated seedlings (control) (Table 2). However, alkaline-stressed seedlings pre-treated with EBL significantly ($p \leq 0.05$) decreased $H_2O_2$ production by ~ 23% and 27%, respectively in comparison with control and only $NaHCO_3$-exposed seedlings. Compared with

**Table 1** Effect of 24-epibrassinolide (EBL) on root and coleoptile length of red rice seedlings treated with 25 mM of $NaHCO_3$, measured 5 days after exposure

| Treatments | Root length (cm) | Coleoptile length (cm) |
|---|---|---|
| 0 (control) | $8.3 \pm 0.33^a$ | $8.9 \pm 0.55^a$ |
| EBL (0.01 μM) | $10.7 \pm 0.27^b$<br>(+ 28.9%) | $11.4 \pm 0.40^b$<br>(+ 28.1%) |
| 25 mM $NaHCO_3$ | $6.2 \pm 0.15^c$<br>(− 25.3%) | $6.5 \pm 0.29^c$<br>(− 27.0%) |
| $NaHCO_3$+EBL | $9.7 \pm 0.67^{ab}$<br>(+ 16.9%) [+ 56.5%] | $9.6 \pm 0.41^{ab}$<br>(+ 7.9%) [+ 47.7%] |

Data represented as mean ± SE of three replications. Different letters in a column represent significant differences among the treatments, according to post hoc Tukey's test at $p \leq 0.05$. Values within the bracket indicate the percent increase (+) or decrease (−) in root and coleoptile length, relative to the control, whereas those in square bracket indicate percent increase [+] compared with $NaHCO_3$ alone-treated seedlings

**Table 2** Effect of 24-epibrassinolide (EBL) on the contents of hydrogen peroxide ($H_2O_2$), malondialdehyde (MDA), and superoxide anions ($O_2^{\bullet -}$) in red rice seedlings treated with 25 mM $NaHCO_3$, measured 5 days after exposure

| Treatments | Hydrogen peroxide content (nmol/g DW) | Malondialdehyde content (nmol/g DW) | Superoxide anions (μmol/g DW) |
|---|---|---|---|
| 0 (control) | $7.9 \pm 0.06^a$ | $7.7 \pm 0.05^a$ | $0.14 \pm 0.05^a$ |
| EBL (0.01 μM) | $6.1 \pm 0.62^b$<br>(− 22.8%) | $5.0 \pm 0.27^b$<br>(− 35.1%) | $0.12 \pm 0.04^a$<br>(− 14.28%) |
| 25 mM $NaHCO_3$ | $10.4 \pm 0.21^c$<br>(+ 31.6%) | $9.4 \pm 0.18^c$<br>(+ 22.1%) | $0.81 \pm 0.01^b$<br>(+ 478.57%) |
| $NaHCO_3$+ EBL | $7.6 \pm 0.27^{ab}$<br>(− 3.8%) [− 26.9%] | $5.6 \pm 0.29^b$<br>(− 27.3%) [− 40.4%] | $0.58 \pm 0.04^c$<br>(+ 314.29%) [− 28.4%] |

Data represented as mean ± SE of three replications. Different letters in a column represent significant differences among the treatments, according to post hoc Tukey's test at $p \leq 0.05$. Values within the bracket indicate the percent increase (+) or decrease (−) in root and coleoptile length, relative to the control, whereas those in square bracket indicate percent increase [+] compared with $NaHCO_3$ alone-treated seedlings

untreated seedlings, $O_2^{\bullet -}$ accumulated at an exceptionally higher rate ($p \leq 0.05$) in rice seedlings exposed to $NaHCO_3$ solution. However, priming with EBL caused a significant reduction of 28.4% compared with $NaHCO_3$-stressed seedlings, when observed 5 days after exposure (Table 2). Thus, the data demonstrated that pre-treatment with EBL significantly diminishes $NaHCO_3$-induced ROS accumulation in the roots and protects the seedlings from oxidative injuries.

### EBL pre-treatment reduces lipid peroxidation

Roots of alkaline-stressed seedlings exhibited 22.1% increase in the MDA content, relative to the control ($p \leq 0.05$). However, the amount of MDA declined significantly ($p \leq 0.05$) by 40.4% in $NaHCO_3$+EBL-treated seedlings, as compared with only $NaHCO_3$ treatment and 35.1% in comparison with the control, 5 days after the treatment (Table 2).

### EBL modulates antioxidant enzymes

The relative increase in CAT activity over the control was 82.4% for the alkaline-stressed rice seedlings ($p \leq 0.05$) (Fig. 1a). Pre-treatment of EBL, however, resulted in an enhancement in CAT activity by ~ 52%, as compared with the 25 mM alkaline-stressed seedlings. In addition, unstressed seedlings exposed to EBL showed an increase in CAT activity by ~ 53%, in comparison with the control (Fig. 1a).

EBL pre-treatment enhanced APX activity by ~ 89% over that in the unstressed seedlings. Similarly, a significant ($p \leq 0.05$) increase of ~ 50% in APX activity was noticed for $NaHCO_3$+EBL-treated seedlings, relative to that of the seedlings only treated with $NaHCO_3$ (Fig. 1b).

Application of EBL to unstressed rice seedlings did not alter SOD activity (Fig. 1c). On the contrary, a decrease of ~ 32% in SOD activity was recorded in $NaHCO_3$-treated seedlings relative to the untreated control seedlings ($p \leq 0.05$). However, application of EBL to alkaline-stressed seedlings reduced the decline and thus, promoted SOD activity by 9.1%, compared with only $NaHCO_3$-treated seedlings (Fig. 1c).

## Discussion

The present study explored the protective role played by EBL in shielding red rice plants against alkaline stress. The results show that alkaline stress imposed by 25 mM $NaHCO_3$ caused a significant decline in the overall growth of rice seedlings as noticed by a reduction in the root and coleoptile length. This observation is in agreement with previous reports where a decline in the relative growth of rice, especially in the roots, was reported with increasing alkaline stress (Wang et al. 2011). Previously, alkaline stress has been correlated with higher accumulation of $Na^+$ ions that promote osmotic stress due to water deficit, and thus, impair the intracellular ion homeostasis within plants (Mir et al. 2018). Higher pH of alkaline soil inhibits root-mediated uptake of nutrients and declines photosynthetic rate, thereby preventing the accumulation of biomass and interfering with stem and root elongation (Guo et al. 2010). However, priming with EBL improved the plant growth and biomass in alkaline-stressed seedlings. This is in agreement with earlier works in which exogenously supplemented 24-EBL restored growth parameters in saline-stressed rice (Zdemir et al. 2004), pepper plants (Houimli et al. 2010), wheat (Dong et al. 2017), cotyledon and hypocotyl explants of tomato (Yilmaz-Gokdogan and Burun 2017), and cotton (Surgun et al. 2015).

Alkaline stress resulted in an enhancement of $H_2O_2$ content and $O_2^{\bullet -}$ generation rate, thereby resulting in oxidative stress due to an excessive generation of ROS. Similar results were

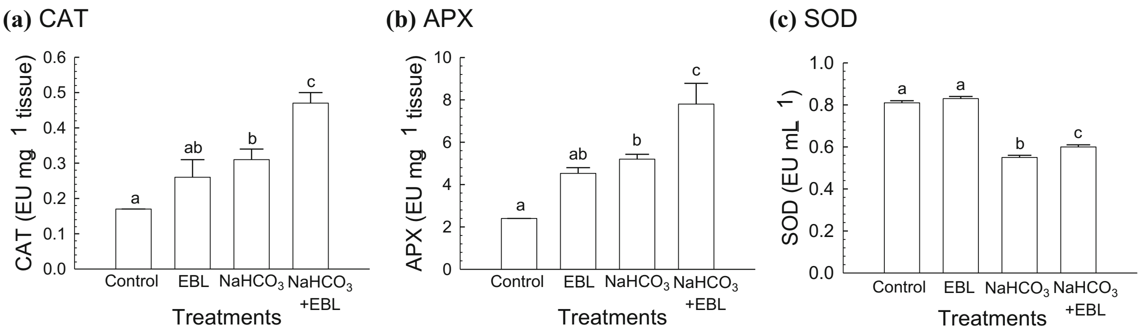

**Fig. 1** Effect of pre-treatment of 24-epibrassinolide (EBL, 0.01 μM) on the activities of catalase (CAT) (**a**), ascorbate peroxidase (APX) (**b**), and superoxide dismutase (SOD) (**c**) in red rice seedlings treated with 25 mM $NaHCO_3$, measured 5 days after exposure. Data represented as mean ± SE of three replications. Different letters represent statistically significant differences among the treatments, according to post hoc Tukey's test at $p \leq 0.05$

obtained in NaCl-treated wheat plants (Dong et al. 2017), $Na_2CO_3$-treated maize seedlings (Mir et al. 2018), and mulberry plants (Ahmad et al. 2014). In addition, exposure to alkalinity increased MDA content, resulting in lipid peroxidation and oxidative stress injury. This is consistent with the observation of Ahmad et al. (2014) who described that increasing the severity of alkaline stress is responsible for an increase in the MDA content in mulberry plants. Undoubtedly, pre-application of EBL to alkaline-stressed seedlings decreases the production of $H_2O_2$ and other ROS, thus preventing the disruption of membranes. These results are in agreement with the previous observations reporting lesser accumulation of MDA and thus, confirming significant reduction in lipid peroxidation in EBL-treated rose-scented geranium under cadmium stress (Rao and Raghu 2017) and zinc-stressed radish (Ramakrishna and Rao 2012). Therefore, supplementation of EBL to alkaline-treated plants protects the membrane from oxidative injury as evinced by the decreased $H_2O_2$ and MDA contents, lowered rate of generation of superoxide radicals, and increased enzymatic activities of the antioxidants such as CAT, APX, and SOD. Plant responses to oxidative stress are chiefly orchestrated by a group of several ROS-protective enzymes including SOD, CAT, and APX that detoxify abiotic stress-induced ROS accumulation. Superoxide dismutase acts as a frontline defense system for the dismutation of superoxide radicals to $H_2O_2$, which is subsequently metabolized to water by the action of CAT, APX, and POD (Singh et al. 2008). The results of our study indicated an enhancement in the enzymatic activities of CAT and APX in alkaline-stressed rice seedlings. This increase corroborates the findings of Rao and Raghu (2017) in rose-scented geranium plants exposed to cadmium stress. However, our study unveiled that alkaline stress reduced SOD activity, which suggests that there is an overproduction of ROS and the oxidative stress-induced membrane injury might not be sufficiently counteracted by SOD activity (Dong et al. 2017). This observation is supported by the results of recent studies that depicted a decrease in SOD activity in wheat (Dong et al. 2017) and perennial ryegrass (Wu et al. 2017) exposed to sodium chloride (NaCl) solution. However, EBL application modulated the enzymatic activities, thereby maintaining SOD, APX, and CAT activities above the untreated control level and also improving the activity in alkaline-treated seedlings. Our findings are consistent with a study by Wu et al. (2017) who demonstrated that application of EBL alleviated the decline in SOD and CAT activity in perennial ryegrass under salt stress. Similarly, another study by Shang et al. (2006) speculated that exogenously applied EBL boosted the levels of SOD and CAT in cucumber seedlings treated with NaCl. Zdemir et al. (2004) also evidenced the improved activities of antioxidant enzymes in untreated as well as salt-stressed rice plants supplied with EBL.

In conclusion, this study showed the potential of EBL in masking the effect of alkaline stress as an effective approach for enhancing alkalinity tolerance in rice plants and thereby, increasing agricultural productivity. Pre-application of EBL improves the growth and development in plants under alkaline stress and also protects the plants from oxidative stress by harmonizing the biochemical activities with antioxidant enzymes. Thus, our results suggest that pre-treatment of EBL at low concentration to alkaline-stressed rice plants may prove to be a sustainable approach in contributing towards stress amelioration and enhancing crop yield in agricultural lands affected with alkalinity.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflicts of interest.

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