

A NOVEL ABA FUNCTIONAL ANALOGUE B2 ENHANCES SALINITY TOLERANCE IN WHEAT

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Abstract. Salinity stress is considered to be the most damaging type of abiotic stress, with devastating effects on wheat production. Abscisic acid (ABA) is a plant hormone that plays an important role in inducing the plant's response to abiotic stresses. Based on the structures of ABA, pyrabactin (PYR) and coronatine (COR), a target chemical compound, B2, was designed in this study. In a previous study, B2 enhanced drought tolerance in wheat by enhancing the ROS capacity, which was achieved by improving the antioxidant enzymes activity and the osmotic adjustment ability. Furthermore, it had a significant effect on improving photosynthesis and increasing the endogenous hormones in wheat under stress conditions. Two wheat cultivars (*Triticum aestivum* L.), JIMAI22 (JM22) and SHANNONG12 (SH12), were grown under hydroponic conditions to explore the effect of B2 in mitigating salt stress. The results showed that B2 had a function analogous to ABA, especially at a concentration of 0.1 $\mu\text{mol}\cdot\text{L}^{-1}$. Under a salinity stress of 150 mM NaCl, treatment with 0.1 $\mu\text{mol}\cdot\text{L}^{-1}$ B2 increased the leaf water content in both wheat cultivars, improving their photosynthetic efficiency and the antioxidant enzyme activity. Moreover, B2 enhanced the expression level of ABA-responsive genes (*TaABAOH2*, *TaNCED1*, and *TaAREB3*). Furthermore, it enhanced the expression level of salt-responsive genes (*TaMYB3R1* and *TaERF3*). Moreover, B2 also induced the endogenous plant hormone (ABA) signaling pathway, which led to an improvement of the salt tolerance in the wheat cultivars.

Keywords: *abscisic acid alternatives, salt stress, ABA and salt-responsive genes, hormonal signaling antioxidant, Triticum aestivum*

Introduction

Wheat is recognized as one of the most commonly cultivated food crops throughout the world and the primary source of proteins and carbohydrates. Current wheat production does not meet the demands of a rapidly growing global population. While plant breeders are working hard to improve wheat production, it is difficult to achieve the required production level due to complex issues, especially abiotic stresses (Jatoi et al., 2011; Moaveni, 2011).

Currently, soil salinity and drought are considered the most damaging abiotic stresses, affecting overall agricultural production. Salinity plays a crucial role in limiting plant production, especially in barren and semi-barren regions (Ashraf et al., 2004; Hussain et al., 2009). The main cause of salt stress in crops could be the use of saline water for irrigation purposes, due to the excessive amount salt in the ground water. Plants have internal mechanisms that help them realize the incoming stresses and regulate their physiological and metabolic processes accordingly to face the calamities (Zhang et al., 2006).

The plant root system rapidly senses the salt stress, which reduces the plant growth. In the short term, the water availability to the plant is hindered by increasing osmotic stress due to a high salt content, while in the long term, it is hampered by the raised ion toxicity, causing a nutrient deficit in the cytosol (Munns, 2005). A high salt concentration leads to a shortage in plant water uptake, even in well-watered soil, due to a reduction in the osmotic potential of the soil solutes, which makes it difficult for the plant roots to absorb water from the soil solution (Rengasamy, 2006). A high level of salinity also substantially reduces the pigment content of the leaves (Al-Sobhi et al., 2006).

High concentrations of NaCl, such as 125 mM and 150 mM, have a completely inhibitory, rather devastating, effect on most of the wheat cultivars (Amor et al., 2005), as most of the growth processes are badly affected. Growth rates and biomass production are efficient indicators to evaluate the level of the stress caused by salt and a plant's ability to resist it (Amor et al., 2005).

The higher salinity concentrations show clear effects on root and shoot lengths, since the roots are directly exposed to salt. A high level of NaCl causes a reduction in root and shoot development due to its toxic effects and an imbalanced nutrient uptake by plants. It also inhibits root elongation and development by reducing the water uptake by plants.

High salt concentrations decrease the water potential of plants and affect the water availability for the plant physiological functions (Jakab et al., 2005). The osmotic stress, as a result of salt accumulation, leads to a reduction of water absorption through roots and hence creates a water deficit in wheat leaf (Rahnama et al., 2010).

One of the main defense strategies in plants is their antioxidant system. This includes enzymatic and non-enzymatic compounds, which act as detoxification agents, scavenging toxic free radicals and helping the plants overcome destructive oxidative stress (Parida et al., 2004; Li et al., 2011). The major antioxidant defense enzymes are superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD). SOD can scavenge the toxic O₂⁻ radicals, and its enzymatic action results in the formation of H₂O₂, which is subsequently converted to H₂O by POD (Li et al., 2011).

ABA is one of the plant hormones that has the ability to regulate the plant physiological responses. It is known as a stress hormone because of its rapid accumulation in response to stresses and its mediation of stress responses, which helps plants to overcome stresses (Zhang et al., 2006). Exogenous ABA application accumulates proline by activating its biosynthesis and inhibiting its degradation pathways, which in turn plays a protective role in salt-stressed plants through scavenging free radicals (Wang et al., 2017). Recent studies show that plant growth responses to abiotic and biotic stresses can be regulated by plant hormones. These hormones have interrelated growth, inducing or inhibiting activities, which leads to a modulation of different plant biosynthesis and physiological responses (Peleg and Blumwald, 2011).

High plants have developed strategies to understand stress signals and modulate the specific stress-responsive gene expressions to acclimate to the stress conditions. Many genes induced by stress have been identified, such as key enzymes of the ABA biosynthesis pathway, osmotic adaptation proteins, cell protective enzymes and transcription factors (TFs). TFs, including AP2/ERF proteins, ABA responsive element (ABRE)-binding proteins and MYB, which play an important role in controlling stress-related gene expressions. Previous studies declare that different ERF members play various functions in plant responses to abiotic stress (Rong et al., 2014).

To synthesize the B2 compound, the sub active structure splicing method of integrating ABA, Pyrabactin (PYR), and coronatine (COR) was used, based on the similarities of the amide structure of COR and the sulfonamide-structure of PYR (Zhou et al., 2019) (*Fig. A1* in the *Appendix*).

In a previous study, B2 enhanced drought tolerance in wheat by enhancing the ROS capacity, which was achieved by improving the antioxidant enzymes activity and the osmotic adjustment ability. Furthermore, it had a significant effect on improving photosynthesis and increasing the endogenous hormones in wheat under stress conditions (Zhou et al., 2019).

Jimai22 is widely known as salt tolerant genotype (Dugasa et al., 2019) however Shannong12 was used as test material as it is not investigated for its salinity tolerance previously.

In the present study, the efficiency of compound B2 in enhancing wheat tolerance to salinity stress was investigated. This was because the function of this compound is analogous to that of ABA. The study was conducted to observe the morphological and physiological changes, occurring in different wheat cultivars. Moreover, the variation of the expression level of the related genes, with the use of B2 under high saline conditions, was also investigated.

Materials and methods

Wheat seedlings preparation and salt stress treatment

The experiment was conducted in Research Institute China Agricultural University, Beijing, China. Wheat seeds were sterilized by soaking them in 5% sodium hypochlorite solution for 10 min. The seeds were then washed 3–4 times with distilled water and planted in a plastic net, placed in a hydroponic box. At the one-leaf stage, after germination, the seedlings were transferred into plastic hydroponic boxes, containing Hoagland solution (Hoagland and Arnon, 1950). All the growth-boxes were placed in a greenhouse, set at 22–25 °C, 50% humidity and 14 h light and 10 h dark conditions, with a light intensity of 400 mol·m⁻²·s⁻¹. Two wheat cultivars, Jimai22 (JM22) and Shannong12 (SH12), were grown to study their performance. After 48 h, when the seedlings were at the two-leaf stage, the chemical compound B2 and ABA treatments were applied by soaking the roots in the treatment solutions for 48 h. The ABA concentration was 0.01 μmol·L⁻¹. B2 was added to the concentrations of 0.1 μmol·L⁻¹, 0.01 μmol·L⁻¹ and 0.001 μmol·L⁻¹. After the treatment, the seedlings were transplanted into Hoagland nutrient solution. Five days later, seedlings were transplanted into Hoagland nutrient solution, containing 150 mM NaCl for salt stress treatment. For the purpose of conducting different analyses, leaf samples were taken from the seedlings, including those under the normal conditions (NC) and NaCl treatments. The treatments were replicated three times, average values of five seedlings were used for each replicate, and samples were collected for physiological measurements or RNA extraction.

Plant growth and biomass accumulation

For the measurement of fresh weight and dry matter accumulation, shoot, and root were separated and weighed for their fresh weight. The shoot and root dry weight was

recorded after drying in hot air oven at 65 °C for 72 h. All the related traits were recorded after eight days of planting.

Root characteristics

Three plants from each treatment were selected, shoot and roots were separated. The roots of each plant were scanned, (the roots of the same plant were submerged in a water tray to ensure separation of roots and to reduce their overlap, and the roots were scanned by using Epson photo scanner (Epson, Long Beach, CA). Scanned images of roots of respected treatments were analyzed using WinRHIZO Pro image analysis software (Regent Instruments, Inc., Quebec City, QC) to calculate the total root length (sum of the lengths of all roots from the same plant), root volume and total root surface area.

Relative water content (RWC)

Three leaves from each treatment were taken for relative water content (RWC) measurement. The fresh leaf weight (FW) was recorded. The leaves were then dipped in distilled water, for 24 h. The leaves were taken out of the water to record the turgid weight (TW). To record the dry leaf weight (DW), the leaves were dried in an electric oven, set at 65 °C for 72 h. The relative water content (RWC) of the leaves was calculated as per the formula given by Cornic (1994).

Antioxidant enzymes extraction and assays

The supernatant, used for the detection of SOD, POD and CAT activities, was according to the protocol described by Parida et al. (2004), where 0.5 g of leaf tissue was homogenized in 4 mL of 50 mM sodium phosphate buffer (pH = 7.0), containing 1% (w/v) PVP, 0.1 mM EDTA-Na₂, 1 mM·L⁻¹ iso ascorbic acid and 0.05% (w/v) Triton X-100 in an ice bath. The homogenate was centrifuged at 12000 rpm and 4 °C for 15 min.

The soluble proteins content was quantified using the Bradford (1976) method, absorbance values, obtained by a standard curve was prepared with bovine serum albumin.

ABA was extracted and purified using the methods of Bollmark et al. (1988) and Yang et al. (2001) with some modifications. Quantifications were conducted using the ELISA approach. The mouse monoclonal antigens and antibodies, used in ELISA, were produced at the Phytohormones Research Institute China Agricultural University (checked and proven).

Malondialdehyde (MDA) and hydrogen peroxide (H₂O₂) content

For the determination of lipid peroxidation in shoots was done by, thiobarbituric acid (TBA) test, which determines Malondialdehyde (MDA) as an end product of lipid peroxidation (Heath and Packer, 1968). Fresh leaf material (500 mg) was homogenized in 5 ml 0.1% (w/v) TCA solution. The homogenate was centrifuged at 10000× g for 20 min, and 0.75 ml of the supernatant was added to 1.5 ml of 0.5% (w/v) TBA in 20% TCA. The mixture was incubated in boiling water for 30 min, and then reaction stopped by placing the reaction tubes in an ice bath. Then the samples were centrifuged at 10000×g for 5 min, and the absorbance of the supernatant was recorded at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The amount of MDA-TBA complex (red pigment) was calculated from the extinction coefficient 155 mM⁻¹ cm⁻¹.

$$\text{MDA (nmol g}^{-1} \text{ fresh weight)} = (\text{Abs532} - \text{Abs600})/155 \quad (\text{Eq.1})$$

H₂O₂ levels were determined according to Alexieva et al. (2001). The absorbency of the supernatant was recorded at 390 nm. The content of H₂O₂ was given on a standard curve.

Proline content

Free proline content in shoots of wheat seedling was extracted with 3% sulfosalicylic acid and centrifuged at 12 000×g. An aliquot was mixed with glacial acetic acid and acidic ninhydrin for 30 min at 100 °C. The reaction was terminated in an ice bath, and chromophore was extracted with five mL of toluene. The chromophore-containing toluene was warmed to room temperature, and absorbance was recorded at 520 nm (Bates et al., 1973).

Determination of photosynthetic parameters

Photosynthetic speed using a portable photosynthetic apparatus LI-6400 (LI-Cor Inc, Lincoln, NE, USA) to measure the photosynthesis rate, stomatal conductance, intercellular CO₂ concentration and transpiration rate. Measurement conditions: leaf surface temperature: 25-28 °C, light intensity 1000 μmol/m² s, flow rate 500 mL/s. Each treatment was repeated 3 times, and the newly developed leaves of the seedling leaves were selected at the measurement site.

Statistical analysis

Two separate experiments were done each one contains one cultivar using complete randomized design (CRD) was used with three replications for each treatment. Analysis of variance (ANOVA) was carried out using Proc Mixed of SAS package version 9.2 (SAS 2008) and means were compared by Duncan's multiple range test at 5% level of probability (Steel and Torrie, 1980).

Extraction of RNA and quantitative RT-PCR

Total RNAs were extracted from the leaves using an Easy Pure Plant RNA Kit (Trans Gene Biotech, Beijing). A Nano-Drop2000 spectrophotometer was used to determine the concentrations. Two μg of the total RNA was used to synthesize cDNA, as per the method described in detail by Zhou et al. (2019). Each sample had three independent replicates, and each replicate had three biological repetitions. The 2-ΔΔCt formula of Livak and Schmittgen (2001) was used to calculate the relative expression level, where reference gene was β-actin gene (Gene Bank ID: AB181991.1) (*Table A1* in the *Appendix*).

Results

B2 effect on plant growth and biomass accumulation, root characteristics and relative water content (RWC) under salt stress

Significant differences were found among different treatments, according to statistical analyses, in terms of the plant dry weight and root/shoot ratio of wheat seedlings under salinity stress conditions.

The data regarding the total plant dry weight of seedlings pretreated with B2 showed that cv. JM22 had a 36.8% increase, and cv. SH12 had a 22.7% increase, in plant dry

weight (compared with the control) under salt stress conditions. An increase in the root dry weight was also observed due to the pretreatment of B2. Compared with the control, an increase of 56.7% and 35.2% in the root weight was found for cv. JM22 and cv. SH12, respectively, under high saline conditions. Moreover, a significant increase of 20.8% for cv. JM22 and 14.8% for cv. SH12, in terms of the root/shoot ratio, was also observed in the seedlings pretreated with 0.1 $\mu\text{mol}\cdot\text{L}^{-1}$ B2 under salt stress conditions (Table 1). On the other hand, the lower concentrations of B2 were not effective under salinity conditions for both wheat cultivars, while the 0.001 $\mu\text{mol}\cdot\text{L}^{-1}$ B2 was effective, compared with the control, in cv. JM22 under normal conditions (NC) in terms of the total dry weight and the root dry weight traits.

The data clearly showed that the seedlings treated with ABA or B2, with different concentrations, performed better under salt stress, compared with the untreated ones. It was also observed that 0.1 $\mu\text{mol}\cdot\text{L}^{-1}$ B2 not only increased the total root length but also enhanced the total root volume and root total surface in JM22 and SH12 (Table 1).

The plant physiological state can be reflected by the relative water content (RWC) of the leaves under salt-stressed conditions. Table 1 shows that the cv. SH12 leaves of seedlings treated with B2 0.1 and 0.001 $\mu\text{mol}\cdot\text{L}^{-1}$ had a substantially higher RWC than the control plants, while the cv. JM22 leaves of seedlings treated with B2 0.1, 0.01 and 0.001 $\mu\text{mol}\cdot\text{L}^{-1}$ had a significant improvement in RWC under the same concentration of salt. Additionally, ABA-treated seedlings had a significant improvement in both cultivars. ABA and B2 enhanced the RWC of both wheat cultivars, SH12 and JM22, under both saline and non-saline conditions.

Based on the preliminary presented data 0.1 $\mu\text{mol}\cdot\text{L}^{-1}$ of B2 was the best effective concentration among the three investigated concentrations. Therefore, the other two concentrations of B2 were excluded from the following experiments.

Influence of B2 on wheat leaf endogenous ABA level

Under the salinity stress conditions, the ABA endogenous hormone level increased significantly with B2 treatment. The ABA content in leaves, for the treated seedlings (with 0.1 μM B2 and ABA), increased, compared with the control, and the endogenous ABA content increased by 88% and 22% in JM22, respectively, and increased by 106% and 76% in SH12, respectively (Fig. 1).

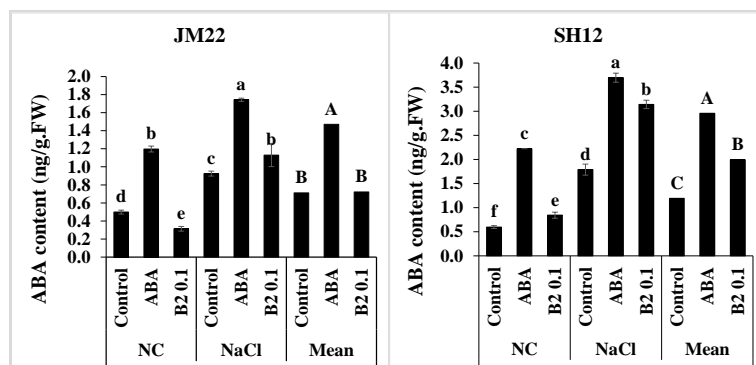


Figure 1. Effect of B2 on the content of endogenous hormone ABA in both wheat cultivars Jimai22 and Shannong12. Normal conditions (NC), under salt stress (NaCl) and the mean of each treatment under both conditions (Mean). The data are expressed as the mean \pm SE of three replicates. Means are compared using Duncan's test at 5% level of significant. Bars labeled with same letters are not significantly differ from each other

Table 1. Effect of B2 on Plant growth and biomass accumulation, root characteristics and relative water content (RWC) in both wheat cultivars Jimai22 and Shannong 12. Normal conditions (NC) and under salt stress (NaCl). The data are expressed as the mean \pm SE of three replicates. Means are compared using Duncan's test at 5% level of significant. Values labeled with same letters are not significantly differ from each other

Treatment		Plant dry weight (mg/plant)	Root dry weight (mg/plant)	Root/shoot ratio	Total root length (cm)	Root surface (cm ²)	Root volume (cm ³)	RWC %	
Jimai 22	NC	Control	421.8 ^c	118.5 ^d	0.39 ^b	2087.8 ^d	189.3 ^e	1.4 ^f	90.0 ^b
		ABA	553.3 ^a	165.8 ^a	0.43 ^a	2268.7 ^c	213.5 ^d	1.7 ^{de}	93.3 ^a
		B2 0.1	495.8 ^{ab}	148.2 ^b	0.43 ^a	2487.9 ^b	249.5 ^b	1.9 ^c	91.7 ^{ab}
		B2 0.01	338.8 ^d	98.7 ^e	0.4 ^{ab}	1901.1 ^{ef}	231.3 ^c	2.3 ^a	92.8 ^{ab}
		B2 0.001	510.2 ^a	144.8 ^b	0.4 ^{ab}	2988.0 ^a	284.0 ^a	2.2 ^b	92.2 ^a
	NaCl	Control	315.7 ^d	82.7 ^f	0.4 ^c	1136.1 ^g	121.0 ^f	1.1 ^g	81.0 ^d
		ABA	444.7 ^{bc}	132.2 ^c	0.4 ^a	2051.0 ^{de}	203.8 ^{de}	1.6 ^e	82.6 ^{cd}
		B2 0.1	432.0 ^c	129.5 ^c	0.4 ^a	2007.0 ^{de}	205.1 ^{de}	1.7 ^{de}	83.7 ^c
		B2 0.01	314.7 ^d	90.5 ^{ef}	0.4 ^{ab}	1939.9 ^{def}	208.0 ^d	1.8 ^d	84.1 ^c
		B2 0.001	331.8 ^d	96.2 ^e	0.4 ^{ab}	1815.2 ^f	203.1 ^{de}	1.8 ^{de}	83.4 ^c
	Mean	Control	368.8 ^C	100.6 ^D	0.37 ^C	1611.9 ^D	155.2 ^D	1.2 ^E	85.5 ^B
		ABA	499.0 ^A	149.0 ^A	0.43 ^A	2159.9 ^B	208.6 ^C	1.7 ^D	87.9 ^A
		B2 0.1	463.9 ^A	138.8 ^B	0.43 ^A	2247.5 ^B	227.3 ^B	1.8 ^C	87.7 ^A
		B2 0.01	326.8 ^B	94.6 ^D	0.40 ^B	1920.5 ^C	219.6 ^B	2.1 ^A	88.5 ^A
		B2 0.001	421.0 ^D	120.5 ^C	0.40 ^B	2401.6 ^A	243.5 ^A	2.0 ^B	87.8 ^A
Shannong 12	NC	Control	326.7 ^b	95.2 ^c	0.41 ^{cd}	854.7 ^d	101.3 ^d	1.0 ^c	89.3 ^b
		ABA	417.5 ^a	132.0 ^a	0.46 ^a	1971.3 ^a	193.8 ^a	1.6 ^a	92.0 ^a
		B2 0.1	357.5 ^b	109.0 ^b	0.44 ^{ab}	1057.3 ^c	116.9 ^c	1.0 ^c	92.0 ^a
		B2 0.01	350.7 ^b	104.0 ^{bc}	0.42 ^{bc}	1542.8 ^b	149.0 ^b	1.2 ^b	92.4 ^a
		B2 0.001	284.8 ^c	61.5 ^e	0.28 ^e	563.9 ^e	60.1 ^g	0.5 ^f	91.9 ^a
	NaCl	Control	263.3 ^{cd}	74.8 ^d	0.40 ^{cd}	385.5 ^f	50.2 ^g	0.5 ^f	83.6 ^{cd}
		ABA	325.3 ^b	101.7 ^{bc}	0.45 ^a	845.3 ^d	87.9 ^e	0.7 ^d	88.3 ^b
		B2 0.1	323.2 ^b	101.2 ^{bc}	0.46 ^a	788.3 ^d	77.5 ^{ef}	0.6 ^{ef}	85.3 ^c
		B2 0.01	247.0 ^d	70.3 ^d	0.40 ^{cd}	633.5 ^e	73.6 ^f	0.7 ^{de}	81.5 ^d
		B2 0.001	207.3 ^e	58.3 ^e	0.39 ^d	837.3 ^d	109.7 ^{cd}	1.2 ^b	85.2 ^c
	Mean	Control	295.0 ^C	85.0 ^C	0.40 ^B	620.1 ^E	75.7 ^E	0.7 ^D	86.4 ^C
		ABA	371.3 ^A	116.8 ^A	0.46 ^A	1408.3 ^A	140.8 ^A	1.1 ^A	90.1 ^A
		B2 0.1	340.2 ^B	105.1 ^B	0.45 ^A	922.8 ^C	97.2 ^C	0.8 ^C	88.7 ^{AB}
		B2 0.01	298.8 ^C	87.2 ^C	0.41 ^B	1088.2 ^B	111.3 ^B	0.9 ^B	86.9 ^C
		B2 0.001	246.1 ^D	59.9 ^D	0.33 ^C	700.1 ^D	84.9 ^D	0.8 ^C	88.5 ^B

B2 effect on the wheat leaves soluble protein content under high salinity levels

After going through the salt stress, the recorded plant content of soluble protein was higher in B2- and ABA-treated leaves, compared with the control. It increased significantly, by 17% in cv. JM22 and 31% in cv. SH12 B2-treated seedlings, in comparison with the control seedlings, while it increased by 9 and 15% in JM22 and SH12, respectively, in ABA-treated seedlings (Fig. 2). Pre-treatment with both

0.1 $\mu\text{mol}\cdot\text{L}^{-1}$ B2 and ABA efficiently enhanced the wheat leaf content of soluble protein under high saline conditions.

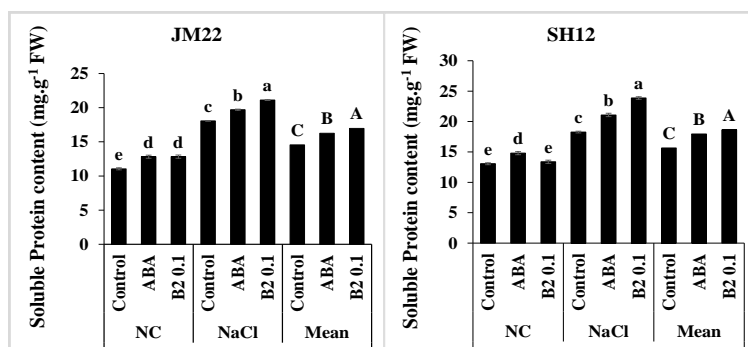


Figure 2. Effect of B2 on the soluble protein content in both wheat cultivars Jimai22 and Shannong12. Normal conditions (NC), under salt stress (NaCl) and the mean of each treatment under both conditions (Mean). The data are expressed as the mean \pm SE of three replicates. Means are compared using Duncan's test at 5% level of significant. Bars labeled with same letters are not significantly differ from each other

B2 influence on the antioxidant enzymes activity under saline conditions

Three days after the salt application, the antioxidant enzyme activity was analyzed. The antioxidant activity of CAT, SOD and POD enzymes was obviously affected by salt stress. The SOD activity significantly increased due to B2 in the leaves of both wheat cultivars. The rate of increase was 17 and 18% for cv. JM22 and cv. SH12, respectively. Furthermore, there was a significant enhancement in the POD activity, where the rate of increase reached 16% in cv. JM22 and 23% in cv. SH12. Additionally, the antioxidant enzyme CAT activity increased significantly in both cv. JM22 and cv. SH12, at 15 and 10%, respectively. In addition, ABA significantly enhanced the antioxidant enzyme activity of SOD, POD and CAT in both cultivars, JM22 and SH12, and the rates of increase were 23 and 16% for the SOD activity, 17 and 8% for the POD activity and 34 and 30% for the CAT activity, respectively, compared with the untreated seedlings under the saline condition (Fig. 3a, b, c).

Proline content

After application of salt stress, it was notable that the plant content of proline become higher in leaves. It increased significantly by 14% and 15% in JM22 and SH12 treated seedlings with B2 and 19% and 26% with ABA compared with the non-treated seedlings in the same concentration of salt (Fig. 4). Pre-treatment with both 0.1 $\mu\text{mol}\cdot\text{L}^{-1}$ B2 and ABA could efficiency enhance leaves content of proline in both cultivars of wheat crop under the high-level salt conditions.

Lipid peroxidation and ROS accumulation

Under salt stress conditions, data clearly presented that ABA and the chemical compound B2 have improving effect on MDA in both wheat cultivars JM22 and SH12, both treatments successfully decreased the MDA level by 16% and 33% with ABA treatment and 14% and 30% with B2 treatment in both JM22 and SH12 respectively (Fig. 5a).

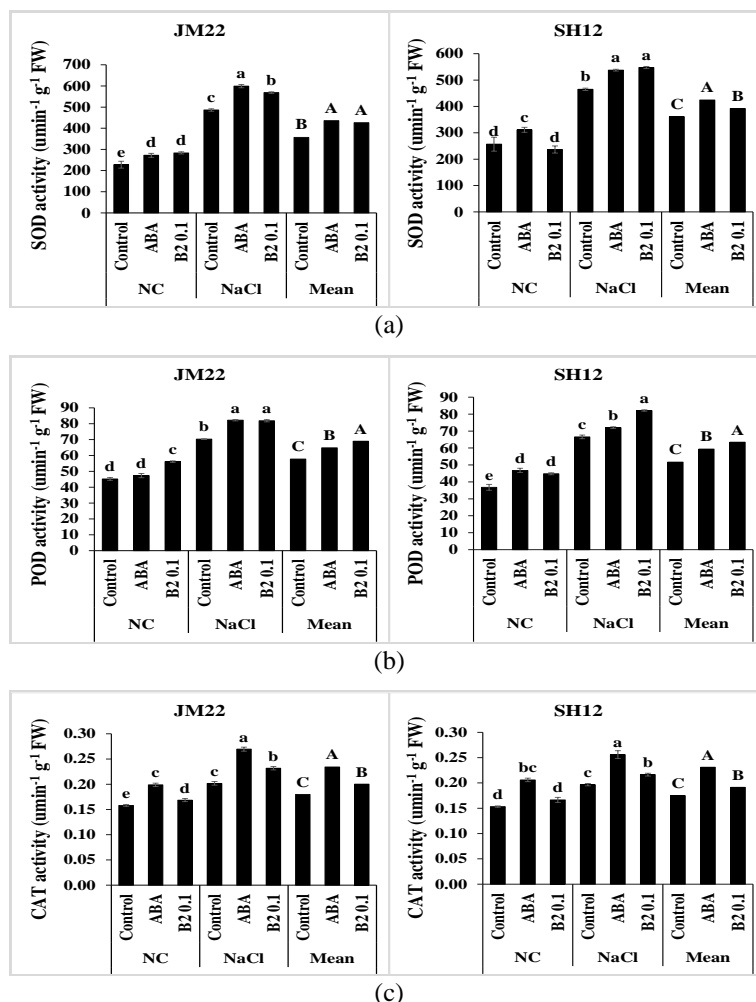


Figure 3. Effect of B2 on the antioxidant enzyme activity. (a) Superoxide dismutase (SOD). (b) Peroxidase (POD). (c) Catalase (CAT) in both wheat cultivars Jimai22 and Shannong12. Normal conditions (NC), under salt stress (NaCl) and the mean of each treatment under both conditions (Mean). The data are expressed as the mean \pm SE of three replicates. Means are compared using Duncan's test at 5% level of significant. Bars labeled with same letters are not significantly differ from each other

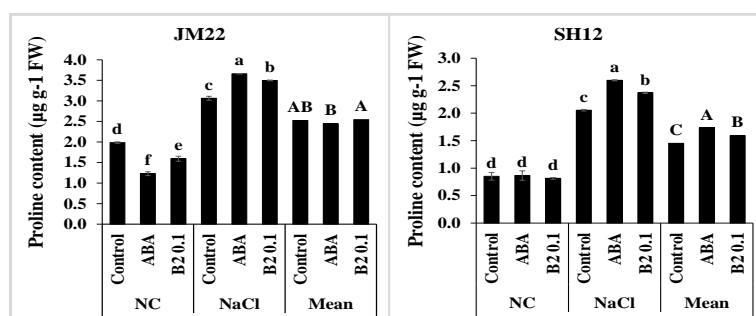


Figure 4. Effect of B2 on proline content in both wheat cultivars Jimai22 and Shannong12. Normal conditions (NC), under salt stress (NaCl) and the mean of each treatment under both conditions (Mean). The data are expressed as the mean \pm SE of three replicates. Means are compared using Duncan's test at 5% level of significant. Bars labeled with same letters are not significantly differ from each other

Both ABA and B2 treatments had significantly showed lower accumulation of H₂O₂ in salt conditions. Where, ABA and B2 reduced the level of H₂O₂ in both wheat cultivars leaves, the decreasing rate were 29% and 44% with ABA and 32% and 71% with B2 in JM22 and SN12 respectively (Fig. 5b).

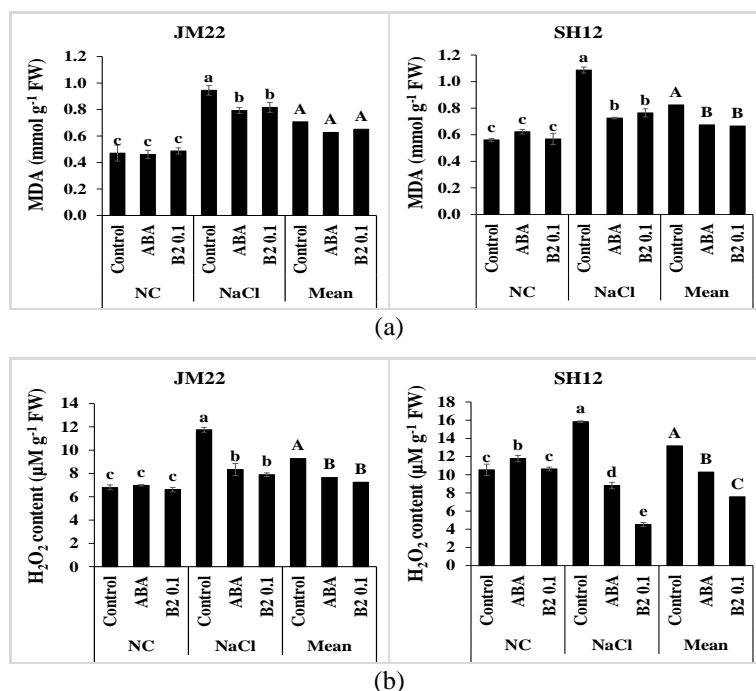


Figure 5. Effect of B2 on MDA (a) and H₂O₂ (b) content in both wheat cultivars Jimai22 and Shannong12. Normal conditions (NC), under salt stress (NaCl) and the mean of each treatment under both conditions (Mean). The data are expressed as the mean ± SE of three replicates. Means are compared using Duncan's test at 5% level of significant. Bars labeled with same letters are not significantly differ from each other

Effect of B2 on wheat leaf photosynthetic rate under salinity stress

The data showed that seedlings treated with ABA or B2 had a high photosynthesis rate, as compared with the control treatment, while growing under salt stress conditions. The photosynthesis rate of ABA- and B2-pretreated seedlings increased by 43 and 39% in cv. JM22 and by 37 and 28% in cv. SH12, respectively, compared with the non-treated seedlings under a high salinity. Additionally, there was a notable effect of ABA and B2 on wheat leaf stomatal conductance and transpiration rate under salt stress in both the cultivars. Moreover, there was a significant increase in B2-treated seedlings in both indicators, Stomatal conductance (Sc) and Transpiration rate (Tr), in the wheat cultivar, SH12, under salt stress conditions, and it increased by 84 and 68%, respectively, while the effect was not notable in JM22 under the stress conditions. On the other hand, the increase rate was not significant for ABA-treated seedlings in both indicators, Stomatal conductance (Sc) and Transpiration rate (Tr), in both SH12 and JM22 (Fig. 6a, b, c).

Effect of ABA and B2 on the expression of salt and ABA-responsive genes

With the B2 and ABA pre-treatment of wheat seedlings, the expression level of salt-responsive genes (i.e., *TaERF3* and *TaMYB3R1*), along with the ABA-responsive genes

(i.e., *TaABAOH2*, *TaNCED1*, and *TaAREB3*), increased significantly under salt stress. However, the time for each gene to reach the highest expression level was different, depending on the treatment and cultivar. The expression level of *TaERF3* increased with B2 and ABA treatments, compared with the control, under salt stress, based on time. Here, B2 was comparable with ABA in terms of the increase in the expression level of the mentioned gene. In cv. JM22, B2 increased *TaERF3* expression at 12, 24, 48 and 72 h, and its effect was higher than that of ABA from 24 to 72 h (Fig. 7a, b). However, in cv. SH12, the significant increase started at 6 h for the treatment with both B2 and ABA (Fig. 7d). On the other hand, an initial increase in the expression level of *TaMYB3R1* was seen in cv. JM22 at 3 h due to ABA treatment. However, the expression dropped down with time (Fig. 7c). In the case of cv. SH12, *TaMYB3R1* expression increased linearly at 24, 48 and 72 h due to the effect of B2, unlike the nonlinear influence of ABA (Fig. 7d). Under salt stress, B2 improved the expression level of *TaABAOH2* in cv. JM22 and cv. SH12, especially at 72 and 24 h, respectively (Fig. 8e, f). Furthermore, like ABA, B2 enhanced the expression level of *TaNCED1* in cv. JM22 at 72 h and significantly enhanced the expression of the same gene in cv. SH12 at 24 h (Fig. 7g, h). The effect of B2 and ABA was almost similar on the expression of *TaAREB3* in cv. JM22, which reached its peak at 72 h. However, in cv. SH12, only ABA affected the gene expression at 12 h, with no significant effect of B2, compared with the control (Fig. 7i, j).

Discussion

Soil salinity and drought are considered to be the most damaging abiotic stresses affecting agricultural production. It has previously been reported that ABA affects plant growth and development and has a role in plant responses to abiotic stresses, including salt, cold and drought stresses (Zhu, 2002).

Under saline conditions, applying a proper concentration of ABA enhances the salinity tolerance and increases the growth rate in sorghum and rice crops (Gurmani and Bano, 2007). Shoot fresh and dry weight decreases with an increase in salinity, but the application of ABA decreases the inhibitory effect of salt on shoot weight to some extent. The root weight of wheat also increases, if the seed is pretreated with ABA (Gurmani and Bano, 2007). These previous studies are in accordance with the present study. Here, both the treatments of ABA and B2 enhanced the total dry weight, root/shoot ratio, and root growth rate.

The plant physiological state is reflected by the relative water content (RWC) of its leaves. The reduction of the leaf water potential (LWP) and relative water content (RWC) were significantly moderated by ABA treatment under saline conditions. Additionally, ABA priming enhanced the RWC and LWP of salt-acclimated plants under subsequent salt stress. This indicates that ABA helps in maintaining a better water status of wheat plants under salinity stress (Davies and Zhang, 1991). The positive effect of ABA-induced antioxidant activity and the reduction in oxidative stress was further manifested by an increase in RWC, plant growth and membrane stability index (Gong and Li, 1998).

In this study, both B2 and ABA promoted the RWC of both wheat cultivars. In accordance with these observations. B2 enhanced the root growth, and plant total fresh and dry weight, leading to an improved water absorption capacity of wheat seedlings and hence an improved RWC (Karcher et al., 2008).

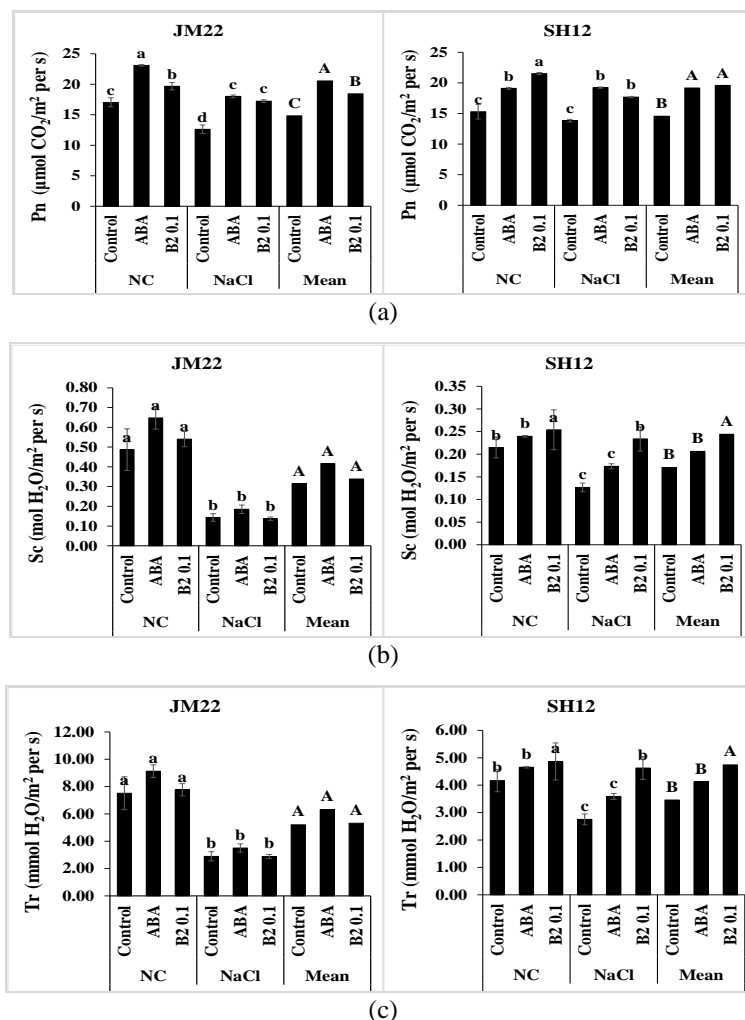


Figure 6. Effect of B2 on the (a) Photosynthesis rate (Pn) (b) Stomatal conductance (Sc). (c) Transpiration rate (Tr) in both wheat cultivars Jimai22 and Shannong12. Normal conditions (NC), under salt stress (NaCl) and the mean of each treatment under both conditions (mean). The data are expressed as the mean \pm SE of three replicates. Means are compared using Duncan's test at 5% level of significant. Bars labeled with same letters are not significantly differ from each other

ABA accumulation in plant cell decreased the level of Na^+ , regardless of whether these plants were salt acclimated. The endogenous ABA content notably increased with the salt treatment. A reduced Na^+ concentration and increased ABA level were caused by the combination of salt acclimation and ABA priming. ABA wheat plant priming improves the endogenous ABA content, which leads to a reduction in Na^+ uptake and accumulation (Davies and Zhang, 1991).

In the present study, the endogenous ABA level increased under salinity conditions. This indicates that B2 induced ABA accumulation in wheat plants. It also contributes to antioxidant enzyme's resisting system regulation and osmotic stress adjustment and enhancement, thus reducing oxidative damage.

One of the main defense strategies in plants is their antioxidant system. This includes enzymatic and non-enzymatic compounds, which act as detoxification agents of toxic-free radicals and therefore help the plant to overcome the destructive oxidative process

(Li et al., 2011; Zhang et al., 2016). The major antioxidant defense enzymes are SOD, CAT, and POD. POD decomposes H₂O₂ through the oxidation of co-substrates, such as phenolic compounds and/or other antioxidants, whereas CAT breaks down H₂O₂ into H₂O and O₂ (Mittler, 2002; Li et al., 2011). An increase in SOD activity decreases free radicals, especially superoxide radicals that harm cell membranes, increase plant tolerance to oxidative stress and scavenges O₂⁻ radicals (Manjili et al., 2012).

Many studies indicate that the antioxidant system has a critical role in plant resistance to oxidative damage caused by salinity stress (Mittler, 2002; Xie et al., 2008; Qiu et al., 2014). Therefore, improving the antioxidant enzyme activities in plants results in an enhancement of the plant tolerance to salt stress. ABA has a positive effect in inducing the POD, SOD, and CAT activity in wheat plants exposed to a high salt stress (Davies and Zhang, 1991). ABA has a positive effect on adaptation responses in wheat (Agarwal et al., 2005).

The present results show that B2 mimics ABA and can enhance the scavenging capacity of reactive oxygen species (ROS) by improving the antioxidant enzymatic activities in wheat seedlings under saline conditions. Therefore, B2 could diminish the oxidative damage, which was also confirmed by the improvement in the soluble protein level, SOD, POD, and CAT, which led to the enhancement in the osmotic adjustment potential of the wheat seedlings.

Plants show many physiological and biochemical responses to cope the environmental abiotic stresses such as the accumulation of proline (Delauney and Verma, 1993). In fact, free proline accumulates in a wide variety of higher plants, such as tobacco, soybean, barley, wheat and rice (Yoshida et al., 1999). Proline play a major role in free radical scavenging, adjusting cell osmotic and a compatible solute that protects enzymes (Verbruggen et al., 1993). The metabolism of proline is essential for the plant stress resistance (Hare et al., 1999). Furthermore, it has been confirmed that accumulation of proline in cells improve osmotic and salt stress tolerance in transgenic plants (Kavi-Kishor et al., 1995; Zhu, 2002), although there also existed the regards that proline was an alternative result from adaptive or detrimental processes responding to osmotic stress (Larher et al., 2003). Proline also has a main function as protecting the enzymes from dehydration and salt accumulation (Thomas, 1990), and because of increasing abscisic acid concentration in response to various stresses (Unyayar et al., 2004), it seems that abscisic acid spraying increases proline content as a defense mechanism. ABA increased the proline content of flag leaf in the high salt level conditions (Flower and Yeo, 1989).

One of the ABA functions is enabling the plant to rapidly respond to salt stress by inducing stomatal closure. This is achieved through the modulation of the cytosolic CA²⁺ concentration, which leads to the depolarization of the guard cell, as a result of an improved ABA level (Wang et al., 2017). It has been proved that salt causes the inhibition of photosynthesis, which could be due to stomatal or non-stomatal limitations. Gas stomata conductance (gs) decreases significantly due to salt stress, while ABA application improves the gs level in wheat plants under saline conditions (Davies and Zhang, 1991).

ABA has a photosynthesis regulation function in wheat plants adapted to salt through the regulation of non-stomatal parameters. ABA plays an effective role in the plant's resistance to salt stress by decreasing the water loss and adjusting the osmotic stress through stomatal control. ABA priming treatment improved the carbon absorbance and water use efficiency of the adapted wheat plants under salt stress (Davies and Zhang, 1991).

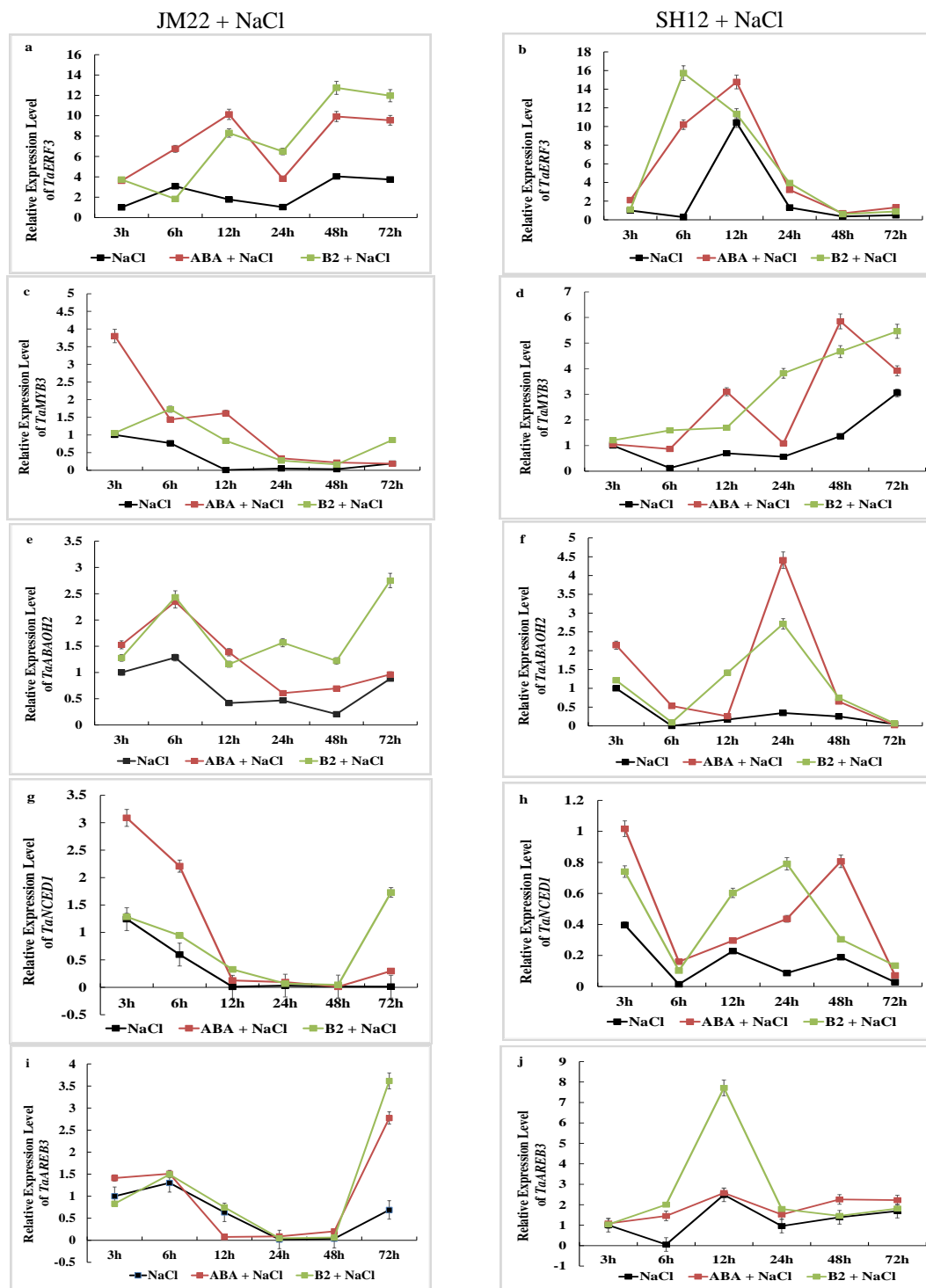


Figure 7. Expression of salt- and ABA-responsive genes in wheat seedlings at 3 h, 6 h, 12 h, 24 h, 48 h, and 72 h under salt stress conditions. Control under salt conditions (NaCl); ABA treatment of wheat seedlings under salt conditions (ABA + NaCl); B2 treatment of wheat seedlings under salt conditions (B2 + NaCl). (a) *TaERF3* gene expression level in JM22, (b) *TaERF3* gene expression level in SH12, (c) *TaMYB3R1* gene expression level in JM22, (d) *TaMYB3R1* gene expression level in SH12, (e) *TaABAOH2* gene expression level in JM22, (f) *TaABAOH2* gene expression level in SH12, (g) *TaNCED1* gene expression level in JM22, (h) *TaNCED1* gene expression level in SH12, (i) *TaAREB3* gene expression level in JM22, and (j) *TaAREB3* gene expression level in SH12

It is well known that the salt-induced inhibition of photosynthesis is attributed to stomatal and non-stomatal limitations (Wang et al., 2017). It might be that ABA improves the photosynthesis of wheat plants under salt stress due to the regulation of some other non-stomatal parameters. Nevertheless, stomatal control by ABA appears to be an efficient strategy for decreasing water loss and the negative effects of osmotic stress, caused by salinity (Wang et al., 2017).

A comparison of the influence of both ABA and B2 analogues on the expression of some genes related to salt stress and the ABA response was also performed. The effect of B2 on the expressions of these genes was found to be similar and sometimes superior to that of ABA, which reflected its efficiency as a stress regulator. However, the activation of gene expression was mainly based on the gene, time, and cultivar. The results showed that B2, like ABA, simulated the expression level of all the studied genes. In addition, among the tested genes, some were involved in drought and salt stress, such as transcription factor *TaERF3* (Zhang et al., 2007a), which was previously isolated and characterized in wheat. This transcription factor may directly regulate the expressions of different stress-related genes by interacting either with their promoters or with other TFs (Buttner and Singh, 1997; Zhang et al., 2007b). Additionally, *TaERF3* may indirectly regulate the expression of other stress-related genes involved in proline and chlorophyll accumulation, redox homeostasis (H₂O₂ reduction), and stomatal closure, which consequently become the induction of abiotic stress tolerance (Rong et al., 2014). It is well known that ERF proteins possess a vital role in regulating the expression of specific stress-related genes. Moreover, ERF subfamily members were primarily involved in biotic stresses as well (Berrocal et al., 2002; Onate et al., 2007; Chen et al., 2008). On the other hand, recent studies show that ERF members are also involved in diverse functions in plant responses to abiotic and biotic stresses (Rong et al., 2014). MYB proteins play multifunctional roles in the regulation of gene expressions (Rosinski and Atchley, 1998; Jin and Martin, 1999). For instance, they control the cell shape and morphogenesis involvement in abiotic and biotic stress responses to ABA (Cai et al., 2011). The *TaMYB3R1* gene expression was up-regulated, and its expression remained high under both cold and salt stresses. This suggested that *TaMYB3R1* may take part in abiotic stress regulation in wheat. In the present study, the *TaMYB3R1* gene was affected by ABA and B2, based on the time and plant genotype. However, B2 was more efficient than ABA in enhancing the gene expression in cv. SH12 in a linear mode through time. This result proved that the novel analogue (B2) has a good influence in enhancing stress tolerance in wheat.

Conclusions

From this study, it is apparent that B2 and ABA have a similar performance in improving the active oxygen radical scavenging efficiency by improving the antioxidant enzyme activity and enhancing the osmotic adjustment ability in wheat under salt stress. Moreover, the photosynthesis rate and the plant endogenous ABA content also increased, which confirms that B2 has a special regulating physiological mechanism. Suggestion that the findings could be beneficial in eradicating the salt stress problem in agricultural field crops is premature. Further studies should be performed to explain the effect of B2 on other crop plants such as rice since it considered as the main food crop in China, and investigate the effect of B2 based on the molecular analysis.

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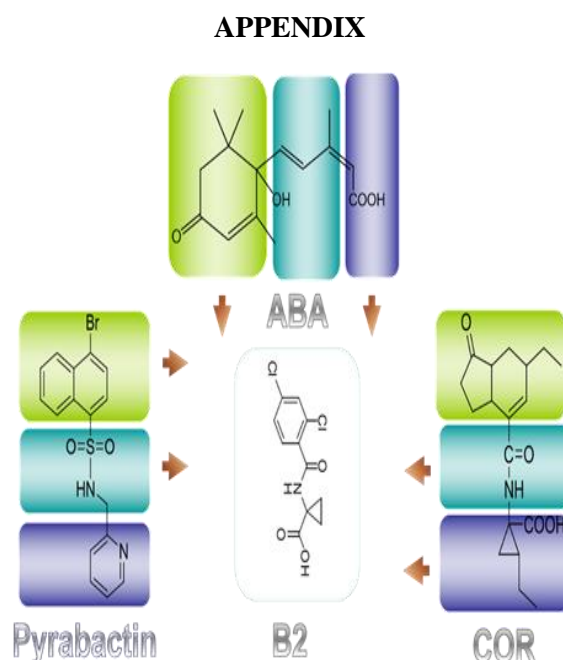


Figure A1. The synthesis process of B2. We adopted sub active structure splicing method, integrating ABA, Pyrabactin and Coronatine, and then synthesized compound B2

Table A1. Sequences of primers used in this study

Gene name	Primer	Sequence (5'-3')
Actin	F	GACCCAGACAACCTCGCAACT
	R	CTCGCATATGTGGCTCTTGA
TaNCED1	F	CGACGGGTACATTCTCACCT
	R	CCTGAGATTCGAGCTCCTTG
TaABAOH2	F	TACAGGTGGGAGGTTGTTGG
	R	TCGTAGTCGTCATCAGTCGG
TaAREB3	F	CTCAAGGACACGAGCGATGCTG
	R	CATTCAGCGGCTGAGGGACAAAC
TaMYB3R1	F	ACGAGAAAGACCGACACCTGC
	R	AACCCAGTGACAGAAAGGAAGCA
TaERF3	F	AGCAATCAGGCAAAGCAACC
	R	ACGACTCAGAAGGAACCACGAC