β-Aminobutyric acid raises salt tolerance and reorganizes some physiological characters in *Calendula officinalis* L. plant

ABSTRACT

Salt stress is one of the main factors limiting plant growth and yield globally. Seed priming technique with different chemicals including β-amino butyric acid (BABA) is found to be effective in enhancing plant growth and development under biotic and abiotic stresses. Scarce reports have been found about BABA seed priming in medicinal plants under stress conditions; however, several studies have been conducted on other crops but have not made an in depth study to investigate biochemical and physiological changes. In current study the shoot growth, relative water content (RWC), chlorophyll content, nutrient content, proline, antioxidants enzymes, lipid peroxidation and membrane permeability were investigated in *Calendula officinalis* L leaves due to BABA seed priming and/or salt stress treatment. Salt stress treatment significantly reduced the growth characters, inflorescence number as well as its fresh and dry weights, N, P and K contents in leaves, RWC, chlorophyll content, stomatal conductance, membrane stability index (MSI) and total phenolic and flavonoids contents of pot marigold. However, proline content, MDA accumulation, H_2O_2 content and antioxidant enzyme activity (CAT, SOD and POD) were increased due to salt stress. On the other hand, seed priming with BABA significantly improved the growth characters, inflorescence attributes and the previously mentioned physiological and biochemical parameters investigated relative to the control. Applying seed priming under salt stress conditions significantly mitigated the negative effects of salinity and enhanced the growth and productivity of pot marigold and therefore was suggested to be an effective technique prior cultivation.

Keywords: Antioxidant, Enzymes, Flavonoids, Lipid peroxidation, Proline, Salt stress, Seed priming.

1. INTRODUCTION

Pot marigold or marigold (*Calendula officinalis* L) is a medicinal herb belonging to Asteraceae family, which is widely used in traditional medicine [1]. Marigold contains two classes of pigments (flavonoids and carotenoids) therefore, it can be used as a colorant, which may be used as orange and yellow natural colors. Natural colors are gaining great attention since several synthetic colorants have given rise to allergic, toxic and carcinogenic effects [2, 3]. Moreover, flavonoids have antioxidant activities, which play a considerable role in human health and food preservation by resisting damage caused by oxidizing agent. It has been also that marigold plants reported to possess several pharmacological activities including, anti-inflammatory [4], antioxidant [5], antifungal [6], antibacterial [7], antiviral [8] and wound healing activity [9].

Abiotic stress is one of the major factors limiting plant growth and yield globally. Salt stress is utmost environmental challenge affecting sorely plant growth and production. Salinization is a considerable problem for different areas around the world that renders prohibitive growth and crop production [10]. The prejudicial effects of salt on plants growth are connected to ionic, osmotic and oxidative stresses [11]. Ionic component of salinity is associated with toxicity of particular ionic species (e.g. Na^+ or Cl^- stress) to plants as well as nutrient imbalance. NaCl decreased fresh and dry masses, relative growth rate, and K^+ and Ca^{2+} content but it increased Na^+ content and Na^+/K^+ ratio [12, 13].

High soil salt concentration decease soil water potential resulting in water deficit or osmotic stress. Salt stress also induces fashioning superoxide anion, singlet oxygen, hydroxyl radical and H_2O_2 and hence causes oxidative stress in several plants [14]. Salinized plants

evidenced a decreasing in plant growth characters such as shoot length, root length, fresh and dry weights as well as the contents of photosynthetic pigments (chlorophyll a and b, and carotenoids) and in the activity of catalase (CAT) against control plants [15,16].

Salt stress resulted in significant changes of macro and micronutrients content and sugars [10]. Salinity stress triggered a concentration-dependent overproduction of ROS and a concurrent up regulation of the expression of different antioxidants [17,18]. The promotion in ROS production resulted in oxidative stress in plants [19]. NaCl treatment significantly increased ROS and malondialdehyde (MDA) content, and electrolyte permeability in several species [20, 21, 22, 23, 24, 25].

MDA content is a sign of membrane damage at the cellular level in stressed plants [26]. Subsequently, MDA accumulation can avail as an important oxidative stress index [27]. In contrast, salinity stress boosted the contents of several organic solutes (soluble protein, soluble sugar, proline and total free amino acids), malondialdehyde (MDA), ascorbic acid and Na, as well as the activities of superoxide dismutase (SOD), peroxidase (POD), and ascorbate peroxidase (APX) in stressed plants relative to lupine control plants [16, 24, 28].

There are scarce efforts to ameliorate salt stress of medicinal plants by traditional breeding programs, but few successes have been reported. Increasing the toleration of these plants to salt or developing tolerance species by exogenously application of different treatments is therefore imperative. The germination of seed and the growth of seedling are the two critical periods for the establishment of crops [29]. These stages are the most sensitive to abiotic stress [30]. Jisha and Puthur [31] reported that seed priming is low cost, easy and low risk technique for enhancing growth and development particularly, in plants grown under reverse environmental conditions. The priming treatments by natural and/or synthetic compounds resulted in the inducement of physiological state in plants. Seed priming

enhanced seed performance, expands faster and coincided germination with definite biochemical, physiological, cellular and molecular changes [28, 33, 34, 35, 36]. These changes involve elongation and cell division, plasma membrane fluidity, the induction of stress-responsive proteins, changes in transcriptome and proteome, H^+-ATPase activity [37, 38, 39] and changes in the activity of antioxidant enzymes system [28, 39, 40, 41].

Seed priming has been shown to neutralize the adverse effects of different abiotic stress [42, 43, 44]. The priming of seeds has been proved to be a successful approach to enhance crop plants salt alleviating [44, 45, 46, 47]. Moreover, different materials or chemicals were utilized in seed-priming technique from time to other, such BABA, KCl, Si and $CaCl_2$, are usually used for the seed priming techniques [48, 49, 50]. One of these chemicals is BABA; it is a non-protein amino acid and is an isomer of naturally occurring non-protein amino acid called γ-amino butyric acid (GABA). BABA is a synthetic compound [51, 52] and its natural occurrence is scarce. Interestingly, when BABA was applied exogenously, it was seen to play a considerable role in increasing plant tolerance versus both biotic and abiotic stresses like salt, drought and heat shock [53]. The priming with BABA gains greater attention as a priming agent. However, although Vijayakumari et al. [54] reported that the promoted effect of BABA on plants metabolism is may be due to a potentiation of natural defense mechanisms against abiotic stresses, the accurate mode of action of priming with BABA in plants is yet a mystery, though it prominence as signaling molecules during stress is un-doubtful.

Seed priming by BABA increased proline content and antioxidant enzyme activities of stressed *Oryza sativa* plants [55], induced a major reorganization in solute content of flax (*Linum usitatissimum*) leaves which resulted in increased accumulation of carbohydrates and proline contents, a decrease in inorganic solutes and induced reduce in osmotic potential and a change in water status of leaves [56]. Moreover, BABA priming

significantly reduced MDA content in rice seedlings and also enhanced the activity of nitrate reductase enzyme and activities of antioxidant enzymes like guaiacol peroxidase and superoxide dismutase [31]. Although BABA priming effects on abiotic stress tolerance of different plants have been investigated, there are little reports with concern to different medicinal plants. Furthermore, most of the earlier studies with regard to other crops have not made an in depth study of biochemical and physiological changes correlated with abiotic stress of the particular plants exhibited to BABA priming.

To best of our knowledge, no information on how BABA treatment regulates physiological/biochemical processes of marigold plants subjected salt stressed. Therefore, this study is an attempt to ameliorate salt stress of this plant using non-protein amino acid (i.e. BABA) especially the mechanisms involved in this tolerance are still poorly understood.

2. MATERIALS AND METHODS

2.1. Plant materials and treatments

Pot marigold (*Calendula officinalis* L.) was used in current study as the plant material. Uniform sizes of healthy seeds were selected for seed priming treatment. Seeds were prewashed with Triton X-100 (0.25 %) for 1 min to remove uncleanness. Thereafter, seeds were washed twice with distilled water for 2 min and surface-dried. The seeds were kept under shade at 25 °C for 48 h to achieve the content of original moisture [31]. After washing, the seeds were divided into four groups; group one was presoaked in distilled water for 12 h (non-primed seeds as a control). Group two was presoaked in 1.5 mM β-aminobutyric acid (BABA) for 12 h in screw cap bottle as seed priming treatment. The BABA concentration was selected from a preliminary experiment. Group three was presoaked in distilled water for 12 h and was prepared for salt stress treatment after 45 days from planting. The last group was firstly primed with BABA for 12 h and plants raised from

primed seeds were exposed to salt stress treatment. Therewith, seeds were cultivated in pots (30 cm) containing soil mixture (sand: peat: clay; 1:1:1) that was previously wet with tap water. All pots were irrigated with tap water and maintained under greenhouse condition. After 45 days from planting, the plants raised from the third and fourth groups were exposed to salt stress with NaCl at 8 dSm^{-1} at three days intervals and the soil was washed with tap water every week to prevent salt accumulation during the four weeks treatment period. The salt stress concentration was selected from a preliminary investigation. Control and primed seeds (first and second groups) were irrigated by tap water every 3 days. The experiments were carried out in complete randomized design and each treatment was replicated 4 times and each replicate consists of 5 pots.

2.2. *Experimental site and growth conditions*

This experiment was conducted at the greenhouse of Faculty of Science, Taif University, Saudi Arabia (21°26'02.4"N 40°29'36.9"E) during September - December, 2016 and was repeated in 2017 at the same conditions. The experimental site is located at an altitude of 1200 m having temperate mild climate. The average temperature, relative humidity and rainfall were 20.9 ˚C, 35-57 and 14.84 mm, respectively during the study.

The physical and chemical characteristics of the soil used in this study were (sand, 77.08%, silt 7.42% and clay 16.50 %) and chemical properties were (pH, 7.85, EC, 2.16 dsm^{-1}, OM, 0.19%, 0.79%, Na^{+}, 3.26 ($meqL^{-1}$), Total $CaCO_3$, SO_4^{-2}, 48.87 ($meqL^{-1}$), Cl^{-}, 0.58 ($meqL^{-1}$), HCO_3, 2.07 ($meqL^{-1}$), total N^{+}, PO_4^{-3}, K^{+} were 0.31, 0.051 and 0.062%, respectively).

2.3. Investigated growth characters

Plant height (cm), branch number, herb fresh and dry weights were determined by the end of the experiment. For leaf area determination (cm^2), blade area was measured using digital image analysis according to Matthew et al. [57] method. Leaf blade digital image was

created in digital format using a Hewlett- Packard scanner (Hewlett Packard, Cupertino, ca), image was scanned at dot/inch (100 dpi), the blade area was measured using public domain software.

2.4. Assessment of inflorescence attributes

During the flowering period, total inflorescence number were recorded and weighted to obtain the total inflorescence fresh weight (g/plant) and then oven dried at 70˚C for 24 h to determine the inflorescence dry weight (g/plant). At the flowering stage, the samples were taken for subsequent physiological and biochemical investigations.

2.5. Relative water content (RWC)

The following relationship as described by Weatherley [58] was used for leaf midday relative water content determination and calculation:

$(W_{fresh}-W_{dry})$ / $(W_{turgid}-W_{dry})$ x 100, where W_{fresh} is the sample fresh weight, W_{turgid} is the sample turgid weight after saturating with distilled water for 24 h at 4°C, and W_{dry} is the oven-dry (70°C for 48 h) weight of the sample.

2.6. Chlorophyll and carotenoids contents

Leaf samples were isolated randomly for chlorophyll determination. Extraction in acetone was repeated until all pigments extracted. The absorbance of extracts was determined according to Sadasivam and Manickam [59]. They were measured by using spectrophotometer at wave length of 663 nm for chlorophyll (a), 644 nm for chlorophyll (b).

2.7. Stomatal conductance

Stomatal conductance (mol H_2O m^{-2} s^{-1}) was determined in pot marigold leaves using Delta T AP4 leaf porometer, UK.

2.8. Proline content

For free proline determination, the method described by Bates et al. [60] was used. Frozen leaf tissue (0.5 g) was homogenized with 10 mL of 3% sulfosalicylic acid at 4 °C.

Then, the obtained extract was filtered with Whatman No. 2. Mixture of 2 mL of filtrate, 2 mL of acid-ninhydrin, and 2 mL of glacial acetic acid was mixed in a test tube and incubated at 100°C for 1 h. The reaction was terminated on ice, and the reaction mixture then extracted with 4 mL of toluene. The chromophore-containing toluene was separated from the hydrated phase. The absorbance at 520 nm was spectrophotometrically determined with toluene as the blank. The proline concentration was calculated based on a standard curve and was expressed as µmol g^{-1} FW.

2.9. Hydrogen peroxide (H_2O_2) content

The content of H_2O_2 in leaves tissue was assayed according to Patterson et al. [61] method with little modifications. 0.5 g of leaves were homogenized with 6 mL of chilled 100% acetone and centrifuged at 12,000 × g for 10 min at 4°C. The obtained extracted solution (1 mL) was mixed with 0.1 mL of 5% Ti $(SO_4)_2$ and 0.2 mL of concentrated NH_4OH solution. The titanium-peroxide complex precipitated and this sediment was dissolved in 4 mL of 2 M H_2SO_4 after centrifugation at 3000 × g for 10 min. The absorbance of the titanium-peroxide complex was monitored at 412 nm. Finally, the absorbance was calibrated to a standard curve generated using known concentrations of H_2O_2 and was recorded calculated as µmol g^{-1}

2.10. Malondialdehyde determination (MDA)

Leaf MDA content was spectrophotometrically measured by the method of Hodges et al. [62]. The concentration of MDA was estimated by using the formula: MDA content = $6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}$, where A_{450}, A_{532} and A_{600} are the absorbance at 450, 532 and 600 nm, respectively and will be expressed as µmol mL^{-1}.

2.11. Membrane stability index (MSI)

MSI was determined by the method of Sairam et al. [63]. Two leaf samples (0.2 g) were taken and placed in 20 mL of double distilled water in two different 50 mL flasks. The

first one was kept at 40°C for 30 min while the second one will be kept at 100 °C in boiling water bath for 15 min. The electric conductivity of the first (C_1) and second (C_2) samples was measured with a conductivity meter. The leakage of ions was expressed as the membrane stability index according to the following formula, MSI = [1- (C_1/C_2)] x 100.

2.12. Antioxidant enzyme activity

Antioxidant enzyme activity was determined as the method previously described by Hassan and Mahfouz [64]. The resulting supernatant was used as an enzyme extract to determine superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) activities. Soluble protein contents of the enzyme extract were assayed according to the method of Bradford [65].

SOD (Ec 1.15.1.1) activity was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). SOD activity was expressed as SOD units min^{-1} mg^{-1} protein. One unit of SOD was considered to be the amount of enzyme required to inhibit NBT reduction by 50% as described by Giannopolitis and Ries [66] by measuring the absorbance at 560 nm by a spectrophotometer.

CAT (Ec 1.11.1.6) activity was spectrophotometrically estimated by method of Clairbone [67]**,** following the disappearance of H_2O_2 at 240 nm. The level of enzyme activity was expressed as µmol min^{-1} mg^{-1} protein.

POD (Ec 1.11.1.7) activity was tested according to Shanon et al. [68]. Sodium acetate buffer (0.1M) and 0.5% guaiacol was added to the enzyme extract. The reaction was started with 0.1% H_2O_2. The rate of change in absorbance was spectrophotometrically measured at 470 nm and the level of enzyme activity was expressed as µ mol min^{-1} mg^{-1} protein.

2.13. Total phenol content assay

Samples of 1 g powder from dry inflorescences were stirred with 50 mL of methanol (80%) at room temperature for 48 h. The extract was kept below 4°C after removing the

solvent and the amount of total phenol content was assayed as reported by McDonald et al. [69]. The flower diluted extract (0.5 mL of 1:10 g mL^{-1}) or standard phenolic compound (gallic acid) was mixed with the Folin-Ciocalteu reagent (5 mL, 1:10 diluted with distilled water) and aqueous Na_2CO_3 (4 mL, 1 M). The total phenol content was spectrophotometrically determined at 765 nm and measured as gallic acid equivalent per gram of plant extract (mg GAE g^{-1} DW).

2.14. Flavonoids content

The inflorescence flavonoids content was spectrophotometrically measured as described by Dewanto et al. [70]. Initially, 5% $NaNO_2$ solution was added to each test tube; after five minutes, 10% $AlCl_3$ solution was added followed by 1.0 M NaOH. Absorbance of resulting colored solutions was recorded at 510 nm against the blank (distilled water). Flavonoids content was expressed as mg catechin equivalent per g fresh weight (mg CE g^{-1} FW).

2.15. Leaf nutrient elements

The wet digestion procedure for dried sample (0.5 g) was performed using sulphuric and perchloric acids method to determine nutrient content according to Jackson [71]. Nitrogen percentage in leaves was determined in the digestion using the micro-Kjeldhl method [72]. Phosphorus, potassium, sodium and chloride contents were determined as described by Jackson [70] and Johnson and Ulrich [73].

2.16. Statistical analysis

The results of this study were combined for the two experiments ($n=8$) and analysis of variance (ANOVA) was performed. SPSS 13.3 program was used for data analyzing to compare means by Duncan multiple range test at $P=0.05$ level.

3. RESULTS

3.1. Growth characters

The growth characters of pot marigold were significantly reduced in salt stress treatment since the shortest plants, lowest branch number, lowest leaf area, lightest fresh and dry weights were recorded when NaCl at 8 dSm^{-1} was applied (Table 1). On the other hand, seed priming with BABA significantly improved the growth characters relative to the control. Moreover, applying seed priming treatment under salt stress conditions significantly mitigated the negative effects of salinity and enhanced the growth characters. The branch number, leaf area and dry weight of plants raised from primed seeds and grown under salinity were increased by 40.52, 35.61 and 12.06%, respectively compared with plants exposed to salt stress only.

Table 1. Effects of seed priming with β-aminobutyric acid (BABA) on plant height, branch number/plant, leaf area, herb fresh and dry weights of *Calendula officinalis* L. grown under salt stress conditions.

Treatments	Plant height (cm)	Branch number/plant	Leaf area (cm^2)	Herb FW (g/plant)	Herb DW (g/plant)
Control	32.62 ±0.83b	11.02 ±0.56b	7.63 ± 0.25b	692.36 ±2.87b	163.18 ±1.27b
Priming	37.26 ±0.97a	12.84 ±0.42a	8.86 ± 0.32a	723.41 ±1.98a	171.36 ± 1.48a
NaCl stress	21.87 ± 0.74d	7.23 ±0.54d	5.42 ± 0.28c	589.58 ±2.14d	136.24 ± 1.35d
Priming + NaCl stress	30.39 ±0.72c	10.16 ± 0.58c	7.35 ± 0.36b	654.25 ± 2.39c	152.68± 1.56c

Values are means ± S.E. ($n = 8$). Means within a column had different letters are significantly differ from each other according to Duncan multiple range test at $P = 0.05$. Priming means primed seeds with 1.5 mM BABA, NaCl stress means plants were exposed to 8 dSm^{-1} NaCl.

3.2. Inflorescence yield attributes

Seed priming with BABA significantly enhanced inflorescence number as well as its fresh and dry weights. However, a significant retardant in these characters was observed under salt stress treatment relative to the control (Table 2). BABA seed priming led to a prominent increase of inflorescence attributes when plants were exposed to salt stress. The inflorescence number, inflorescence fresh weight and inflorescence dry weight were

increased by 75.95, 131.21 and 156.38%, respectively relative to salt stress treatment when seeds were primed with BABA and then plants were grown under salt stress condition.

Table 2. Effects of seed priming with β-aminobutyric acid (BABA) on flower yield attributes of *calendula officinalis* L. grown under salt stress conditions.

Treatments	Inflorescences number/plant	Inflorescences FW (g/plant)	Inflorescences DW (g/plant)
Control	12.89 ± 0.62b	20.52± 0.53b	5.69 ± 0.21b
Priming	15.36 ± 0.54a	26.14 ± 0.54a	7.54± 0.27a
NaCl stress	4.32 ± 0.45d	9.69 ± 0.56d	2.15 ± 0.26e
Priming + NaCl stress	9.12 ± 0.43c	17.24± 0.62c	4.08 ± 0.34c

Values are means ± S.E. ($n = 8$). Means within a column had different letters are significantly differ from each other according to Duncan multiple range test at $P = 0.05$. Priming means primed seeds with1.5 mM BABA, NaCl stress means plants were exposed to 8 dSm^{-1} NaCl.

3.3. Leaf nutrient elements

Data in Table (3) clearly show that the contents of N, P and K were significantly reduced due to salinity stress relative to the control. Seed priming with BABA improved the contents of those elements in pot marigold leaves. Applying seed priming under salt stress conditions considerably increased the content of investigated macro-elements compared with salt stressed plants. Although Na and Cl contents were increased when salt stress treatment was applied, seed priming significantly decreased the content of both elements when interacted with salinity treatment. Hence, application of seed priming under salt stress treatment enhanced the Na/K ratio and reached to 0.77 compared with 0.68 that observed in plants grown under salt stress.

3.4. Relative water content (RWC)

The treatment of salt stress significantly decreased the RWC while seed priming with BABA maintained it whether it was solely applied or even under salinity (Fig 1A). The

lowest RWC was recorded by NaCl stress treatment however seed priming suppressed this reduction and increased the RWC when interacted with salts stress application.

Table 3. Effects of seed priming with β-aminobutyric acid (BABA) on leaf nutrient elements (mg g^{-1} DW) of *Calendula officinalis,* L grown under salt stress conditions.

Treatments	N	P	K	Na	Cl
Control	20.27±0.73b	3.49±0.38b	16.24±0.52b	1.98 ± 0.28c	2.98 ±0.32c
Priming	22.53±0.82a	3.73±0.52a	19.26±0.54a	1.96 ± 0.14c	2.94 ±0.28c
NaCl stress	13.37±0.75d	2.06±0.51d	10.47±0.57d	4.63 ± 0.23a	6.78 ±0.36a
Priming + NaCl stress	17.63±0.82c	2.99±0.41c	14.56±0.62c	2.52 ± 0.17b	3.25 ±0.27b

Values are means ± S.E. ($n = 8$). Means within a column had different letters are significantly differ from each other according to Duncan multiple range test at $P = 0.05$. Priming means primed seeds with1.5 mM BABA, NaCl stress means plants were exposed to 8 dSm^{-1} NaCl.

3.5. Chlorophyll content

Salt stress treatment significantly decreased the total chlorophyll content relative to the control or seed priming treatments. However, application of BABA seed priming significantly ameliorated the negative effects of salinity and therefore retarded the chlorophyll decrease under salt stress condition (Fig 1B). The total chlorophyll content was increased by 41.56% due to seed priming application under salinity relative to salt stress treatment.

3.6. Stomatal conductance

The stomatal conductance was considerably decreased under salt stress. However, seed priming application with BABA significantly improved the stomatal conductance in pot marigold leaves grown under salinity (Fig 1C).

3.7. Proline content

The proline content in pot marigold leaves was significantly enhanced in salt stressed plants. Application of seed priming treatment solely did not cause any increase in proline

content compared to the control. However, when seed priming was applied before salt stress treatment, a considerable increase in proline accumulation was observed (Fig 2A).

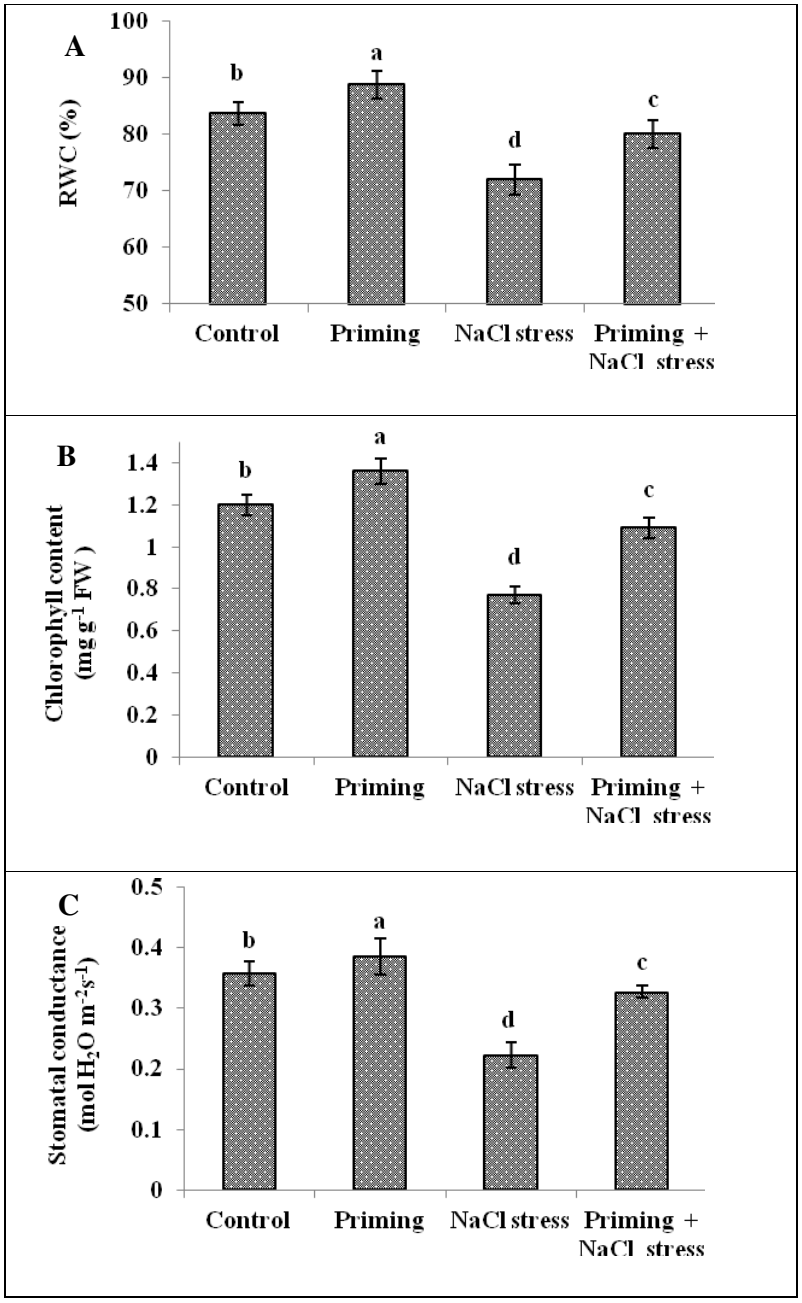
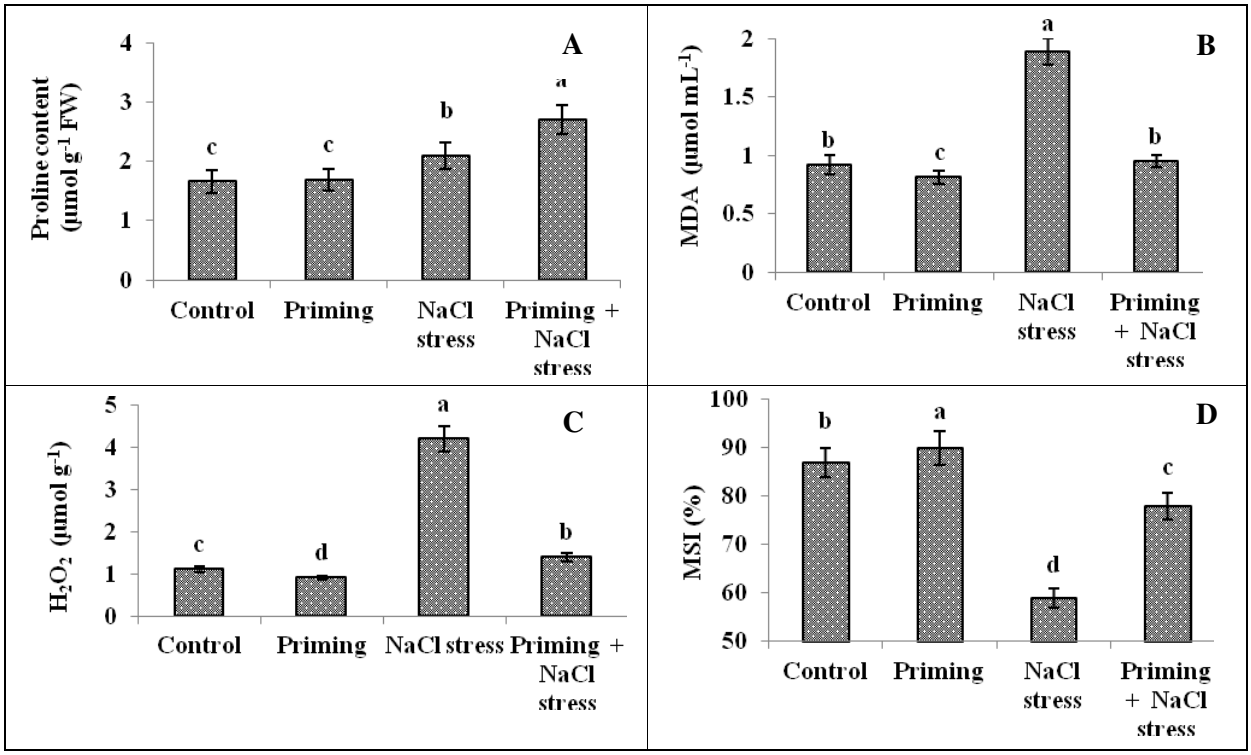


Fig. 1. Relative water content (A), chlorophyll content (B) and stomatal conductance (C) of *Calendula officinalis* L. in response to β-aminobutyric acid seed priming under salt stress conditions. Columns (means ± SE, n = 8) had different letters show a significant difference at $P \leq 0.05$.

3.8. Malondialdehyde content (MDA)

Data presented in Fig (2B) indicated that MDA accumulation was significantly increased in salt stressed plants compared with priming or untreated control. Priming treatment significantly reduced the MDA content relative to the control. The impact of priming treatment in MDA reduction was very clear when it was applied under salt stress conditions since priming treatment decreased MDA by 98.95 % relative to the salt stress treatment.

Fig. 2. Proline content (A), malondialdehyde (B), H_2O_2 content (C) membrane stability index (D) of *Calendula officinalis* L. in response to β-aminobutyric acid seed priming under salt stress conditions.



Columns (means ± SE, n = 8) had different letters show a significant difference at $P \leq 0.05$.

3.9.Hydrogen peroxide (H_2O_2) content

The content of H_2O_2 in pot marigold leaves was significantly increased in plants grown under salt stress relative to the control. On the other hand, seed priming application significantly reduced H_2O_2 content compared to salt stress or even untreated plants. When seed priming was applied prior cultivation and plants exposed to salt stress, the priming treatment retarded the increase in H_2O_2 that observed in salt stressed plants (Fig 2 C).

3.10. Membrane stability index (MSI)

It is very clear from data in Fig (2 D) that salt stress treatment significantly decreased the MSI relative to priming or control treatments since the lowest value of MSI was observed in pot marigold leaves applied with salt stress. However, the highest value of MSI was observed in plants raised from primed seeds. Additionally, priming treatment mitigated the effects of salinity and enhanced the MSI when it was applied under salinity.

3.11. Antioxidant enzyme activity

Data in Fig (3) revealed that the antioxidant enzyme activity (CAT, SOD and POD) were significantly increased due to salt stress treatment compared to the control or priming treatments. The enzyme activity was also increased due to priming treatment relative to the control. The highest activities of assessed enzymes were recorded when priming treatment was combined with salt stress.

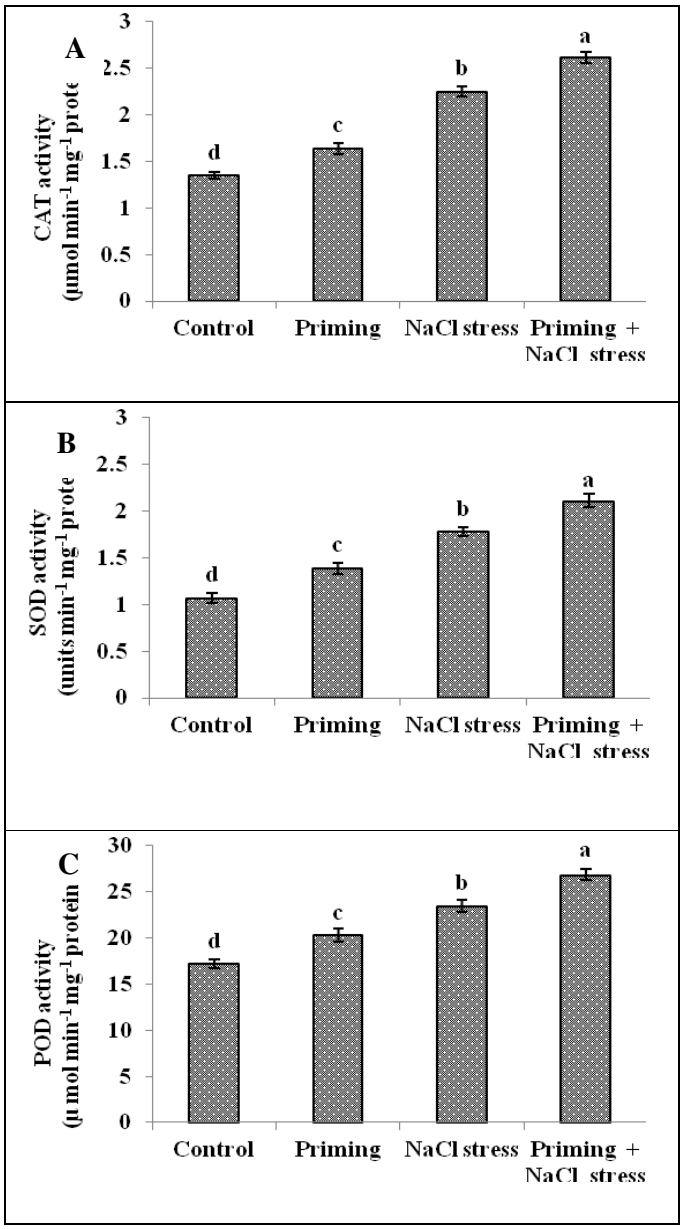


Fig. 3. Antioxidant enzyme activities (A, CAT, B, SOD and C, POD) of *Calendula officinalis* L. in response to β-aminobutyric acid seed priming under salt stress conditions. Columns (means ± SE, n = 8) had different letters show a significant difference at $P \leq 0.05$.

3.12. Total phenolic and flavonoids contents

Salt stress treatment significantly reduced both total phenolic and flavonoids contents in pot marigold inflorescence compared to the control. On the other hand, both parameters were considerably increased by seed priming with BABA. Seed priming treatment alleviated

the adverse effect of salinity when applied in combination with salt stress treatment and gave higher values of total phenolic and flavonoids contents relative to plants raised from salt stress only (Fig 4). Moreover, the recorded values obtained from combined treatment of seed priming and salinity were insignificant compared to the control.

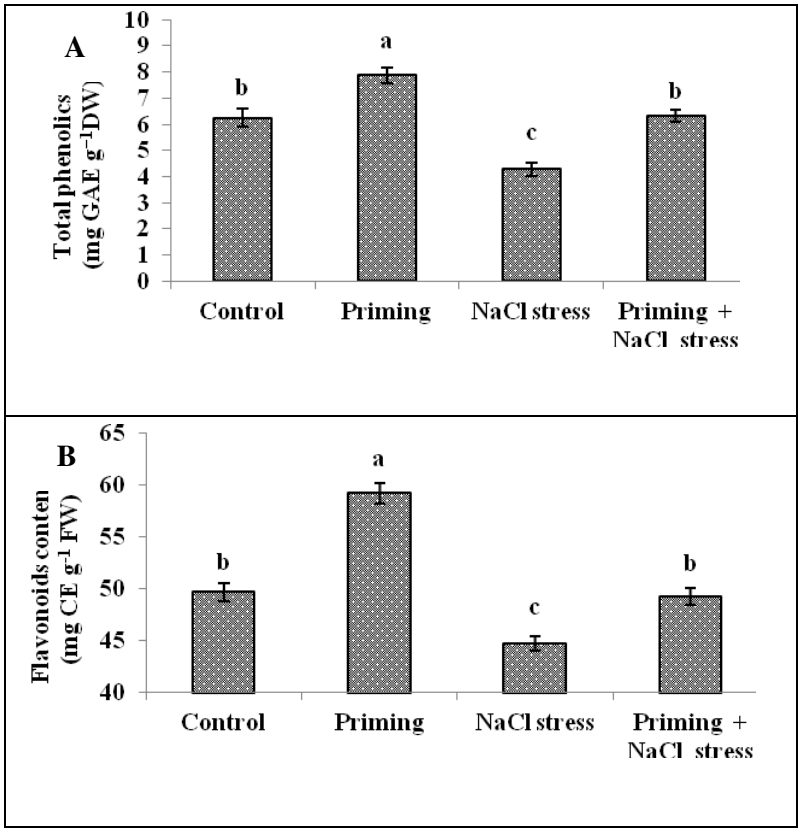


Fig. 4. Total phenolic and flavonoids contents of *Calendula officinalis* L. in response to β-aminobutyric acid seed priming under salt stress conditions. Columns (means ± SE, *n* = 8) had different letters show a significant difference at $P \leq 0.05$.

DISCUSSION

The obtained results showed that the growth variables (plant height, branch number, fresh and dry weights, leaf number as well as leaf area) and inflorescence yield of salt stressed pot marigold plants were decreased compared with untreated plants. Our findings are in accordance with others obtained by Moghimi and Ghavami [74, 75] on *Calendula officinalis* and Hassan et al. [24] on *Rosmarinus officinalis* who found that the salt stress

treatments seriously affected the growth and development. Nevertheless exogenously applied BABA effectively mitigated the salt stress hurtful effects and promoted the plant growth characteristics. It has been reported that BABA seed priming was found to be helpful for several plants to neutralize the adverse effects of different abiotic stresses [44].

In this concern, exogenously applied of BABA play an important role in increasing plant tolerance versus abiotic stresses like salt [53, 54]. Pretreatment of rice seeds with BABA enhanced radicle length and seedling growth under stressed conditions compared to control [76]. Salt stress usually led to drought stress, drought stress is characterized by tissues dehydration and its influence could be better evaluated by determine the RWC which signal the water cells status. In our research, a decrease in RWC was observed on exposure to salt stress. Notably, the reduction in RWC of pot marigold leaves was more retarded in BABA-treated plants (Fig 1a).

In our study, stomatal conductance and chlorophyll content were significantly reduced due to salt stress however; BABA seed priming treatments improved the stomatal conductance under salinity. It has been reported that salt stress significantly reduced the chlorophyll content in pot marigold [74, 75]. Decreasing RWC, chlorophyll and stomatal conductance under salt stress has been documented [24, 77]. It is well known that the stomatal closure of leaves is evidence to water shortage caused by salt stress. Previous studies have shown that BABA pretreatment triggers ABA accumulation, resulting in stomatal closure thus enhancing stress tolerance [51, 78]. BABA-mediated partial closing of stomata might be responsible for maintaining such high relative water content under salt-deficit condition (Fig 1c). Furthermore, Jisha and Puthur [32] found that BABA priming considerably improved the photosynthetic pigments in rice compared to non-primed seeds. Improving the photosynthetic system is considered a logic reason for growth promotion that reflected in increasing the inflorescence yield of pot marigold in current study.

Salt stress treatments increased H_2O_2 production and lipid peroxidation measured by MDA accumulation that resulted in membrane deterioration and therefore reduced MSI. Otherwise, pot marigold plants grown under salinity stress showed an increase in both proline and antioxidant enzyme activity. On the other hand, seed priming with BABA effectively reduced the adverse effects of salt stress on the previous physiological and biochemical attributes and therefore maintained the membrane and hence increased the tolerance of pot marigold plants to salinity. MDA is well known as lipid peroxidation indicator in oxidative stressed plants under water deficit [48]. Xu et al. [79] reported that the accumulation of MDA showed the aggravation of membrane lipid peroxidation while, BABA increased proline content and reduced MDA and hence may protect the integrity of plasma membrane [32]. It is well known that salt stress activates ROS production leading to lipid peroxidation of the membrane. The high level of H_2O_2 caused the membrane lipid peroxidation and consequently MDA content was increased under salt stress. However, BABA seed priming can counteract the oxidative damage and protect the cell membranes [80, 81]. In current experiment, reducing the production of H_2O_2 and MDA content suggest that BABA seed priming decreased the plasma membrane damage by decreasing the production of ROS. Moreover, the accumulation of free proline is a possible mechanism to protect the cells against oxidative damage [82]. Proline that promoted due to BABA priming may detoxify plants through ROS scavenging [83]. The growth reduction under salt stress in current study could ascribed to the reduction in nutrient uptake (N, P and K) under salt stress [24] and as a consequence the increase of ROS level which caused lipids oxidative damage and hence increased the MAD content was observed. The enhancement in growth of pot marigold plants raised from primed seeds could be explained by the role of BABA in improving the nitrogen metabolism through the activation of nitrate reductase that reported by Jisha and Puthur [32].

The antioxidant enzyme activities (CAT, SOD and POD) were increased in salt stressed pot marigold plants relative to the control (Fig. 3). Improving the antioxidant system is a critical factor against oxidative stress in plants [84]. Additionally, CAT, SOD and POD enzymes are effective scavengers of ROS and play a crucial role in alleviating oxidative damage [85]. Interestingly, BABA improved the activities of CAT, SOD and POD enzymes hence reduced the H_2O_2 level leading to improve the membrane properties. ROS Scavenging by antioxidant enzymes when exogenous BABA was applied has been previously reported [32, 81, 86].

In this study, total phenolic and flavonoids contents of *Calendula officinalis* L. were significantly reduced under salinity stress conditions however; applying BABA seed priming enhanced the both characters relative to the control (Fig. 4). Ashraf et al. [87] reported that phenolics have acquired much importance because of their properties of plant health promoting. Furthermore, BABA may directly/indirectly affect the metabolic processes in such a way that it enhanced the internal phenolic and flavonoid contents in pot marigold. However, the exact mechanism is really unknown. Otherwise, BABA seed priming promoted the photosynthetic system that resulted in improving the photosynthetic pigments in pot marigold [74, 75] therefore the flavonoids content may be enhanced.

CONCLUSION

BABA seed priming improved the plant growth and development under salinity. This treatment also maintained RWC, chlorophyll content, stomatal conductance and nutrient content. In addition, it also enhanced the activities of CAT, SOD and POD enzymes as well as proline content which reduced H_2O_2 production and limited MDA accumulation therefore retained the membrane stability and alleviated the salt stress damage. BABA treatment increased the phenolic and flavonoids contents in plants grown under salt stress. Improving

the antioxidant machinery as well as total phenolics and flavonoids may play a role in defense system against salt stress in pot marigold. Further studies are required to support the current results and to provide information about the roles of BABA that may have for phenolics and flavonoids promotion.

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