Cotton Seed Priming with Brassinosteroid Promotes Germination and Seedling Growth

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Abstract: Cotton (Gossypium hirsutum) is the largest fibre crop globally and an important oilseed crop. Rising temperatures and declining water supplies, which are also impacting soil salinity, threaten cotton plant productivity. Germination, emergence and young seedling stages in cotton are highly sensitive to salinity and heat stresses. Brassinosteroids (BRs) are plant steroid hormones that are essential for proper plant growth and development and also promote tolerance to a range of environmental stresses. Cotton seeds were primed with BR (24-epibrassinolide) alone or in combination with other hormones (abscisic acid, auxin and gibberellic acid) and tested for germination and early seedling growth. BR promoted germination under no stress as well as under salinity and heat stress conditions, while other hormones were ineffective under stress conditions. BR also promoted cotyledon opening and the development of lateral roots in germinated seedlings. The ability of BR to positively impact seedling growth across different stress conditions suggests that priming cotton seeds with BR may help in early and successful establishment of seedlings, which may benefit the plant through its lifecycle.

Keywords: brassinosteroid; cotton; seed priming; germination; salt stress; heat stress

1. Introduction

As the world’s primary natural fibre crop and the third largest oilseed crop, cotton (Gossypium hirsutum) is a major agricultural commodity for several global economies. Grown in more than 100 countries, the world cotton market was estimated at USD $77 billion for 2014/15 [1]. Around 25 million tonnes of cotton is produced globally per annum, with India, China and the US being the top three cotton producers. Global cotton production is projected to grow by 1.5% per annum to reach approximately 30 million tonnes by 2029 [2]. This growth is expected to come from an increase in average global yields and from expansion of cotton growing area. Despite the 1% increase in global yields, cotton yield growth remains a challenge in many countries. More resilient genetics and better agronomic approaches are needed to sustainably enhance cotton production.

Rising temperatures and declining water supplies, which are also impacting soil salinity, stand to threaten cotton plant productivity. Although cotton is regarded as a crop of hot, semi-arid regions, its growth and yield are negatively correlated with higher temperatures particularly during flowering and early boll development [3,4]. Another stage very sensitive to temperature is seedling growth three weeks post-emergence [5]. While the temperature optimum for cotton growth varies with cultivars and stage of development, temperatures between 20–30 °C are considered conducive for cotton growth [6]. Temperatures above 35 °C inhibit photosynthesis and consequently vegetative growth and yield [7].

Major cotton-growing regions are dry and some of them are managed by irrigation. High evaporation, drought and poor agricultural practices have led to salt accumulation...
Cotton is considered to have medium salt-tolerance, but growth, productivity and fibre quality of the plant is adversely affected by salinity [9]. Germination, emergence and young seedling stages are considered to be more sensitive to salinity stress than other stages; seed germination and emergence are delayed and reduced under salinity stress [10]. Osmotic stress resulting from reduced water uptake by the root system under saline conditions, followed by ion toxicity are the main mechanisms through which salinity stress reduces plant growth and yield. The physiological changes that occur due to osmotic stress include inhibition of photosynthesis, nutrient ion imbalance due to high levels of Na$^{+}$ and Cl$^{-}$ that reduce the uptake of essential nutrients such as K$^{+}$, NO$_{3}^{-}$, PO$_{4}^{3-}$, and reactive oxygen species (ROS) production that negatively affects cellular integrity and metabolism, such as damage to membranes and to cellular components proteins, lipids, and DNA [11]. Several agronomic management processes targeted at reducing soil salinity, as well enhancing salt tolerance of cotton are used to combat the negative effects of salinity stress [12], including seed priming [9,13] to overcome germination problems. Seed priming is an economical hydration technique to stimulate rapid germination and homogenous seedling emergence. Numerous factors such as reduction in imbibition time, activation of pre-germinative enzymes, enhancement of metabolite production and osmotic adjustment contribute to increased and uniform germination of primed seeds [14]. Priming also induces numerous molecular changes such as de novo synthesis of DNA and proteins, DNA repair, ATP production, and accumulation of osmolytes and antioxidant metabolites. Depending on the priming material, the priming methods are classified as hydro-priming, osmo-priming, hormopriming and others [15]. Hydro-priming involves pre-soaking of seeds in water and may or may not be followed by drying to original moisture. Osmo-priming is when seeds are soaked in an osmotic solution (sugar, mannitol, polyethylene glycol and others) followed by air drying, and hormopriming is when seeds are soaked in phytohormone solutions (auxin, gibberellic acid (GA), cytokinin and others) before sowing these and other methods have been reviewed in [14–16].

Brassinosteroids (BRs) are plant steroidal hormones that are essential for proper plant development as well as plant stress responses [17,18]. BRs regulate seed size, shape, and yield through regulation of seed developmental pathways [19]. The role of BR in promoting germination is also well established. Treatment with BR could rescue the germination defect in gibberellin (GA) biosynthetic mutants [20], override the inhibitory effects of abscisic acid (ABA) on germination in the model plant Arabidopsis [21], and help overcome salt stress mediated inhibition of germination in Brassica napus [22]. A recent proteomic analysis of germinating rice seeds identified >800 BR-responsive proteins, including 88 proteins with high confidence [23]. A large number of these proteins were associated with protein biosynthesis and carbohydrate metabolism, which is consistent with protein synthesis and reactivation of metabolism during germination. We previously demonstrated that BR has a major effect on protein synthesis under stress by affecting the stability and synthesis of the components of the translational machinery [24,25]. With such anabolic effects together with the ability to impact cell elongation and division, it is not surprising that BR promotes the growth potential of the plant from germination to maturity.

In cotton, BR is required for fibre initiation and elongation [26–28], and it promotes fibre maturation through deposition of cellulose into the secondary cell wall [29]. However, BR has not been tested in cotton for its effects on germination, early seedling growth, and seedling tolerance to stresses. In the present study, we subjected cotton seeds to hormopriming with 24-epibrassinolide (EBR), a BR, either alone or in conjunction with other phytohormones (ABA, auxin or GA) and studied their effects under no stress and stress (salinity and high temperature) conditions. ABA and auxin inhibit germination while GA promotes germination. The results of the present study demonstrate that BR promotes germination in cotton under both no stress and stress conditions, and helps to overcome ABA-induced inhibition of germination. BR also promotes cotyledon opening and the development of lateral roots in germinated cotton seedlings. The ability of BR to positively impact seedling growth across different stress conditions suggests that pre-soaking of
cotton seeds in BR solution before sowing may help in early and successful establishment of seedlings, which may benefit the plant through its lifecycle.

2. Materials and Methods
2.1. Plant Material

The experiments were conducted using two cotton varieties, Sicala V-2 with excellent Verticillium wilt tolerance and premature senescence tolerance but poor heat tolerance [30], and Sicot 730, a modern conventional commercial cultivar [31].

2.2. Hormone Preparation and Seed Treatment

Stock solutions of EBR (Sigma-Aldrich, Castle Hill, NSW, Australia) at 20 mM, and of ABA, indole-3-acetic acid (IAA) or GA\textsubscript{3} at 100 mM, were prepared in absolute ethanol and stored at \(-20^\circ\text{C}\). Uniformly sized cotton seeds, selected based on visual observation, were soaked at 40 seeds/30 mL in 0, 0.5, 1.0 and 2.0 \(\mu\text{M}\) EBR for 6 h in the dark at room temperature. For hormone combination experiments, seeds were soaked in EBR and either ABA, IAA or GA\textsubscript{3} at concentrations indicated in the figure legends.

2.3. Germination and Seedling Growth under Heat Stress (HS) and Salt Stress

EBR-treated seeds were placed on the surface of moistened thick blotting paper (Bio-Rad Laboratories, Gladesville, NSW, Australia) contained in square Petri plates (100 \(\times\) 100 \(\times\) 20 mm). For each EBR concentration there were three replicate plates with 10 seeds/plate. For germination under heat stress (HS), the Petri plates were separately incubated at room temperature, 35 \(^\circ\text{C}\) and 40 \(^\circ\text{C}\). For studying the effects of EBR on germination in seeds exposed to short-term but acute HS, EBR-treated and untreated seeds were placed in falcon tubes containing 30 mL deionised water preheated to 50, 60, 70 and 75 \(^\circ\text{C}\) and exposed to these temperatures for 15 min in water baths maintained at these temperatures. The HS exposed seeds were incubated for germination in Petri plates at room temperature.

For salt stress experiments, untreated and EBR-treated seeds were incubated for germination at room temperature on blotting paper wetted with 30 mL of 0, 100, 150 and 200 mM NaCl solution. For all experiments, Petri plates were placed in a cabinet and the numbers of germinated seeds were observed on the 2nd, 4th, 10th and 13th days, while seedling growth (hypocotyl length, root length, seedling fresh weight, number of seedlings with fully open cotyledons, number of seedlings with lateral roots, number of lateral roots per seedling, and length of the longest lateral root) were noted on either the 10th or 13th day, depending on the experiment. Following data collection, the seedlings were dried at 70 \(^\circ\text{C}\) and seedling dry weight was measured when constant weight was reached (72 h). A seed with an emerged radicle measuring about 2 mm was considered germinated. The number of lateral roots were counted using a Leica MZ6 stereo microscope. Seed treatment with ABA (0, 100 and 200 \(\mu\text{M}\)), IAA (0 and 5 \(\mu\text{M}\)) and GA\textsubscript{3} (0 and 50 \(\mu\text{M}\)) were carried out in combination with EBR at 0 or 2 \(\mu\text{M}\) under conditions described above. The controls (no hormone) were supplemented with ethanol at volumes equal to that of hormone stock solution added to treatment solutions. Experiments were replicated three times but since day (15–23 \(^\circ\text{C}\)) and night room temperatures varied considerably over the period that experiments were conducted, seedling phenotypes varied between replicate experiments although trends remained the same in all repetitions. For this reason, data of three replicate plates (10 seeds/plate) incubated at the same time has been represented in the figures. For the same reason, and the fact that two different varieties were used in this study, inter-experimental comparison of data obtained in different experiments for the same growth parameters is not possible. We believe that in addition to temperature variability, changes in day-length may have also contributed to differences in growth parameters. Experiments involving priming with IAA and GA\textsubscript{3} were conducted at the same time.
2.4. Statistical Analyses

Data were checked for approximate normality and approximate equal variances among the treatment groups using quantile comparison plot and box plot. The homogeneity of variances was also determined for different data parameters using Levene’s test. Data were subjected to two-way analysis of variance (ANOVA) followed by Tukey’s test for pairwise comparison of means to determine the interaction between effects of seed priming with either EBR or hormone combinations (EBR+ABA, or EBR+IAA, or EBR+GA) and salt concentrations. The diagnostics plots were also tested for the confirmation of approximate normality of the residuals. All data were analysed using R (version 3.3.0) [32]. Statistical significance was determined at 5% level of significance ($p < 0.05$).

3. Results

3.1. Effect of 24-Epibrassinolide (EBR) on Cotton Seed Germination and Seedling Growth under Salinity Stress

To investigate the effect of EBR on cotton seed germination under salinity stress, seeds of Sicala V-2 were pre-soaked in 0, 0.5, 1 and 2 µM EBR solution for six hours and then put to germination in the presence of 0, 100, 150 and 200 mM NaCl. Germination percent, counted on the 4th day was significantly reduced in the presence of 200 mM NaCl (Figure 1a). EBR enhanced germination under all concentrations of NaCl, but the most noticeable effect was under 200 mM NaCl with a 2.5-fold increase in the presence of 1 µM EBR as compared to untreated control (0 µM). Measured on the 13th day after sowing, EBR-treated seedlings showed increased hypocotyl length under both no stress and salinity conditions but had little effect on the root length (data not shown). The dry weight of EBR primed seedlings (Figure 1b) was significantly higher than untreated seedlings particularly under the 200 mM NaCl condition (Figure 1b).

![Figure 1. Cotton seed germination and seedling growth under salt stress following pre-treatment with 24-epibrassinolide (EBR). Percent germination (a) and seedling dry weight (b) in response to 0, 0.5, 1 and 2 µM concentrations of EBR under 0, 100, 150 and 200 mM NaCl were recorded for seedlings on the 4th and 13th day, respectively. Error bars represent standard error (SE) of the mean for three replicates ($n = 30$). Different letters above the bars indicate significant differences in two-way analysis of variance (ANOVA, $p < 0.05$) followed by Tukey’s post hoc test.](image)

EBR promoted cotyledon opening in seedlings under all conditions (no stress and salt stress), with the most pronounced effect being in response to 2 µM EBR pretreatment under 100 mM NaCl (Figure 2a, compare (cf.) 27% of untreated (0 µM EBR) seedlings vs. 90% of 2 µM EBR-treated seedlings with fully open cotyledons, indicating a >3-fold increase). Results represented in Figure 2a should be noted in conjunction with seedling phenotypes shown in Figure 2e to fully capture the effects of salt, EBR or both. For instance, seedlings not treated with EBR exhibit a similar proportion of seedlings with open cotyledons across 0–200 mM NaCl (Figure 2a), but a clear inhibitory effect of salt on germination and cotyledon emergence can be seen in Figure 2e.
Although EBR had no major effect on root length, percent seedlings with lateral roots (Figure 2b), the average number of lateral roots per seedling (Figure 2c) and the average length of longest lateral roots (Figure 2d) were considerably higher in EBR-treated seedlings under no stress condition (0 mM NaCl). The length of lateral roots was sensitive to EBR concentration with 0.5 μM promoting a 2.4-fold increase in length but 2 μM being slightly inhibitory as compared to 0 μM control (Figure 2d). EBR promoted lateral root growth under salt conditions but the results were highly variable and statistically insignificant. At 200 mM NaCl, lateral roots were not visible under the experimental conditions. EBR-regulated phenotypes of cotton seedlings can be seen in Figure 2e. Overall, these results indicate that cotton seeds treated with EBR have significantly better germination and cotyledon opening than untreated seeds under salt stress, but the positive effect of EBR on visible lateral root formation is restricted to no stress condition.

3.2. Combined Effect of EBR and Abscisic Acid (ABA), Indole-3-Acetic Acid (IAA) or Gibberellin (GA) on Germination and Seedling Growth under Salinity Stress

During seed formation, ABA serves as an endogenous inhibitor of precocious germination. However, treatment of non-dormant seeds with ABA also inhibits germination mainly by restricting availability of energy and metabolites for growth [33]. To investigate the effect of EBR on another cotton cultivar, as well as to see the combined effects of EBR and ABA on cotton seed germination under salinity stress, seeds of Sicot 730 were treated with combinations of EBR (0 and 2 μM) and ABA (0, 100, 200 μM) and then put for germination in the presence of 0, 150 and 200 mM NaCl. In this cultivar, EBR treatment enhanced germination...
under both no stress and salt stress conditions (Figure 3a,b). As would be expected, ABA inhibited germination under all conditions and EBR helped to overcome ABA-induced inhibition of germination (Figure 3a,b). For example, the 2.8-fold and 3.5-fold decrease in germination by 100 µM ABA under 150 and 200 mM NaCl conditions, respectively, was almost completely recovered by 2 µM EBR (Figure 3a, cf. 0 µM ABA/EBR with 100 µM ABA/0 µM EBR and 100 µM ABA/2 µM EBR). Seedling dry weight was also inhibited by ABA under all conditions, which was overcome by EBR almost completely under no stress conditions and to a lesser extent under salt stress conditions (data not shown). Interestingly, ABA increased hypocotyl length of seedlings under no stress conditions (Figure 3b), an effect also observed in tomato seedling grown in the dark [34].

Figure 3. Cotton seed germination and seedling growth under salt stress following pre-treatment with EBR or ABA alone or together. Percent germination (a) and seedling phenotypes (b) in response to pre-treatment with EBR (0 and 2 µM), ABA (100 and 200 µM), and ABA + EBR (as indicated in figures) under 0, 150 and 200 mM NaCl were noted on the 4th and 13th day, respectively. Error bars represent standard error (SE) of the mean for three replicates (n = 30). Different letters above the bars indicate significant differences in two-way ANOVA (p < 0.05) followed by Tukey’s post hoc test.

Similar to Sicala V-2, EBR enhanced cotyledon opening in Sicot 730, while ABA at 200 µM completely inhibited cotyledon opening under all conditions (Figures 3b and 4a). Inclusion of 2 µM EBR with 200 µM ABA (200 µM) led to 27% of seedlings with opened cotyledons under no stress condition (Figure 4a). Lateral root formation was completely inhibited in Sicot 730 in the presence of salt (Figure 4b–d) as was also the case in Sicala V-2. ABA at 200 µM had an inhibitory effect on lateral root formation under no stress conditions, however, the presence of EBR increased the number of seedlings with lateral
roots (Figure 4b), average number of visible lateral roots per seedling (Figure 4c), and lateral root length (Figure 4d). For instance, soaking seeds in 2 µM EBR enhanced percent seedlings with lateral roots by ~3-fold as compared to water alone under no stress condition, and by ~2.6-fold in combination with 200 µM ABA as compared to 200 µM ABA alone (Figure 4b). Together these results indicate that EBR not only overcomes ABA-induced inhibition of germination, but can also promote post-germination seedling growth characteristics.

Figure 4. Cotyledon opening and lateral root (LR) formation in seedlings under salt stress following pre-treatment with either EBR or ABA alone or together. Percentage of seedlings with fully open cotyledons (a); percentage of seedlings with LRs (b); average number of visible LRs per seedling (c); average length of the longest LR in seedlings (d), in response to pre-treatment with EBR (0 and 2 µM), ABA (100 and 200 µM), and ABA + EBR (as indicated in figures) under 0, 100 and 200 mM NaCl were recorded on the 13th day after seeds were put to germination. Error bars represent standard error (SE) of the mean for three replicates (n = 30). Different letters above the bars indicate significant differences in two-way ANOVA (p < 0.05) followed by Tukey’s post hoc test.

Auxin and GA are plant growth-promoting hormones. While auxin inhibits germination [35], GA promotes it [36]. The germination percentage was not influenced by 5 µM IAA (auxin) alone, and the combination of IAA with EBR showed the same increase as with EBR alone under no stress and salt stress conditions (Figure 5a). In contrast, the two hormones appeared to act synergistically in increasing hypocotyl length under no stress conditions (Figure 5b,d). A small but statistically significant increase was also seen on primary root length under no stress conditions (Figure 5c). Effects of IAA in combination with EBR were similar to those of EBR alone on cotyledon opening (Figure 5d) and lateral root formation (data not shown).

Seed treatment with 50 µM GA₃ alone did not affect germination percent as compared to the untreated control (Figure 6a). Interestingly, the 2-fold increase in germination percent by 2 µM EBR under salt stress condition (200 mM NaCl) was reduced to that of untreated control when EBR was combined with GA₃ (Figure 6a). GA₃ treatment alone increased hypocotyl length (Figure 6b,d) and cotyledon opening (Figure 6c,d). The effect of GA₃ on cotyledon opening under no stress condition was profound with 76% of GA₃ primed seedlings having fully opened cotyledons as compared to untreated seedlings, which in this experiment showed only partial emergence of cotyledons from the seed coat (Figure 6c,d). However, the effect of GA₃ on hypocotyl length and cotyledon opening was suppressed when GA₃ and EBR were used together.
Figure 5. Cotton seed germination and seedling growth under salt stress following treatment with either EBR or IAA alone or together. Percent germination (a); hypocotyl length (b); primary root length (c); and seedling phenotypes (d), in response to pre-treatment with EBR (0 and 2 µM), IAA (0 and 5 µM) and IAA plus EBR (as indicated in figures) under 0 and 200 mM NaCl were recorded on the 4th and 13th days, respectively. Error bars represent standard error (SE) of the mean for three replicates (n = 30). Different letters above the bars indicate significant differences in two-way ANOVA (p < 0.05) followed by Tukey’s post hoc test.

Figure 6. Cotton seed germination and seedling growth under salt stress following treatment with either EBR or GA3 alone or together. Percent germination (a); hypocotyl length (b); percentage of seedlings with fully open cotyledons (c); and seedling phenotypes (d), in response to pre-treatment with EBR (0 and 2 µM), GA3 (0 and 50 µM) and EBR plus GA3 (as indicated in figures) under 0 and 200 mM NaCl were noted on the 4th and 13th day, respectively. Error bars represents standard error (SE) of the mean for three replicates (n = 30). Different letters above the bars indicate significant differences in two-way ANOVA (p < 0.05) followed by Tukey’s post hoc test.
GA is reported to both negatively and positively regulate lateral root formation [37,38]. In our studies, treatment with GA$_3$ alone produced a minor increase in the number of lateral roots per seedling and lateral root length only under no stress conditions (not shown). Overall, treatment with none of the three hormones ABA, IAA or GA$_3$ produced any positive effects on germination and seedling growth under salt stress as compared with EBR.

3.3. Effect of EBR on Seed Germination and Seedling Growth under Heat Stress

Sicala V-2 seeds untreated and treated with EBR were germinated in Petri plates incubated at room temperature, 35 °C and 40 °C. EBR-treated seeds had significantly higher germination rates than untreated seeds under HS conditions (Figure 7a). Percent germination of EBR-treated seeds (2.0 µM) on day 2 were 77% and 20% at 35 °C and 40 °C, respectively, as compared to 14% and 3% of untreated seeds under the same conditions. More than 70% germination at 35 °C were recorded for 0.5 and 1.0 µM EBR-treated seeds, indicating >5-fold increase in germination rate over untreated control (Figure 7a). All concentrations of EBR enhanced lateral root growth under room temperature and 35 °C, and 2 µM EBR also promoted lateral root formation under 40 °C (not shown). These results indicate that 2 µM EBR was most effective in promoting germination and lateral root formation at 40 °C.

![Figure 7](image-url)
HS, seeds were placed in Petri plates for germination at room temperature. Germination percent was reduced by >2-fold in untreated seeds subjected to 70 and 75 °C, but treatment with 1 or 2 µM EBR recovered germination to the same level as of untreated seeds at room temperature (non-stressed) (Figure 7b). These results indicate that EBR is more effective in promoting tolerance against severe stress conditions but that the effects are EBR concentration- and HS condition-dependent.

In addition to its role in seed dormancy, ABA is also a stress hormone. We tested its effect alone or in combination with EBR on germination of seeds exposed to 75 °C for 15 min (Figure 7c). Interestingly, while ABA inhibited germination at room temperature, it had no further reduction on the high temperature-mediated decrease in germination. The addition of 2.0 µM EBR in the 200 µM ABA solution enhanced the germination of seeds exposed to 75 °C by 2.8-fold as compared to ABA alone (Figure 7c). It can be deduced from these results that ABA-induced inhibition of germination in response to stress depends on the stress treatment (cf. trends in Figures 3a and 7c) and that EBR consistently overrides ABA effects on germination.

Seeds exposed to 75 °C were also assessed for open cotyledons. Similar to effects noted before (Figure 6c), EBR and GA3 enhanced cotyledon opening with GA3 having the most notable effect in both unstressed and HS seeds (not shown).

4. Discussion

With rapid germination and growth, which relates to strong early plant vigor, crops can both recover from early season damage due to temperature fluctuations, short durations of drought, diseases and pests, as well as tolerate certain levels of soil salinity. The development of a robust root system during seedling emergence influences the further course of plant growth and the ability to tolerate environmental stresses. Similarly, the rapid enlargement, opening and greening of the cotyledon allows for the seedling to become active in photosynthesis, a process vital for life. The dynamic balance and interactions between various phytohormones controls seed dormancy and germination, and seed stress tolerance [39].

In the present study, we tested the effects of BR (EBR) on cotton seed germination and seedling growth under no stress and stress conditions. A relatively new class of phytohormones, BRs have been demonstrated to promote seed germination in various plant species and counteract the inhibitory effect of ABA, the phytohormone primarily responsible for seed dormancy [20–22]. Pre-treatment of cotton seeds with EBR, in most experiments, enhanced percent germination under no stress conditions as compared to untreated seeds, but there were distinct positive effects on seedling dry weight, cotyledon opening and lateral root formation, all of which indicate strong seedling vigour. Inter-experimental comparison of data in different experiments is not possible since two different varieties were used, and there was considerable variation in ambient temperature. As a result, some variability in growth parameters was observed across the different experiments. However, the patterns of effects were consistent. The effect of EBR on germination and seedling dry weight was even more striking under salt stress conditions (200 mM NaCl) (Figure 1), but lateral roots remained invisible in seedlings of both EBR-treated and untreated seeds under this condition (Figure 2). Salt stress causes remodeling of the root system architecture (RSA), including reduction of main root length and alteration to lateral root development [40]. Considering that the root is the first organ to sense salt and plays a role in ion exclusion, it is possible that a short and stubby primary root with no visible lateral roots is an adaptation to aid plant performance under salinity stress. According to Julkowska et al. [40], the correlation between main root length and number of lateral roots in Arabidopsis remains unaltered by salt stress. More detailed studies of cotton seedling RSA in the future will reveal whether or not this holds true in cotton, and also whether BR-mediated changes to RSA confer benefit to seedling growth or survival under stress conditions.
The ability of BR to override inhibition of germination by ABA is well documented in other plant species [20–22]. The present study demonstrates that EBR could overcome ABA-induced inhibition of germination not only under no stress conditions but also under salt stress (Figure 3a), and to some extent ABA-induced inhibition of cotyledon opening (Figure 4a). EBR treatment also maintained its positive effects on lateral root formation in seedlings in the presence of ABA (Figure 4b,c). BR has a vital role in regulating the initiation of lateral root primordia and it promotes lateral root initiation by increasing acropetal auxin transport [41], whereas ABA-induced lateral root inhibition is mediated by an auxin-independent pathway [42]. In the present study, priming with only the highest concentration (200 µM) ABA inhibited LR traits in cotton seedlings under no stress condition (Figure 4b–d); this is likely because following initial treatment with ABA, seeds were germinated and grown in the absence of the hormone.

Auxin plays a major role in seedling development but alongside ABA, auxin also promotes seed dormancy [35]. Treatment of cotton seeds with 5 µM IAA showed neither any inhibition nor enhancement of germination (Figure 5). Furthermore there was no effect on lateral root traits, but, as previously noted [43], IAA and EBR acted synergistically to enhance hypocotyl length. Prior to identification of BR’s role in seed germination, GA was recognised as the major determinant of seed germination. More recent studies have shown that BR and GA interact in controlling seed germination, which in rice occurs via common protein targets that function in metabolism reactivation, coleoptile elongation in rice, and mobilisation of reserves [44]. BR and GA also perform other overlapping functions such as cell elongation, flowering, senescence and fertility [45]. Pre-treatment of cotton seeds with GA3 alone led to greater increases in hypocotyl length and cotyledon opening than pre-treatment with EBR alone or EBR plus GA3 (Figure 6b,c), while EBR had the best effect on germination, especially under salt stress conditions (Figure 6a). Thus, taking into account the various cotton seed treatments used in this study, if one was to choose seed priming for best germination and early seedling growth results under salt stress, EBR emerges as the most promising hormone.

Cotton is regarded as a crop of hot, semi-arid regions with some regions characterised with daytime maximum temperatures >40 °C [46]. Nevertheless, high temperature stress can drastically impact cotton seed germination, early growth, flowering and boll formation. The optimum temperature for cotton seed germination and seedling growth is generally taken to range between 28–30 °C, although some studies have indicated 33–36 °C as being optimal for germination and 39 °C to 40 °C as the maximum temperature under which cotton seed can germinate [47]. In our study, percent germination was highest at 35 °C and it was dramatically enhanced by pre-treatment with EBR (Figure 7a) under all temperatures tested. EBR also enhanced lateral root formation, including at 40 °C. The effect of EBR on seedling heat stress tolerance is well studied [18,25]; extrapolating from these studies it is postulated that BR maintains or even enhances the metabolic and mitotic activities required for germination by protecting and upregulating the enzymes and proteins involved in these activities.

Cotton seeds are exposed to high air temperatures during the delinting process. Germination results of seeds exposed to between 50 °C and >80 °C have indicated that temperatures above 70 °C decrease germination, and temperatures as high as 60 °C increase germination [48]. Our results are consistent with these observations in that exposure of seeds to 50 and 60 °C enhanced germination as compared to seeds that received no heat treatment, and exposure to 70 °C and 75 °C decreased percent germination (Figure 7). Since EBR enhanced germination of seeds exposed to 70 °C and 75 °C, the same rationale presented above can be applied for how EBR may confer protective benefit under heat stress. These results not only confirm the beneficial effects of EBR against heat stress [18,25], but also add a new facet to the role of EBR in germination under suboptimal conditions, urging serious consideration for BR to be used in cotton seed priming before sowing.
In addition to the previously mentioned studies, several other works have highlighted the role of brassinosteroids in stress resistance. For instance, Rhaman et al. (2008) explored seed priming methods and their application in field crops, showing promising results in managing stress tolerance. Similarly, Divi et al. (2013) demonstrated that brassinosteroids confer stress tolerance in Arabidopsis thaliana and Brassica napus, indicating their potential for improving stress resistance in crop plants.

Moreover, the role of brassinosteroids in improving the efficiency of agronomic practices in combating salinity stress is also noteworthy. Dong (2020) reviewed the role of agronomic practices in reducing the impact of salinity stress on cotton, emphasizing the importance of these practices in maintaining crop productivity under adverse conditions.

In summary, the research community has made significant progress in understanding the role of brassinosteroids in stress resistance and their potential applications in enhancing crop stress resistance. Further research is needed to fully harness the potential of these phytohormones in improving crop productivity and sustainability.
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