ACTA SCIENTIARUM POLONORUM Acta Sci. Pol. Hortorum Cultus, 20(5) 2021, 15–23

https://czasopisma.up.lublin.pl/index.php/asphc ISSN 1644-0692 e-ISSN 2545-1405 https://doi.org/10.24326/asphc.2021.5.2

ORIGINAL PAPER

Accepted: 9.09.2020

THE INFLUENCE OF EXOGENOUS GIBBERELLIC ACID (GA_3) AND 24-EPIBRASSINOLIDE (24-EpiBL) ON SEED GERMINATION AND THE EXPRESSION OF GENES INVOLVED IN GA AND BR SYNTHESIS/SIGNALLING IN PEPPER (*Capsicum annuum* L.)

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ABSTRACT

Gibberellins (GAs) and brassinosteroids (BR) are the plant hormones involved in various physiological processes including seed germination. In this study, the effects of exogenous gibberellic acid (GA_3) and 24-epibrassinolide (24-EpiBL) treatments on the expression of key genes involved in GA and BR synthesis/signalling during seed germination were investigated in pepper (*Capsicum annuum* L).

The expressions of *BES1* and *BRI1* involved in BR synthesis/signalling pathway as well as *GA3OX1* and *GA20OX1* associated with gibberellic acid biosynthesis in plants were determined. Exogenous GA_3 treatments increased *BES1* expression and the highest increase was determined with 10^{-8} M BR + 100 µM GA_3 ($P < 0.05$). On the contrary, the expression of *BRI1* gene was significantly decreased by 10^{-8} M BR + 100 µM GA_3 ($P < 0.05$). The expression of *GA3OX1* gene was induced with BR and GA_3 treatments ($P < 0.05$). *GA20OX1* gene expression was generally higher compared to the expression of *GA3OX1* and significantly increased by the GA_3 treatments. Our findings are expected to bring an insight to the influence of BRs during seed germination together with the expression of associated genes.

Key words: brassinosteroids, *Capsicum annuum*, expression, germination, gibberellins, pepper

INTRODUCTION

Gibberellins (GAs) and brassinosteroids (BRs) are the two prominent plant hormones associated with growth promoting activities. GAs are tetracyclic diterpenoid phytohormones regulating many phases of plant growth and development. They stimulate cell elongation and are involved in seed germination, hypocotyl elongation, induction of flowering, anther, pollen and fruit development [Miransari and Smith 2014]. GAs are required for seed germination in many plant species. Endogenous GAs influence seed germination directly by increasing the growth potential of embryo by the induction of hydrolytic enzymes [Kucera et al. 2005].

Brassinosteroids are a group of plant-specific polyhydroxylated steroidal hormones that regulate a wide range of growth and developmental events including

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cell proliferation, cell division and differentiation, root and stem growth, flower, seed and fruit formation [Gudesblat and Russinova 2011]. Research findings reveal the growth-promoting roles of BRs in plants [Cheon et al. 2013]. BRs are reported to play a role in promoting germination and hypocotyl elongation in *Arabidopsis* [Steber and McCourt 2001].

Recent studies suggest an interaction between GAs and BRs during signalling and/or synthesis [Ross and Quittenden 2016, Unterholzner et al. 2015]. While some researchers report that BRs and GAs interact with each other at the plant signalling level [Bai et al. 2012, Li and He 2013, Wang et al. 2014] some other researchers suggest that BRs are the main regulators of gibberellic acid biosynthesis [Tong et al. 2014, Unterholzner et al. 2015]. In the latter hypothesis, it is suggested that BRs stimulate growth by affecting the gibberellic acid (GA_3) levels in the plant. Tong et al. [2014] reported that brassinosteroid mutants in rice show sensitivity to gibberellic acid. It is stated that this information supports the views that BRs stimulate growth by increasing the GA_3 levels in the plant. Further evidence was provided by the application of GA_3 to the brassinosteroid mutant (bri1-301) in *Arabidopsis* [Unterholzner et al. 2015].

GA 20-oxidase (*GA20OX*) and *GA 3-oxidase* (*GA3OX*) gene families are involved in gibberellin biosynthesis and modulate conversion of precursor GAs into a bioactive form [Qin et al. 2013]. In *Arabidopsis*, *GA20OX* is reported to modulate conversion of GA12/GA53 to GA9 /GA20 while *GA3OX* modulating the conversion of GA9/GA20 to GA4/GA1 [Yamaguchi and Kamiya 2000].

Brassinosteroid insensitive 1 (*BRI1*) gene, Brassinazole-resistant 1 (*BZR1*) and BRI1-EMS suppressor 1 (*BES1*) transcription factor are the most studied genes and transcription factors associated with BR biosynthesis [Hategan et al. 2014]. *BRI1* is the BR receptor that is responsible for initiating the events of BR signalling and is the key component of the pathway in *Arabidopsis* [Hategan et al. 2014]. *BES1/BZR1* are the two key transcription factors in the BR signalling pathway that directly regulates the expression of target genes and positively mediate BR responses [Li et al. 2018]. *BES1* is the closest homolog of *BZR1*, with 88% overall amino acid sequence identity [Zhao et al. 2002]. *BES1/BZR1* are reported to bind to the promoters of a large number of genes involved in a wide range of cellular activities and biological processes [Li and He 2013].

Both GAs and BRs are known to be involved during seed germination. The objectives of this research were (i) to understand how GA and BR treatments and their combination influence seed germination in pepper (*Capsicum annuum* L.) (ii) to reveal how hormone treatments change gene expression profiles in the light of previous literature findings with model organisms.

MATERIALS AND METHODS

Plant material. In this study, a standard pepper cultivar (*Capsicum annuum* L. 'Sera Demre 8') (Biogen Seeds) was used for the germination tests and the expression analysis. The experiment included a total of 10 treatments including BR and GA_3 applications and the water control. Each hormone treatment had 3 biological replicates, each replicate containing 2 petri dishes with 50 seeds each. The seeds were surface sterilized with 1% sodium hypochlorite for 10 minutes. rinsed with double distilled water for 30 seconds and allowed to dry for 30 minutes prior to the experiment. Sterilised filter papers were placed in 9 cm sterile plastic petri dishes and 50 seeds were placed in each petri dish.

Brassinosteroid and giberellic acid treatments. 24-Epibrassinolide (22R, 23R, 24R-2α, 3α, 22,23-Tetrahydroxy-B-homo-7-oxa-5α-ergostan-6-one $C_{28}H_{48}O_6$, ≥85%, MW: 480.68) and GA_3 ($C_{19}H_{22}O_6$, ≥90%, MW: 346.37) purchased from Sigma® were used in the study. 24-Epibrassinolide (24-EpiBL) was prepared at a concentration of 10^{-8} M according to Da Silva et al. [2015] and gibberellic acid concentrations were 10 µM, 50 µM and 100 µM according to previous studies [Ma et al. 2018]. The hormones were prepared and maintained in dark conditions and covered with aluminium foil to avoid exposure to light. Hormone concentrations were as follows: (i) 10^{-8} M BR (ii) 10^{-8} M BR + 5 µM GA_3 (iii) 10^{-8} M BR + 10 µM GA_3 (iv) 10^{-8} M BR + 50 µM GA_3 (v) 10^{-8} M BR + 100 µM GA_{33} (vi) 100 µM GA_3 (vii) 50 µM GA_3 (viii) 10 µM (ix) 5 µM GA_3 and the water control.

The seeds in each petri dish were moistened with hormone solution (4 ml). In case of combined treatments, 2 + 2 ml (BR + GA) from each solution was ap-

plied in order to reach to a total volume of 4 ml in each petri dish. Hormone treatments were performed in dark conditions. Each petri dish was placed in sealed boxes and germinated in an incubator adjusted to 25°C [ISTA 2017].

Seed germination assays. The germinated seeds (2 mm radicle emergence) were counted at every 4 h. The highest germination rate (%) was determined at 108 h upon imbibition in pepper. Therefore, germination rate of seeds was recorded for each treatment at that particular time point.

RNA isolation and real-time qPCR (qRT-PCR) analysis. Germinating seed samples were collected at 108 h for the gene expression analysis. The collected samples were immediately transferred to liquid nitrogen and placed in –80°C freezer prior to RNA analysis. RNA isolation was performed using 30 mg ground tissue with the Promega® SV Total RNA Isolation System kit (Madison-USA) according to the manufacturer's protocol. Total RNAs were extracted separately from each biological replicate and visualised on 1% agarose gel. Promega GoScript™ Reverse Transcription System (Madison-USA) was used for cDNA synthesis. The amount of cDNA was measured with the Nanodrop ND-1000 spectrophotometer and the cDNA samples were used to determine target gene expression levels.

The expressions of *BRI1* (Brassinosteroid Insensitive 1) and *BES1* (BRI1-EMS-Suppressor 1) were used together with the *GA3OX1* (Giberellin 3-oxidase-1) and *GA20OX1* (Giberellin 20-oxidase-1) were determined with qRT-PCR analysis.

In the present study, the primer sequences of the *GA20OX1* and *BRI1* genes are designed from *Capsicum annuum* and *BES1* transcription factor and *GA3OX1* gene primer sequences were designed from the conserved regions between *Arabidopsis* and *Solanaceae.* The *Capsicum annuum Actin* gene was selected as the housekeeping control gene for qRT-PCR analysis. The primer sequences and gene IDs are presented in Table 1.

Real time quantitative PCR amplifications were performed using Roche Light Cycler 480 and all the reactions run three times. The PCR was performed in 8.5–13 µL of reaction mixture composed of 2–4 µl of cDNA, 10 pmol forward and reverse primer, 5 µl of LightCycler® 480 SYBR Green I Master (Roche) and ddH_2O. The standard curve was prepared from serial dilutions of control cDNAs. PCR conditions were performed as follows: 95°C for 10 min, followed by

Table 1. Primer sequences designed for qRT-PCR analysis in pepper

Primer sequences		Gene ID
BRI1		
Forward	5′-CTGTGTACAAAATCCAGGGTGC-3′	XM_016688118.1 *Capsicum annuum BRI1*-like (LOC107843741), mRNA
Reverse	5′-AGTTGCCAAGATCCACACGA-3′	
BES1		
Forward	5′-CCTGTCACTCCACCAGTGTC-3′	NM_101792.4 *Arabidopsis thaliana* (BZR1) family protein (BES1), mRNA
Reverse	5′-AGACACCGCATAAAACGGGT-3′	
GA3OX1		
Forward	5′-AGATATTGATGTGGCCGGGA-3′	XM_006356115.2 : *Solanum tuberosum GA3OX1*-like (LOC102585516), mRNA
Reverse	5′- GCATGTTCAATTGGGGAGCC-3′	
GA20OX1		
Forward	5′-TAGGCATAGAGAGGAGCCACTT-3′	XM_016709248.1 : *Capsicum annuum GA20OX1*-like (LOC107863364), mRNA
Reverse	5′-TTGAAGCCCTCCAACGCA-3′	
ACTIN		
Forward	5′-TGTTATGGTAGGGATGGGTC-3′	XM_016683691.1 : *Capsicum annuum* actin-100 (LOC107840006), mRNA
Reverse	5′-TTCTCTCTATTTGCCTTGGG-3′	

45 cycles at 95°C for 10 sec, 53–57°C for 10 sec, 72°C for 5 sec. The specificity of the PCR amplification was checked with a melting curve analysis (from 95°C to Tm) following the final cycle of the PCR. PCR conditions were optimized for high amplification efficiency >95% for all primer pairs used. The *Ct* (Cycle threshold) values on the amplification curves were obtained between the 20th and 35th cycles. In melting curve analyses, overlapping single peak images were obtained and dimer presence was not detected. *Ct* values were determined by using of the peak profiles.

Statistical analysis. The experiment was conducted according to a completely randomised design. For the germination tests, the significance of differences among different hormone treatments were tested using the SPSS software package and the significance was evaluated at $P < 0.001$ level. The relative expression levels were calculated by using the $2^{-\Delta\Delta CT}$ (the delta-delta-Ct or ddCt) algorithm [Livak and Schmittgen 2001]. Reaction efficiency (RE) was considered as 1 (one). $P < 0.05$ were considered statistically significant.

RESULTS

The germination rate (%) of pepper seeds treated with GA_3 and 24-EpiBL. The seed germination rate was determined as 75% with 10^{-8} M BR treatment followed by the water control seeds (70.66%) at 108 h upon imbibition. The germination rate of GA_3 applied as 10^{-8} M BR + 5µM GA_3, 10^{-8} M BR + 10 µM GA_3 were 66.33% and 61%. Seeds applied with 5µM GA_3 germinated at a rate of 66%, germination was not obtained at higher concentrations of GA_3 alone or in combination with BR at 108 h ($P < 0.001$) – Figure 1.

Influence of GA_3 and BR treatments on the expression of *BES1, BRI1, GA3OX1, GA20OX1* genes during seed germination in pepper. Expression of *BES1*, the homolog of *BZR1* regarded as the major transcription factors in brassinosteroid biosynthesis pathway was determined in germinating pepper seeds with qRT-PCR analysis in samples taken at 108 h. According to the findings, the highest and significant

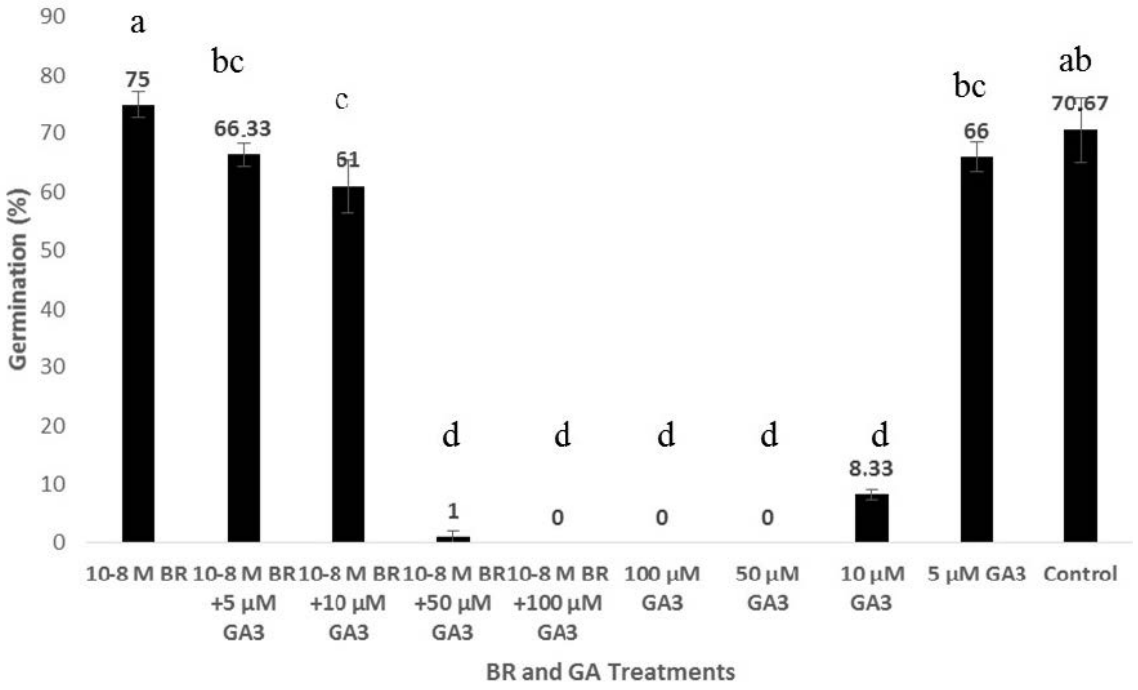

Fig. 1. Mean germination (%) of pepper seeds defined as 2 mm radical emergence treated with different BR and GA_3 concentrations at 108 h

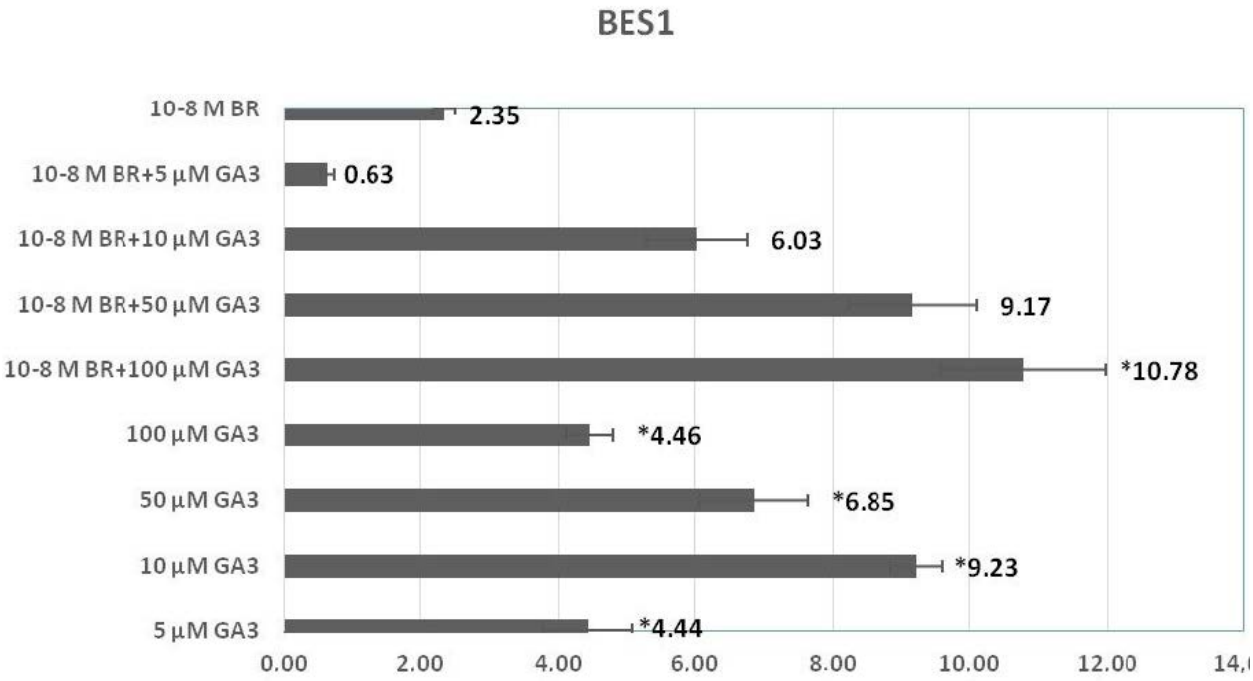

Fig. 2. *BES1* expression of BR and GA treated pepper seeds relative to control (Fold change)

* Statistically significant according to $P < 0.05$ by Student's T-Test

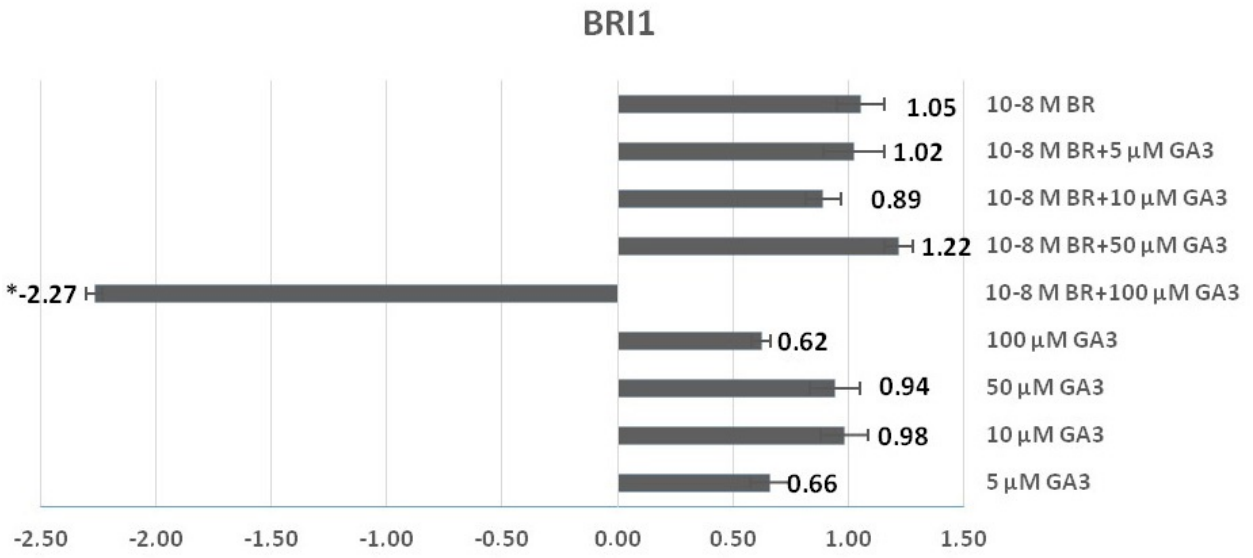

Fig. 3. *BRI1* expression of BR and GA treated pepper seeds relative to control (Fold change)

* Statistically significant according to $P < 0.05$ by Student's T-Test

increase in the expression of *BES1* was determined with 10^{-8} M BR + 100 µM GA_3; followed by 10 µM GA_3, 50 µM GA_3, 100 µM GA_3 and 5 µM GA_3 treatments ($P < 0.05$). The increase in *BES1* expression of 10^{-8} M BR + 50 µM GA_3; 10^{-8} M BR + 10 µM GA_3 and 10^{-8} M BR treatments was not statistically significant compared to the control. The slight increase in *BES1* expression in 10^{-8} M BR + 5 µM GA_3 treatment was not significant compared to other treatments and the control ($P > 0.05$) – Figure 2.

The expression of *BRI1* gene was generally low and was not different among different treatments

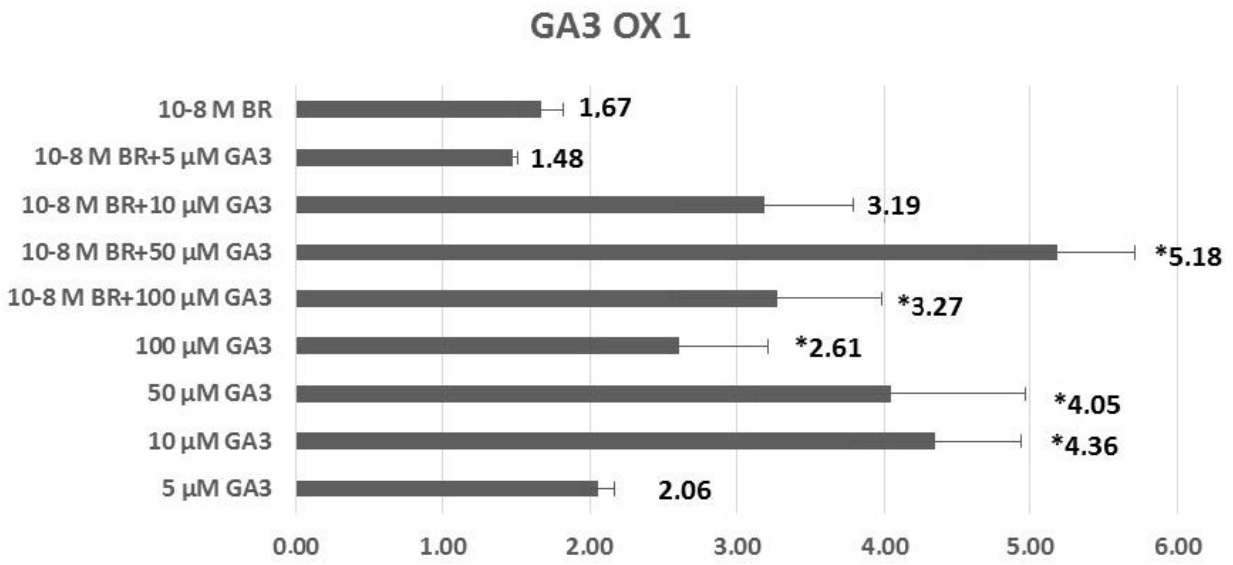

Fig. 4. *GA3OX1* expression of BR and GA treated pepper seeds relative to control (Fold change)

* Statistically signi icant according to $P < 0.05$ by Student's T-Test

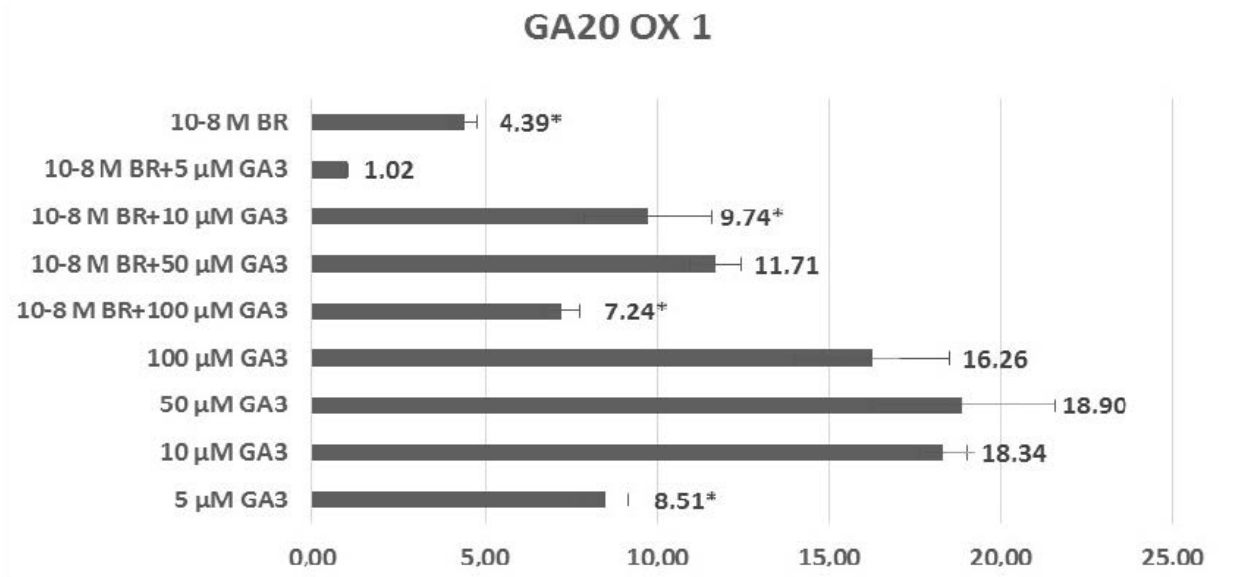

Fig. 5. *GA20OX1* expression of BR and GA treated pepper seeds relative to control (Fold change)

* Statistically significant according to $P < 0.05$ by Student's T-Test

($P > 0.05$). Only the 10^{-8} M BR + 100 µM GA_3 treatment was found to decrease *BRI1* gene expression significantly ($P < 0.05$) – Figure 3.

GA3OX1 gene expression was highest with 10^{-8} M BR + 50 µM GA_3 treatment followed by 10 µM GA_3, 50 µM GA_3, 10^{-8} M BR + 100 µM GA_3 and 100 µM GA_3 ($P < 0.05$). However, the increases with 10^{-8} M BR + 10 µM GA_3 and 5 µM GA_3 treatments were not statistically significant compared to the control and other treatments ($P > 0.05$) – Figure 4.

The expression of *GA20OX1* was significant with 10^{-8} M BR + 10 µM GA_3; 10^{-8} M BR + 100 µM GA_3; 5µM GA_3 and 10^{-8} M BR ($P < 0.05$). However, although the expressions of 50 µM, 10 µM, 100 µM GA_3 treatments and 10^{-8} M BR + 50 µM GA_3, 10^{-8} BR + 5 µM GA_3 were significantly increased, this increase was not statistically different than control based on the Ct values (Fig. 5).

DISCUSSION

GAs are essential for germination in many plant species and recent evidence suggest the influence of BRs during germination in *Arabidopsis* [Steber and Mc Court 2001, Yang et al. 2011]. BRs have been previously shown to rescue the germination phenotype of severe GA mutants and of the GA-insensitive mutant *sleepy1* in *Arabidopsis* demonstrating the role of BRs on seed germination [Steber and Mc Court 2001]. With these findings, these researhers suggest that the BR signal is required to reverse ABA-induced dormancy and stimulate germination in *Arabidopsis*. Barboza da Silva [2015] revealed the improved germination performance of bell pepper seeds primed with 24-EpiBL.

In order to understand the influence of exogenously applied GA_3 and BR on germination together with the key genes involved in GA_3 and BR biosynthesis, we conducted germination assays and applied BR in the active form of 24-EpiBL and GA_3 to pepper seeds at varying concentrations. The findings are given on the basis of countings at 108 h upon imbibition. The results suggested a positive influence of BR treatment on seed germination in pepper. We applied varying concentrations of GA_3, and the results indicated a concentration dependent response of GA_3. The highest germination was determined at 5 µM, alone or with 10^{-8} BR. However, it should be noted that these results are taken at 108 h since this was the time point we have observed the highest seed germination upon imbibition. The seeds were germinated in all hormone applications according to our follow up experiment which was continued until the 14th day.

Key genes involved in BR and GA synthesis/signalling pathways were analysed. *GA20OX1* and *GA3OX1* are the key gene families involved in gibberellin biosynthesis pathway catalysing sequential conversions towards the synthesis of bioactive GAs. *BRI1* gene is responsible for initiating the events of BR signalling and *BES1,* the closest homolog of *BZR1,*involved in BR biosynthesis. Recent studies suggest that BRs control a wide range of biological processes through the direct regulation of gene expression via the *BZR1/BES1* family of transcription factors [Li et al. 2018]. In our study, *BES1* transcription factor was mostly increased with 10^{-8} M BR + 100 µM GA_3 followed by GA_3 treatments. Interestingly, seed germination was not obtained at these particular treatments at the time of sampling. Contrarily, when the germination was highest with 10^{-8} M BR, the *BES 1* expression was not statistically significant ($P > 0.05$). Germination of seeds treated with 10 µM GA_3, was 8.33% and *BES1* induction was significantly high (9.23 fold) ($P < 0.05$). Whereas germination of seeds treated with 10^{-8} M BR + 10 µM GA_3 was 61% and the expression of *BES1* was not statistically different than the control ($P > 0.05$). Ross [2016] report that DELLA proteins which are negative regulators of GA signaling are cleaved in the presence of bioactive GA and physically interact with the *BZR1* transcription factor.

BRI1 gene expression was generally lower in all hormone treatments however, 10^{-8} M BR with 100 µM GA_3 was found to significantly downregulate *BRI1* gene expression ($P < 0.05$). *BRI1* encodes a critical component of the BR receptor [Li and Chory 1997]. Studies with dominant *bzr1-1D mutants suggested that* increases in *BZR1* (*BES1*) protein accumulation suppressed the BR-insensitive *bri1* and *bin2* mutants [Wang et al. 2002]. Similarly, Chung and Choe [2013] reported that when the BR signal is activated, BR-specific transcription factor, *BZR1* (*BES1*), inhibits transcription of BR biosynthetic genes through feedback downregulation mechanisms. In line with these reports, our findings with 10^{-8} M BR + 100 µM GA_3

treatment gave the highest *BES1* expression. while suppressed *BRI1* (Figs 2 and 3).

The expression of *GA3OX1* gene was induced with most of the treatments ($P < 0.05$). However, the induction with 10^{-8} M BR + 50 µM GA_3 and 5 µM GA_3 was not statistically different than the control and the induction with 10^{-8} M BR + 5 µM GA_3 and 10^{-8} M BR was not significant according to T-test analysis ($P > 0.05$). The expression of *GA20OX1* gene was generally higher compared to *GA3OX1* gene expression. However, the induction was not statistically significant with most of the treatments. *GA20OX1* and *GA3OX1* are associated with the conversion of GA to their bioactive forms. Studies report that *GA3OX1* is induced in the cortex and endodermis of the embryo axis of germinating seeds [Mitchum et al. 2006]. Therefore, the induction of both genes could be attributed to the developmental stage of germinating seeds during seedling development, hypocotyl and root elongation in each hormone treatment while sampling.

Unterholzner et al. [2015] reported a joint regulation of plant growth and development by GAs and BRs in *Arabidopsis* suggesting that *BES1/BZR1* may regulate *GA20OX1* expression evidenced by BR treatment inducing *GA20OX1* expression in *Arabidopsis* mutants. Same researchers conclude that since DELLA proteins are degraded with GA as reported by several other researchers, an induction of GA would stimulate *BES1/BZR1* activity downstream in the signalling pathway.

In the present study, we have demonstrated the influence of exogenous BR and GA treatments during seed germination in pepper together with the expression of key genes associated with BR and GA biosynthesis.

CONCLUSION

BRs and GAs are plant growth promoting hormones that have crucial roles in plant physiology. Although the influence of GAs on seed germination is well known and described, less information is available about the influence of BRs on seed germination. In this research we aimed to understand how GA_3 and 24-EpiBL treatments and their combination influence seed germination in pepper in the light of the expression analysis of major genes associated with GA and BR biosynthesis. Our findings demonstrated that like GA_3, 10^{-8} M 24-EpiBL also had a positive impact on seed germination. Although seeds in all treatments germinated within a two weeks' interval, we selected 108 h time point upon imbibition for qPCR analysis. We presented the germination values and the results obtained by the expression analysis of some major genes at 108 h. In the light of these findings, it is ex-pected that our findings might provide an insight for future research prospects.

ACKNOWLEDGEMENTS

We thank Dr. K. Kazan for useful comments on the manuscript.

The authors have no conflict of interest.

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