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Brassinosteroids Effectively Enhance Sunflower Resistance against Parasitic Weed (*Orobanche cumana*) Infection

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Abstract: Broomrape (*Orobanche cumana*), root parasitic weed, negatively affects sunflower, thereby causing severe yield losses and required controls on *O. cumana* infestation. Brassinosteroids (BRs) plays key roles in plant growth and provides resilience to weed infection. This designed study evaluated the mechanisms by which BRs ameliorates *O. cumana* infection in sunflower (*Helianthus annuus*). The pretreatment of sunflower seeds with various concentrations of BRs (1, 10, and 100 nM) were applied with and without *O. cumana* infestation. Under *O. cumana* infection, a significant reduction in plant height (38.61%), fresh/dry biomass, photosynthesis and regulation in plant defense (POX, GST), BRs signaling (*BAK1, BSK1* to *BSK4*) and synthesis (*BRI1*, *BR6OX2*) associated genes. Moreover, *O. cumana* elevated the levels of MDA, OH^-, H_2O_2 and $O_2^{\bullet-}$ in leaves/roots by 77%/112%, 62%/104%, 57%/98% and 55%/89.4% and caused ultrastructural damages in leaves/roots, suggesting the severe oxidative or cellular damages in sunflower. In response, plants activate activities of SOD and POD enzymes as well as reduced or oxidized glutathione, but unable to stimulate APX and CAT activities. Addition of BRs, especially at 10 nM notably lowered the production of oxidative stress, activated the enzymatic and non-enzymatic antioxidants. The downregulation in the reported genes by BRs attributed to the increased resilience of sunflower (via susceptible reaction). In nutshell, the pretreatment of BRs enhanced the sunflower resistance to *O. cumana* infection by improving the planta growth and biomass, photosynthesis, activating the antioxidant defense system, lowering the oxidative stress, cellular damages and modulating the expression of BR synthesis and signaling genes.

Keywords: Brassinosteroids; *Orobanche cumana*; reactive oxygen species; sunflower; antioxidants defense

1. Introduction

Sunflower (*Helianthus annuus* L.) is a popular oilseed crop grown all over the world as a major contributor to livestock feed. Sunflower cultivation is more competitive in some countries, such as Asia and South Africa, than other crops i.e. maize, soybeans, and sorghum [1]. The requirement for obtaining sunflower seeds (edible oil) and by-products has grown due to the continual expansion in human population. To meet demand, it is necessary to step up efforts to increase sunflower

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production. Sunflower, along with soybean and rapeseed, are the three most major oilseed crops farmed today. It has long been recognized as a key source of high-quality edible oil [2].

Broomrape species (*Orobanche* and *Phelipanche* spp., *Orobanchaceae*) are particularly harmful to agricultural crops, including tobacco, tomato, and sunflower. These weeds drastically reduced the crops quality and production [3]. Broomrape (*Orobanche cumana*), root parasitic weed, is a significant barrier to sunflowers cultivation mainly in Middle East, Southeast Europe, and Asia [4]. The invasion of *O. cumana* have potential to reduce the sunflower yield up to 80% [5]. The basic infection mechanism is that *O. cumana* interferes with planta metabolism, restricts the uptake of essential mineral nutrients, impairs the photosynthetic performance, extra generation of reactive oxygen species, lipids peroxidation, imposes the ultrastructural cellular damages and synchronizes the antioxidants defense system [6–8]. Various approaches have been utilized for the management of root parasitic plant (*O. cumana).* For instance, some herbicides such as imazamox and glyphosate displayed herbicidal effects on *O. cumana* [9]. But, sometimes they cause adverse effects on the crop productivity [10]. Therefore, effective management of *O. cumana* infection is required. Recent investigations have documented the involvement of different plant hormones in plant growth promotion and management of *O. cumana* infestation [7,11]. Brassinosteroids (BRs), plant steroid hormone, are typically linked with plant developmental process and facilitates planta immunity to biotic stresses [12]. Being a signaling hormone, BRs induces the hypersensitive response, the expression of genes associated to pathogenesis, and ensured systemic resistance acquired by plants [13]. However, it is rarely known how BRs improve the plant tolerance to *O. cumana* invasion in sunflower plants. Hence, our main objective was to evaluate the protective roles of BRs against *O. cumana* invasion by targeting the plant growth traits, photosynthetic machinery, oxidative stress, antioxidants defense, expression of different genes and cellular ultrastructural changes in sunflower seedlings.

2. Results

2.1. Exogenous BR Improved the Plant Phenotype and Growth Attributes against O. cumana Infection

To asses the protective roles of BRs in controlling the *O. cumana* infestation, we checked the impacts of different levels of BR on the plant phenotype, plant height, fresh biomass, dry biomass and number of *O. cumana* spikes in sunflower roots under *O. cumana* infection (Figure 1A–H). A clear phytotoxic effects in the form of chlorosis of leaves, yellow color of leaves, plant growth inhibition (Figure 1A) and higher number of *O. cumana* (Figure 1B) facilitated by *O. cumana* infection were observed. While, BRs treatments minimized these visual toxic symptoms as noticed by the better plant growth performance and less number of *O. cumana* attached with sunflower roots (Figure 1A and B). The alone application of *O. cumana* significantly declined the stem fresh weight (SFW) (34.36%), stem dry weight (SDW) (32.24%), root fresh weight (RFW) (34.02%), root dry weight (RDW)(53.33%) and plant height (PH) (38.61%) and number of *O. cumana* spikes when compared with relative controls. In contrast, the exogenous applications of BR reversed the *O. cumana* infection and notably improved the SFW (30.66%), SDW (29.66%), RFW (19.37%), RDW (28.57%) , PH (42.49%) and reduced the *O. cumana* spikes attached with roots as compared to alone *O. cumana* infection (Figure 1C–H).

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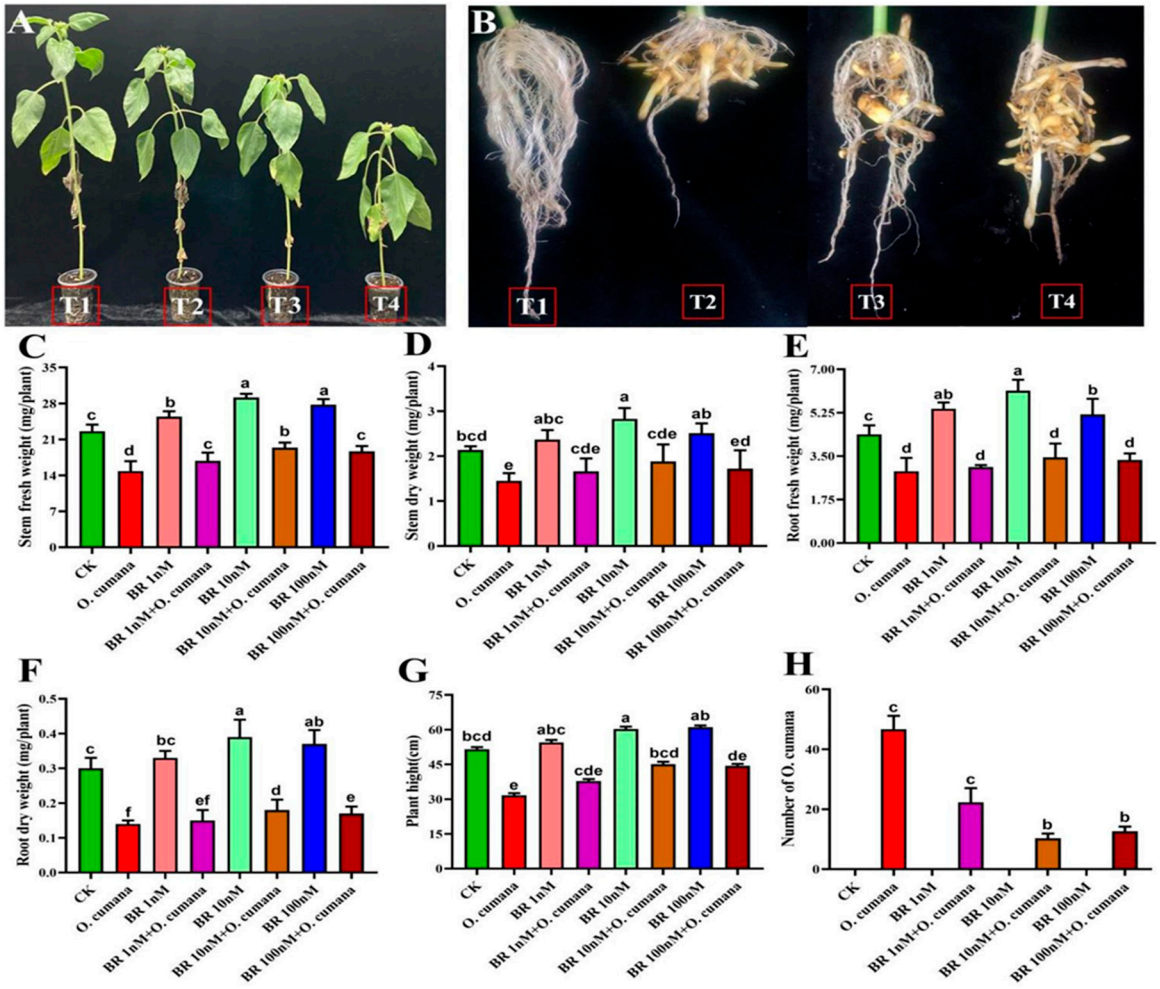

Figure 1. Effects of different concentrations of BR (1, 10, 100 nM) and *O. cumana* infection on **(A)** plant biomass **(B)** visual appearance of roots (T1 represent the control, T4 represent *O. cumana* while T1, T2 represent *O. cumana* + BR 10 nM and *O. cumana* + BR 100 nM) **(C, D)** stem fresh/dry weight **(E, F)** root fresh/dry weights, **(G)** plant height (cm) and **(H)** number of *O. cumana* spikes in sunflower.

These findings verified that supplementation of BR (especially at 10 nM) was more effective than BR (100 nM) in attenuating the *O. cumana*-induced toxic effects on sunflower growth performance. While, the BRs mediated reduction in *O. cumana* spikes in sunflower roots indicated the involvement of BRs in planta defense responses.

2.2. Exogenous BRs Improved the Chlorophyll Contents and Photosynthetic Apparatus against O. cumana Infection

The potential effects of exogenously applied BRs on chlorophyll's and gas exchange parameters (stomatal conductance, net photosynthetic rate, transpiration rate and intercellular CO_2 concentration) were noticed in the leaves of sunflower plants under *O. cumana* infection (Figure 2A–G). The alone applications of *O. cumana* greatly reduced the levels of *Chl a* (33.53%), *Chl b* (35.82%), carotenoids (43%), stomatal conductance (32.92%), net photosynthetic rate (37.91%), transpiration rate (33.61%), and intercellular CO_2 concentration (39.41%). In contrarily, applications of BR (particularly at 10 nM) effectively improved the photosynthetic attributes including stomatal conductance (30.77%),

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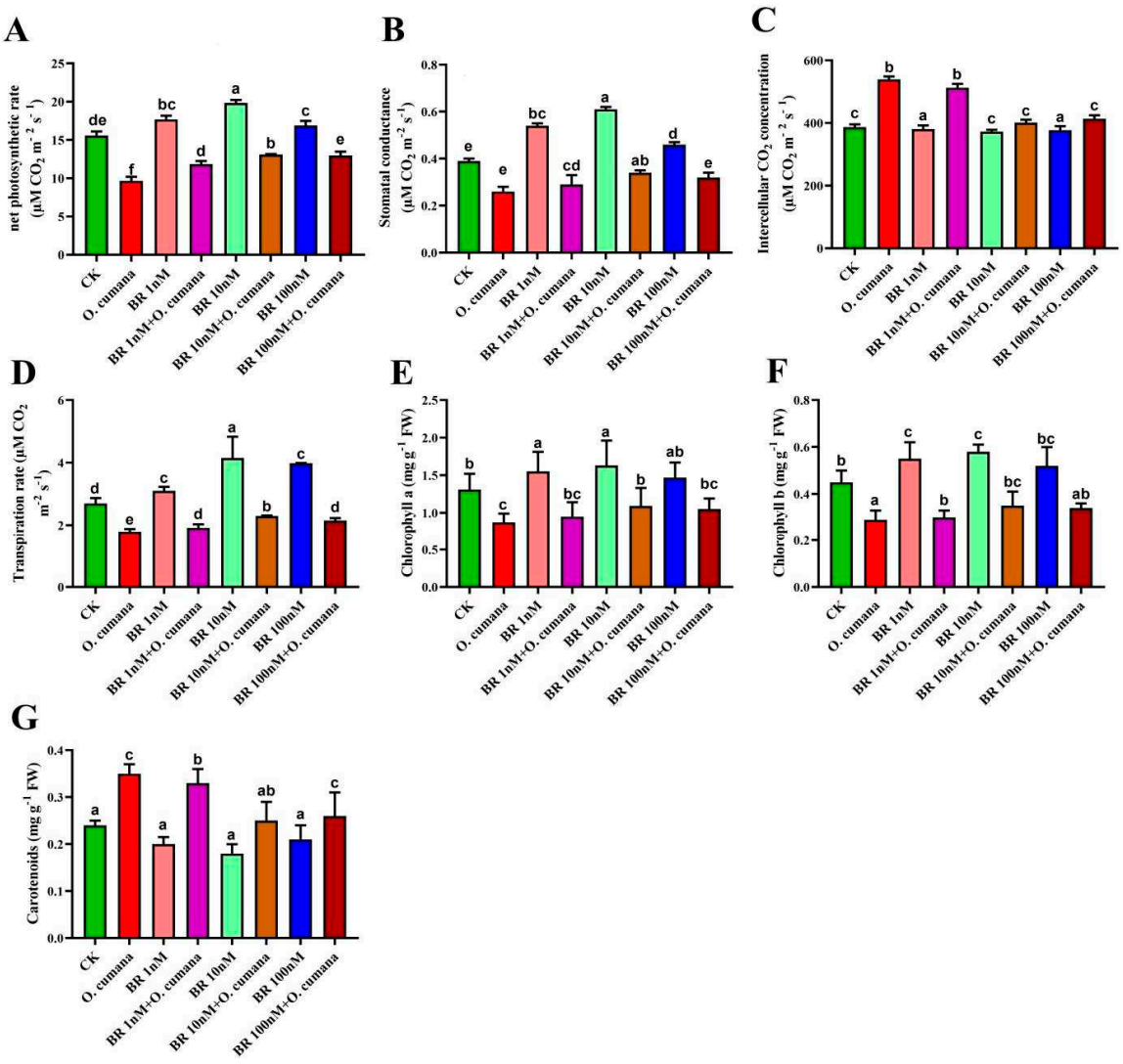

Figure 2. Effects of different concentrations of BR (1, 10, 100 nM) and *O. cumana* infection on the photosynthetic traits including **(A)** net photosynthetic rate **(B)** stomatal conductance **(C)** intercellular CO_2 concentrations **(D)** transpiration rate and chlorophyll content **(E)** Chl a **(F)** Chl b **(G)** carotenoids in sunflower.

net photosynthetic rate (decreased 35.38%), transpiration rate (28.74%), intercellular CO_2 concentration (25.61%), *Chl a* (24.71%), *Chl b* (21.03) and carotenoids (28.57%) under *O. cumana* infection (Figure 2A–G). These findings suggested the protective roles of BR in alleviating the *O.cumana* indulged photosynthetic inhibition in sunflower.

2.3. Exogenous BRs Reduce the Oxidative Damages Induced by O. cumana Infection

Under alone infection of *O. cumana*, MDA, OH^-, H_2O_2 and $O_2^{\bullet-}$ levels were dramatically increased in leaves/roots by 77.22%/112.76%, 62.46%/104.41%, 57.56%/98.31% and 55.02%/89.44%, respectively when compared with untreated control treatments (Figure 3A–F). Comparatively higher MDA, OH^-, H_2O_2 and $O_2^{\bullet-}$ levels were observed in roots than leaves which indicated that *O. cumana* induced more oxidative stress in roots than leaves. In responding to alone *O. cumana* exposure, the treatments of BR (1, 10 and 100 nM) notably the reduce the accumulation of MDA, H_2O_2 and $O_2^{\bullet-}$ both in leaf and root cells. Markedly, the supplementation of BR (10 nM) enormously restricted the extra accumulation of MDA (24.14%/34.11%), OH^- (30.28%/35.43%), H_2O_2 (31.08%/34.18%) and $O_2^{\bullet-}$ (28.04%/36.53%) levels in leaves/roots against *O. cumana* infection (Figure 3A–F). To further confirm the *O. cumana* induced lipids peroxidation and oxidative stress via extra generation of MDA, H_2O_2

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and $O_2^{\bullet-}$, and their alleviation effects by BR, sunflower leaves were treated with 3,3-diaminobenzidine (DAB) and nitro-blue tetrazolium (NBT), respectively (Figure 3G and H). The DAB staining showed dark brown color (indicator of H_2O_2) and NBT staining displayed dark blue (indicator of $O_2^{\bullet-}$) in leaf tissues at the exposure of *O. cumana* infection as compared to bright leaves in control treatments. In response, the exogenous supplementation of BR substantially minimized the intensity of dark brown and dark blue colors, indicating the reduction in oxidative stress induced by H_2O_2 and $O_2^{\bullet-}$. These findings verified that BRs greatly minimized the *O. cumana*-induced lipids peroxidation and oxidative stress in leaf and root tissues of sunflower seedlings.

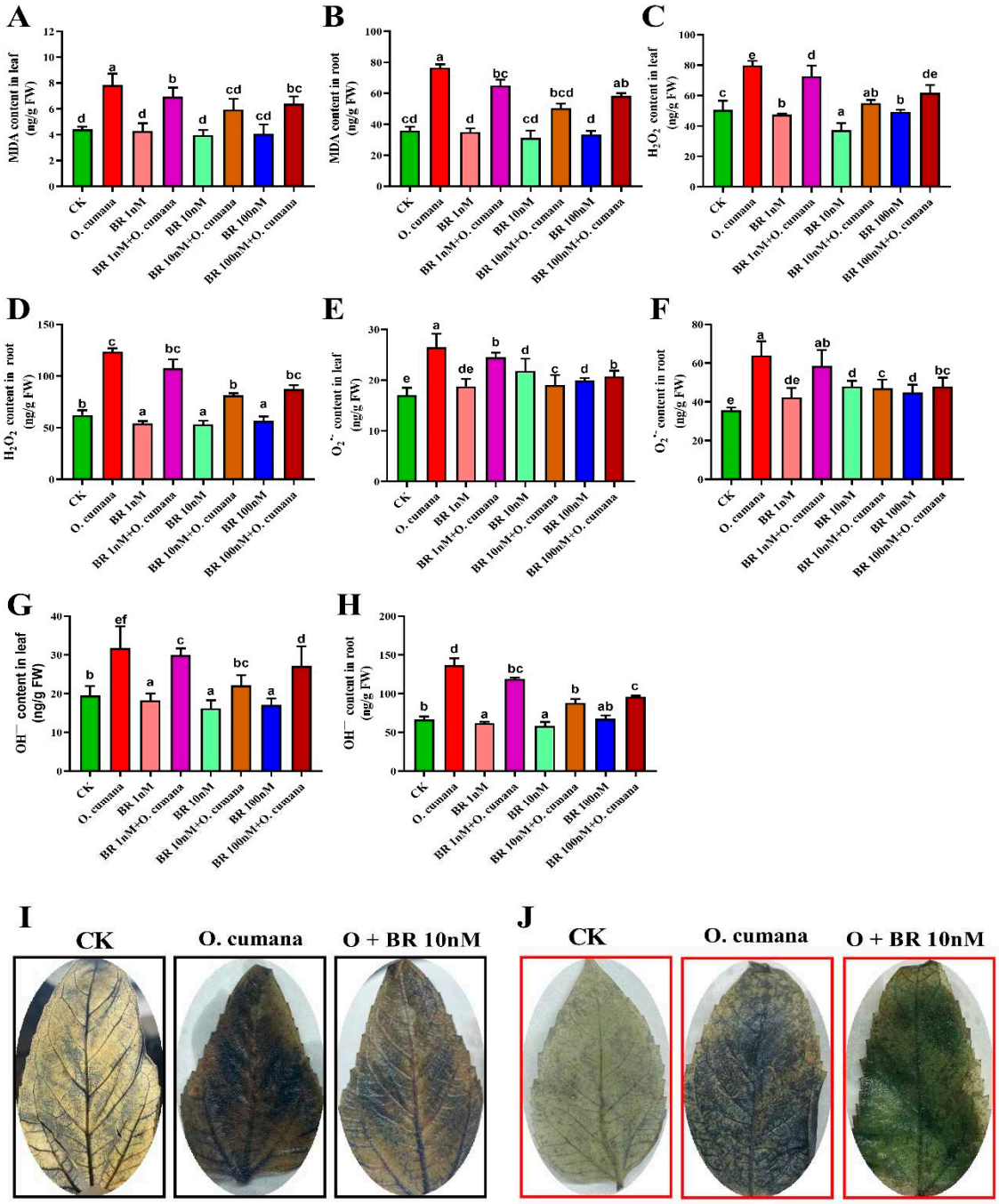

Figure 3. Effects of different concentrations of BR (1, 10, 100 nM) and *O. cumana* infection on **(A, B)** melondialdehyde (MDA) **(C, D)** hydrogen peroxide (H_2O_2) **(E, F)** superoxide radicle ($O_2^{\bullet-}$) **(G, H)** hydroxide ion (OH^-) content in leaves and roots and **(I)** leaf staining with nitro-blue tetrazole (NBT) and **(J)** 3,3-diaminobenzidine (DAB) in leaf and roots of sunflower of sunflower.

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2.4. Influence of BR on GSH, GSSG and GSH/GSSG Ratio against O. cumana Infection

In comparison to untreated controls, *O. cumana* infection caused a dramatic upsurge in the contents of GSSG (82/65%), GSH (109/97%), GSH/GSSG ratio (65/57%) in leaves/roots of sunflower (Figure 4A–F). The addition of BRs (especially at 10 nM) improved the GSH, GSSG and GSH/GSSG levels by 33/29%, 19/23% and 96/82% in leaves/roots, respectively under *O. cumana* infection. While, GSSG displayed in decreasing trends in leaves or roots accumulation when compared with untreated control plants. However, further increase in BRs (100 nM) reduced the GSH contents and increase the GSSG. This increase GSSG/GSH ratio indicated the generation of oxidative stress. These findings revealed that BRs (at 10 nM) was found most effective in enhancing the GSH/GSSG ratio and GSH levels in the leaves and roots of sunflower plants against *O. cumana* induced infection.

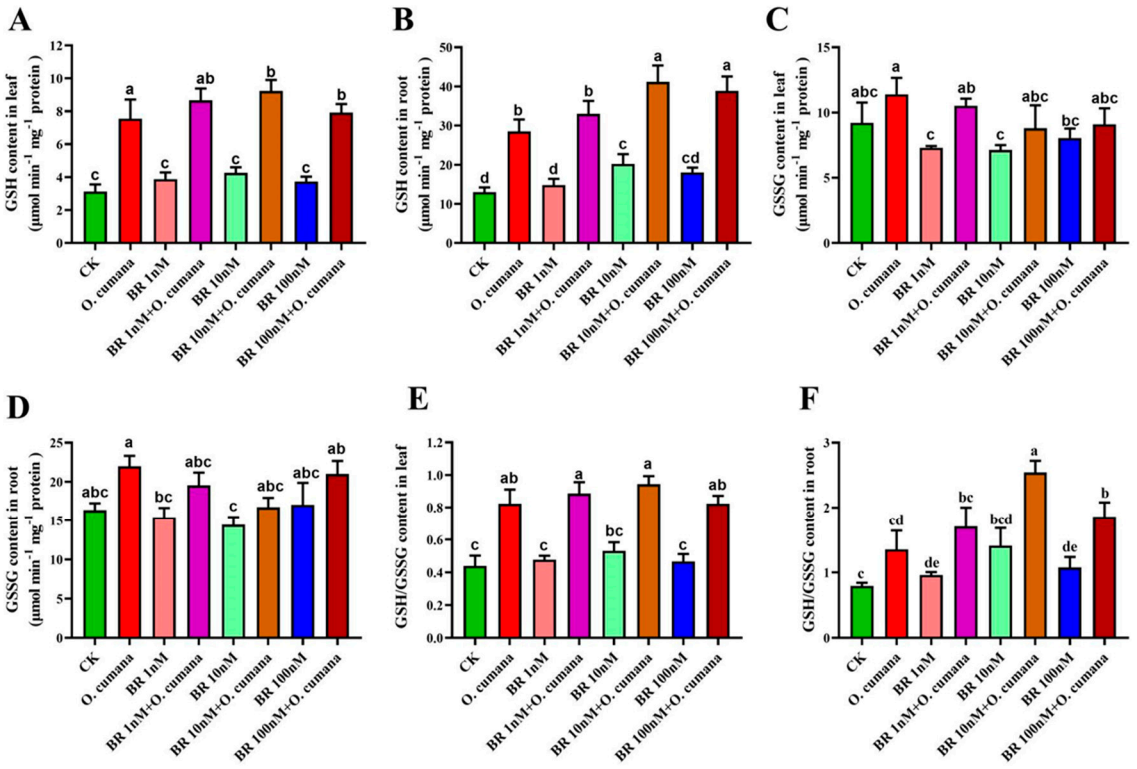

Figure 4. Effects of different concentrations of BR (1, 10, 100 nM) and *O. cumana* infection on the **(A, B)** reduced glutathione (GSH) **(C, D)** oxidized glutathione (GSSG) **(E, F)** the ratio of reduced glutathione/oxidized glutathione (GSH/GSSG) content in leaf and roots of sunflower.

2.5. Exogenous BR Activate the Antioxidant Defence System against O. cumana Infection

Under alone *O. cumana* treatment, the potential effects of different levels of BR on the activity of the antioxidant enzymes (SOD, POD, CAT, and APX) in sunflower plants were assessed (Figure 5A–D). The activities of SOD, POD, were elevated by 26.84%/32.77%, 38.72%/41.03%, while CAT and APX were decreased by 27.56/31.72% and 32.04%/36.28% in leaves/roots, respectively by *O. cumana* infection alone when compared to the corresponding controls. Under the supplementation of BR at different concentrations (1, 10 and 100 nM), it was observed that the activities of SOD, POD were increased to maximum level at the exposure of BRs from 1 to 10 nM, and then displayed decreasing trends upon the 10 to 100 nM BRs treatments. In parallel to this, the CAT and APX activities were substantially decreased and maximum decrease were recorded under 10 nM BR by 31.19%/30.33% and 40.55%/45.41, respectively and then starts increasing at the exposure of 100 nM BRs (Figure 5c and d). These observations indicated that SOD and POD activity participate in plant defense system to manage the *O. cumana* infection. While, the decreasing trends in CAT and APX activities illustrated

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the excessive oxidative stress indulged by *O. cumana* infection which severely hampered the sunflower defense system.

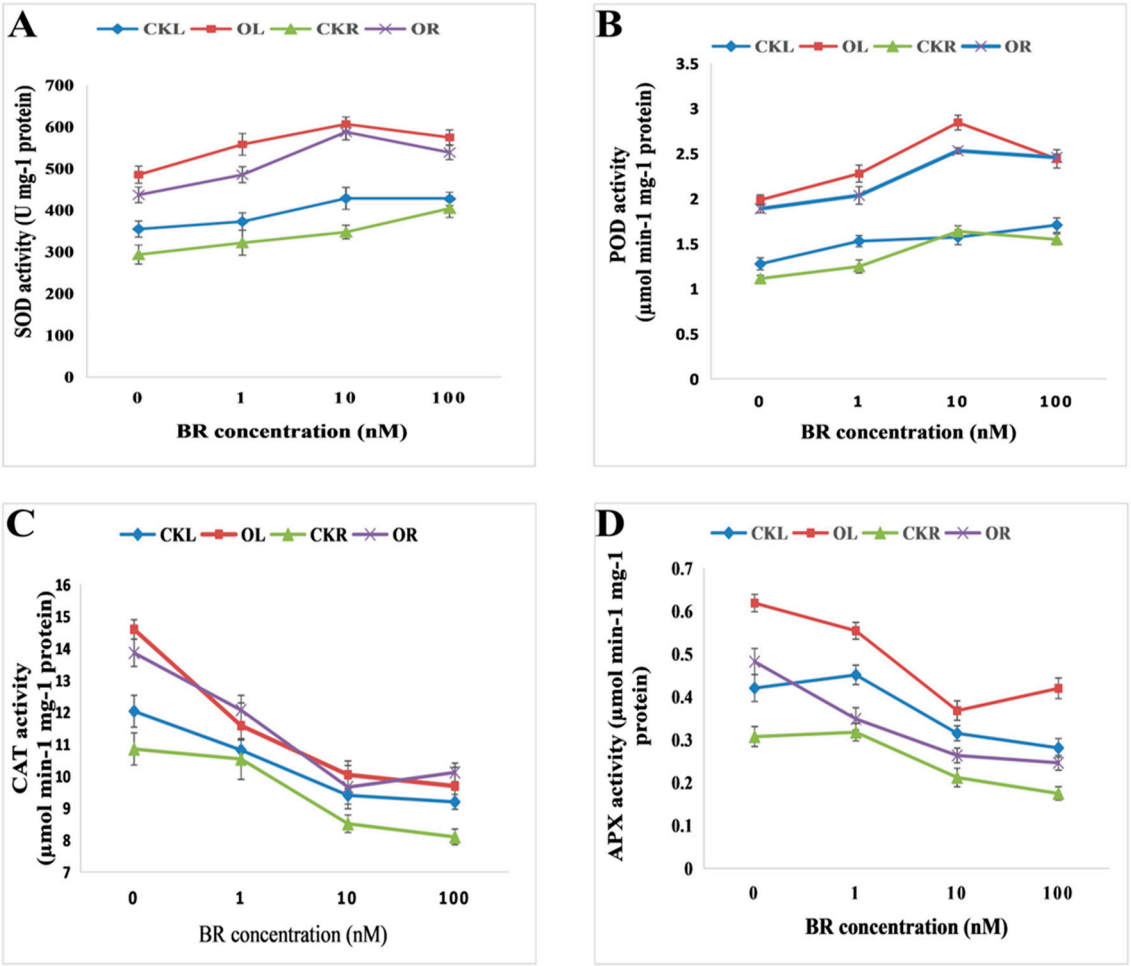

Figure 5. Effects of different concentrations of BR (1, 10, 100 nM) and *O. cumana* infection on the activity **(A)** superoxide dismutase (SOD) **(B)** peroxidase (POD) **(C)** catalase (CAT) **(D)** ascorbate peroxidase (APX) in the leaf and roots of sunflower.

2.6. Influence of BR in Regulating Different Transcript Levels against O. cumana Infection

The expression profiles of different antioxidant defense (POX, GST), BRs signaling (*BAK1*, *BSK1*, *BSK2*, *BSK3* and *BSK4*) and synthesis (*BRI1*, *BR6OX2*) genes were analyzed in sunflower leaves exposed and not-exposed to *O. cumana* infection (Figure 6). In response to *O. cumana* infection alone treatment, transcript levels of antioxidants defense, signaling and synthesis genes were downregulated, except *POX*, *GST* and *BSK3* as compared to non-stressed controls. Alone supply of BRs significantly enhanced the expression profile of *GST*, *BR6OX2* and *BSK1*, indicating the crucial involvement of these genes in planta defense, and BRs signaling and synthesis. The co-tretaments of BRs and *O. cumana* further escalated the expression profiles of most of the above-reported genes. This suggested the involvement of BRs in plant defense, and signaling transduction and endogenous BR synthesis to control the *O. cumana* infection (Figure 6).

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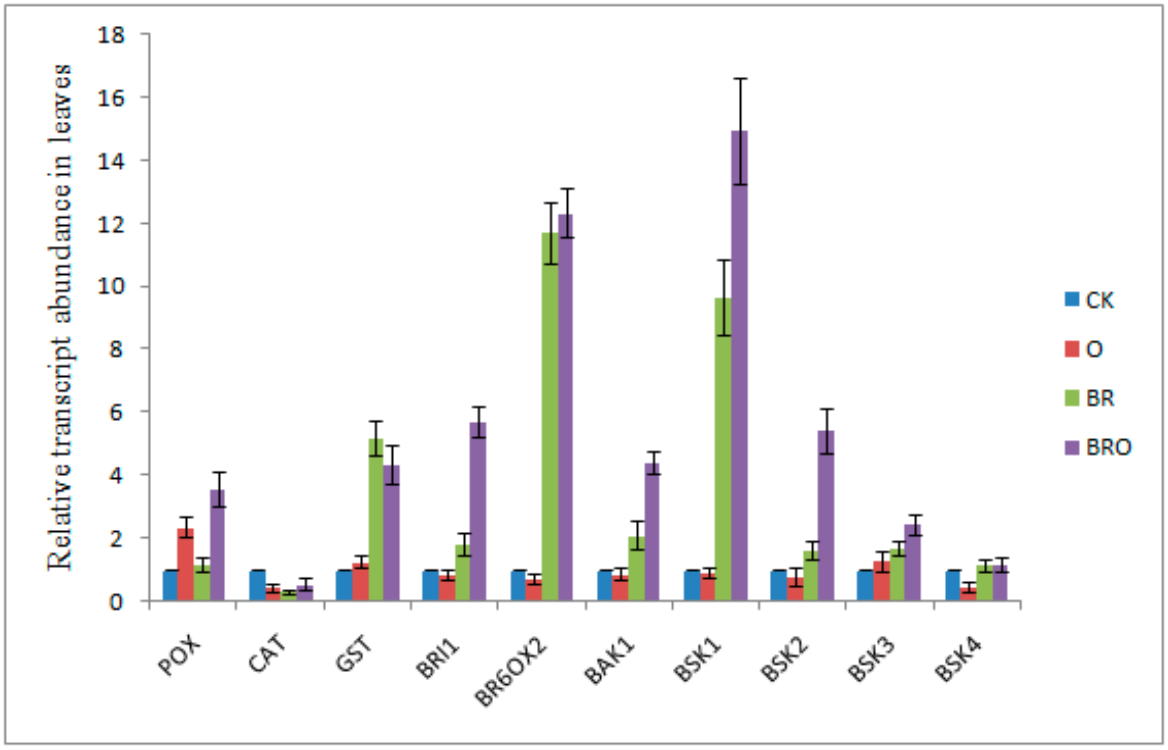

Figure 6. Effects of different concentrations of BR (1, 10, 100 nM) and *O. cumana* infection on the expression of antioxidant, signaling or synthesis related genes in the leaves of sunflower.

2.7. Effects of BRs and O. cumana on Lignin, Phenolics and Endogenous BRs Contents

The lignin contents exhibited a significant increase in response to *O.cumana* infection alone, in comparison to uninfected control (Figure 7A and B). Furthermore, *O. cumana*-infected sunflower plants treated with BRs seed treatment had higher lignin contents. The levels of phenolics in the leaf and roots of sunflower increased under *O. cumana* stress when compared to the control (Figure 7C and D). Notably, the application of BRs (10 nM) significantly increased the phenolic contents irrespective of the *O.cumana* infection. Quantitative analysis revealed that *O.cumana* infection significantly decreased the endogenous levels BRs. However, the application of BRs seed treatment led to an increased in the contents of endogenous BRs with or without infection of *O.cumana*, as compared to their corresponding control (Figure 7E and F).

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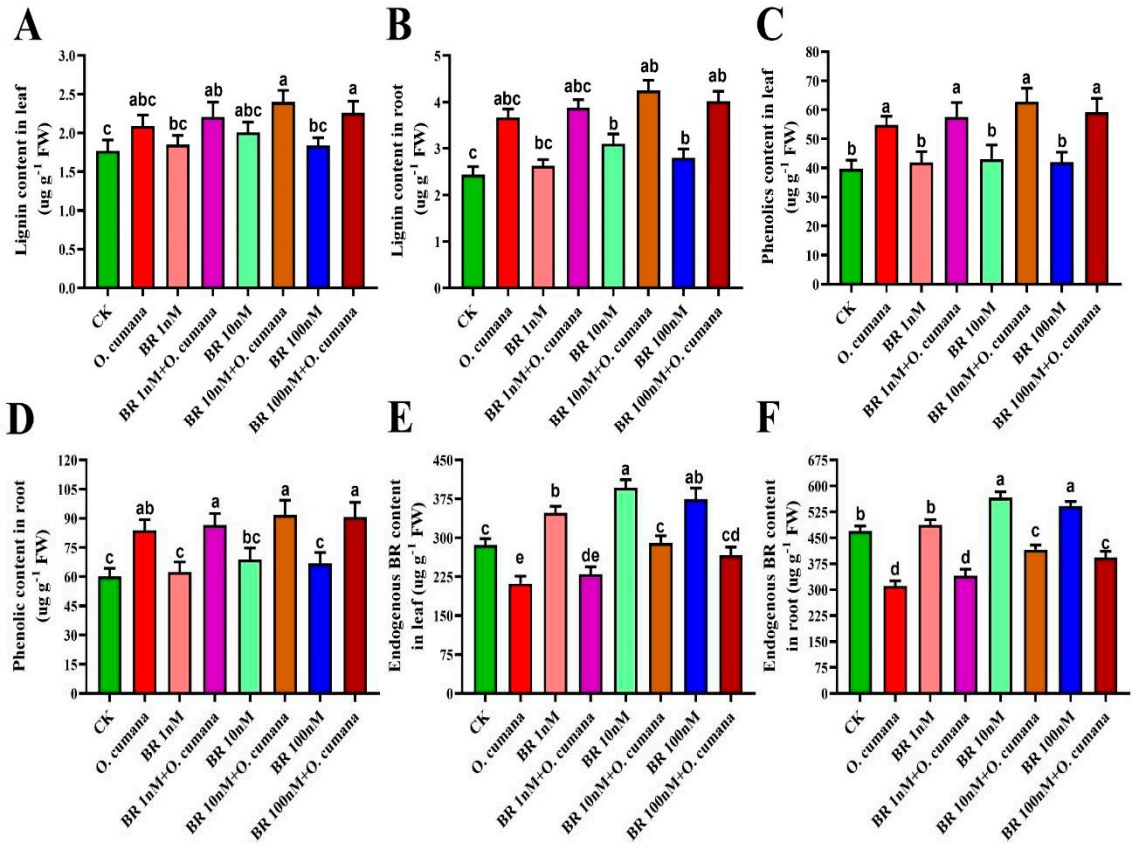

Figure 7. Effects of different concentration of BR (1, 10, 100 nM) and *O. cumana* infection on the lignin, phenolics and endogenous BR contents in the leaf and roots of sunflower.

2.8. Exogenous BRs Minimize the O. cumana Induced Cellular Ultrastructural Damages

TEM images showed severe damages to sub-cellular structures by *O. cumana* infection and their cellular protection by BRs in the leaves and roots of sunflower plants (Figure 8A and B). All the observed cellular organs were well developed and structured under control treatments. Leaf mesophyll cell contained eminent cell wall, cell membrane, healthy mitochondria, numerous starch grains, regular arrangement of thylakoid nuclear membrane in chloroplast and plastoglubuli within cytoplasm (Figure 8A). At the exposure of *O. cumana* infection, visible toxic symptoms such as scattered cell wall, immature plastoglubuli, less size starch grains, swollen thylakoid membrane, cracked cell membrane and broken nuclear membrane were observed. The treatments of BR greatly rescued the sunflower cells from *O. cumana* infection and induced cellular damages as noticed by the recovered and developed cell wall, cell membrane, nuclear membrane, plastoglubuli, thylakoid membrane and higher number of starch grains (Figure 8A). TEM micrograph of root tip cells of sunflower under control treatments showed well-sized nucleus with clear nucleoli as well as nuclear membrane (Figure 8B). Under *O. cumana* infection, TEM micrograph displayed dispersion in nucleus, nucleoli, broken cell wall as well as mitochondria and unclear nuclear membrane. These damages to leaf and root cells were effectively recovered by BRs specially at 10 nM. These observations verified that BRs displayed efficacy to minimize the *O. cumana* induced cellular ultra-structural damages in sunflower tissues.

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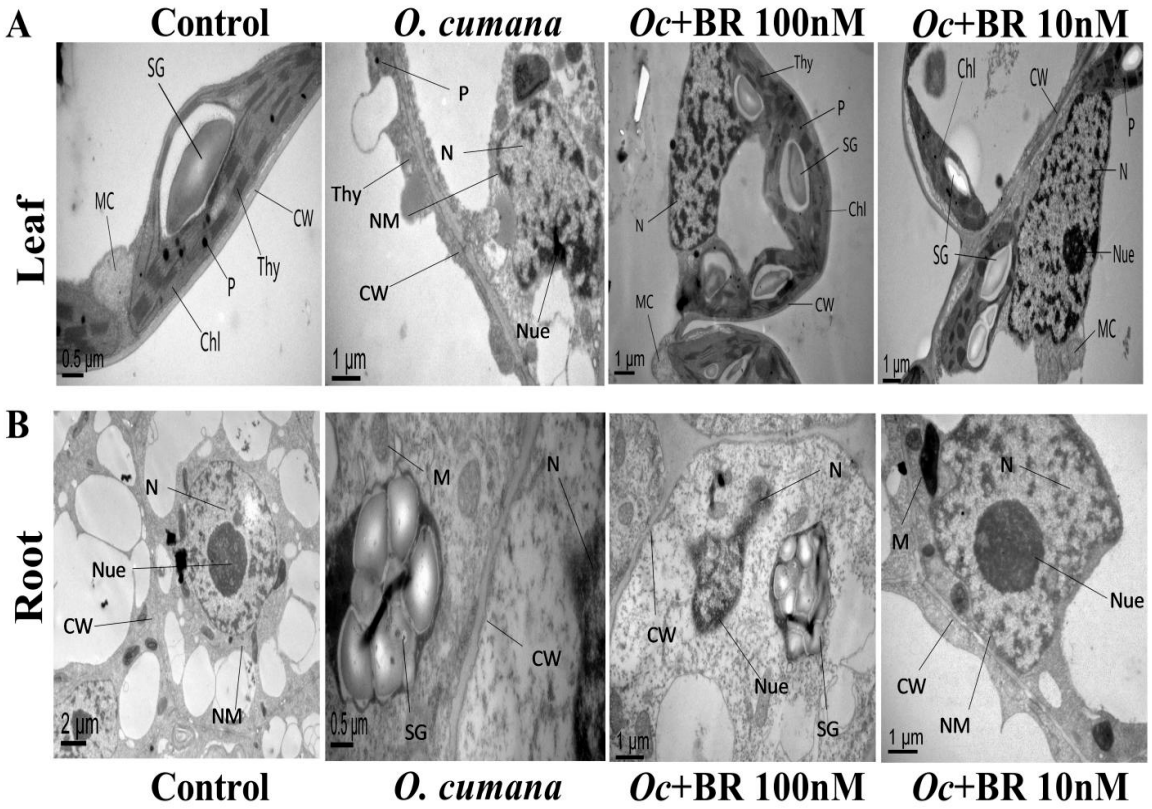

Figure 8. Transmission electron microscopic analysis revealed the toxic effects of *O. cumana* and protective roles of brassinosteroids BR (1, 10, 100 nM) in the leaves and roots of mature sunflower. The TEM micrographs of leaf mesophyll cells displayed well-developed thylakoid membrane (Thy) chloroplast (Chl), and a number of plastoglobuli (PG) under control conditions. the TEM micrographs of leaf mesophyll cells showed clustering of plastoglobuli (P), scattered nucleus (N) with nucleoli (Nue), unclear cell wall (CW) and severe nuclear membrane (NM) damage. TEM micrograph of root tip cells of sunflower under control **(A)** shows larged-size nucleus (N) with clear nucleoli (Nue) and clear nuclear membrane (NM). TEM micrograph under *O. cumana* stress **(B)** shows broken cell wall (CW), scattered Nucleus (N), nucleoli (Nue) and broken mitochondria (M) and unclear nuclear membrane. Interestingly BR decrease the toxic effects of *O. cumana* on the ultra-structure of roots and leaf mesophyll. The combined application of *O. cumana* and BR levels reverse the toxic effects of *O. cumana* in the cell ultra-structure such as normal shape chloroplast, clear cell wall, well developed plastoglubuli and clear nucleus.

3. Discussion

Our study purpose was to evaluate the protective effectiveness of BR in reducing the toxic effects of *O. cumana* infection on sunflower plants by focusing on the plant growth traits, photosynthetic apparatus, alterations in cellular ultrastructures, oxidative stress, and antioxidant defense system. Up to now, there are rare studies on the interactions of BRs with *O. cumana* in sunflower. Our research has shown that *O. cumana* infection caused detrimental impacts on plant growth, and fresh and dry biomasses that may be interlinked to protein inhibition and chlorophyll degradation [25] (Figure 1). In contrast, the harmful effects of *O. cumana* on these growth traits were greatly diminished by the supplementation of BRs. In accordance with our results, [26] reported that BR recovers the deficiency of protein synthesis and boost chlorophylls, ultimately helps to promote the plant growth attributes. Previous investigations have also shown that the exogenous applications of BRs mitigated the biotic and abiotic toxicity by enhancing the growth and biomass production in *Leymus chinensis* [27], *Zea mays* [28]. The number of *O. cumana* attached spikes with host sunflower roots were significantly increased at the exposure of alone *O. cumana* infection. While, exogenous treatments of BRs reduced

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the number of *O. cumana* spikes in sunflower roots. It was obvious that BRs may delays the formation of *O. cumana* tubercles and restricts *O. cumana* attachment with sunflower roots. Similarly, a recent study reported that salicylic and indole acetic acid treatments retarded the development of tubercles of *O. crenata* on *Vicia faba* roots [29]. This indicated the particular defense response of these hormones. Alone treatments of *O. cumana* limited the functionality of photosynthetic machinery (Figure 2A–G) as noticed in earlier studies [7,8]. There is strong possiblity that *O. cumana* infection degraded the photosynthetic pigments which may results in impairment of electron transport chain and photochemical efficiency (PS I and PS II) [7]. In current study, BR significantly reduced the deterioration of chlorophylls caused by *O. cumana* and enhanced the planta ability to photosynthesize under both control and *O. cumana* infection (Figure 2). Former studies demonstrated that BRs significantly increased the photosynthetic efficiency and suppressed the chlorophylls degradation in *cucumis sativus* [30] and cereal crops [31]. Leaf gas exchange parameters were significantly decreased at the exposure of *O. cumana* infection. While, BRs notably recovered these photosynthetic damages (Figure 2A–D). The inhibitory effects of *O. cumana* on these gas exchange parameters were directly correlated with the reduction in photosynthetic pigments. It seems that *O. cumana* compromised the efficiency of carbon assimilation and reduces the chlorophyll synthesis under severe environmental conditions. While, BRs may reinforce the respiration intensity and photosynthetic rates that helps in the improvement of photosynthetic pigments [31,32]. A significant generation of MDA, H_2O_2, $O_2^{\bullet-}$ and OH^- levels by *O. cumana* infection caused lipids peroxidation and oxidative stress. While, BRs notably repaired the oxidative and cellular membrane damages (Figure 3A–F). The overproduction of these oxidative stress markers could be the reason behind the growth retardation of sunflower plants under *O. cumana* infection as observed by [33] in tomato. The extra generation of ROS has been associated with growth reduction and lower crop yields at the exposure to environmental constraints [34]. Given the involvement of ROS in acclimatization pathways in response to plant stress, we suggested the role of ROS signaling from leaves to roots that limits the induction of MDA and ROS (H_2O_2, $O_2^{\bullet-}$ and OH^-) in leaves as compared to roots. This can be associated with the role of ROS in influencing the expression of genes involved in systematic signaling and antioxidants defense responses (Figure 6) as reporter in earlier study [35]. To control the extra accumulation of ROS and their induced cellular damages, sunflower plants activated their antioxidants defense system in the form of enzyme activities (mainly SOD and POD) (Figure 4A and B). In accordance with current study, a pronounced upsurge in the activities of antioxidant enzymes were observed under *O. cumana* infection. Generally, the activation in antioxidants signals the extra production of oxidative stress. The exogenous supply of BRs escalated the activities of SOD and POD enzymes as compared to *O. cumana* infection which displayed that BRs had effectively reduced the ROS production. While, BRs decreased the CAT and APX as compared to *O. cumana* infection (Figure 5A–D) suggested that *O. cumana* desynchronized the antioxidant defense and limits their involvements in plant defense system. Similar antioxidants resistance mechanisms were observed under *O. cumana and O. ramosa* infestation by ALA and SA in sunflower [7] and tomato [33] plants. Thus, BR has the potential to act as a signaling molecule, facilitating enhancement of the antioxidant defense system and reducing the oxidative damages caused by *O. cumana*. On the other hand, plants enhance their non-enzymatic free-radical scavengers (GSH and GSSG) to tackle the extra generation of ROS. In current study, the elevated levels of GSH and GSSH in planta leaves and roots against *O. cumana* infection (Figure 4A–F) cause a reduction in ascorbic acid levels which indicated the induction of oxidative stress. The higher production of GSH and GSSH under co-exposure of BRs and *O. cumana* infection might be due to the involvement of BRs in redox homeostasis and sunflower tolerance mechanisms to *O. cumana* infection. It was noticed that BRs regulated the antioxidant defense (*POX*, *GST*), BRs signaling (*BAK1*, *BSK1*, *BSK2*, *BSK3* and *BSK4*) and synthesis (*BRI1*, *BR6OX2*) related genes under *O. cumana* infection (Figure 6). Alone exposure to *O. cumana* infection caused the downregulation in the expression levels of most of the genes associated with plant defense, infection signaling or BRs synthesis. While, alone BRs applications significantly enhanced the expression profiles. The co-treatments of BRs and *O. cumana* further escalated the expression levels of most of the above-reported genes. This indicated the crucial participation of BRs in plant defense, synthesis of endogenous BRs

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in plant tissues and signaling due to *O. cumana* infection. Alike the current outcomes, earlier studies have documented that defense associated genes such as GST and POX greatly detoxify the oxidative stress [35]. In our study, higher GST and POX levels indicated the induction of detoxification that leads to better plant growth. The induction of BRs synthesis genes under alone BRs or combined treatment (BRs + *O. cumana* infection), relatively higher synthesis, indicating higher BRs production might be connected to sunflower against *O. cumana* invasion. While, the induction in BRs-responsive genes under *O. cumana* and further escalation under co-treatments of BRs and *O. cumana* revealed the BRs related upsurge in the defense responses of sunflower plants against *O. cumana* infection. The current investigations revealed that exogenous supply of BRs enhanced the lignin accumulation in *O.cumana*-infected sunflower seedlings (Figure 7A and B). These findings suggested that BRs helps in reducing the attachment of *O. cumana* to sunflower tissues via lignification as reported in earlier findings [36]. They found that SA enhance the resistance of host plants by inducing the roots lignification during the interaction of *O. minor* infection and red clove. These outcomes revealed that BRs mediated lignification to host resistance by serving as a mechanical barrier that strengthens the plant cell wall. Phenylalanine ammonia lyase (PAL) is a crucial enzyme in the synthesis of phenolic compounds. The induction in phenolic contents *O.cumana*-treated sunflower plants and further escalation by BRs treatment suggested the up-regulation in the transcript levels of PAL synthesis genes which is directly correlated with the endogenous BRs synthesis (Figure 7C-F). These results indicated that the acquired resistence by BRs may be partially participated in BRs-mediated synthesis or activation of PAL genes. Earlier study also noticed the induction of PAL genes in grape berry by salicylic acid [37]. In present study, the cellular ultrastructural changes against *O. cumana* infection and protective roles of BRs were observed. When compared with controls, the severe structural damages in planta cells were noticed under *O. cumana* infection (Figure 8A and B). These cellular damages were directly correlated with the excessive production of ROS and indulged oxidative damages. Importantly, BRs supply alleviated the damages to cellular components and provide cellular protection to *O. cumana* infection (Figure 8A and B). These cellular changes suggested that proper concentration of exogenous BRs can recover the leaf mesophyll cellular structures in response to *O. cumana* exposure. Earlier studies illustrated that the exogenous supplementation of phytohormones (ALA) and herbicide (imazapic) could diminished the sunflower infection to *O. cumana* induced cellular damages and enhance the plant tolerance [7, 38]. However, more deeper cellular and molecular investigations are required to further validate the protective roles of BRs in improving the sunflower tolerance to *O. cumana,* pathogen infectation.

4. Materials and Methods

4.1. Experiment Design and Growth Conditions

The seeds of sunflower (cv. TK0409) and *O. cumana* (race G) were acquired from Inner Mongolia Academy of Agricultural and Animal Husbandry Sciences (Hohhot, China). Before treatment, seeds sterilization was carried out with 1% $KMnO_4$ for 2 min, then 95% ethanol was applied for 1 min, followed by distilled water washing. The sterilized seeds were allowed to germinate in dark for two to three days in Petri dishes containing filter paper. The sprouted seeds (having uniform length) were shifted into pots consisting of peat and vermiculite in 1:1 v/v as substrate. For inoculation, 0.1 g of *O. cumana* seeds were added with 0.5 kg substrate. The conditions were maintained as follows: 14/10 h photoperiod, relative humidity as 65% and 200 μM $m^{-2}s^{-1}$ light intensity. A factorial design was employed to create a total of eight treatment groups, consisting of four levels of BRs (0, 1, 10, and 100 nM) combined with two levels of *O. cumana* (with and without). Each treatment group consisted of at least three replicates. After four-weeks of post-inoculation stage, sunflower plants (leaves and roots) were individually separated and harvested to carried out the morphological, physio-biochemical, cellular alterations and transcription profiling.

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4.2. Measurement of Agronomic Parameters and Photosynthesis Traits

Immediately following harvesting, the number of *O. cumana,* fresh biomass of stem and roots, and plant height were measured manually. After oven-drying the samples 70 °C until constant weight and dry analysis was performed. Chlorophyll a as *Chl a*, chlorophyll b as *Chl b*, and carotenoids were estimated according to earlier protocols [14]. To record the gas exchange parameters, net photosynthetic rate (*Pn*), transpiration rate (*Tr*), intercellular CO_2 concentration (*Ci*), and stomatal conductance (*gs*) was determined from the fully-stretched leaves by using LiCor-6400 portable photosynthesis system during periods of maximum light intensity (between 10:00 and 12:00 am).

4.3. Estimation of Lipids Peroxidation and Reactive Oxygen Species

Lipids peroxidation denoted as MDA was estimated by 2-thiobarbituric (TBA) method as reported in earlier study [7]. Superoxide ($O^{2\bullet-}$) was estimated by following the method performed by [15] with slight changes. Fresh plant samples (0.5 g) were intermixed in 3 mL of 70 mM potassium phosphate buffer (PBS) (pH 7.8), followed by centrifugation for 10 min at 5000 *g* in iced-cold conditions. Then, 1 mL supernatant was mixed with 0.9 mL of 70 mM PBS and 0.1 mL of 10mM hydroxylaminde hydrochloride. Right after 24 h of incubation under 25°C, 1 mL of 17 mM sulphanilamide and 1 ml of 7 mM α-naphthylamine were mixed with 1ml solution for 20 min at 25°C. After this, same volume of n-butanol was affixed and centrifuged at 15000 *g* for 5 min. A standard curve determined the $O^{\bullet-2}$ generation and absorbance was measured 530 nm. For the determination of H_2O_2 contents in leaves and roots, the established method was used [16]. In addition, the accumulation of H_2O_2 and $O^{\bullet-2}$ was confirmed by leaf staining with NBT (nitroblue tetrazolium) and DAB (diaminobenzidine tetrahydrochloride) dyes, respectively.

4.4. Determination of Antioxidant and Non-enzymatic Antioxidants

0.5g fresh leaf and root samples were separately intermixed in 50 mM PBS (8 mL) of pH 7.8 at iced-cold conditions. To evaluate the enzyme activities, homogenized supernatant was undergone centrifugation for 15 min at 10,000 *g* under 4°C. Following the method of [17], catalase (CAT) activity was determined by estimating the degradation of H_2O_2 (extinction coefficient of 39.4 $mM^{-1}\,cm^{-1}$) for 1 min at 240 nm. Superoxide dismutase (SOD) activity was established as nitro blue tetrazolium (NBT) mediated photochemical inhibition [18]. For estimation of peroxidase (POD) activity, guaiacol mediated variation in absorbance were measured at 470 nm as reported previously [19]. Ascorbate peroxidase (APX) was estimated by following the earlier established method [49]. Regarding the non-enzymatic antioxidants, the measurements of reduced glutathione (GSH) and oxidized glutathione (GSSG) were carried out by following the previous methods [20].

4.5. Gene Expression Analysis

Total RNA was extracted from 0.1 g of leaf and root samples (each) using Plant RNA extraction Kit (TakaRa MiniBEST). A total 200 ng of RNA was reversed transcripted utilizing TaKaRa PrimeScript™ RT Master Mix reagent kit with gDNA eraser. The CFX96TM Real-Time System was utilized to conduct polymerase chain reactions (PCRs) using SYBR Premix Ex Taq II (Ti RNaseH Plus, TakaRa) reagent. To confirm the relative expression of particular genes, the designed primers were listed (Table 1). For the analysis of relative gene expression, Ct method with four replicates was performed [21] and *EF-1a* gene was taken as a reference gene.

Table 1. Oligonucleotide primers used in the present study.

Gene ID	Gene Name	Forward	Reverse
EF-1α	Elongation factor-1 alpha (reference gene)	GGATACAACCCCGACAAA	CCTGAAGTGGGAGACGAA
POX	Peroxidase	GGACCGTAAGACTTGGAG	ATAAGGCGACCATTTCTC
CAT	Catalase	CGTCTTGGACCGAACTATT	CAAACCACCCACAACTCTG

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GST	Glutathione	GCATTCCCGCATTATTC	ACTTACTGCCTTTCACTTTC
BRI1	Brassinosteroid homeostasis	GTGAAACTAAACTGGTCCA CAC	GTTGAACACCTGAAACTTTG GT
BR6OX2	Cytochrome p450 enzyme	GGGAGTTTCTTCAAGTCTCA CA	GCAACAAGTCCTTTCGATTC AT
BAK1	BRI1-associated receptor kinase	ACTTCTTTGATGTACCAGCT GA	CAACTTGTAGTTCACGCAAT GA
BSK1	BR-signaling kinase 1	GATTTCAAAACAGCCATCG ACT	AGTAAGTAGCATAGACTTC GCC
BSK2	BR-signaling kinase 2	AATACTACTCCAAGTTGGTG GG	TCATTAAGTAGGAGAAAGC CCG
BSK3	BR-signaling kinase 3	TGATGAAGAATAGTCGGGA TGG	TCACTATCAGAAAACTGCC CAT
BSK4	BR-signaling kinase 4	GATCTTTCGTTTATGTGGAC	AGACTGGAGATACAATGCA

4.6. Determination of Phenolics, Lignin and Endogenous Brassinosteroids (BRs) Levels

Total phenolic content was determined using the methodology of [22]. At a wavelength of 725 nm, phenolic concentrations were measured and determined by using p-coumaric acid as a standard. Lignin content was quantified according to the [23] method, which involved by measuring the absorbance of lignin-like thioglycolic acid (LTGA) derivatives at wavelength of 280 nm. The content of different BRs was determined through their extraction, purification, and subsequent analysis using ultra-high performance liquid chromatography (UHPLC) followed by mass spectrometry. This analytical approach was conducted following the methodology established by [24].

4.7. Transmission Electron Microscopy (TEM)

Healthy and matured leaves of sunflower plants were selected to observe the cellular changes induced by *O. cumana* and BRs (Figure 7 A and B). For that, leaf fragments in 1 mm^2 without veins were placed in 2.5% (v/v) glutaraldehyde in 0.1 M PBS (pH 7.4), followed by washing (three-times) with same earlier used buffer. Afterwards, samples were fixed with 1% osmium tetraoxide (for 1 h) in PBS solution and washed again with 0.1 M PBS (pH 7.4) for 15 min. Later on, samples were dehydrated twice with 30, 50, 70, 80, 90, 95, and 100 % ethanol concentrations for 15-20 min and finally washed with acetone for 20 min. Right after performing overnight embedding in pure resin, samples were heated at 70°C for 8 h and ultra-thin sections were observed under transmission electron microscopy (TEM, JOEL-30EX instrument Ltd. Tokoyo, Japan) [7].

4.8. Statistical Analysis

The statistical tool SPSS version 20.0 (SPSS, Chicago, IL, USA) was employed to analyze the data. Two-way analysis of variance (ANOVA) was performed. Fisher's least significant test (LSD) was used for multiple comparison to calculate the significant difference (P 0.05) between individuals. All reported values in results are the mean of at least three replicates ± standard error.

5. Conclusions

Taken together, our findings revealed that exogenous supplementation of BRs provides tolerance to sunflower plants against *O. cumana* infection as explained in schematic diagram (Figure. 9). In details, alone exposure to *O. cumana* drastically inhibited the plant growth traits, fresh or dry biomass production, photosynthesis-related parameters, induced the oxidative or cellular damages and desynchronized the overall antioxidant defense system in sunflower tissues. In response, BRs treatments (especially at 10 nM) significantly minimized the photosynthetic inhibition, plant growth retardation, and cellular damages due to oxidative stress by up-regulating the enzymes related or not-related antioxidants defense system. Moreover, BRs recovered the *O. cumana* mediated damages to cellular ultra-structures (leaf mesophyll cells) and activated the genes linked to plant defence, and

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BRs signaling or synthesis indicates high response to planta immunity. These observations provide novel approaches to minimize the *O. cumana* infestation on sunflower or other agricultural crops.

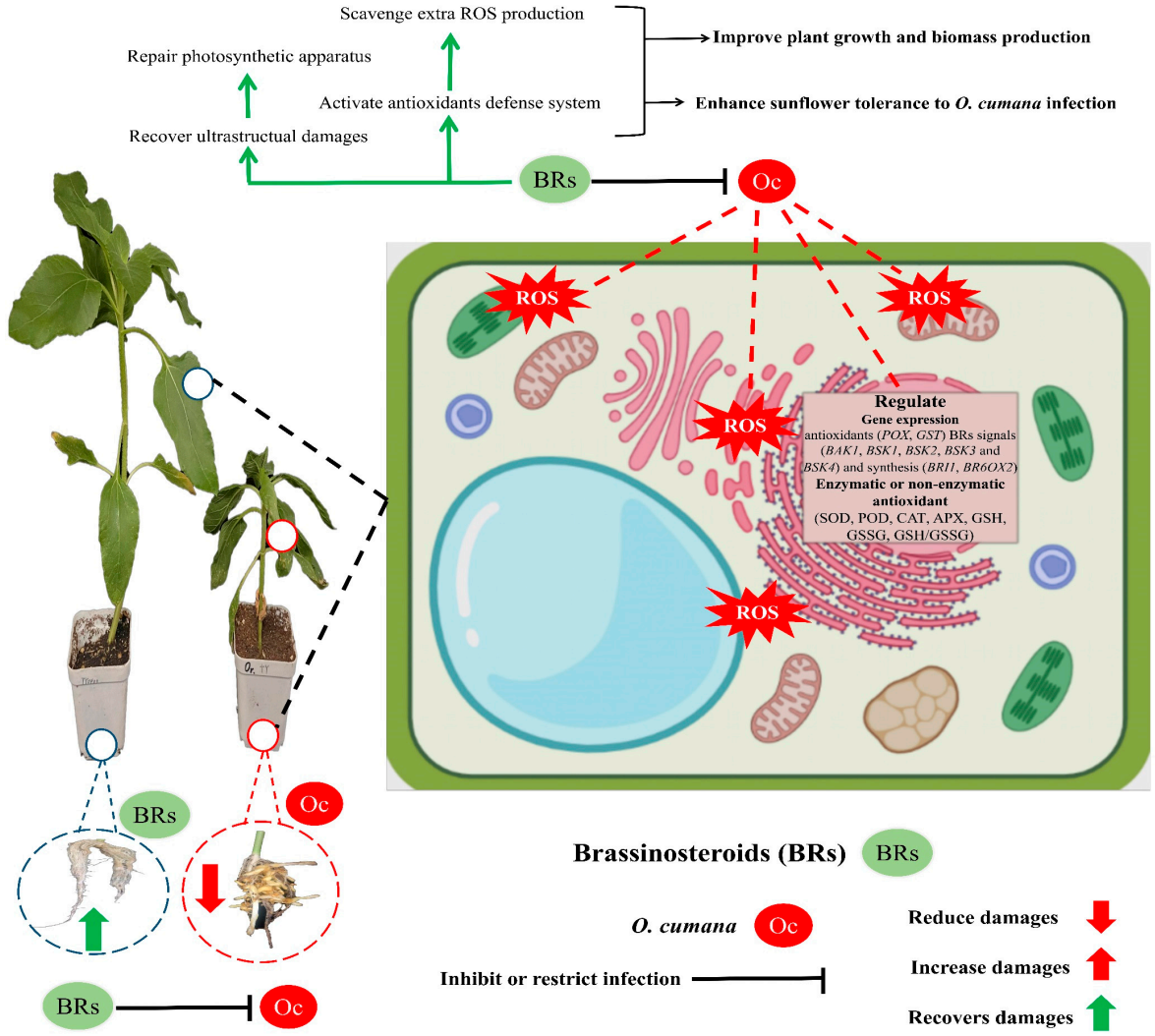

Figure 9. Protective roles of BRs in inducing the sunflower resistance to *O. cumana* indulged infection.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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