Plant-Microorganism Interactions

Gibberellins hypersensitivity hinder the interaction of Bipolaris sorokiniana (Scc.) under cross talks with IAA and transzeatin

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ABSTRACT

Gibberellins are considered to play an important role in modulating plant–pathogen interactions. In order to find this, we studied the effect of GA4 on the interactions of Bipolaris sorokiniana (Scc.) (BIPOL) with Triticum aestivum. After inoculating the roots of hydroponically grown T. aestivum seedlings, the increased level of GA4 elicited a hypersensitive response (HR) in the T. aestivum seedlings. The elicitation in HR was confirmed through measurement of HR-inducing signaling compounds (i.e. c-di-GMP, cAMP, phosphatidic acid, pure and esterified oxo-phytodeinoic acid, and jasmonic acid). The concentration of HR-inducing signaling compounds in the treated T. aestivum seedlings was 31% higher compared to the control seedlings. Moreover, the induced HR response lowered the indole-3-acetic acid (IAA) level by 15% and the transzeatin (TZn) level by 22%. However, the application of 10 mM uniconazole through aerial spray inhibited GA4 biosynthesis in T. aestivum seedlings. Also, BIPOL colonized the roots of T. aestivum seedlings after uniconazole treatment and promoted seedling growth through increased relative growth rate (14%) and net assimilation rate (27%).

Abbreviations: BIPOL: Bipolaris sorokiniana (Scc.); C: control; GA4: gibberellin; IAA: indole-3-acetic acid; JA: jasmonic acid; LE and RE: leaf extract and root exudates; OPDA: pure oxo-phytodeinoic acid; PA: phosphatidic acid; SD: spore density; TZn: trans zeatin (cytokinin); U: Uniconazole; U-B: Uniconazole–bipolaris.

Background

Plant–microbe interactions elicit several hypersensitive reactions, including hormonal biosyntheses and initiation of subsequent signal transduction mechanism (Kim et al. 2018). The signal transduction in the host later develops the expression of genes, which further decide the fate of microbe interaction with host (Ramirez-Prado et al. 2018). Recently, hypersensitivity accompanied by their signal transduction processes has emerged as a critical component of plant–microbe interactions (Kim, Lotter-Stark, Rybicki, Chikwamba and Palmer 2018). However, very little information is available about the cross-talk between GAs and other phytohormones in plant under stress conditions, such as pathogen attacks (Zhang et al. 2018). On the other hand, gibberellins signaling are widely manipulated by the proteolytic activity of DELLA proteins (Xiang et al. 2018), which might activate hypersensitive response (HR) in plants species (Groškinsky et al. 2016).

Certainly, HR in plant species can be triggered after the interaction of undesired microbe(s) with the host plant species (Matč et al. 2016). HR in the plant is characterized by the production of hypersensitive molecules, including c-di-GMP, and cAMP (Cadby et al. 2019). Other signaling molecules, such as phosphatidic acid (PA), free or esterified oxo-phytodeinoic acid (OPDA), and jasmonic acid (JA), can also be produced after HR in host plant species (Hou et al. 2016). These molecules might activate the host defense response against the attacking microbes (Lim et al. 2017). Besides, the host plant species can also produce phytoalexins (Suárez et al. 2018) and exhibit high protease activity (Asai and Shirasu 2015).

During HR, a decrease in various growth parameters of the host plant species has been noted that can be linked with Relative Growth Rate (RGR) and Net Assimilation Rate (NAR) (Vasseur et al. 2018, Koch et al. 2019). HR may also lead to the higher production of metabolites in host leaves and roots in order to combat stress (Yang et al. 2018). The commonly produced metabolites by the plants under stress includes proline (Silva et al. 2018), flavonoid contents (Tohge et al. 2018), phenyl propanoids (PPs) (Hiruma 2019), and glucosinolates (GLs) (Czerniawski and Bednarek 2018). Phyto-hormonically, the HR-inducing microbes suppress the activity of E3 ligase to inhibit GA4 production from degrading DELLA protein (Alsanius et al. 2019). In fact, most of the arbuscular fungi (AF) are known to utilize GA4 signaling in order to produce nodulation in plants (Mamontova et al. 2019). Pea mutant cry-s is known to have high AF colonization due to reduce level of GA4 and high DELLA protein activity (McGuiness et al. 2019). Fungal sporulation specifically needs low GA signaling in host under normal conditions (Bedini et al. 2018). It has been noticed that plant host usually discourages the growth of biotroph in their tissues by releasing GA4 and associated signals (Yimer et al. 2018). It is also worth
mentioning that the surface elicitors of the microbes also contribute to gibberellins hypersensitivity (Mott et al. 2018). Surface elicitors that are responsible for gibberellins hypersensitivity in host include glycoproteins and glycolipid (Chalila et al. 2018). Higher production of GA₄ in host plant species antagonizes its relationship with other plant growth hormones through cross talk, thus affecting the growth and development of the host plant species (Feurtado and Ker-mode 2018, Fuentes et al. 2019).

Bipolaris is a genus of higher fungi that can be found frequently in plant debris and soil (Kurosawa et al. 2018). Although various species of Bipolaris are reported to have growth promoting activity in plants, but some species (Bipolaris hawaiensis, Bipolaris spicifera, and Bipolaris australiensis) are pathogenic in telomorphic stage (Nandhini et al. 2018, Nur Ain Izzati et al. 2019). Bipolaris sorokiniana is one of the dubious species of Bipolaris that acted as a hemibirotroph in T. aestivum and barley (McDonald et al. 2018), whereas Khan, Ali, et al. (2015) reported it as a beneficial endophyte. This shows a wider research gap to define the mode of action of B. sorokiniana (BIPOL).

Presently, we have analyzed the GA₄ hypersensitivity and its cross talks with IAA and cytokinins in T. aestivum seedlings after BIPOL treatment. Furthermore, the mechanism of GA₄ cross talks with IAA and transzeatin was studied. Also, the effect of cross talks on T. aestivum growth responses after root colonization by BIPOL was evaluated.

Materials and methods

Plant materials

Seeds of T. aestivum (cultivar 711) obtained from Bio Care Service Seeds (BCSS) were kept for vernalization at 4°C for 21 days (Müller et al. 2017). At the time of the experiment, the seeds were surface sterilized by treating them with a dilute solution of HgCl₂ (0.1%) for 10 s, followed by dipping it in 70% ethanol for 10 min (Sen et al. 2013). The sterilized seeds were washed with autoclaved distilled water to rinse off any surface adsorbent. The seeds were then shifted to Petri plates having filter paper moistened with distilled autoclaved water. Afterward, the Petri plates were wrapped in an aluminum foil and incubated at 25°C for 3 days. Uniformly germinated seedlings were selected and shifted to pots having standardized Hoagland solution as described earlier (Hassan 2017). The seedlings were acclimatized in the Hoagland solution for 3 days and then shifted to four different sets of treatment. The first set of T. aestivum seedlings were treated with 2 mL of BIPOL spores (B). Leaves of the second set of T. aestivum seedlings were sprayed with uniconazole (U). The third set of T. aestivum roots were inoculated with BIPOL after 12-h pretreatment of uniconazole (U-B). The control group consisted of T. Aestivum seedlings that did not receive any of the above-mentioned treatments (C). A working solution of uniconazole (10 mM) was made in absolute acetone and applied to the leaves of T. aestivum seedling @2 mL. The growth kinetics of the T. aestivum seedlings was expressed in terms of RGR and NAR as described earlier (Yousaf et al. 2021).

Preparation of fungal inoculum

The culture of previously isolated, identified, and preserved BIPOL was refreshed by using PDA medium (Asaf et al. 2019). After the growth of BIPOL on PDA medium, 0.02 g of the fungal spores was picked and transferred to a 500-mL flask containing 250 mL of potato dextrose broth. The flask was incubated at 25°C for 5 days in a shaking incubator operated at 150 rpm (Khan, Ullah, et al. 2015). On the 5th day, 1 mL of the inoculum from the spore suspension was taken and transferred to a fresh potato dextrose broth. The fresh culture broth was kept overnight at 25°C in a shaking incubator. Prior to inoculation, the spore was washed thrice with sterile distilled water by centrifugation at 10°C and 10³ xg for 30 min (Huttenlocher et al. 2019). Optical density (OD) of the washed spore’s suspension was measured at 600 nm and adjusted to OD₀.₂ or OD₀.₄ or OD₀.₆ with the help of sterilized distilled water. Germinated spores were used to inoculate the T. aestivum seedlings to accelerate the colonization potential of B. sorokiniana (Selvakumar et al. 2018).

Determination of fungal root colonization

Root segments of fungal treated T. aestivum seedlings were kept on PDA media in a Petri plate, and the plate was incubated at 25°C. To calculate the fungal colonization frequency, a relative number of the root segments occupied by the fungus were observed.

Sample preparation for biochemical analyses

For phytochemical analyses, fresh leaves of T. aestivum seedlings were ground to powder in liquid nitrogen with the help of mortar and pestle. Absolute methanol (200 mL) was then added to the fine powder (2 g), and the solution was transferred to the Soxhlet apparatus for the extraction of phytochemicals (Dhawan and Gupta 2017). The obtained leaf extract (LE) was filtered, and the filtrate was concentrated in the rotary evaporator (Wahyuningsih et al. 2017). Moreover, T. aestivum root exudates (RE) were obtained by filtering the hydroponic solution in which roots of T. aestivum were submerged during the course of the experiment. The filtered hydroponic solution was then concentrated in the rotary evaporator.

Estimation of metabolites in T. aestivum

Samples (1 mL) of LE or RE were mixed with 3% sulfo-salicylic acid (4 mL) and centrifuged for 5 min at 6077 rcf. After centrifugation, 2 mL of ninhydrin reagent was added to the samples and the contents were shaken vigorously. Acid ninhydrin reagent was prepared by mixing 1.25 g of ninhydrin in pure glacial acetic acid (30 mL) and 20 mL of phosphoric acid (6 M). The reaction mixture was heated for 1 h at 100°C, and the pellet formed was then disappeared after the addition of toluene (4 mL) (Lee et al. 2018). OD of the samples was monitored at 520 nm by using UV/Vis–NIR Spectrophotometer (UV-3600i Plus, Shimadzu, Japan). For the determination of total flavonoid contents, 0.5 mL of LE or RE samples was mixed with 10% potassium acetate (100 µL), 10% aluminum chloride (100 µL), and 70% ethanol (4.3 mL). The mixture was incubated at room temperature for 30 min, and the OD was measured at 450 nm by using spectrophotometer (Gil-Ramirez et al. 2016). The final concentration of flavonoids and proline in the samples was
calculated with the help of calibration curves. Quercetin and proline (10–1000 µg/mL) were used as standards.

High-performance liquid chromatography technique was used to determine the phenylpropanoids and glucosinolates in the RE or LE. Ferulic acid (Genovese et al. 2018) and indolyl glucosinolates (Aghajanzadeh et al. 2019) were used as standards. The samples (RE or LE) were initially filtered through a 0.2-µm membrane filter fitted in a micro-syringe of HPLC. The filtrate (20 µL) was then loaded to a C18 reverse-phase HPLC column and eluted with 75% methanol (in 10% acetic acid) through an isocratic pump. The eluate was monitored through a UV detector set at 212 and 287 nm for phenylpropanoids and glucosinolates, respectively (Jeon et al. 2018). The final values were obtained using the calibration curves drawn against the known quantities of the standard ferulic acid and indolyl glucosinolates (10–800 µg/mL).

**Determination of enzymatic activities in T. aestivum**

Oxidase and catalase activities were determined in the LE and RE samples of *T. aestivum* (Röcker et al. 2016). For the determination of oxidase activity, sample (200 µL) of LE or RE was mixed with 1.5 mL of phosphate buffer (50 mM), 200 µL of ascorbic acid (0.5 mM), and 200 µL of H₂O₂ (0.1 mM) in a cuvette. OD was measured with an interval of 30 s at 290 nm. The collected data were averaged for each sample and expressed in enzyme units per gram of tested sample (U/g). For catalase activity, the reaction mixture of (10–100 ng/mL) using HPLC (Thermo Fisher Scientific), coupled with MS (UltiMate 3000 HPLC) (Pu et al. 2018). Thermo Xcalibur software 2.10 was used to monitor the system. The samples were eluted in the gradient system consisting of 1% formic acid (v/v), and acetonitrile concentration was changed from 20% to 70% (v/v) within 20 min. The flow rate was maintained at 0.20 µL/min, and the temperature was kept at 30°C. Sample fractions were collected at different time intervals and characterized by their retention time. Pure c-di-GMP and cAMP were used as standards. The final reading was acquired by comparing with the calibration curve drawn against known quantities of the standard pure c-di-GMP and cAMP levels (10–800 ng/mL) using HPLC.

**Quantification of hypersensitive inducing molecules**

Quantification of hypersensitive inducing molecules as pure o xo-phytodeinoic acid (OPDA), esterified OPDA, phosphatic acid (PA), and jasmonic acid (JA) was carried out. Root or leaf tissues (2 g) were taken in a glass tube containing 5 mL of distilled water and agitated in an orbital shaker. After removing the tissues, samples were acidified by adding 50 µL HCL (1.6 M). Phase separation was done by mixing ethyl acetate (2 mL), and the samples were then dried passing it through a stream of nitrogen gas. The dried samples were resuspended in methanol (50 µL) and subjected to HPLC (Thermo Fisher Scientific), coupled with MS (UltiMate 3000 HPLC). IAA, GA₄, and TZn activity in the LE and RE of *T. aestivum* seedlings were observed through HPLC–ESI–MS/MS. LE and RE (10 mL) were centrifuged at 6744 rpm for 20 min. The obtained supernatants were concentrated to 5 mL in a vacuum concentrator (Heto-Holten, Denmark). The concentrate was first filtered through 0.22-mm disposable cellulose acetate membrane and subsequently evaluated by HPLC–ESI–MS/MS. The HPLC–ESI–MS/MS consisted of agilent 1260 HPLC connected with an elegant 6410B mass spectrometer equipped with an electrospray ionization source (4000 V, 45 psi) and Park nitrogen generator. The instrument ran in a negative mode with a flow rate of nitrogen at 11 mL/min. The mobile phase was made by adding acetonitrile in acidic water (0.1% formic acid) in a ratio of 2:1 at a flow rate of 0.5 mL/min with a linear gradient length of 20, 30, or 40 min. Hormonal analysts (IAA, GA₄, TZn) were monitored at 312.9, 391.6, and 379.4 m/z, respectively.

**HPLC–ESI–MS/MS analyses of phytohormones**

IAA, GA₄, and TZn activity in the LE and RE of *T. aestivum* leaf or root tissues was observed by spectrophotometric instrument (FLX800, BioTek) with succ-LLYV-NH-AMC (sigma fluorogenic substrate for the general protease activity) at excitation wavelength 350 nm and emission wavelength 470 nm. Z-LRR-amino luciferin at excitation wavelength 370 nm and emission wavelength 495 nm was used to estimate the serine protease activity. Caspase-3 Substrate I at an excitation wavelength of 355 nm and emission wavelength of 485 nm was used to

**Measurement of protease activity**

Protease activity in the root or leaf tissues was observed by spectrophotometric instrument (FLX800, BioTek) with succ-LLYV-NH-AMC (sigma fluorogenic substrate for the general protease activity) at excitation wavelength 350 nm and emission wavelength 470 nm. Z-LRR-amino luciferin at excitation wavelength 370 nm and emission wavelength 495 nm was used to estimate the serine protease activity. Caspase-3 Substrate I at an excitation wavelength of 355 nm and emission wavelength of 485 nm was used to
estimate the cysteine protease activity. The reaction mixture was made up by adding 50 µg ground sample (leaf or root) into 220 µL proteolysis buffer (2 mM ATP, 10 mM KCl, 100 mM HEPES-KOH, 5 MM MgCl₂, pH adjusted at 7.5). The general protease activity was monitored through the release of amino-methyl-coumarin (AMC), luciferin, and Capase-3 as a result of enzyme–substrate reaction.

Quantification of phytoalexins in *T. aestivum*

Phytoalexins in *T. aestivum* seedlings were quantified by using the HPLC/MS/MS (Fu et al. 2018). RE or LE (2 mL) was mixed with 10 mL of extraction buffer [HCl:H₂O:2-propanol 37%, in a ratio of 1:2:0.002 (v/v/v)] and the mixture was vortexed for 25 s. After rigorously shaking, dichloromethane (DCM) was added to the mixture in a 1:2 ratio. The mixture was centrifuged for 40 min at 6077 rcf. The lower phase was transferred to a fresh tube with the help of a Pasteur pipette. The contents of the tube were passed through N₂ and later heated at 42°C. The dried samples (2 mg) were then mixed in 2 mL of methanol in water (95.9%). The samples were filtered by spin-X centrifuge tube at 10,000×g. The filtrate as phytoalexins (20 µL) was then loaded to the reverse-phase column (Acclaim120 C18) of HPLC (Ultimate 3000) coupled with MS. The temperature of the system was kept at 35°C, and the flow rate was maintained at 0.2 mL/min. The mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in methanol (solvent B). Phytoalexins were then identified and quantified by electro spraying the HPLC effluent into the mass spectrometer (Orbitrap XL) with R = 30,000. Measurements were recorded at a mass range of m/z 110–460 in positive ionization mode at 4.7 kV, 280°C capillary temperature, 35 au sheath gas flow rate, and 28 au Aux Ga flow rate. The final readings obtained were compared with the calibration curve drawn against the known quantities (10–800 ng/mL) of the standard phytoalexins and subsequently analyzed using HPLC coupled MS (Ding et al. 2018).

Electrolytic leakage

Electrolytic leakage (EL) was measured as described by Orrego et al. (2019). Briefly, 0.3 g of leaf from every individual plant was washed with deionized water and then placed in 15 mL of falcon tube containing 15 mL of deionized water. The samples were incubated for 2 h at 25°C (13), and the electrolytic conductivity of the sample (L₁) was recorded. Samples were then autoclaved at 120°C for 20 min, cooled down to 25°C, and the EC (L₂) was measured again.

The final read was obtained using the formula:

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EL (%) = \frac{L_1}{L_2} \times 100
\]
Determination of marker genes involved in HR at GA₄ cell signaling perception and repression.

Data available for GA₄ cell signaling perception and repression under host–microbe interaction at Expression Angler 2016 (http://bar.utoronto.ca/ExpressionAngler/) were extracted using the co-related gene expression with r-cut-off range from 0.7 to 1.0 and −0.1 to −0.7 (Holland and Jez 2018). The reference microbes used to extract the data were Pseudomonas syringae vs tomato DC3000, a bacterial hemibiotroph (Narusaka and Narusaka 2017), Botrytis cinerea, a fungal necrotroph (Petrasch et al. 2019), Phytophthora infestans, a fungal hemibiotroph (Zuluaga et al. 2016), and Erysiphe orontii, a fungal biotroph (Bheri et al. 2019) to obtain the universal marker genes at GA₄ cell signaling perception and repression in the above-mentioned r-cut-off range. Using yED Graph Editor, raw data were formatted and visualized (Reissmann and Muddukrishna 2018). In such visualization, red balls indicated the upregulating genes with the subject gene (GID1 or RGR1) and blue balls represented downregulating gene. The role of each co-expressed genes significant at our subject study was determined on TAIR (The Arabidopsis Information Resource) (www.arabidopsis.org) (Consortium et al. 2019). Homologs of nine marker genes expressed in Arabidopsis thaliana were obtained in T. aestivum seedling using T. aestivum genome database (www.T.aestivum gdb.org) (Portwood et al. 2018). The expression of such nine genes was analyzed using qRT-PCR technique in the root sample of 11-day-old seedlings of T. aestivum.

Extraction of RNA and subsequent qRT-PCR analysis

RNA extraction from the T. aestivum root was carried out by using Spectrum Plant Total RNA Kit (Sigma Aldrich). DNase I was used to remove any genomic DNA in the sample during RNA extraction (Yin et al. 2016). Primers (Table S1) of oligo (dTs) with Super-Script III Frist Strand Synthesis SuperMix (Invitrogen) were used to generate cDNA library.
The qRT-PCR invitrogen 1× SYBR Green I was done in a CFX96 Real time PCR (Bio-Rad detection system) (Amaral et al. 2017). In order to normalize the expression level of target genes, expression of housekeeping genes (DPP9) was used for comparison (Amaral, Santos, Oliveira and Mafra 2017). The primers for genes (Table S1) were designed using online Oli2go (http://oli2go.ait.ac.at/) at desired temperature and primer length (Hendling et al. 2018).

Statistical analysis

The experiments were repeated three times under the same conditions and with the same materials. The data were analyzed by the analysis of variance (ANOVA) followed by a Duncan’s Multiple Range Test (DMRT) using SPSS software (IBM SPSS Statistics 21) to determine the significance level ($P = 0.05$). Graphs were plotted using Graph Pad Prism (Version 5.03).

Results

Growth promotion under BIPOL inoculation and GA$_4$ inhibition at spore density of OD$_{0.6}$

Uniconazole-treated *T. aestivum* seedlings showed a low level of GA$_4$ after inoculation with BIPOL (Figure 1(A,B)). The RGR (0.26 ± 0.03 g/g/day) and NAR (33 ± 1.17%) of *T. aestivum* seedlings were improved with the inhibition of GA$_4$ after BIPOL inoculation at SD of OD$_{0.6}$ (Figure 1(C,F)). However, the RGR (0.14 ± 0.01 g/g/day) and NAR (6 ± 0.04%) was found to be very low till 72 h of uniconazole treatment (Figure 1(A,D)). Surprisingly, when we challenged the host with BIPOL alone at SD of OD$_{0.6}$, the growth of the seedlings was highly compromised (RGR: 0.04 ± 0.001 g/g/day, NAR: 2 ± 0.08) (Figure 1(B,E)). As expected, EL (47% ± 1.7) was low in U-B seedlings at SD of OD$_{0.6}$ (Figure 1(I)), whereas the uniconazole pretreated seedlings of *T. aestivum* had high EL (67% ± 3.5) till 72 h that got back to normal afterward (Figure 1(G)). On the contrary, EL of
BIPO- treated *T. aestivum* seedlings were very high (95 ± 6.1%) at SD of OD$_{0.6}$ (Figure 1(H)).

**Hormonal cross-talks under BIPOI interaction with host at spore density of OD$_{0.6}$**

In case of BIPOI interaction with *T. aestivum*, an essential hormonal cross-talks between GA$_4$, IAA, and TZn were noticed. In uniconazole-treated *T. aestivum* seedlings, no changes were noticed in the levels of IAA and TZn till 72 h (Figure 2(A,B,F,G,K,L)). In U seedlings, GA$_4$ levels were initially inhibited till 72 h, but the levels GA$_4$ (176 ± 16 ng/g) were restored afterward (Figure 2(C–E)). The *T. aestivum* seedlings treated with BIPOI triggered higher levels of GA$_4$ (291 ± 12.3 ng/g) (Figure 2(A–E)), while the observed levels of IAA (0.02 ± 0.005 mg/g) and TZn (12 ± 0.09 ng/g) were very low (Figure 2(F–O)). Interestingly, in U-B seedlings, the level of IAA (11 ± 0.07 ng/g) and TZn remained high with a low GA$_4$ level (Figure 2(A–O)).

**Plant metabolites in host under BIPOI interaction at spore density of OD$_{0.6}$**

The results showed that LE and RE had low contents of total flavonoids (18 ± 0.2 µg/mL) and proline (108 ± 4.8 µg/mL) in growth-promoted U-B seedlings till 120 h (Figure 3(A–J)). Conversely, the concentration of total flavonoids (98 ± 3.9 µg/mL) and proline (293 ± 19.1 µg/mL) was high in the *T. aestivum* seedlings treated with BIPOI at SD of OD$_{0.6}$ (Figure 3(A–J)). However, in Uniconazole-treated seedlings, the concentrations of total flavonoids (83 ± 2.3 µg/mL) and proline (241 ± 12.9 µg/mL) were initially increased till 72 h (Figure 3(A–C,F–G)), followed by a significant drop in the later hours (Figure 3(D,E,I,J)).

We also quantified phenylpropanoids and glucosinolates in the treated *T. aestivum* seedlings. As expected, uniconazole treatment triggered high amounts of phenylpropanoids (352 ± 12.6 µg/mL) and glucosinolates (441 ± 25.3 µg/mL) till 72 h that decreased later on (Figure 3(K–T)). BIPOI-treated *T. aestivum* seedlings accumulated high amounts of phenylpropanoids (457 ± 17.7 µg/mL) and glucosinolates (641 ±
Interestingly, the U-B seedlings exhibited low amounts of phenylpropanoids (126 ± 5.8 μg/mL) and glucosinolates (170 ± 2.19 μg/mL) (Figure 3(K–T)).

Enzymatic activity under BIPOL inoculation at spore density of OD0.6

Different enzymatic activities were observed in the LE and RE of T. aestivum seedlings. The catalase activity was high in the uniconazole-treated T. aestivum seedlings (1.8 ± 0.005 μg/mL) till 72 h, which decreased (1.1 ± 0.008 μg/mL) later (Figure 4(A–E)). In U-B-treated T. aestivum seedlings, the catalase activity remained high (2.4 ± 0.006 μg/mL) (Figure 4(A–E)), whereas in BIPOL-treated seedlings, the recorded catalase activity (1.17 ± 0.002 μg/mL) was very low till 120 h (Figure 4(A–E)). Likewise, an increased oxidase activity (2.4 ± 0.002 μg/mL) was noticed in uniconazole-treated T. aestivum seedlings till 72 h that decreased (1.6 ± 0.004 μg/mL) later. In contrast, the oxidase activity remained low (0.5 ± 0.0009 μg/mL) in U-B-treated T. Aestivum seedlings (Figure 4(A–E)). However, BIPOL inoculation at SD of OD0.6 increased oxidase activity (2.81 ± 0.0046 μg/mL) in T. aestivum seedlings (Figure 4(A–E)). As indicated in Figure 4(K–T), NAD⁺ (72.6 ± 0.85 nmol mg/prot/min) and FAD⁺ (80.9 ± 0.35 nmol mg/prot/min) were highly induced under uniconazole-treated T. aestivum seedlings till 72 h, which later decreased. BIPOL inoculation at SD of OD0.6 triggered the highest production of NAD⁺ (92.6 ± 0.43 nmol mg/prot/min) and FAD⁺ (99.0 ± 3.8 nmol mg/prot/min) till the end of the experiment. The U-B-treated T. aestivum seedlings exhibited low concentrations of NAD⁺ (28.1 ± 0.33 nmol mg/prot/min) and FAD⁺ (35.3 ± 0.19 nmol mg/prot/min) (Figure 4(K–T)).
Hypersensitive inducing biomolecules under BIPOL inoculation at spore density of OD$_{0.6}$

The results deduce that under uniconazole treatment, the concentrations of the hypersensitive molecules c-di-GMP, cAMP, PA, pure OPDA, esterified OPDA, and JA remained same as control (Figure 5(1)A–J, (2)A–T). On the contrary, after BIPOL inoculation at SD of OD$_{0.6}$, the concentrations of the hypersensitive molecules in *T. aestivum* seedlings shot up and reached peaks. However, in U-B-treated seedlings, the concentration of hypersensitive molecules remained very low (Figure 5(1)A–J, (2)A–T). Additionally, the results revealed that uniconazole treatment did not cause any changes in the protease activity of the *T. aestivum* seedlings (Figure 5(2)A–O). However, the BIPOL treatment triggered high protease activity, whereas...
the U-B seedlings showed low protease activity (Figure 5 (2)A–O). As shown in Figure 5(3)A–T, the concentration of phytoalexins (zealexin A4, kauralexin A4, DIMBOA, and HDMBOA) remained unchanged in uniconazole-treated *T. aestivum* seedlings. However, in BIPOL-inoculated *T. aestivum* seedlings, high amounts of phytoalexins were observed. In U-B seedling on the other hand had extremely low amounts of phytoalexins (Figure 5(3)A–T).

**Interfering activity of GA₄ signaling under BIPOL inoculation at spore density (SD OD₀.₆)**

We observed interfering activity of GA₄ cell signal perception and cell signal repression by using co-expression data on GA₄ perception and repression during host–microbe interaction (Figure 6(A)). The descriptions of the genes were extracted from the online Arabidopsis database available (TAIR [www.arabidopsis.org](http://www.arabidopsis.org)). The principle genes included in this case were WI2 and TRX5. The product of WI2 and TRX5 increased the redox potential in the host cell, thus discouraging the microbial interaction. Similarly, the co-expression of SNF7, EDA16, ALA3 and PTF1 decreased the vacuolar transportation in the host cell to reduce fungal colonization in plant tissues. Moreover, in the same manner, other cell stabilizing activities (electron transfer activity, cell apoptosis, and modification of sugar molecules) were also induced by SAG14, RSW10, PEP12, and ADT5 expressions (Figure 6(A)).

Additionally, the co-expression of upregulated DELLA (RGA1) determined the high expression of DPMS2, whose products eliminated the oxidative burst in order to facilitate microbial interactions (Figure 2(B)). Similarly, the products of TAT3, SPE3, and LOL3 commenced the aminotransferase like enzymatic activity to aid microbe colonization of the host tissues. Other transcriptional facilitating processes (WRKY15, RABA1, and ERD2) used for cytoplasmic streaming to promote the microbial interactions were also upregulated (Figure 6(B)).
We analyzed the expression of selected 9 marker genes in 11-day-old *T. aestivum* seedlings under various treatments. qRT-PCR results indicated that expressions of WII2 (*Zm00001d002065*), TRX5 (*Zm00001d002690*), and PTF1 (*Zm00001d045046*) were highly reduced in U-B seedlings, while the same were upregulated in BIPOL-treated *T. aestivum* seedlings (Figure 7(A–E)). A non-significant (*P*=0.05) difference was found in uniconazole-treated *T. aestivum* seedlings compared to the control (Figure 7(A–E)). On the contrary, expressions of DPMS2 (*Zm00001d034682*), TAT3 (*Zm00001d053107*), and WRKY15 (*Zm00001d036542*) were high in U-B seedlings, while low in BIPOL-treated *T. aestivum* seedlings (Figure 7(F–J)). The results of uniconazole treatment were non-significant (*P*=0.05) compared to the control (Figure 7(F–J)). However, the expressions of GID1 (*Zm00001d010308*), RGA1 (*Zm00001d041362*), and ga20ox2 (*Zm00001d007894*) were remained unaffected in uniconazole-treated *T. aestivum* seedlings (Figure 7(K–O)). Furthermore, the expression of GID1 (*Zm00001d010308*) and ga20ox2 (*Zm00001d007894*) was very high, while RGA1 (*Zm00001d041362*) was low in BIPOL-treated seedlings (Figure 7(K–O)). The U-B seedlings followed an opposite trend concerning the expression of GID1, RGA1, and ga20ox2 genes compared to the BIPOL-treated seedlings. The expression of GID1 and ga20ox2 was very low in BIPOL-treated seedlings, while the expression of RGA1 was high (Figure 7(K–O)).

**Root colonization of BIPOL under high GA4 concentration at spore density of OD$_{0.6}$**

Fungal colonization was noted very low (19 ± 0.02% colonization) in BIPOL-treated seedlings, whereas it was relatively high (88 ± 3.51% colonization) in U-B seedlings (Figure 8).

**Analyses of BIPOL interaction at spore density of OD$_{0.2}$ and OD$_{0.4}$**

To elucidate and validate the biological model for BIPOL-*T. aestivum* interactions at GA$_4$ hypersensitivity, we conducted parallel experiments. Initially, we inoculated the *T. aestivum* roots with BIPOL at a spore density of OD$_{0.2}$ or OD$_{0.4}$. After inoculating *T. aestivum* roots with only BIPOL or in combination with uniconazole at SD of OD$_{0.2}$ or OD$_{0.4}$, the growth of the seedlings was not significant (*P*=0.05) compared to the control (Figure S1A–S1D). Similarly, non-significant changes were observed in EL at SD of OD$_{0.2}$ or OD$_{0.4}$ (Figure S1E–S1F). Likewise, the BIPOL application at SD of OD$_{0.2}$ or OD$_{0.4}$ did not elicit significant (*P*=0.05) production of total flavonoids and proline contents in BIPOL- and U-B-treated seedlings compared to control (Figure S2A–S2B & 2E–2F) (Figure S2C–S2D & 2G–2H). The amount of phenylpropanoids and glucosinolates in *T. aestivum* seedlings from various treatments were also the same as control (Figure S2I–S2J; S2M–S2N; S2G–S2L)
& S2O–S2P). Additionally, the SD at OD0.2 or OD0.4 did not trigger high amounts GA4, IAA, and TZn in T. aestivum seedlings from all treatments compared to the control (Figure S3A–S3F). In GA4-inhibited seedlings, the BIPOL application at SD of OD0.2 or OD0.4 also failed to restore GA4 levels in T. aestivum (Figure S3A–S3F).

Regarding enzymatic activities, BIPOL inoculation of T. aestivum seedlings at SD of OD0.2 or OD0.4 did not induce catalase and oxidase activity in T. aestivum from all tested treatments (Figure S3A–S3B, Figure S4E–S4F, Figure S3C–S3D & S4E–S4F). The concentrations of the enzyme co-factors (NAD or FAD) under BIPOL inoculation at SD of OD0.2 or OD0.4 remained the same in all the tested treatments (Figure S4I–S4J & S4M–S4N). Similarly, BIPOL inoculation at SD of OD0.2 or OD0.4 did not able to reduce the elevated levels of co-factors in U seedlings (Figure S3K–S3L & S3O–S3P). The results regarding HR-inducing molecules in T. aestivum seedlings inoculated with BIPOL at SD of OD0.2 or OD0.4 revealed non-significant (P = 0.05) changes in all treatments including control (Figure S5A–S5L & S5S–S5X). Moreover, the BIPOL inoculation at SD of OD0.2 or OD0.4 did not exhibit high protease activity in uniconazole-treated T. aestivum seedlings (Figure S5M–S5R). Also, a non-significant (P = 0.05) difference was noted in all treatments regarding the percent colonization of BIPOL when applied at SD of OD0.2 or OD0.4 (Figure S6).

**Discussion**

HR in the plant can be elicited in plant species in response to various pathogenic or non-pathogenic microbes (Hatsugai et al. 2018, Ger and Chang 2019). High HR was noticed in the 11-d-old T. aestivum seedlings exposed to BIPOL. The HR further affected the RGR and NAR of the T. aestivum seedlings and deterred the colonization of BIPOL. However, elicitation of HR was noticed only in the T. aestivum seedlings inoculated with BIPOL at SD of OD0.6. On the contrary, no such response was observed in seedlings inoculated with BIPOL at SD of OD0.2 or OD0.4, which means that BIPOL could elicit HR in the host plant species at a specific SD. In our case, BIPOL induced HR in T. aestivum seedlings at SD of OD0.6.

Most of the plant hormones act as a phytosignalling molecules in order to help the host plant species after exposure to microbes (Li et al. 2019). We observed high levels (P = 0.05) of GA4 in T. aestivum leaf and root exudates after application of BIPOL at SD of OD0.6. Interestingly, this high level of GA4 inhibited the production of IAA and TZn in host leaf and roots, which clearly indicated that high levels of GA4 promoted negative cross talks with IAA and TZn. After blocking the GA4 biosynthesis through uniconazole application, we observed growth promotion in T. aestivum seedlings inoculated with BIPOL at SD of OD0.6. In fact, BIPOL colonized the roots of T. aestivum seedlings and promoted its growth in the absence of GA4. On the other hand, inoculation of T. aestivum seedlings treated with BIPOL at SD of OD0.2 or OD0.4 did not promote growth in uniconazole-treated or GA4-deficient seedlings. This means that BIPOL can promote the growth of the uniconazole-treated host plant species under certain SD.

We also examined the effect of uniconazole on the growth and metabolites production by T. aestivum seedlings treated with BIPOL at SD of OD0.6. Certainly, plant species can produce metabolites under biotic or abiotic stress at the expense of their normal growth and development to cope with stress conditions (Yang, Wen, Ruan, Zhao, Wei and Wang 2018). In the case of T. aestivum seedlings inoculated with BIPOL at SD of OD0.6 and treated with uniconazole, the levels of proline, total flavonoids, PPs (phenylpropanoids), and GLs (glucosinolates) were significantly (P = 0.05) high. However, the levels of above-mentioned metabolites came to normal after 72 h of uniconazole treatment. This means that the effect of uniconazole last for 72 h and T. aestivum seedlings may need 3 days to settle down and act normally. Besides, plant metabolites, several biotic and abiotic stresses also induce oxidase activity in plant species. High oxidase activity may result in stunted growth of the plant species for the time being, but it helps the host plant to eliminate the stress conditions (Selinski et al. 2018). As expected, the application of uniconazole to T. aestivum seedlings increased oxidase activity and subsequently decreased plant growth till 72 h. High catalase activity on the other hand helps in the prolific growth of plant species (Poli et al. 2018). We observed low catalase activity and low plant growth in uniconazole-treated T. aestivum seedlings till 72 h. After 72 h, the oxidase and catalase activity of the T. aestivum seedlings came to normal.
which indicated toward the successful defense of the *T. aestivum* against stress.

Plant species exposed to biotic and abiotic stress can produce higher amounts of HR-inducing molecules, such as PA, pure OPDA, esterified OPDA, and JA levels (Schuman et al. 2018). As expected, the *T. aestivum* seedlings with high GA$_4$ (B seedlings treated with BIPOL at SD of OD0.6) produced a high level of HR-inducing molecules, while the seedlings with low GA$_4$ (U-B seedlings treated with BIPOL at SD of OD0.2 or OD0.4) produced low concentrations of these molecules. This deduces that BIPOL inoculation at SD of OD0.2 or OD0.4 is non-effective; i.e., at such SD, BIPOL may not cause virulence or act as a plant growth promoter in *T. aestivum*. Protease activity is common in plant species after inoculation by microbes, these proteases can break down the proteins that involves in the nutrient transportation and microbial colonization (Havé et al. 2018). In the

Figure 7. Determination of gene expression in the leaf tissues of 11-day-old *T. aestivum* seedlings exposed to different treatments including C (control); U (uniconazole), B (BIPOL), U-B (uniconazole-BIPOL) for the different time duration. Duncan’s test was performed, and different alphabetic letters show a significant difference. The experiment was repeated at least three times independently.

Figure 8. Determination of fungal colonization on *T. aestivum* root under the stress of BIPOL (BIPOL) and UNI-BIPOL (uniconazole-BIPOL) in spore density (OD$_{600}$) on the roots of *T. aestivum* seedlings. Duncan’s test was performed, and different alphabetic letters show the significant difference. Experiment was repeated at least three times independently.
present study, the activity of three marker proteases (universal protease, serine protease, and cysteine protease) was monitored. High protease activities with elevated HR were noticed in *T. aestivum* seedling stressed with BIPOL at SD of OD0.6. As HR can be triggered in host plants due to microbe interaction, cell death-inducing substances, such as phytoalexins, might be biosynthesized in host plants (Komives and Kiraly 2019). In our case, we monitored the concentrations of four different phytoalexins (*Zealexins* A4, kauralexin A4, DIMBOA, and HDMBOA) commonly found in *T. aestivum* (Li, Zhao, Zhai, Yuan, Zhang, Wu, Lu, Peng, Sun and Lin 2019).

Using an in-silico approach, we finally extracted data regarding gene expressions in *A. thaliana* under the interaction with model biotrophs and necrotroph at GA4 hypersensitivity. Based on the data, we analyzed the expression of nine markers genes in *T. aestivum* seedlings using qRT-PCR. The result showed that genes (*WII2, TRX5, PTF1, DPMS2, TAT3, WRKY15, GID1, ga20ox2, and RGR1*) that are responsible for the inhibition of microbial colonization were highly upregulated in the *T. aestivum* seedlings with high GA4 levels. On the contrary, such genes were downregulated in Y-B, thus facilitating BIPOL interaction. The description of the genes was taken from the online available database of Arabidopsis (TAIR) (Consortium, Doherty, Friesner, Gregory, Loraine, Megraw, Provart, Slotkin, Town and Assmann 2019). After overall inspection, we also determined BIPOL colonization frequency on the *T. aestivum* roots as roots of most plant species serve as a site for colonization (Hugoni et al. 2018). As expected, the BIPOL at SD of OD0.6 colonized only the roots of the uniconazole-treated *T. aestivum* seedlings. It might be because of the low levels of GA4 that may interfere with the root colonization by BIPOL. Inhibition of GA4 by uniconazole in *T. aestivum* seedlings may lead to a higher accumulation of IAA and tranzeatin. In fact, IAA and tranzeatin are known to have a role in the fungal colonization of the host plant roots (Kabbara et al. 2018, Mehmood et al. 2018). In the *T. aestivum* seedlings with high GA4, the BIPOL colonization was hindered. This could be due to the high level of GA4, which might develop a cross-talks with IAA and tranzeatin, thus decreasing its production in host plant species.

**Conclusion**

Besides growth-inducing molecules, GA4 also indicated the compatibility of host–microbial interactions. Inoculation of BIPOL mainly caused GA4 hypersensitivity at SD of OD0.6. This hypersensitivity decreased the relative growth rate and net assimilation rate of the plant roots (Kabbara et al. 2018, Mehmood et al. 2018). In *T. Aestivum* seedlings with high GA4, the BIPOL colonization was hindered. This could be due to the high level of GA4, which might develop a cross-talks with IAA and tranzeatin, thus decreasing its production in host plant species.

**NAR and RGR.** This further supported our hypothesis that GA4 hypersensitivity hinders the *T. aestivum* root colonization by BIPOL through cross-talks with IAA and cytokinins.

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Conceptualization, AH and MJY; methodology, AH, MH, and AI; validation, MJY and AH; formal analysis, MJY; resources, AH, MH, and IJL; data curation, MJY, AH, AI; writing—original draft preparation, MJY, AI; writing—review and editing, MH, IJL, AH, and AI; supervision, AH, MH, and IJL; project administration, AH; funding acquisition, IJL. All authors have read and agreed to the published version of the manuscript.

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**Data availability statement**

We confirm that all the data generated and material used in this current study are available within this manuscript and its supplementary materials. Moreover, we included in-silico analyses in this study to link to our in-vitro lab experiments. The in-silico data were taken and analyzed using the bioinformatics tool provided on the Bio-Analytic Resource for Plant Biology (BAR; http://bar.utoronto.ca) and *T. aestivum* genome database (www.T.aestivumgdb.org).

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