Diaporthe atlantica improves tomato resistance against the vascular pathogen Fusarium oxysporum f. sp. lycopersici

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Abstract

Background

Fungal pathogen attacks are a major threat to crop growth and productivity, with *Fusarium oxysporum* f. sp. *lycopersici* being particularly menacing to tomato plants by causing vascular wilt disease. *Diaporthe atlantica* is a main component of the root microbiome of *Festuca rubra subsp. pruinosa*, a grass that inhabits sea cliffs. This fungus can confer drought and salinity tolerance to some agricultural plant species. In this study, we evaluated the efficacy of *Diaporthe atlantica* in conferring resistance against *Fusarium* in tomato plants in a greenhouse experiment.

Results

A significant reduction in Fusarium wilt symptoms was observed in plants inoculated with *Diaporthe atlantica*. Furthermore, *Diaporthe* suppressed *Fusarium* colonisation, thereby mitigating vascular browning and improving plant growth, chlorophyll content and nutrient acquisition. In addition, the effect of *Diaporthe atlantica* on plant defence against *Fusarium* seems to not directly involve classical induced systemic resistance or systemic acquired resistance mechanisms.

Conclusion

These findings underscore the potential of *Diaporthe* as a biocontrol agent to enhance plant resistance against Fusarium wilt.

Background

Fungal pathogens cause enormous losses in global agricultural activities and food production due to their destructive effects on crops (1, 2). Although chemical treatments have demonstrated efficacy in controlling fungal pathogens, they have undesirable consequences, such as chemical residues, contributing to environmental pollution and potential threats to human health (3). Furthermore, the widespread development of fungicide resistance in fungal pathogens requires effective disease management (4). Consequently, developing alternatives or complements to fungicides is a key challenge for economically and environmentally sustainable crop production.

Symbiotic associations between plants and fungi range from beneficial to pathogenic. For example, the *Fusarium oxysporum* species complex has been grouped into 106 *formae speciales* (5), which can cause vascular diseases in important crops, such as bananas, cotton, soybean or tomato, resulting in enormous agricultural and economic losses every year (6, 7). *Fusarium oxysporum* f. sp. *lycopersici* (Fol) causes a destructive vascular wilt disease in tomato (*Solanum lycopersicum* L.), limiting its production worldwide (8). Fol penetrates roots and spreads through vascular tissue, colonising and damaging xylem

vessels, eventually causing severe water stress and wilting (9). In contrast, some *Fusarium oxysporum* strains with an endophytic lifestyle can benefit plants (10, 11).

Unlike pathogens, certain endophytic fungi can establish a beneficial symbiotic relationship with plants, improving their performance. This phenomenon is attracting much interest in plant science and agriculture (12–14). Some plant-endophyte relationships enhance plant defence against pathogens through direct mechanisms, where endophytes produce antimicrobial metabolites or compete for resources with pathogens, or indirect mechanisms, where endophytes stimulate plant resistance mechanisms (15–18). These particular characteristics of fungal endophytes position them as promising alternatives for phytopathogen control. For this reason, the search for beneficial microorganisms for the biological control of Fusarium wilt in tomato has increased (10, 19, 20).

The fungal genus *Diaporthe* comprises both pathogens and endophytes associated with numerous plant species (21). *Diaporthe atlantica* is a component of the core microbiome of *Festuca rubra* subsp. *pruinosa* roots, a perennial grass that inhabits sea cliffs of the Atlantic coasts (22, 23). Intriguingly, *D. atlantica* ameliorates salt stress when inoculated in non-host grasses, increasing proline accumulation and nutrient uptake and promoting plant growth (24). Moreover, *D. atlantica* has been shown to increase tolerance to drought stress in tomato plants, ameliorating photosynthesis, nutrient content, and enzymatic antioxidant response (25).

Beneficial microorganisms such as endophytes can activate Induced Systemic Resistance (ISR) or Systemic Acquired Resistance (SAR) against pathogens through hormonal signalling pathways (16, 26). These pathways typically involve hormones such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET), abscisic acid (ABA) (27), or a combination of them, which play crucial roles in activating systemic resistance against Fol. However, a previous study revealed that endophyte-mediated resistance against Fol can occur independently of hormonal signalling pathways (10). This suggests that there may be alternative mechanisms through which endophytes confer resistance against pathogens, highlighting the complexity of plant-microbe interactions.

In this study, we investigated the potential of a strain of the fungus *Diaporthe atlantica* to mitigate Fusarium wilt in tomato plants in a greenhouse experiment.

METHODS

Fungal material

The *Diaporthe atlantica* strain EB4 was originally isolated from *Festuca rubra* subsp. *pruinosa* roots collected in a natural population on the northern coast of Galicia, Spain (22). *D. atlantica* inoculum was produced in 30 g of sugar beet pulp pellet mixed with 9.0 g of $CaCO_3$, 4.5 g of $CaSO_4$ and 60 mL of water, which were autoclaved in wide-mouth glass bottles for 30 minutes at 121°C (28). Each flask containing beet pulp substrate was inoculated with four plugs of mycelium previously cultured in potato dextrose agar (PDA) and incubated at room temperature (20–22°C) for four weeks.

Fusarium oxysporum f. sp. *lycopersici* (Fol) strain Fol029 was kindly provided by Dr. Martijn Rep (University of Amsterdam). To produce spores to be used as inoculum, this strain was cultured in a minimal medium composed of 0.17% yeast nitrogen base without amino acids and ammonium sulfate, 3% sucrose, and 100 mM KNO_3 at 25°C and 250 rpm for one week. Cultures were filtered through Miracloth (Millipore), and spores were diluted to yield a microconidial inoculum of 1.0×10^7 spores/mL (29).

Experimental setting

To determine the effect of *D. atlantica* EB4 as a biocontrol agent against Fol in tomato plants, a bioassay with two variables was designed: *Diaporthe* (inoculated and uninoculated) and Fol (inoculated and uninoculated). Each of the four treatments had 12 single plant replicates. To obtain plants inoculated with *D. atlantica*, tomato seeds cv. Marmande was sown in a plastic tray containing seven parts of peat and perlite (1:1, v:v) previously treated at 80°C for 24 h and one part of *D. atlantica* EB4 inoculum. Seeds were sown in a tray containing only the peat and perlite substrate to obtain uninoculated plants. All plants were grown in a greenhouse with a controlled day-night temperature of 25°C. To inoculate plants with Fol, roots of 10-day-old seedlings (24 uninoculated and 24 *Diaporthe*-inoculated) were trimmed, leaving approximately 1 cm of roots to facilitate Fol inoculation. Then, one-half of the seedlings of each treatment were dipped in a Fol spore suspension (1.0×10^7 spores/mL) for 5 min, and the other half were dipped in water (29). All seedlings were then individually transplanted to 2.5 L plastic plots. The *Diaporthe*-uninoculated seedlings with and without Fol inoculation were potted in a mix of peat and perlite (1:1, v:v), and *Diaporthe*-inoculated seedlings with and without Fol inoculation were potted in a mix of seven parts of peat and perlite with one part (v:v) of *D. atlantica* EB4 inoculum.

Six weeks after Fol inoculation, plant disease symptoms were visually assessed, and plants were harvested. On each plant, a sample of three leaves from the same branch was collected and immediately immersed in liquid nitrogen to store at − 80°C for gene expression analysis. The remaining plant material was separated into leaves, stems, and roots and lyophilised. The dry weight of the lyophilised samples was measured, and chemical analyses were performed.

Evaluation of Fol disease symptoms

At harvest, the plant disease severity was estimated using the disease index (DI) (30), adding an extra score value of 5. Thus, the DI was visually scored on a scale from 0 to 5 as follows: 0: no symptoms (no wilt, dwarfing or brown vessels); 1: one or two brown vessels and development not affected; 2: lowest fully developed leaves showing chlorosis; 3: all fully developed leaves showing chlorosis; 4: all leaves showing chlorosis, including the rosette of newly developed leaves; and 5: plants are dead or very small and wilted.

To detect the presence of Fol in the vascular system, 0.5 cm thick stem pieces were sectioned at the crown and cotyledon level, surface-sterilised with 70% ethanol, rinsed twice with sterile water, and placed on 9 cm plates containing potato dextrose agar (PDA) medium. Plates were incubated in the dark at

25°C for 4 d, allowing the fungus to grow from the stem sections (29). The Fol outgrowth was assessed at crown and cotyledon levels through morphological identification of Fol, and the percentage of fungus-infected stem pieces on each plate was recorded.

Detection of Diaporthe atlantica in inoculated plants

Root samples collected at harvest were analysed by microscopy to determine the presence of *D. atlantica*. Fresh root fragments were cleared in 5% KOH at 90°C for 15 min, neutralised with 1% HCl at 20°C overnight, stained with trypan blue (31), and visualised by light microscopy.

Measurements of plant physiological and biochemical parameters

Photosynthetic parameters

The chlorophyll content was determined in the leaves of six-week-old tomato plants 24 hours before harvesting using a leaf-clip sensor (Dualex Force, Orsay, France) in six plant replicates per treatment. For each plant, three leaves of the third branch from the top were selected, and the average chlorophyll content was obtained from three measurements performed at the central position of each leaf. Chlorophyll analysis of Fol-uninoculated plants was carried out on six randomly selected replicates. However, in Fol-inoculated plants, chlorophyll was only determined in live plants with leaves large enough for optimal measurement with the leaf-clip sensor.

Analysis of mineral element content

Six replicates of leaves from each treatment were analysed for N, P, K, Ca, Mg, Fe, Zn, and Mn content. Freeze-dried and ground samples were calcined at 450°C for 8 h, and ashes were dissolved in $HCl:HNO_3:H_2O$ (1:1:8). Th content of P, K, Ca, Mg, Fe, Zn and Mn were determined by inductively coupled plasma atomic emission spectroscopy (ICP-OES) on a Varian 720-ES spectrometer (Agilent, USA). Nitrogen content was determined using the Dumas combustion method in a C-N analyser (Leco CHN-628, USA).

RNA extraction and gene expression analysis

To determine the potential involvement of JA, SA, ABA and ET in the response against Fol observed in plants inoculated with *D. atlantica*, the expression level of seven gene markers was analysed. *Allene oxide synthase 1* (*AOS1*) is a gene involved in the biosynthesis of JA (32). JA regulates the expression of the *proteinase inhibitor II* (*PiII*) gene (33). *Phenylalanine ammonia-lyase* (*PAL*) is a key enzyme in the synthesis of SA (34). *Pathogenesis-related protein 1* (*PR-1*) is a marker for salicylic acid-dependent systemic acquired resistance (35). The gene *NCDE1* encodes *9-cis-epoxy carotenoid dioxygenase*, a key enzyme in the biosynthesis of ABA (32), and *late-embryogenesis protein 4* (*Le4*) involves ABA signalling and cell protection against stress (32). The *Pti5* gene encodes a pathogen-inducible ET response factor, which acts as a transcription factor to regulate ET-mediated responses upon pathogen attack (36). The

elongation factor 1α (*EF1α*), a constitutive expression gene, was used to normalise the gene expression level of the marker genes related to hormonal pathways (37).

At harvest, three leaves of tomato plants from the same branch were pooled for RNA extraction. Total RNA was extracted as described by Oñate-Sánchez & Vicente-Carbajosa (38). The quality and quantity of RNA were checked using a Nanodrop ND-1000 spectrophotometer. First-strand cDNA was synthesised from 1 µg of purified total RNA using RevertAid H Minus Reverse Transcriptase (ThermoFisher Scientific) according to the manufacturer's instructions.

Gene expression was analysed by real-time qRT-PCR using TB Green Premix Ex Taq (Takara) and the gene-specific primers described in Supplementar1 in a 7900HT Fast Real-Time PCR System (Applied Biosystems). The qRT-PCR conditions were 30 s at 95°C followed by 40 cycles of 95°C for 5 s, 60°C for 34 s and 95°C for 15 s and, a single step of 60°C for 1 min and 95°C for 15 s. Relative quantification of specific mRNA levels was made using the comparative method of Livak & Schmittgen (39).

Statistical analyses

The data were evaluated for distribution assumptions of the ANOVA using the Shapiro-Wilk normality test and Levene´s equal variance test. The effects of *D. atlantica* and Fol inoculation on plant parameters were analysed by means of a two-way ANOVA. Post hoc comparisons for differences among treatment means were evaluated with Tukey's test. All statistical analyses were conducted using the R programming language.

RESULTS

Detection of Diaporthe atlantica

No visual symptoms of diseases were observed in plants inoculated with *D. atlantica*. Furthermore, fungal structures were not detected by light microscopy in the roots of *Diaporthe*-inoculated plants. This suggests that the presence of *D. atlantica* EB4 in tomato plants might be limited to the rhizosphere than being established as an endophyte.

Effect of Diaporthe atlantica and Fol on plant growth

Fol had a strong visible effect on tomato plants, causing a severe reduction in growth. However, this detrimental effect was partly counterbalanced in plants previously inoculated with *D. atlantica* (Fig. 1). Fol inoculation and a [*Diaporthe* × Fol] interaction significantly influenced shoot length (Table 1, Fig. 2A). In the absence of Fol, *D. atlantica* reduced shoot length; however, the opposite occurred in *Diaporthe* + Fol plants. Both shoot and root biomass were significantly affected by *D. atlantica*, Fol, and their interaction (Table 1, Fig. 2B, 2C). Specifically, there was a remarkable reduction in shoot and root biomass in plants inoculated solely with Fol. In contrast, in *Diaporthe* + Fol plants, the shoot and root biomass were similar to plants inoculated only with *D. atlantica*.

Figure 1 – Six-week-old tomato plants uninoculated and inoculated with *Diaporthe atlantica* EB4 in the absence or presence of *Fusarium oxysporum* f. sp. l*ycopersici* (Fol) (no-Fol and Fol).

Figure 2 - Six-week-old tomato plants uninoculated (green) or inoculated with *Diaporthe atlantica* (orange) in the absence or presence of *Fusarium oxysporum* f. sp. *lycopersici* (Fol) (No-Fol or Fol). (A) Shoot length, (B) shoot biomass and (C) root biomass. Different letters indicate different means (Tukey $p < 0.05$) for the [*Diaporthe* × Fol] interaction. Values are means + SE (n = 10). Level of significance: ***$p < 0.001$.

Effect of Diaporthe atlantica on Fol disease severity

The Disease Index (DI) of plants inoculated with Fol was significantly reduced ($p < 0.001$) in *Diaporthe* + Fol plants (Fig. 3). Fol plants showed severe symptoms: 50% of them had chlorosis in all the leaves (DI = 4), and the remaining plants were either dead or small and wilted (DI = 5). However, in *Diaporthe* + Fol plants, the disease symptoms diminished: 42% of them were symptomless (DI = 0), 25% showed mild symptoms (DI = 1–3), and only 33% showed severe symptoms (DI = 4–5). Summarising, the mean DI score in Fol-inoculated plants was 4.5, but it decreased to 1.8 in *Diaporthe* + Fol.

Figure 3 – Quantification of the Disease Index (DI) in tomato plants inoculated with *Fusarium oxysporum* f. sp. *lycopersici* (Fol) and uninoculated and inoculated with *Diaporthe atlantica*. Percentage of plants in each DI category: DI = 0, no symptoms; DI = 1, one or two brown vessels and development not affected; DI = 2, lowest fully developed leaves show chlorosis; DI = 3, all fully developed leaves show chlorosis; DI = 4, all the leaves show chlorosis, including the rosette of newly developed leaves; and DI = 5 the plants are dead or tiny and wilted. Above each column represents the mean DI score of each treatment.

In order to determine to what extent *D. atlantica* inhibited Fol colonisation, surface-sterilised crown and cotyledon-level stem sections were placed on PDA media. Stem pieces from uninoculated and *D. atlantica* plants showed no fungal outgrowth at the crown or cotyledon level (Fig. 4). Fol outgrowth was observed in all stem sections from plants inoculated with Fol at the cotyledon level and 92% at the crown level. In contrast, 58% of the *Diaporthe* + Fol plants showed Fol infection at the cotyledon level and only 35% at the crown level. In 42% of plants, no infection of any stem piece was observed (Fig. 4).

Figure 4 – *Fusarium oxysporum* f. sp. l*ycopersici* (Fol) in stem pieces at the cotyledon level of tomato plants: **(A)** – Stem pieces from plants with several levels of Fol infection from asymptomatic (without xylem vessel infection by Fol) (left) to severe Fol wilt symptoms (with xylem vessel infection by Fol) (right). **(B)** Stem pieces from each treatment group were incubated for 4 days on a minimal medium. Uninoculated plants and *Diaporthe*-inoculated plants showed no fungal outgrowth. Fol-inoculated plants showed fungal outgrowth in all pieces and different levels of fungal outgrowth; *Diaporthe* + Fol plants showed different levels, ranging from no fungal outgrowth to fungal outgrowth in some pieces.

Effect of Diaporthe atlantica and Fol on chlorophyll content

A significant effect of *D. atlantica*, Fol, and their interaction was detected on the chlorophyll content of leaves (Table 1). Compared to uninoculated plants, the chlorophyll content increased significantly with *D. atlantica* inoculation regardless of Fol (Fig. 5). This was associated with a more intense green colour of the leaves of plants inoculated with *D. atlantica* (Fig. 1).

Figure 5 – Chlorophyll content of tomato plants uninoculated (green) or inoculated with *Diaporthe atlantica* (orange) in the absence or presence of *Fusarium oxysporum* f. sp. *lycopersici* (Fol) (No-Fol or Fol). Different letters indicate different means (Tukey $p < 0.05$) for the [*Diaporthe* inoculation × Fol] interaction. Values are means + SE (n = 12). Level of significance: ***$p < 0.001$.

Effect of Diaporthe atlantica and Fol on Mineral Content

The response patterns of N, P, K, Fe and Zn content were rather similar. The content of these elements increased notably in plants inoculated only with *D. atlantica*. However, in *Diaporthe* + Fol plants, its content was lower than in Fol plants. Specifically, N content responded significantly only to *Diaporthe* inoculation (Table 1, Fig. 6A). Conversely, the contents of P, K, Fe and Zn were significantly influenced by the *Diaporthe* x Fol interaction (Table 1). P and Zn content increased significantly in *Diaporthe*-inoculated plants, while differences between Fol-inoculated plants, with or without *Diaporthe*, were not significant (Fig. 6B, 6G). In the absence of Fol, *Diaporthe*-inoculated plants exhibited higher potassium (K) and iron (Fe) content; however, *Diaporthe* + Fol plants showed lower potassium (K) and iron (Fe) content than Fol plants (Fig. 6C, 6F). Furthermore, Mg and Mn exhibited significant increases above all other treatments in Fol-inoculated plants (Table 1, Fig. 6E, 6H). Ca content was significantly influenced by both *D. atlantica* and Fol (Table 1, Fig. 6D).

Figure 6 – (A) Total nitrogen, (B) phosphorus, (C) potassium, (D) calcium, (E) magnesium, (F) iron, (G) zinc, and (H) manganese content in tomato plants uninoculated (green) or inoculated with *Diaporthe atlantica* (orange) in the absence or presence of *Fusarium oxysporum* f. sp. *lycopersici* (Fol) (No-Fol or Fol). Different letters indicate different means (Tukey $p < 0.05$) for the [*Diaporthe* inoculation × Fol] interaction. Values are means + SE (n = 6). Level of significance: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Involvement of phytohormone signalling pathways in plant defences against Fol

To determine if phytohormone signalling pathways play a role in the beneficial effect of *D. atlantica* against Fol, the expression level of several JA, SA, ABA, and ET marker genes was analysed by qRT-PCR (Table S1). Although the changes in the expression level of the marker gene *AOS1* related to JA biosynthesis were not significant, its expression decreased in plants inoculated with Fol without *D. atlantica* and maintained a similar level to Fol-free plants in *Diaporthe* + Fol plants (Fig. 7A). On the other hand, the JA-responsive gene *PiII* exhibited a pronounced response to *D. atlantica*, but only in the absence of Fol (Fig. 7B). Regarding genetic markers related to SA biosynthesis, *PAL* expression significantly decreased in response to Fol (Fig. 7C), and no significant effect was observed on the

expression of the *PR1a* gene, although its expression was higher than in *Diaporthe*-inoculated plants. Both genetic markers related to ABA biosynthesis, *NCED1* and *Le4*, showed a significant decrease in expression in plants inoculated with Fol; in addition, *D. atlantica* also influenced *Le4* expression levels, causing a decrease in its expression. (Fig. 7D, 7E). The genetic marker *Pti5* related to ET responsive exhibited decreased expression in response to Fol (Fig. 7G).

Figure 7 – Relative expression levels of genes involved in hormonal pathways. **(A)** Jasmonic acid (JA) biosynthesis-related gene *AOS1* (*Allene Oxide Synthase 1*), **(B)** JA responsive-related marker gene *Pi II* (*Proteinase Inhibitor II*), **(C)** Salicylic acid (SA) biosynthesis-related gene *PAL* (*Phenylalanine ammonia-lyase*) **(D)** SA *biosynthesis-related* gene *PR1a*, **(E)** Absicic acid (ABA) biosynthesis-related gene *NCED1*, **(F)** ABA responsive-marker gene *Le4* (*Late-Embryogenesis* Abundant protein involved in desiccation protection), **(G)** Ethylene (ET) biosynthesis-related gene *Pti5* in tomato plants uninoculated (green) or inoculated with *Diaporthe atlantica* (orange in the absence and presence of *Fusarium oxysporum* f. sp. *lycopersici* (Fol) (No-Fol or Fol). Values are means + SE (n = 6). Level of significance: *p < 0.05; ***p < 0.001.

Table 1 - Results of a two-way ANOVA analysis showing the effects of the inoculation with *Diaporthe atlantica* and *Fusarium oxysporum* f. sp. *lycopersici* (Fol) and in several parameters measured on tomato plants. Numbers in red mean that the factor significantly affects the variable.

	Diaporthe		Fol		*Diaporthe* x Fol	
	F	P	F	P	F	P
Shoot length	0.378	0.542	50.64	<0.001	30.33	<0.001
Shoot biomass	15.89	<0.001	43.94	<0.001	95.54	<0.001
Root biomass	17.53	<0.001	55.31	<0.001	102.3	<0.001
Chlorophyll	38.41	<0.001	5.971	0.024	8.863	0.007
N	5.042	0.037	3.165	0.091	3.188	0.090
P	6.273	0.020	1.592	0.220	15.42	<0.001
K	0.041	0.840	15.30	<0.001	62.67	<0.001
Ca	11.98	0.002	39.55	<0.001	0.803	1.000
Mg	13.26	0.001	17.83	<0.001	4.729	0.041
Fe	2.133	0.158	4.947	0.037	41.05	<0.001
Zn	33.64	<0.001	3.217	0.087	41.99	<0.001
Mn	10.63	0.004	4.062	0.056	11.17	0.003
AOS1	0.968	0.338	0.412	0.528	0.464	0.504
PiII	4.088	0.057	20.75	<0.001	9.397	0.006
PAL	2.917	0.104	29.80	<0.001	0.840	1.000
PR1a	0.510	0.484	0.020	0.890	0.068	0.797
NCED1	0.1691	0.686	151.4	<0.001	2.340	0.143
Le4	4.909	0.039	7.889	0.011	1.916	0.182

Pti5	0.190	0.667	21.77	<0.001	12.145	0.159

DISCUSSION

An effective management of Fusarium wilt is essential for sustaining global tomato production. Biological control strategies for managing pathogens hold considerable potential due to their cost-effectiveness and environmentally friendly nature (40, 41). In this study, we observed that *D. atlantica* conferred resistance against *F. oxysporum* f. sp. *lycopersici* to tomato plants, leading to a substantial reduction in disease severity, along with improved plant growth and nutrient acquisition.

The symptoms observed in plants inoculated with Fol were consistent with vascular damage, which results in wilting, growth reduction, chlorosis, vascular necrosis and, in severe cases, plant death. Fol crosses the root epidermis and colonises the xylem vessels, disrupting water and nutrient transport, leading to compromised photosynthetic capacity and eventually stunted growth, wilting and even death (42, 43). Surprisingly, despite the fact that the vascular damage hindered nutrient absorption and translocation, we observed an increase in nutrient content in Fol-inoculated plants. This may be related to nutrient mobilisation and redistribution prompted by Fol-induced vascular damage, as well as the release of stored nutrients from the breakdown of cellular components during premature senescence. Furthermore, the observed increase in Mn content may be related to its role in pathogen defence through lignin production and synthesising phenolic compounds toxic to pathogens (44–46).

D. atlantica significantly reduced the disease symptoms caused by Fol, which is a primary criterion for assessing resistance in tomato plants. In addition to disease suppression, *D. atlantica* played a crucial role in increasing specific mineral and chlorophyll content. *Diaporthe*-inoculated plants exhibited enhanced levels of macronutrients (N, P, K, Fe, Ca) and chlorophyll, indicating a positive effect of *D. atlantica* on plant health and physiology. The observed increase in chlorophyll content, particularly in conjunction with improved N assimilation, suggests mechanisms by which *D. atlantica* enhances photosynthetic efficiency and overall plant vigour. Previous studies further support the role of *D. atlantica* in promoting N acquisition and chlorophyll synthesis in host plants (25, 47–49).

Fe is an essential cofactor for chlorophyll synthesis. Our observations suggest that *D. atlantica* contributes significantly to increasing plant Fe availability. *D. atlantica* cultures produce siderophores, which might improve Fe availability at plant roots (24). Despite the observed decrease in Fe content in *Diaporthe* + Fol plants compared to other treatments, the Fe levels remained higher than in uninoculated plants. The higher chlorophyll levels due to *D. atlantica* might reflect the direct effect of Fe availability on chlorophyll accumulation, though the combined presence of *D. atlantica* and Fol may impact Fe availability. This is consistent with previous research demonstrating a positive correlation between Fe status, chlorophyll content, and plant health (50).

The observed inhibition of Fol by *D. atlantica* within the xylem vessels can involve a direct antagonistic interaction, wherein the presence of *D. atlantica* facilitates an inhibitory effect on Fol. This inhibition may

be attributed to the synthesis of antibiotics or analogous bioactive compounds by *D. atlantica*. A previous study (24) reported that *D. atlantica* is capable of producing indole acetic acid (IAA), a phytohormone known for reducing Fol inoculation and promoting plant growth (51, 52). Therefore, the production of IAA by *D. atlantica* could be associated with suppressed Fol proliferation, enhanced root and shoot biomass, improved root-soil contact, and increased water and nutrient uptake. Furthermore, the presence of endophytic fungi is known to increase the production of antioxidants by symbiotic plants, potentially as a response to the generation of reactive oxygen species (ROS) by endophytes (53). This symbiosis would provide *Diaporthe* inoculated with enhanced protection against oxidative stress induced by pathogens such as Fol through the action of these antioxidants. Previous research has demonstrated the ability of *D. atlantica* to enhance enzymatic antioxidant defence against drought stress in tomato plants (25). *Fusarium* infections are well documented to trigger the production and accumulation of ROS in plants, leading to oxidative stress (54). Thus, the antioxidant defence mechanisms promoted by *D. atlantica* could play a crucial role in mitigating the deleterious effects of Fol.

Previous research by our group (25) demonstrated improved growth of tomato plants when their roots were not cut after *D. atlantica* inoculation. However, we observed a growth reduction in plants inoculated with *D. atlantica* compared to uninoculated plants. This might be due to a potential influence of root cutting after *D. atlantica* inoculation. Highlighting a possible link between root manipulation and *D. atlantica* colonisation affecting plant biomass. However, this reduction does not preclude the positive effect of *D. atlantica* on tomato plants, as observed by their healthy appearance and improvement in several physiological aspects, including mineral and chlorophyll content.

Fusarium oxysporum is considered a hemibiotrophic pathogen, transitioning from a biotrophic to a necrotrophic lifestyle (6, 55). This transition involves the manipulation of host defence signalling pathways, including JA, SA, ABA, and ET (27, 55, 56). In our study, Fol suppressed SA, ABA and ET phytohormone pathways, as evidenced by decreased expression of pathway genes such as *PAL, NCED1, Le4*, and *Pti5*, respectively. Notably, the expression level of *AOS1*, a gene involved in JA biosynthesis, remained similar in plants inoculated with *D. atlantica* and Fol compared to plants free of Fol. This observation suggests that *D. atlantica* may counteract the suppressive effect of Fol on JA biosynthesis, maintaining *AOS1* expression levels comparable to Fol-uninoculated plants. Furthermore, *D. atlantica* positively influenced the JA pathway through upregulation of the gene *PiII*, although this effect was not observed in the presence of both *D. atlantica* and Fol. These findings highlight the specific and context-dependent modulation of phytohormone responses by microbial interactions.

Our results suggest that the effect of *D. atlantica* on plant defence against Fol may not directly involve ISR or SAR mechanisms, which typically entail a broad-spectrum activation of defence pathways against pathogens. Instead, the observed modulation of specific phytohormone pathways, particularly JA, in response to *D. atlantica* indicates a more targeted and nuanced interaction. This underscores the complexity of plant-microbe interactions and the need for detailed molecular studies to elucidate the mechanisms underlying *D. atlantica* modulation on plant defence responses. Previous studies also

suggested that endophyte-mediated resistance against Fol was independent of conventional defence pathways (10, 19). Understanding these molecular mechanisms can enhance our understanding of disease control and the independence of conventional hormone-mediated defence pathways.

CONCLUSION

This study shows the potential of *D. atlantica* as a biological control agent against *F. oxysporum* f. sp. *lycopersici* in tomato plants. This research elucidates the underlying dynamics that can confer a beneficial interaction between *D. atlantica* and tomato plants, shedding light on their effectiveness in mitigating Fusarium wilt symptoms and promoting plant growth. *D. atlantica* can mitigate *Fusarium* proliferation in the xylem vessel to improve plant growth, chlorophyll content and nutrient acquisition. Moreover, this study reveals that *D. atlantica* can enhance plant defence through targeted interactions rather than traditional systemic resistance mechanisms, highlighting the complexity of plant-microbe interaction. These findings underline the significance of biocontrol strategies in sustainable agriculture and emphasise the need for further research to optimise the utilisation of *D. atlantica* to enhance crop health and productivity.

Declarations

Ethics approval and consent to participate

All experimental research and studies on plants, including the use of a commercial variety of tomato seeds and production in the greenhouse, comply with relevant institutional, national, and international guidelines and legislation.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no competing interests.

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Author contributions

ECP performed the experiments and analyses and wrote the main manuscript. ECP, BRVA, JBA, IF and IZ designed the experiments, worked on the manuscript and data analyses, and approved the submitted version.

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Figures

Figure 1

Six-week-old tomato plants uninoculated and inoculated with *Diaporthe atlantica* EB4 in the absence or presence of *Fusarium oxysporum* f. sp. l*ycopersici*(Fol) (no-Fol and Fol).

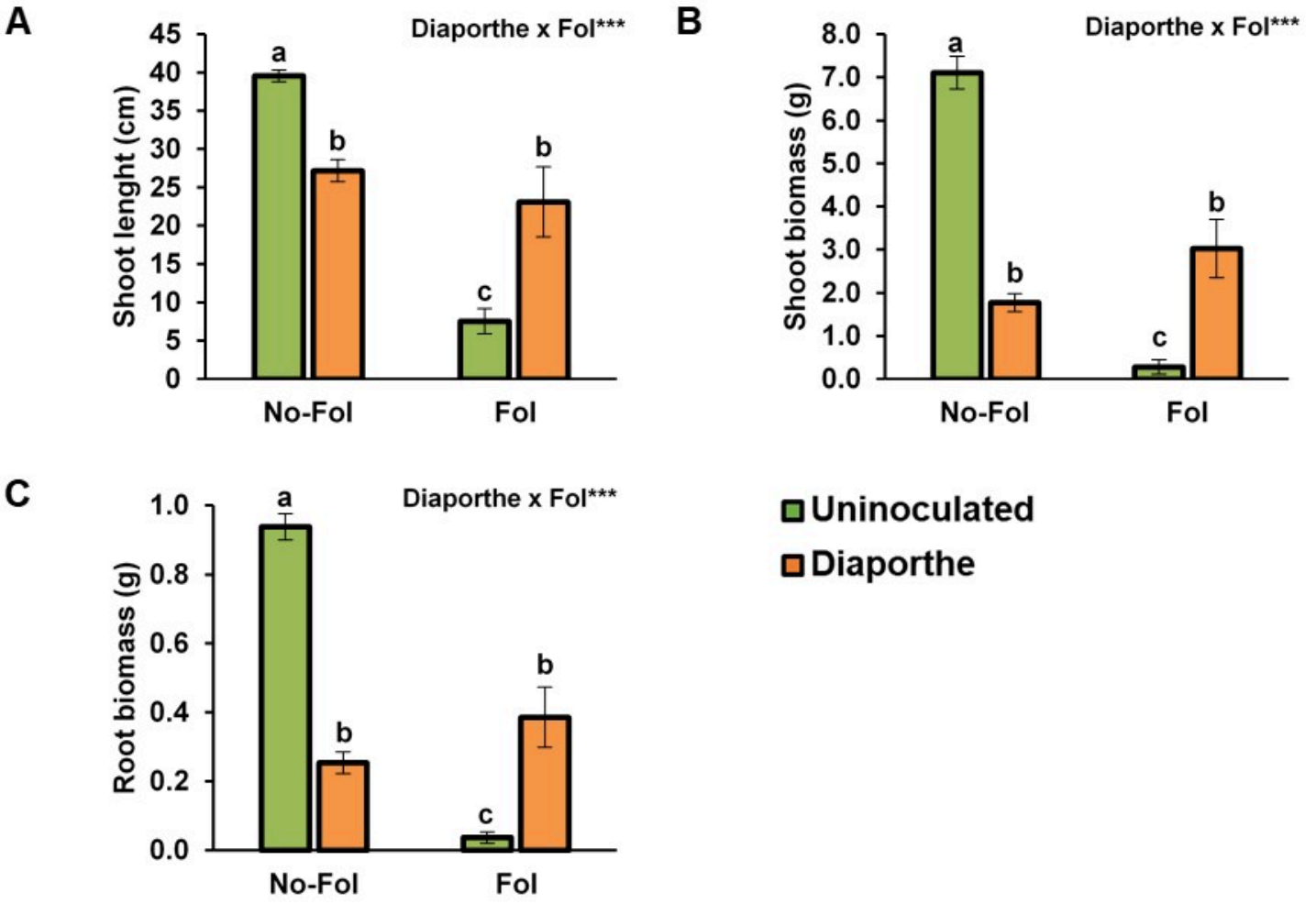

Figure 2

Six-week-old tomato plants uninoculated (green) or inoculated with *Diaporthe atlantica* (orange) in the absence or presence of *Fusarium oxysporum* f. sp. *lycopersici* (Fol) (No-Fol or Fol). (A) Shoot length, (B) shoot biomass and (C) root biomass. Different letters indicate different means (Tukey $p<0.05$) for the [*Diaporthe*× Fol] interaction. Values are means +SE (n=10). Level of significance: ***$p < 0.001$.

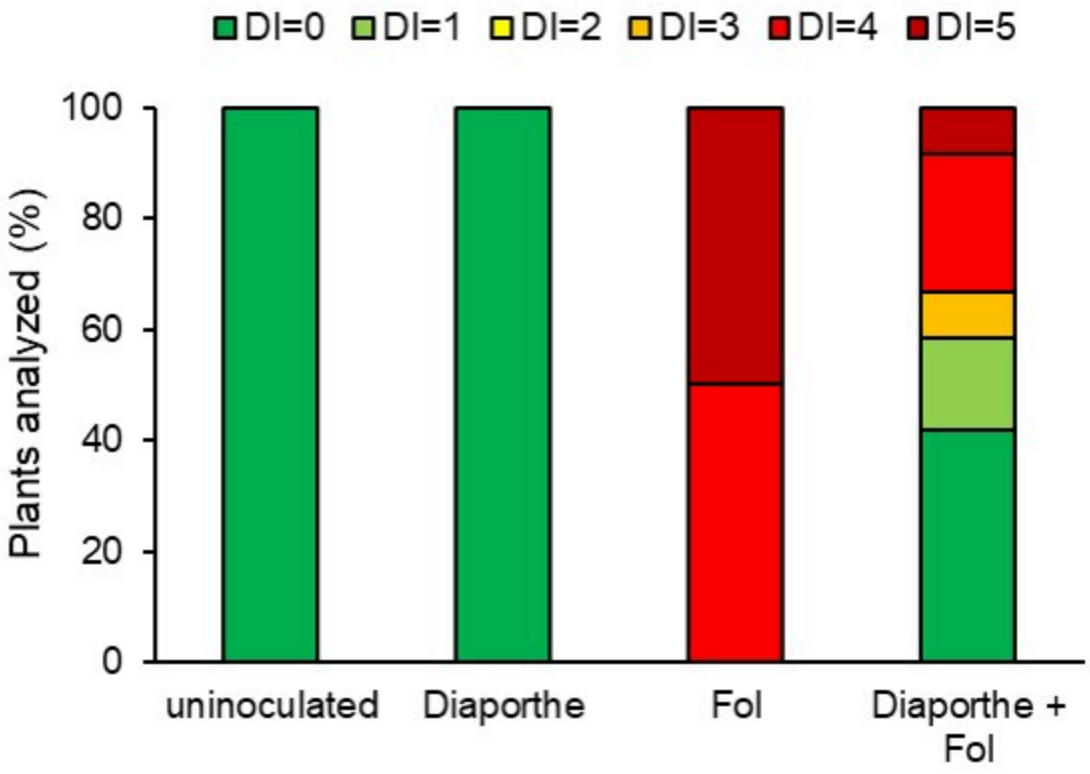

Figure 3

Quantification of the Disease Index (DI) in tomato plants inoculated with *Fusarium oxysporum* f. sp. *lycopersici* (Fol) and uninoculated and inoculated with *Diaporthe atlantica*. Percentage of plants in each DI category: DI=0, no symptoms; DI=1, one or two brown vessels and development not affected; DI=2, lowest fully developed leaves show chlorosis; DI=3, all fully developed leaves show chlorosis; DI=4, all the leaves show chlorosis, including the rosette of newly developed leaves; and DI=5 the plants are dead or tiny and wilted. Above each column represents the mean DI score of each treatment.

Figure 4

Fusarium oxysporum f. sp. l*ycopersici*(Fol) in stem pieces at the cotyledon level of tomato plants: **(A)** – Stem pieces from plants with several levels of Fol infection from asymptomatic (without xylem vessel infection by Fol) (left) to severe Fol wilt symptoms (with xylem vessel infection by Fol) (right). **(B)** Stem pieces from each treatment group were incubated for 4 days on a minimal medium. Uninoculated plants and *Diaporthe*-inoculated plants showedno fungal outgrowth.Fol-inoculated plants showed fungal outgrowth in all pieces and different levels of fungal outgrowth; *Diaporthe* + Fol plants showed different levels, ranging from no fungal outgrowth to fungal outgrowth in some pieces.

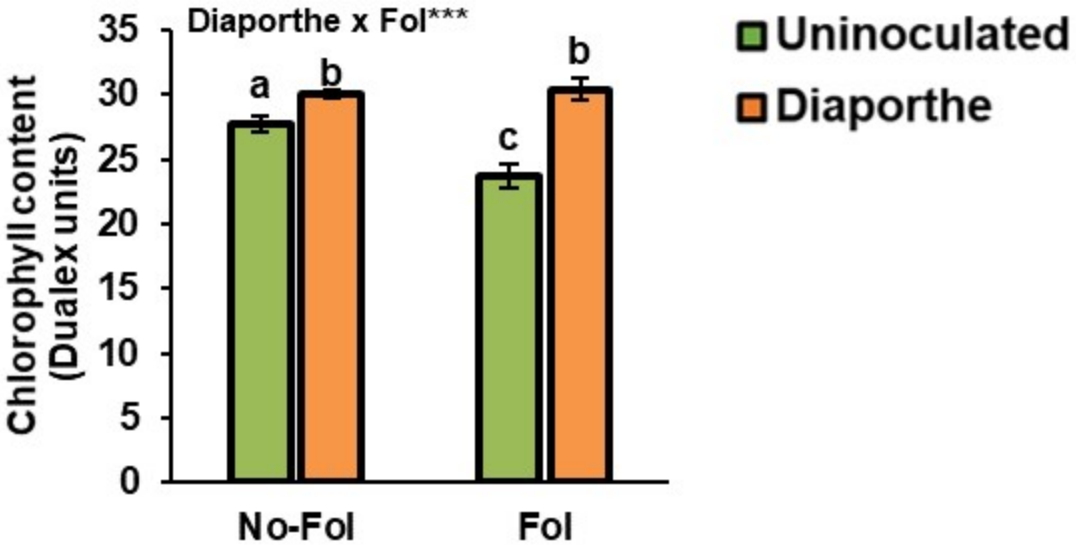

Figure 5

Chlorophyll content of tomato plants uninoculated (green) or inoculated with *Diaporthe atlantica* (orange) in the absence or presence of *Fusarium oxysporum* f. sp. *lycopersici*(Fol) (No-Fol or Fol). Different letters indicate different means (Tukey p<0.05) for the [*Diaporthe* inoculation × Fol] interaction. Values are means +SE (n=12). Level of significance: ***p < 0.001.

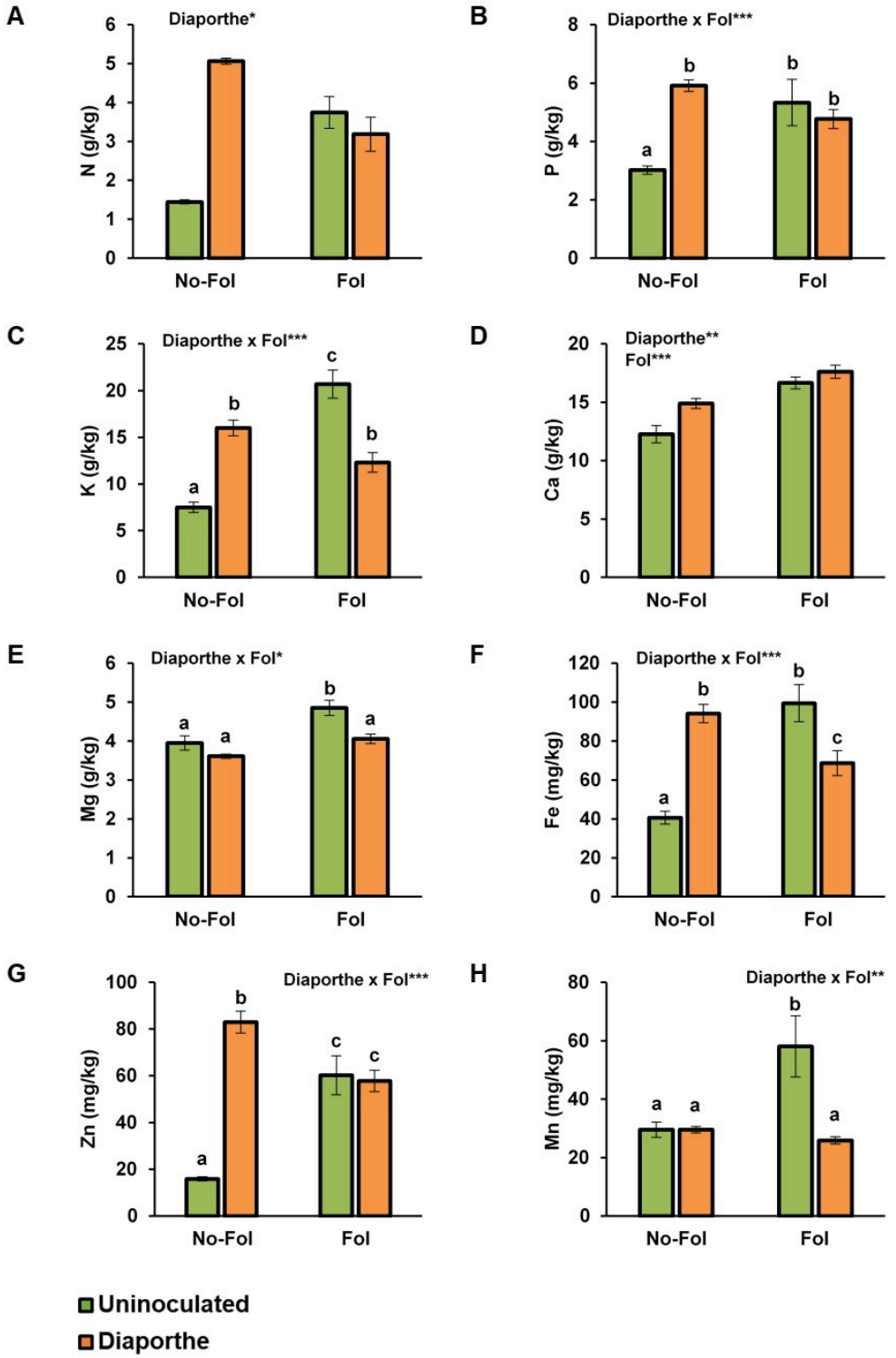

Figure 6

(A) Total nitrogen, (B) phosphorus, (C) potassium, (D) calcium, (E) magnesium, (F) iron, (G) zinc, and (H) manganese content in tomato plants uninoculated (green) or inoculated with *Diaporthe atlantica*(orange) in the absence or presence of *Fusarium oxysporum* f. sp. *lycopersici*(Fol) (No-Fol or Fol). Different letters indicate different means (Tukey p<0.05) for the [*Diaporthe* inoculation × Fol] interaction. Values are means +SE (n=6). Level of significance: *p < 0.05; **p < 0.01; ***p < 0.001.

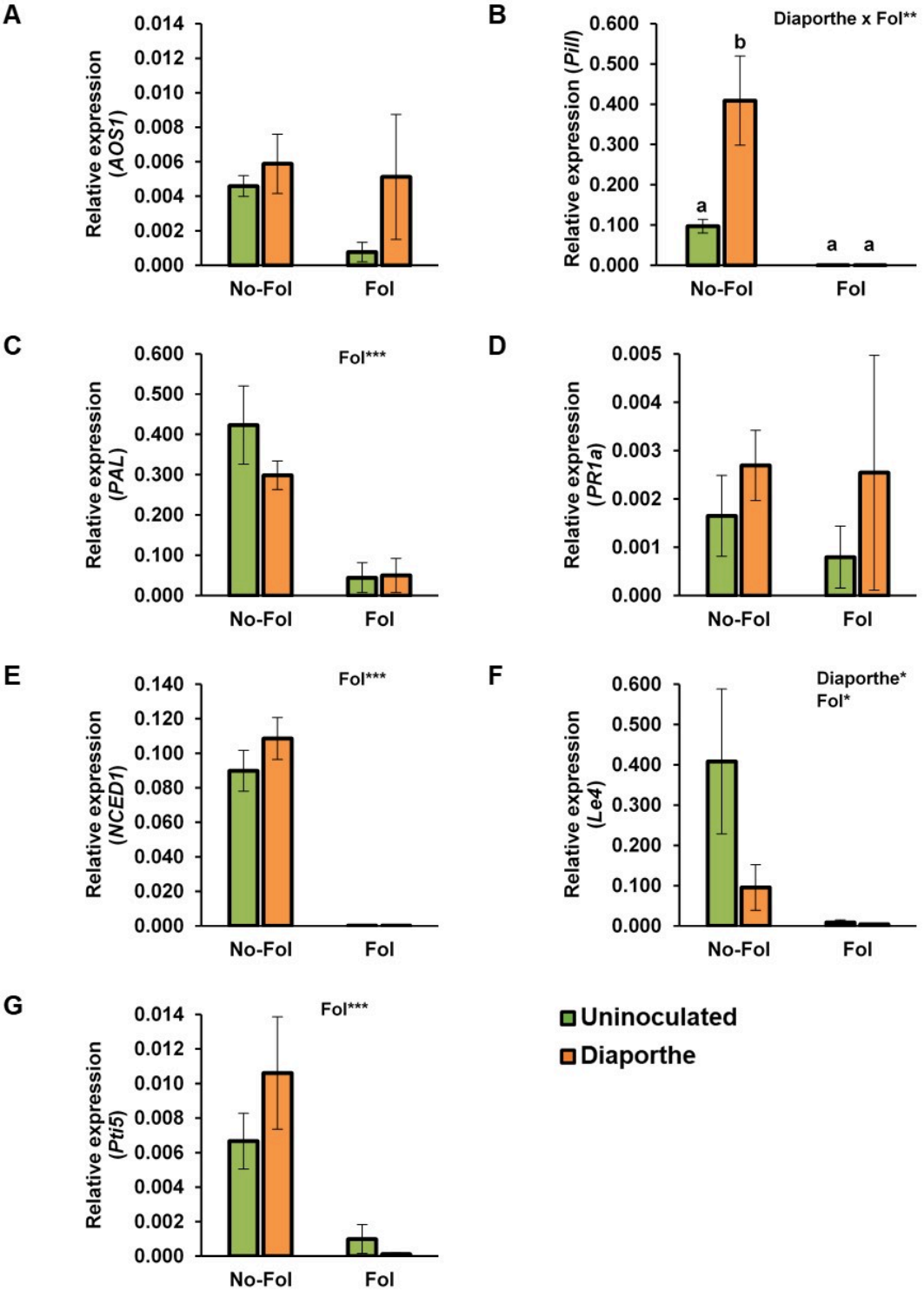

Figure 7

Relative expression levels of genes involved in hormonal pathways. **(A)**Jasmonic acid (JA) biosynthesis-related gene *AOS1* (*Allene Oxide Synthase 1*), **(B)** JA responsive-related marker gene *Pi II* (*Proteinase Inhibitor II*), **(C)**Salicylic acid (SA) biosynthesis-related gene *PAL* (*Phenylalanine ammonia-lyase*) **(D)** SA *biosynthesis-related* gene *PR1a*, **(E)** Absicic acid (ABA) biosynthesis-related gene *NCED1*, **(F)** ABA responsive-marker gene *Le4*(*Late-Embryogenesis* Abundant protein involved in desiccation protection), **(G)**Ethylene (ET) biosynthesis-related gene *Pti5* in tomato plants uninoculated (green) or inoculated with *Diaporthe atlantica* (orange in the absence and presence of *Fusarium oxysporum* f. sp. *lycopersici*(Fol) (No-Fol or Fol). Values are means +SE (n=6). Level of significance: *p < 0.05; ***p < 0.001.

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