



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

Eric C. Pereira^{a,b,*}, Beatriz R. Vázquez de Aldana^a, Juan B. Arellano^a, Ivan Fernandez^a,
Iñigo Zabalgoeazcoa^a

^a Plant-Microorganism Interaction Research Group, Institute of Natural Resources and Agrobiology of Salamanca, Consejo Superior de Investigaciones Científicas (IRNASA-CSIC), Salamanca, Spain

^b Research School of Biology, The Australian National University, Canberra, ACT 2601 Australia

HIGHLIGHTS

- *Diaporthe atlantica* significantly reduced Fusarium wilt severity in tomato plants.
- Inoculation with *D. atlantica* enhanced chlorophyll content and nutrient acquisition.
- *D. atlantica* suppressed Fusarium colonisation in vessels.
- The biocontrol effect of *D. atlantica* was independent of ISR or SAR pathways.

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ABSTRACT

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 which inhabits sea cliffs. This fungus can confer drought and salinity tolerance to some agricultural plant species. This study evaluated the efficacy of *Diaporthe atlantica* in conferring resistance against *Fusarium* in tomato plants in a greenhouse experiment. A significant reduction of Fusarium wilt symptoms was observed in plants inoculated with *Diaporthe*. Furthermore, *Diaporthe* suppressed *Fusarium* colonisation, mitigating vascular browning and improving plant growth, chlorophyll content and nutrient acquisition. In addition, the effect of *Diaporthe atlantica* on plant defence against *Fusarium* does not directly involve classical induced systemic resistance (ISR) or systemic acquired resistance (SAR) mechanisms. These findings underscore the potential of *Diaporthe* as a biocontrol agent against Fusarium wilt.

1. Background

Fungal pathogens cause enormous losses in global agricultural activities and food production due to their destructive effect on crops (Savary et al., 2012; Doehlemann et al., 2017). 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 (Wightwick et al., 2010). Furthermore, the widespread development of fungicide resistance in fungal pathogens requires effective

disease management (Lucas et al., 2015). 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* (Edel-Hermann and Lecomte, 2019), which can cause vascular diseases in important crops, such as banana, cotton, soybean or tomato, producing enormous agricultural and economic losses every year (Michielse and Rep, 2009; Dean et al., 2012). *Fusarium oxysporum* f. sp. *lycopersici* (Fol) causes a

* Corresponding author at: Plant-Microorganism Interaction Research Group, Institute of Natural Resources and Agrobiology of Salamanca, Consejo Superior de Investigaciones Científicas (IRNASA-CSIC), Salamanca, Spain.

E-mail address: eric.pereira@anu.edu.au (E.C. Pereira).

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destructive vascular wilt disease in tomato (*Solanum lycopersicum* L.), limiting its production worldwide (Srinivas et al., 2019). Fol penetrates the roots and spreads through the vascular tissue, colonising and damaging xylem vessels, eventually causing severe water stress and wilting (Gordon, 2017). In contrast, some *Fusarium oxysporum* strains with an endophytic lifestyle can benefit plants, even protect them against Fol (Fravel et al. 2003; Constantin et al., 2019; de Lamo and Tekken, 2020).

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 (White et al., 2019; Chitnis et al., 2020; Verma et al., 2022). Some plant-endophyte relationships can enhance plant defence against pathogens through direct mechanisms, where the endophytes produce antimicrobial metabolites or compete for resources with pathogens, or indirect mechanisms, where the endophytes stimulate plant resistance mechanisms (Zabalgoitia et al., 2008; Pieterse et al., 2014). 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 (Fravel et al. 2003; Li et al., 2018; Constantin et al., 2019; 2020).

The fungal genus *Diaporthe* comprises both pathogens and endophytes of numerous plant species (Gomes et al., 2013). *Diaporthe atlantica* is a component of the core microbiome of the roots of *Festuca rubra* subsp. *pruinosa*, a perennial grass which inhabits sea cliffs (Pereira et al., 2019; Toghueo et al., 2023). Moreover, *D. atlantica* has been successfully inoculated in tomato plants, increasing drought tolerance by enhancing photosynthesis, nutrient content, and enzymatic antioxidant responses (Pereira et al., 2023). Notably, this fungus has also been demonstrated to alleviate salt stress in non-host grasses, enhancing proline accumulation, nutrient uptake, and promoting plant growth under saline conditions (Toghueo et al., 2023). Given these demonstrations, we selected *D. atlantica* for this study to evaluate its potential as a biocontrol agent against Fusarium wilt in tomato.

Beneficial microorganisms such as endophytes can activate Induced Systemic Resistance (ISR) or Systemic Acquired Resistance (SAR) against pathogens through hormonal signalling pathways (Fu et al., 2013; Pieterse et al., 2014). These pathways typically involve hormones such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET), abscisic acid (ABA) (Di et al., 2016), or a combination of them, which play crucial roles in activating systemic resistance against Fol. However, a previous work observed that endophyte-mediated resistance against Fol can occur independently of hormonal signalling pathways (Constantin et al., 2019). 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. The specific aims of this study were: (i) to evaluate the potential of *D. atlantica* to reduce Fol symptoms in tomato; (ii) to assess its effect on plant physiological and biochemical parameters; and (iii) to investigate whether the effect of *D. atlantica* on plant defence against Fol is associated with the activation of classical phytohormone-mediated defence pathways.

2. Materials and methods

2.1. 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 (Pereira et al., 2019). *D. atlantica* inoculum was produced in a growth medium consisting of 30 g of sugar beet pulp pellet mixed with 9.0 g CaCO₃, 4.5 g CaSO₄ and 60 mL of water, which were autoclaved in wide-mouth glass bottles for 30 min at

121 °C (Vázquez-de-Aldana et al., 2020). Each flask containing beet pulp substrate was inoculated with four plugs of *Diaporthe* 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 KNO₃ 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 (de Lamo et al., 2018).

2.2. 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 inoculate plants with *D. atlantica*, seeds of tomato cv. Marmande were sown in a plastic tray containing a substrate composed of 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 in water (de Lamo et al., 2018). All seedlings were then individually transplanted to 2.5 L plastic pots. 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 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 – 80 °C for gene expression analysis. The remaining plant material was separated into leaves, stems and roots, and lyophilised. The lyophilised samples were used to measure dry weight and perform chemical analyses.

2.3. Evaluation of Fol disease symptoms

At harvest time, the plant disease severity was estimated using the disease index (DI) described by Gawehns et al. (2014), 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 show chlorosis; 3: all fully developed leaves show chlorosis; 4: all leaves show 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 (de Lamo et al., 2018). The Fol outgrowth was assessed at the crown and cotyledon levels through morphological identification of Fol, and the percentage of fungus-infected stem pieces was recorded in each plate.

2.4. Detection of *D. Atlantica* in inoculated plants

Root samples collected at harvest time were analysed by microscopy to diagnose the presence of *D. atlantica* in roots. 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 (Berthelot et al., 2016), and visualised by light microscopy.

2.5. Photosynthetic parameters

The chlorophyll content was determined in leaves of six-week-old tomato plants 24 h before harvesting using a leaf-clip sensor (Dualux Force, Orsay, France) in six plant replicates per treatment. In 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.

2.6. 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. For that purpose, freeze-dried and ground samples were calcined at 450 °C for 8 h, and ashes were dissolved in HCl:HNO₃:H₂O (1:1:8). Then, P, K, Ca, Mg, Fe, Zn and Mn contents were determined by inductively coupled plasma atomic emission spectroscopy (ICP-OES) in a Varian 720-ES spectrometer (Agilent, USA). Nitrogen content was analysed using the Dumas combustion method in a C-N analyser (Leco CHN-628, USA). These measurements reflect the total nutrient content in plant tissues, related to nutrient uptake under different treatments.

2.7. 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 (López-Ráez et al., 2010). JA regulates the expression of the *proteinase inhibitor II* (PiII) gene (Uppalapati et al., 2005). *Phenylalanine ammonia-lyase* (PAL) is a key enzyme in the synthesis of SA (Shine et al., 2016). *Pathogenesis-related protein 1* (PR-1) is a marker for salicylic acid-dependent systemic acquired resistance (Breen et al., 2017). The gene *NCDE1* encodes 9-*cis*-epoxy carotenoid dioxygenase, a key enzyme in the biosynthesis of ABA (López-Ráez et al., 2010), and *late-embryogenesis protein 4* (Le4) involves ABA signalling and cell protection against stress (López-Ráez et al., 2010). The *Pti5* gene encodes a pathogen-inducible ET response factor, which acts as a transcription factor to regulate ET-mediated responses upon pathogen attack (He et al., 2001). Relative quantification of gene expression was performed using the comparative 2^{-ΔΔCt} method (Livak & Schmittgen, 2001), with elongation factor 1α (EF1α) as the reference gene for normalisation (Rotenberg et al., 2006).

At harvest time, 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 (2008). 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 Sup. Table S1 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.

2.8. Statistical analyses

The data were evaluated for distribution assumptions of the ANOVA using the Shapiro-Wilk normality test and Levenés equal variance test. The effects of *D. atlantica* and Fol inoculation on plant parameters were analysed by means of a two-way ANOVA. When significant effects were detected, post hoc comparisons among treatment means were conducted using Tukey's Honest Significant Difference (HSD) test. All statistical analyses were performed using R (version 4.4.0; R Core Team, 2024) in RStudio (version 2024.04.0), employing the "stats", "car", and "ggplot2" packages. Statistical significance was considered at $p < 0.05$.

3. Results

3.1. Detection of *D. Atlantica* in inoculated plants

No visual disease symptoms were observed on the roots or leaves of plants inoculated with *D. atlantica*, and fungal structures were not observed by light microscopy in the roots of *Diaporthe*-inoculated plants. Therefore, the association of *D. atlantica* EB4 with tomato plants may be rhizospheric and not endophytic.

3.2. Effect of *D. 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, in *Diaporthe* + Fol plants, the opposite occurred. 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*.

3.3. Effect of *D. 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.

In order to determine to what extent *D. atlantica* inhibited Fol colonisation, surface-sterilised crown and cotyledon level stem sections were placed on PDA plates. 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).

3.4. Effect of *D. 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



Fig. 1. Six-week-old tomato plants uninoculated and inoculated with *Diaporthe atlantica* EB4, with two different *Fusarium oxysporum* f. sp. *lycopersici* (Fol) treatments (no-Fol and Fol).

Table 1

Results of a two-way ANOVA analysis showing the effects of inoculation with *Diaporthe atlantica* and *Fusarium oxysporum* f. sp. *lycopersici* (Fol) on several parameters measured on tomato plants. Numbers in bold mean that the factor significantly affects the variable. F-value indicates the ratio of variance between groups to variance within groups, and *P*-value indicates the probability that the results occurred by chance. Values less than 0.05 are considered significant.

	<i>Diaporthe</i>		Fol		<i>Diaporthe</i> 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
PilI	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

with *D. atlantica* (Fig. 1).

3.5. Effect of *D. Atlantica* and Fol on mineral content

The response patterns of N, P, K, Fe and Zn content are 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 a *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 and 6G). In the absence of Fol, *Diaporthe*-inoculated plants exhibited higher K and Fe content; however, *Diaporthe* + Fol plants showed lower K and Fe content than Fol plants (Fig. 6C and 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).

3.6. Involvement of phytohormone signalling pathways in the plant defence against Fol

To determine if phytohormone signalling pathways play a role in the

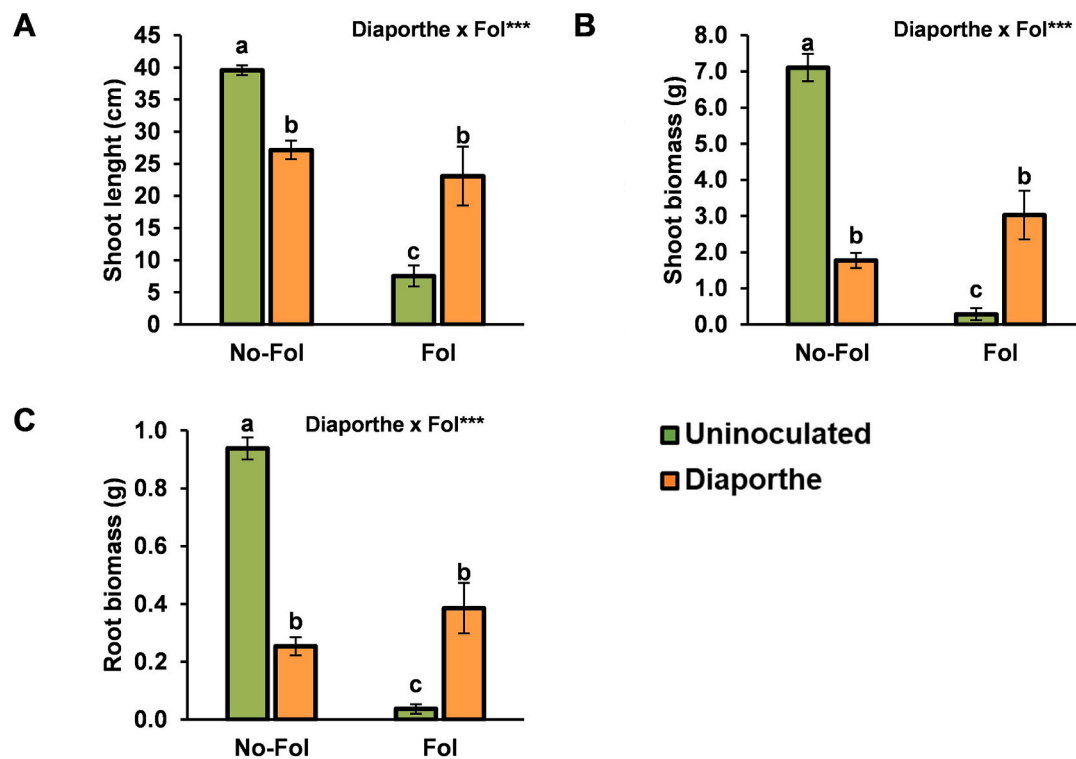


Fig. 2. Six-week-old tomato plants uninoculated (green) or inoculated with *Diaporthe atlantica* (orange) in the absence and presence of *Fusarium oxysporum* f. sp. *lycopersici* (Fol) (No-Fol or Fol). (A) Shoot length, (B) shoot, (C) root biomass. Different letters indicate different means (Tukey $p < 0.05$) for the [*Diaporthe* $\times$ Fol] interaction. Values are means $\pm$ SE ($n = 10$). Level of significance: *** $p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

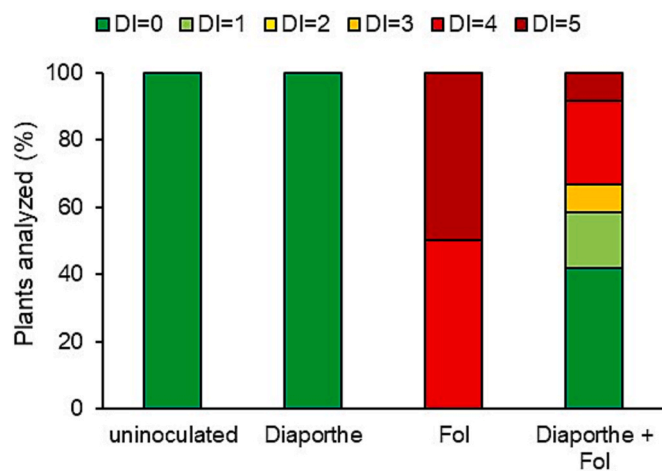


Fig. 3. Quantification of the Disease Index (DI) in tomato plants inoculated with *Fusarium oxysporum* f. sp. *lycopersici* (Fol) and uninoculated and inoculated with *Diaporthe atlantica*. Percentage of plants in each DI category: DI = 0 no symptoms; DI = 1 one or two brown vessels and development not 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; DI = 5 the plants are dead or tiny and wilted. Above each column represents the mean DI score of each treatment.

beneficial effect of *D. atlantica* against Fol, the expression level of several JA, SA, ABA, and ET pathway 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 *PilI* 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 and 7E). The genetic marker *Pti5* related to ET-responsive exhibited decreased expression in response to Fol (Fig. 7G).

4. Discussion

An effective management of *Fusarium* wilt is essential for sustaining global tomato production. Biological control strategies for managing pathogens can hold considerable potential due to their cost-effectiveness and environmentally friendly nature (He et al., 2021a; 2021b). In this study, we observed that *D. atlantica* conferred resistance against *Fusarium 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. 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 (Lorenzini et al., 1997; Nogués et al., 2002). Surprisingly, despite the fact that the vascular damage hindered nutrient absorption and translocation, we observed an increase in total 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



Fig. 4. *Fusarium oxysporum* f. sp. *lycopersici* (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 d 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.

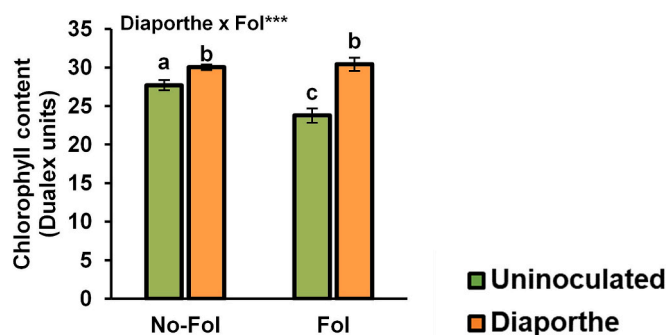


Fig. 5. Chlorophyll content of tomato plants uninoculated (green) or inoculated with *Diaporthe atlantica* (orange) in the absence and presence of *Fusarium oxysporum* f. sp. *lycopersici* (Fol) (No-Fol or Fol). Different letters indicate different means (Tukey $p < 0.05$) for the [*Diaporthe* inoculation $\times$ Fol] interaction. Values are means $\pm$ SE ($n = 12$). Level of significance: *** $p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

components during premature senescence. Furthermore, the observed increase in Mn content may be related to its role in pathogen defence through lignin production and the synthesis of phenolic compounds toxic to pathogens (Graham & Webb, 1991; Dordas, 2009; Orra & Nelson, 2018).

D. atlantica greatly reduced the disease symptoms caused by Fol, which is a primary criterion for assessing resistance in tomato plants. Beyond disease suppression, *D. atlantica* played a crucial role in increasing specific mineral contents and chlorophyll. *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. *D. atlantica* did not show phosphate solubilization activity *in vitro* (Toghueo et al., 2022). However, this strain exhibited strong cellulase activity, and siderophore and ammonium production (Toghueo et al., 2022). The cellulase activity can enhance nutrient availability by decomposing and recycling organic matter. 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 (Yang et al., 2014; 2015; Siddique et al., 2016; Pereira et al., 2023). Fe is an essential cofactor for chlorophyll synthesis. *D. atlantica* cultures produce siderophores, which might improve Fe availability at plant roots (Toghueo et al., 2022). 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 finding is consistent with previous research demonstrating a positive correlation between Fe status, chlorophyll content, and plant health (Ahmed & Holmström, 2014).

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 other bioactive compounds by *D. atlantica*. Toghueo et al. (2022) reported that *D. atlantica* is capable of producing indole acetic acid (IAA), a phytohormone known for reducing Fol inoculation and promoting plant growth (Sharaf & Farrag, 2004; Numponsak et al., 2018). 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 the endophytes (White et al., 2010). This symbiosis would provide *Diaporthe*-inoculated plants 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 (Pereira et al., 2023). *Fusarium* infections are well-documented to trigger the production and accumulation of ROS in plants, leading to oxidative stress (Iqbal et al., 2024). 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 (Pereira et al., 2023) 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, the 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 (Michielse & Rep, 2009; Chen et al., 2014). This transition involves the manipulation of host defence signalling pathways, including JA, SA, ABA, and ET

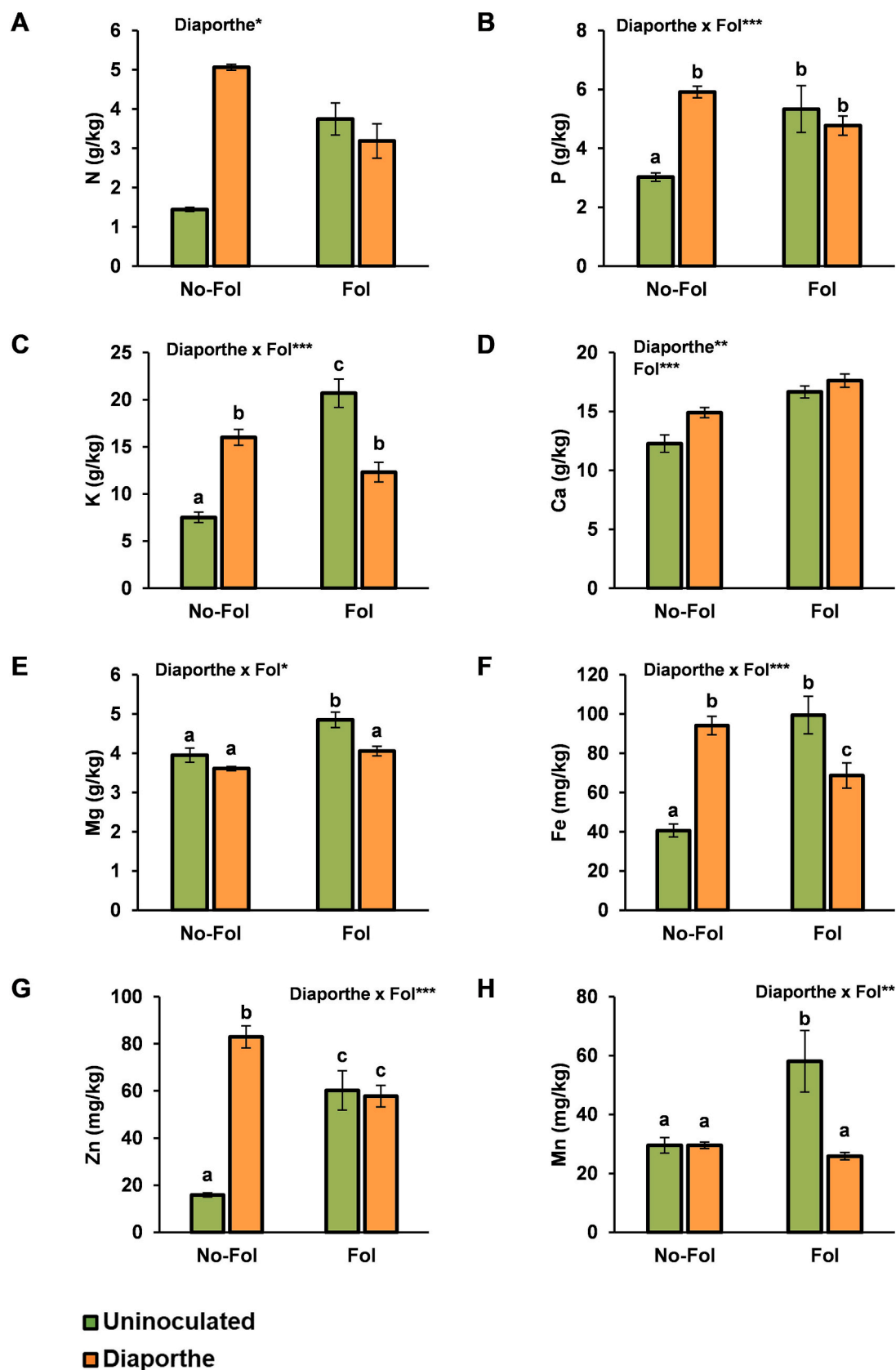


Fig. 6. (A) Total nitrogen, (B) phosphorus, (C) potassium, (D) calcium, (E) magnesium, (F) iron, (G) zinc, and (H) manganese content in tomato plants uninoculated (green) or inoculated with *Diaporthe atlantica* (orange) in the absence and presence of *Fusarium oxysporum* f. sp. *lycopersici* (Fol) (No-Fol or Fol). Different letters indicate different means (Tukey $p < 0.05$) for the [*Diaporthe* inoculation $\times$ Fol] interaction. Values are means $\pm$ SE ($n = 6$). Level of significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

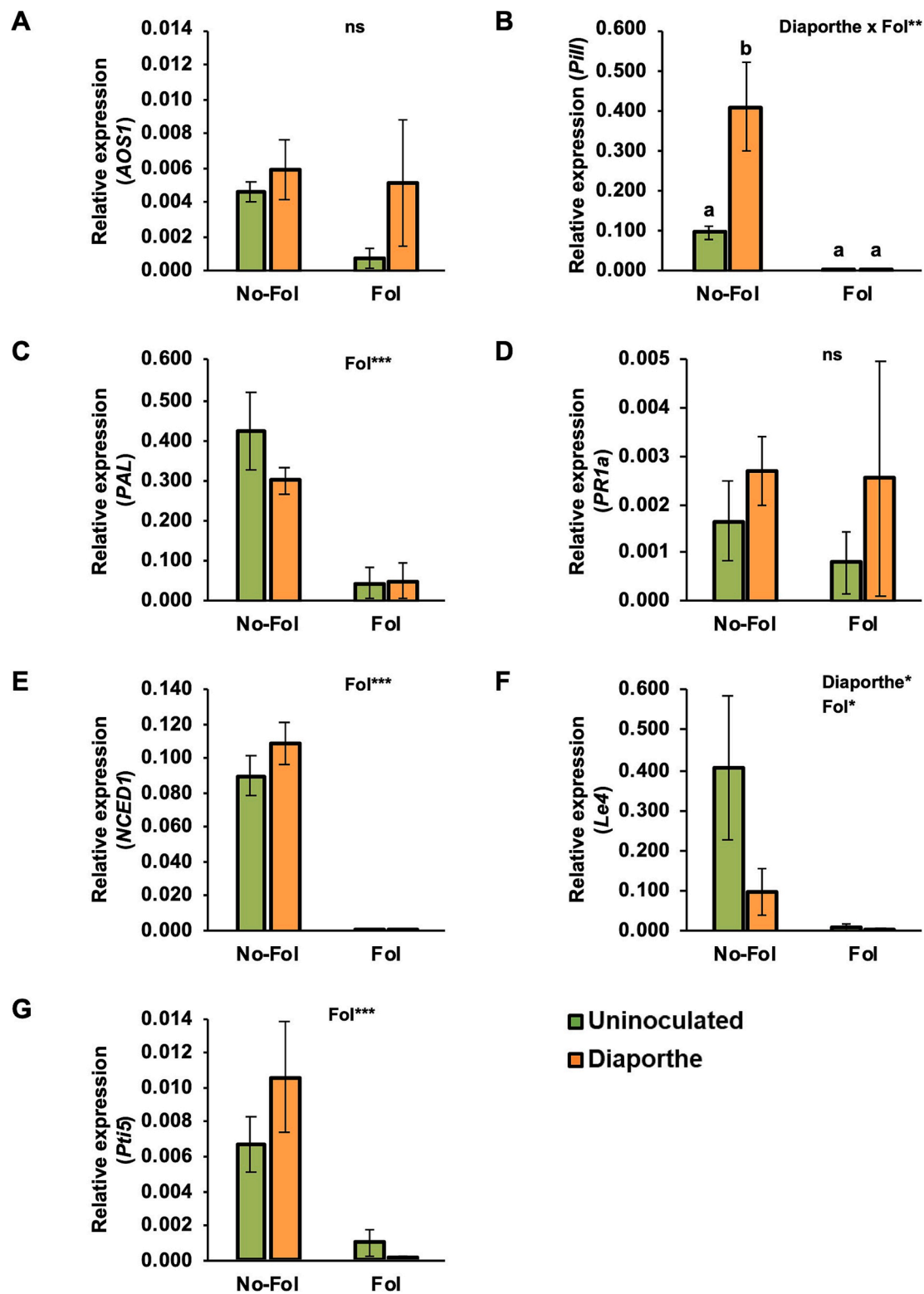


Fig. 7. Relative expression levels of genes involved in hormonal pathways. (A) Jasmonic Acid (JA) biosynthesis-related gene *AOS1* (*Allene Oxide Synthase 1*), (B) JA responsive-related marker gene *Pi II* (*Proteinase Inhibitor II*), (C) Salicylic Acid (SA) biosynthesis-related gene *PAL* (*Phenylalanine ammonia-lyase*) (D) SA biosynthesis-related gene *PR1a*, (E) Abscicic 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. ns = not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Thatcher et al., 2009; Chen et al., 2014; Di et al., 2016). 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.

While *D. atlantica* modulates the expression of certain hormone-responsive genes, its biocontrol activity against Fol does not align with the canonical hormone-dependent ISR or SAR pathways typically observed in classical biocontrol agents. Instead, *D. atlantica* appears to confer protection through a distinct mode of action, likely involving a combination of reduced pathogen colonisation, improved plant physiological status, and specific modulation of plant responses, highlighting its potential as an effective biocontrol agent with a complementary mechanism of action. Previous studies also suggested that endophyte-mediated resistance against Fol was independent of conventional defence pathways (Constantin et al., 2019; 2020). 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.

Recent advances have highlighted the potential of microbiome-based strategies for sustainable plant disease management, such as using synthetic microbial communities (Zhou et al., 2022) or natural antimicrobial compounds (Ren et al., 2025). Our findings contribute to this emerging field by showing that *D. atlantica*, although not a native endophyte of tomato, can establish a beneficial association with this crop under controlled conditions, reducing *Fusarium* wilt severity and improving plant physiological status. This supports the concept that carefully selected non-native endophytes with proven plant-beneficial traits can serve as nature-based tools within microbiome-informed disease management strategies. However, it is essential to recognise that results obtained in greenhouse conditions may not directly translate to field performance. In field settings, factors such as competition with the native soil microbiome, environmental variability, and interactions with local microbial communities can significantly influence the establishment and efficacy of introduced biocontrol agents (White et al., 2019; Köhl et al., 2019). Therefore, future field trials under diverse environmental and soil conditions will be critical to validate the practical applicability of *D. atlantica* for sustainable *Fusarium* wilt control in tomato cultivation.

5. Conclusions

This study shows the potential of *D. atlantica* as a biological control agent against *Fusarium 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 interactions. 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.

Future studies should prioritise a deeper mechanistic characterisation of *D. atlantica* mode of action. This includes comparative analyses

with other endophytes, detailed information of colonisation sites, metabolic profiling, and high-throughput plant sequencing to assess gene expression changes induced by biocontrol agent and pathogen combinations. These insights will help to clarify its role and optimise its application as a biocontrol agent.

Data statement

The R scripts used for statistical analyses in this study are available at https://github.com/EricCPereira/diaporthe_fol_analysis.R/tree/main.

CRediT authorship contribution statement

Eric C. Pereira: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Beatriz R. Vázquez de Aldana:** Writing – review & editing, Validation, Methodology, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Juan B. Arellano:** Writing – review & editing, Validation, Methodology, Supervision, Resources. **Ivan Fernandez:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis. **Iñigo Zabalgogea:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

ECP performed experiments and analyses. All authors designed the experiments, worked on data analyses, and approved the submitted version.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biocontrol.2025.105852>.

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