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Alternative N-forms cause differential response to the soil-borne disease *Fusarium oxysporum* f. sp. *melongenae* in eggplant (*Solanum melongena* L.)

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ABSTRACT

Mineral nutrition may strongly influence plant resistance or susceptibility to disease onset and progression, affecting plant growth and crop yield. Nitrogen (N) affects the plant-microbe interaction by modulating the production of antimicrobial compounds or defense-related enzymes and proteins that can boost or alleviate disease development.

A comparative analysis of alternative N-forms (NO_3^- and NH_4^+) was conducted on the tolerant eggplant line AM199 during infection by *Fusarium oxysporum* f. sp. *melongenae* (*Fom*), a soil-borne pathogen. At 14 Days After Inoculation (DAI), NO_3^--fed plants exhibited significantly reduced disease symptoms and incidence over time compared to NH_4^+-fed plants, which showed more evident symptoms. Root transcriptomic comparative analysis elucidated the AM199 defence mechanisms at the early stage of *Fom*-infection (4 h after inoculation; T1) and the cross-talk between plant responses to the pathogen and the alternative N-forms supplied at long-term (14 days after *Fom*-inoculation; T2).

At T1, we detected a rapid activation of genes involved in early defense, such as chitinases, proteinase inhibitors, cytochrome P450s, and receptor kinases, many of which mediate hypersensitive response and salicylic acid signaling. Furthermore, the plant pathogen recognition complex (LYK), involved in plant-pathogens interactions, was highly upregulated following inoculation. This activation, along with the induction of an Arabinogalactan protein (AGP) and an Aspartic protease (AP), which are involved in cell-to-cell communication signaling, as well as the enhancement of pathogen-associated molecular patterns (PAMP), contributed to an immediate response to *Fom* infection. In NO_3^--fed plants, the initial response to pathogen infection was followed by a stronger defense reaction compared to NH_4^+-fed plants. This was marked by higher expression levels of genes associated with Reactive Oxygen Species (ROS) and Resistance (R) responses, as well as the upregulation of genes involved in cell wall development, including cellulose biosynthesis (CSLG1, CSLH1) and membrane sterol production (SQE2), suggesting a potential thickening of the cell wall to hinder fungal invasion.

Our study provides new valuable insights into the molecular mechanisms induced by *Fom* infection under alternative N-forms supply, contributing to the identification of improved fertilization strategies for disease management in eggplant, in support of more sustainable and low-impact agriculture.

Abbreviations: CYP, *Chromosome P450*; ERF, *Ethylene-response factor*; FDR, *False Discovery Rate*; *Foc*, *Fusarium oxysporum f. sp. cucumerinum*; *Fom*, *Fusarium oxysporum f. sp. melongenae*; LRR, *Leucine-rich repeat*; N, *Nitrogen*; NH_4^+, *Ammonium*; NO_3^-, Nitrate; PCA, Principal Component Analysis; PR, Pathogenesis Related Protein; RIN, RNA integrity number; ROS, Reactive Oxygen Species; TMM, Trimmed Mean of M Values; WGCNA, Weighted Gene Co-expression Network Analysis.

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Introduction

Eggplant: agronomic relevance and stress adaptation

Eggplant (*Solanum melongena* L.), belonging to the Solanaceae family, is one of the most worldwide cultivated vegetable crops with a global production of more than 60,7 Mton over 1.2 million hectares distributed in tropical and subtropical regions, mainly in Asia and Mediterranean basin (Faostat, 2023). Eggplant berries are an important component of human diet, being also rich in health-related compounds (Gürbüz et al., 2018; Mennella et al., 2012). However, its high susceptibility to biotic and abiotic stress caused crop yield losses and food shortages (Daunay and Girard, 2012; Díaz-Pérez and Eaton, 2015; Plazas et al., 2019; Toppino et al., 2022; Wu et al., 2014).

Multiple screenings involving both intraspecific and interspecific genetic resources have been performed to identify the resistance traits to the main stresses in eggplant (Gramazio et al., 2023; Toppino et al., 2022, 2021), revealing several partial resistance traits, not easily exploitable for breeding (Taher et al., 2017; L. Toppino et al., 2021).

Among the main pathogens affecting eggplant, *Fusarium oxysporum* f. sp. *melongenae* (*Fom*) is a soil-borne fungus responsible for one of the most devastating vascular wilt diseases (Altınok, 2005; Urrutia Herrada et al., 2004). *Fom* detects root exudates and initiates germination, forming hyphae that invade the epidermal and cortical tissues through a combination of mechanical force and enzymatic activity, including the secretion of cellulases and pectinases (Altınok, 2005). From the roots, the infection propagates to xylem vessels, leading to vascular blockage and causing progressive wilting and yellowing, followed by necrosis and plant collapse. In several countries, *Fom* has been identified in both open field and greenhouse eggplant cultivation as causal agent of heavy yield losses (Altınok, 2005; Van Steekelenburg, 1976).

Role of nitrogen in plant–pathogen interactions

The development of plant disease is influenced by the complex interaction between the pathogen, the host plant species, the specific organ or tissue colonized, and the type and management of nitrogen (N) fertilization (Fagard et al., 2014; Hoffland et al., 2000, 1999; Lecompte et al., 2010). Mineral nutrition is recognized as a key factor in controlling fungal diseases, as it can either promote or suppress the onset and progression of infection and symptoms development (Dordas, 2008; Huang et al., 2017; Mur et al., 2016; Tripathi et al., 2022). The underlying mechanisms, however, remain only partially understood and may involve both direct effects on pathogen growth and plant vigor, as well as indirect effects mediated by changes in root exudates, rhizosphere dynamics, soil nutrient availability, pH, and the activation of plant defense pathways (Tripathi et al., 2022; Walters and Bingham, 2007). Nitrogen (N), a key element for plant metabolism and growth, is mainly available in soils as organic N compounds, ammonium (NH_4^+) and nitrate (NO_3^-). Its role in modulating plant-microbe interactions and disease development has been well documented (Dietrich et al., 2004; Fagard et al., 2014; Walters and Bingham, 2007). Notably, excessive N supply has been shown to reduce plant resistance to fungal pathogens by enhancing nutrient availability for pathogen proliferation (Jensen and Munk, 1997; Neumann et al., 2004; Snoeijers et al., 2000; Stout et al., 1998; Tripathi et al., 2022; Walters and Bingham, 2007).

N-forms can influence the development of plant diseases by modulating key physiological processes, such as photosynthetic and respiration rate, enzymatic activity, signaling pathway, and water balance (Engelsberger and Schulze, 2012; Guo et al., 2007; Horchani et al., 2010; Patterson et al., 2010; Yang et al., 2012). They also regulate disease tolerance by affecting signaling mechanisms that control pathogen virulence and metabolic adaptation (López-Berges et al., 2010; Solomon et al., 2003). A higher tolerance to *Fusarium oxysporum* f.sp. *cucumberi* (*Foc)* wilt was reported in NO_3^- compared to NH_4^+-fed cucumber plants. Specifically, nitrate supply was associated with a reduced disease index, while ammonium supply led to increased disease severity (Zhou et al., 2017). Furthermore, root colonization by the fungus and disease incidence were negatively correlated with root organic acid levels, which are influenced by the type of nitrogen supplied (Wang et al., 2019).

Genetic resistance to Fom and breeding advances

In eggplant, promising full or partial resistance to *Fom* wilt, identified in cultivated accessions (Miyatake et al., 2016; Mutlu et al., 2008) and allied relatives (Toppino et al., 2008b, 2008a), were successfully introgressed into breeding lines. Molecular assisted selection of segregant progenies was developed through markers linked to the resistance traits (Barchi et al., 2018; Tassone et al., 2022; Toppino et al., 2008a). Germplasm screening for the response to *Fom* revealed lines carrying additional traits of partial resistance which appeared potential useful for genes pyramiding to develop varieties with a durable resistance (Arafa et al., 2022).

The availability of high-throughput genetic maps and completely anchored and annotated eggplant genomes (Barchi et al., 2023, 2021, 2019; Toppino et al., 2020) provided an opportunity for transcriptomic comparative analyses for several traits of breeding interest, including fruit quality and stresses adaptation (Liu et al., 2023, 2021; Mauceri et al., 2021, 2020; Villanueva et al., 2023; Hong Wang et al., 2024; Xiao et al., 2023; Zhang et al., 2020). Although the accessibility to these genomic big data, the cross-talk between N fertilization and eggplant plant reaction to *Fom* infection has not been clarified to date.

Scope and objectives of the study

In the present study, the responses of the tolerant eggplant line AM199 to *Fom* infection were investigated under fertilization with alternative nitrogen forms (NO_3^- or NH_4^+), in order to shed light on the mechanisms underlying the interaction between nitrogen supply and disease tolerance. The early eggplant disease tolerance pattern, that include the activation of plant pathogen recognition complex, cell-to-cell communication and pathogen-associated molecular patterns, was underlined. An enhanced response to *Fom* infection at long time induced by nitrate through a high ROS level and the over- expression of resistance related genes, as well as an increased cell wall thickening, were elucidated. This work provides new insights on the modulation of eggplant resistance to vascular wilt by N-forms, contributing to the development of nutrient-based disease management strategies and the breeding of more resilient cultivars.

Materials and methods

Plant material

Two eggplant (*Solanum melongena* L.) breeding lines, the genome reference 67/3 and AM199, were selected from a large germplasm collection available at CREA-GB which displayed a wide variability for fruit size, shape, colour, and plant growth habit (Barchi et al., 2018; Cericola et al., 2014; Portis et al., 2015; Tassone et al., 2022), as well as different responses to *Fusarium* wilt. The two lines are characterized by partial resistance with a score ranging from 0.4 to 0.6 in a response scale to *Fusarium oxysporum* f. sp. *melongenae* (*Fom*), previously employed (Barchi et al., 2018; Tassone et al., 2022). The assessment of the response to *Fom* inoculation in both lines under two alternative nitrogen sources (NO_3^- or NH_4^+), supplied at rates ranging from 0 to 250 units/ha N equivalent either as a single application or in partitioned fertilization, was previously conducted (Tassone et al., 2022). Although both lines exhibited similar phenotypic responses to *Fom* inoculation when fertilized compared to unfertilized plants (0 N), in the 67/3 line, the improved tolerance was mainly associated with the total N amount supplied. The best responses were observed from 40 N units, whereas significant difference was not detected between the two alternative N-forms (Table S1; Table S2). By contrast, the AM199 line exhibited a distinct better phenotypic response to *Fom* in plants fed with NO_3^- compared to NH_4^+. The most divergent responses were highlighted in plantlets fed with a

partitioned amount of 20 units/ha N equivalent (Table S1; Table S2). Finally, to carry out a molecular overview on the effect of alternative N forms (NO_3^- or NH_4^+) in the responses to the *Fom* infection at short (T1 - 4 h after inoculation) and long (T2 - 15 days after inoculation) term, the AM199 line was adopted (Figure S1; Table S1; Table S2).

Growth conditions, N fertilization and Fom infection

Seeds of AM199 for phenotypic and transcriptomic analyses and 67/3 as phenotypic control were sown on peat (Technic Nr 03 with an organic N content: 0.5 % DW) substrate in plastic 104-hole trays. After germination, the trays were placed in a growth chamber with a day/night temperature of 27/20 ± 2 °C, 65 % relative humidity, and 16 hr of daylight at ~50μE m^{-2} s^{-1} intensity. At the second true leaf, plantlets were transferred into trays of 54-holes each of 72 cm^3 vol and filled with sterilized mix of sand: peat 3:1 (v/v). Partitioned N fertilization (either NH_4^+ or NO_3^-) was weekly supplied using an ammonium bicarbonate (NH_4HCO_3) or calcium nitrate tetrahydrate [$Ca(NO_3)_2 \times 4H_2O$]) solution, for 4 consecutive weeks until reaching the 20 N units amount to each plantlet. The total N input was divided into the 5 progressive doses of 10 % (at the transfer, 0 days), 15 %, 20 %, 25 % and 30 % of the total rate (Figure S1).

Fom isolate, available at CREA-GB, was first incubated on potato dextrose agar medium (PDA) in Petri dishes at 28 °C in the dark for 7 days. Agar cubes containing fungus mycelium were removed from the culture margins, inoculated into 1-liter Erlenmeyer flasks containing 200 mL Czapek's medium, and incubated at 28 °C for 4 days in an orbital shaker (150 rpm). The cultures were filtered through gauze to remove mycelium fragments. Then, the conidia were counted using a Bürker Counting Chambers, and resuspended in sterile tap water at 1.5×10^6 conidia/ml concentration.

Five days after the second N-treatment (when 25 % of the total planned N units was supplied), *Fom* or mock was inoculated by immersion (Tassone et al., 2022) (Figure S1). Plantlets at 2–3rd true leaf stage were gently removed from the plastic trays, their roots were washed under tap running water and immersed 10 min in the *Fom* conidia suspension for infected samples or in sterile tap water for mock-inoculated control plantlets. After inoculation, plantlets were gently dried and transferred again to plastic trays filled with sand: peat 3:1 (v/v) and placed in growth chamber for phenotypic and molecular assays.

Determination of the disease index

From T0 (inoculation time) to T30 (Figure S1), plant height and wilt symptoms were recorded every week using a scale score of symptoms ranging from 0 to1 (Tassone et al., 2022). The disease index was calculated according to the following formula:

$$R = \frac{\sum (plant * score\ assigned)}{total\ n^{o}of\ inoculated\ plants}$$

Statistical analysis

ANOVA statistical analyses to determine the significant differences among genotypes, treatments, and N-form supplies was performed through JASP software. Tukey test ($p = 0.05$) was used for means comparison.

RNA extraction and library construction

AM199 roots were collected at T0 (inoculation), 4 h (T1) and 14 days after inoculation - DAI (T2) with *Fom*. At each sampling time, root of plants treated with mock (water) were also sampled as negative control. Three independent biological replicates (each consisting of three pooled plantlets) were collected per sample and treatment, and immediately frozen in liquid nitrogen and stored at −80 °C until use. Total RNA was extracted and purified from 20 mg of grinded not-fibrous root tissue using ReliaPrep™ RNA Tissue Miniprep System (www.promega.com) following the technical manual (TM394) for Illumina (IL) library preparations. The protocol included an incubation step with DNase I enzyme. The amount and purity of RNA were measured both in agarose gel and using Thermo Scientific™ NanoDrop™ One Microvolume UV–Vis Spectrophotometers. RNA integrity number (RIN) was assessed by an Agilent 2100 bioanalyzer. Library prep was performed using Illumina® Stranded mRNA Prep, Ligation kit (www.illumina.com), according to manufacturer instructions. The resulting products were checked with an Agilent Bioanalyzer DNA 1000 chip and Qubit fluorometer and sequenced on an Illumina NovaSeq6000 Sequencing System through a S4 flow cell (AMES Group SRL).

Reads trimming and mapping

Raw sequences were demultiplexed through bcl2fastq and assessed for quality using FASTQC (Andrews, 2010). Adapters were removed and the quality trimming (Phred score cut-off of 20) was performed using Trimmomatic tool version 0.39 (Bolger et al., 2014). Clean reads were filtered by length and aligned to the last version of 67/3 eggplant reference genome (version 4.1), available at the Solanaceae web portal Solgenomics.net (https://www.solgenomics.net) (L. Barchi et al., 2021). Reads mapping was performed through the function Rsubread in the Bioconductor R package (Liao et al., 2019), allowing a maximum of 7 mismatches and multiple alignments. Mapped reads were assigned to genetic features through feature Counts (Liao et al., 2014).

Differentially expressed genes (DEGs)

Gene expression analysis was carried out through a quasi-likelihood edgeR pipeline (Robinson et al., 2010) and a DESeq2 workflow (Love et al., 2014) to compare transcriptomics, validating the results by using two statistical methods. Counts were filtered by expression levels, excluding genes with a value lower than 10 (in edgeR) or a sum of counts < 10 (in DESeq2).

The counts normalization was performed through TMM (Trimmed Mean of M Values) and in-built normalization methods in the edgeR and DESeq2 pipelines, respectively. A Multidimensional scale Analysis (MDS) was performed and then plotted through the R Bioconductor function *plotMDS*, designed for microarray/RNA-Seq data. Twenty-five pairwise comparisons were performed and the Differentially Expressed Genes (DEGs) showing a log2 Fold Change (log2FC) > 2 or < −2 and a False Discovery Rate (FDR) value < 0.05 for each comparison were considered significant.

Co-expression network analysis and GO enrichment analysis

A Weighted Gene Co-expression Network Analysis (WGCNA) was carried out by using the R package WGCNA (Langfelder and Horvath, 2008), adopting the normalized expression levels of all the genes Differentially Expressed (DE) in at least one pairwise comparison. Module-condition relationship was evaluated for each co-expression module using the Pearson correlation between the eigengenes of each module and a binary matrix representing each condition.

The modules significantly correlated with sampling time ($p < 0.05$) were removed. The Network representation of each module was analyzed by the software Cytoscape v.3.9.1 (Shannon, 2003). Hub genes were identified as the top 5 % of nodes sorted by degree.

A Gene Ontology (GO) enrichment analysis was performed through the R package 'topGO' (Alexa et al., 2006) by adopting the default parameters to identify enriched GO terms within each set of DEGs obtained by pairwise comparisons, belonging to the modules correlated to *Fom* infection and N-forms applied.

qRT-PCR analysis

First-strand cDNA was synthesized from 400 ng of total RNA by ImProm-II Reverse Transcription System (Promega, Madison, WI, USA) and oligo (dT) primers, according to the manufacturer's instructions. qRT-PCR analysis was carried out in 72-Well Rotor with Rotor-Gene Q (QIAGEN, Hilden, Germany). Reactions were performed at 95 °C hold temperature for 2 min, 40 cycles of 95 °C for 10 s, 59 °C for 60 s, with GoTaq qPCR Master Mix in a 10 µl fo final volume. All the reactions were performed in duplicate with three biological replicates, and no-template samples in all the analyses as negative controls were included. Standard curves for all primer pairs were calculated across a 4-fold dilution series of pooled cDNA amplified in technical triplicate. The PCR efficiency was calculated by Rotor-Gene 6000 Series Software, and it was optimised to be in the range 90–100 %. Gene expression of target gene was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), using *SmelGADPH* (Glyceraldehyde 3- phosphate dehydrogenase) housekeeping gene (Barbierato et al., 2017) and T0–0N-mock condition as calibrator sample. Specificity of amplifications was assessed by melt curves analysed for the presence of a single peak. Primers pairs for target genes were designed using Primer3Plus v.3.3.0 (Untergasser et al., 2012).

Results

Nitrogen fertilization and response to soil-borne fungal diseases

To shed light on the central role played by Nitrogen (N) and its alternative forms, in plant responses to disease, short- (4 h) and long-term (14 days) effects of NO_3^- and NH_4^+ on the AM199 and 67/3 eggplant genotypes (characterized for a different response to *Fusarium* wilt, see Methods) were investigated under *Fom* infection (Figure S1). The two accessions exhibited significant differences in disease symptoms especially at 14 days after infection (DAI) (T2) (Figure 1; Figure S2). At T2 significantly more severe disease symptoms were identified in the 67/3 line only in the N-unfertilized compared to the N-fed plants (Figure 1; Figure S2), which did not show any differences between the two N-forms (Table S1; Table S2). By contrast, at 14 DAI a significant contrasting response was observed between the two N-forms for the AM199 genotype, with the NO_3^--fed plants having significantly milder symptoms compared to NH_4^+-fed ones (Table S2), while the N-unfertilized plants exhibited intermediate symptoms (Figure 1; Figure S2). At 21 DAI, a *Fom*-tolerance recovery in NH_4^+-fed plants with the production of new small deep green leaves was observed, although the plantlet still displayed a stunted development and growth (Fig. 1). A significantly different effect on plant height was evidenced between N-forms and genotypes (Figure S2; Table S3; Table S4). NH_4^+- compared to NO_3^--fed and unfertilized plants showed a higher plant height. Surprisingly, the plant height of NO_3^- fed compared to unfertilized plantlets was not significantly different. Finally, 67/3 line showed a higher plant height increase compared to AM199 line. Thus, AM199 was selected for subsequent transcriptomic analyses as it exhibited differential response to *Fom* infection depending on the N-form supplied (see Methods).

Gene expression modification in response to Fom and alternative N-forms

A whole transcriptome analysis was conducted on the AM199 genotype to elucidate the molecular mechanisms underlying its specific tolerance to *Fusarium oxysporum* (*Fom*) infection. Differentially Expressed Genes (DEGs) were identified through pairwise comparisons (Table S5). A Principal Component Analysis (PCA) was performed on whole transcriptome data to highlight the main differences in response to *Fom* and N treatments at each sampling time (T0 - before inoculation; T1 - short effect, 4 h after inoculation; T2 - long effect, 15 days after inoculation -DAI). The samples were separated into three main clusters according to their sampling time, as expected (T0, T1, and T2; Fig. 2A). At T1 *Fom*-inoculated and mock samples formed distinct sub-clusters regardless the N-form, while interestingly at T2 samples were mainly distinguished based on N treatments, with NH_4^+-fed plants grouped in a distinct sub-cluster, including both *Fom*-inoculated and mock samples, as well as the NO_3^--fed mock plants (Fig. 2A).

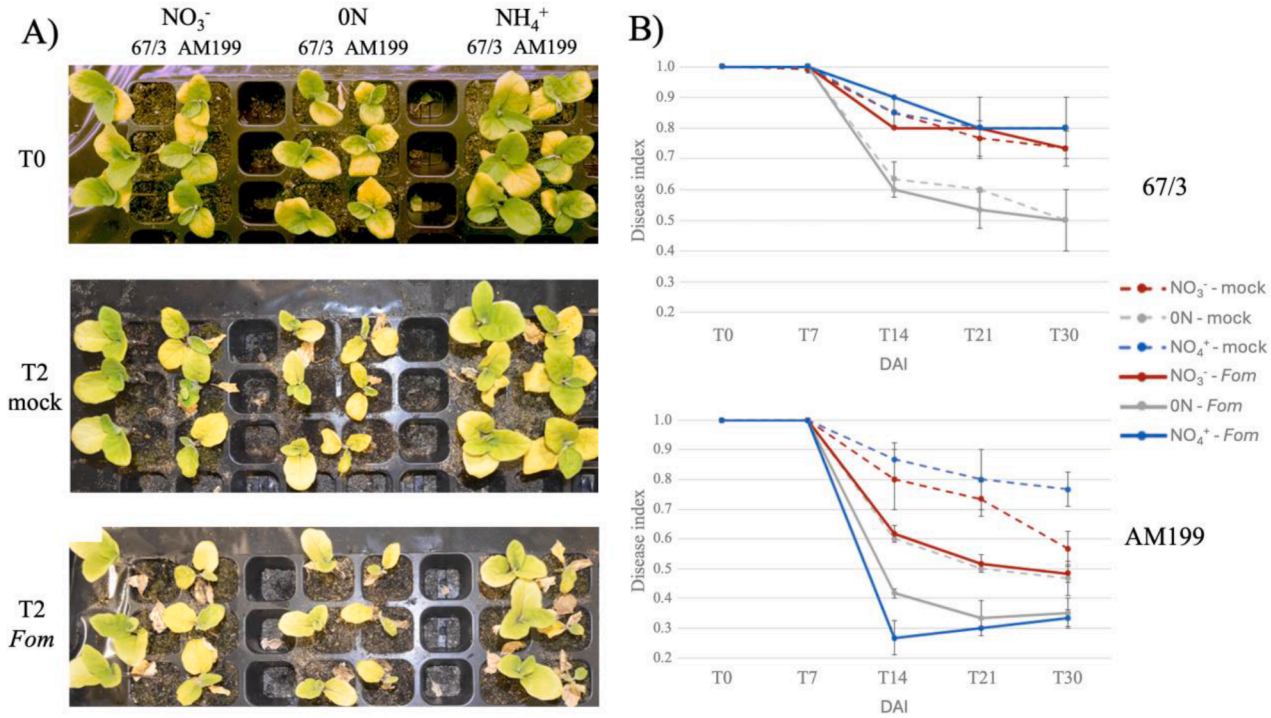

Fig. 1. Monitoring of infection symptoms. **A)** Plants just after artificial inoculation with *Fom* (*i*); Mock-inoculated plants at 14 DAI (*ii*); *Fom*-inoculated Plants at 14 DAI after inoculation with conidial solution (*iii*). From left to right in the plastic trays: two consecutive lanes (67/3 - left and AM199-right), fertilized with NO_3^- (red labels), unfertilized (0 N, white labels), and fertilized with NH_4^+ (light blue labels), respectively. **B)** Disease index caused by *Fusarium oxysporum* f. sp. *melongenae* (*Fom*) infection on 67/3 and AM199 lines grown under different nitrogen sources, compared to the mock-infected controls. The disease index was determined after 7, 14, 21, and 30 Days After Inoculation (DAI) (see Figure S1). Experiments were repeated five times with comparable results.

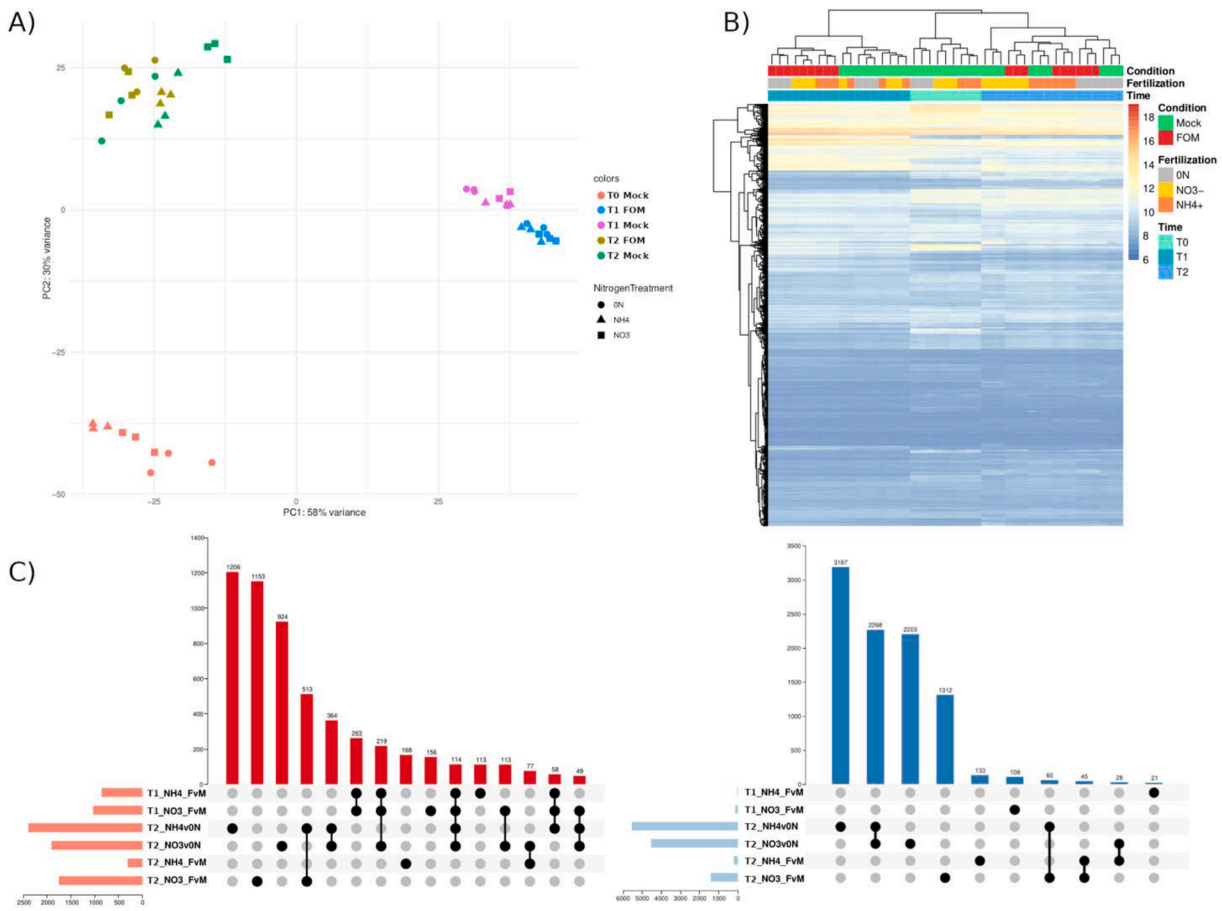

Fig. 2. Differential expression analysis of AM199's root tissue. **A)** Principal Component Analysis (PCA) of Differentially Expressed Genes (DEGs). Different conditions and treatments were highlighted using specific symbols and colors (see figure). **B)** Heatmap of normalized expression levels. Hierarchical clustering was performed on both rows and columns. Sample conditions were highlighted using a color scheme. **C)** Upset plots highlighting DEGs in each comparison. Up-regulated and down-regulated genes for the pairwise comparisons studied were reported in red and blue, respectively. M: mock samples; F: samples inoculated with *Fom*.

The heatmap generated from DEGs expression data revealed three distinct clustering patterns, grouped by sampling time (T0, T1, and T2). These clusters included samples from both treatments (Fom and mock) and all three N fertilization conditions (0 N, NO_3^-, and NH_4^+) (Fig. 2B). At each sampling time, the N-unfertilized samples consistently clustered separately from the fertilized ones. At T1, the *Fom*-inoculated samples were clearly distinct from the mock samples. At T2 samples grouped primarily based on the nitrogen form rather than the infection status, with the mock-NO_3^- samples forming a distinct cluster separate from the others (Fig. 2B).

At T2 the mock-inoculated plants under NH_4^+supply exhibited a higher number of DEGs compared to NO_3^--fed samples (2413 *vs*. 1860 up- and 5515 *vs*. 4499 total down-regulated genes, respectively). At T2, N fertilization modulated 364 up- and 2268 down-regulated DEGs, shared between the two N alternative forms (Fig. 2C). In addition, 1206/924 up-regulated genes, as well as 3187/2203 down-regulated genes were uniquely affected by each N condition, respectively (Figure 2C; Table S5).

At T1, during the early response to *Fom* infection, 263 up-regulated DEGs were shared between NO_3^- and NH_4^+-fed plants, while 156 genes showed a higher expression level only under NO_3^- supply (Fig. 2C). Several Cytochrome P450 (14), Ethylene-response factor (ERF) (10), WRKY (10), LRR (5) and different CYP71D55 (5) encoding the pre-mnaspirodiene oxygenase, all involved in the biosynthesis of sol-avetivone, an effective antifungal phytoalexin, were identified (Table S5; Table S6).

At T2, the *Fom*-inoculated plants showed only a total of 311 genes up-regulated under NH_4^+ supply compared to 1182 genes in the NO_3^--fed plants (Figure 2C; Table S6). In addition, 168 *vs*. 1153 up-regulated genes were unique under two N conditions, respectively. A significant increase of Leucine-rich repeat (LRR) family genes, as well as genes involved in Nicotinamide nucleotides pathways, NADH and NADPH genes, and Cytochrome P450 was observed under NO_3^- supply, mainly at T2 after *Fom* inoculation. In detail, in this condition 57 LRR and 37 NADH/NADPH genes were detected as DEGs and among these 79 % and 81 %, respectively, resulted up-regulated (Table S6). Interestingly, 54 Cytochrome P450 genes (71 %) were differentially expressed between the two N-forms supply.

This evidence suggested that an early response (4 h; T1) to *Fom* infection was induced in the AM199 tolerant genotype and, a more effective disease reaction was shown under NO_3^- compared to NH_4^+ supply at long time (T2). The significant presence of CYP (Chromosome P450) gene family members, genes involved in nicotinamide nucleotides pathways (NADH, NADPH), and genes belonging to LRR class, including a largest subfamily of receptor-like kinases, altogether involved in the plant defence-related processes, among the detected DEGs in the *Fom*-infected AM199 line fed with NO_3^- resulted of large interest (Table S6).

Co-expression network analysis delights key hub genes in response to Fom infection and alternative N-forms

Weighted Gene Co-expression Network Analysis (WGCNA) analysis was performed using 8792 DEGs from all the pairwise comparisons (Table S5). DEGs were included in 20 co-expression modules ranging

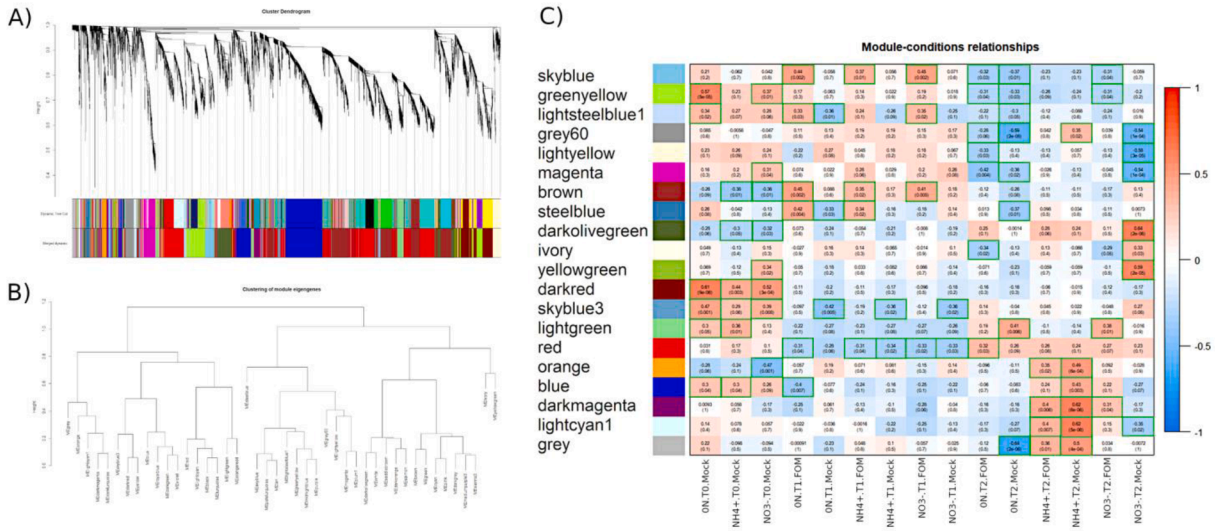

Fig. 3. Weighted Gene Co-expression Network Analysis (WGCNA). **A)** Cluster dendrogram and module merging. **B** Hierarchical clustering of module eigengenes. **C)** Heatmap of the Correlation coefficients between Module Eigengenes and samples. Pearson Correlation coefficient was evaluated using a binary matrix representing each condition (1) against all the others (0) for each sample. Significant correlations were highlighted using green boxes.

from 1839 (red) to 32 genes (ivory). The co-expression modules related to distinct responses to *Fom* under both N-forms were identified through the module-condition correlation assessment (Figure 3A; Fig. 3B). Five modules significantly correlated to different condition/treatment, brown (1755), darkolivegreen (600), skyblue (488), lightgreen (219), and lightsteelblue1 (48), were identified (Fig. 3C). Both brown and skyblue modules were involved in the early response to fungal infection, exhibiting a significant up-regulation at T1 in the *Fom*-inoculated samples under both N-forms (Figure S3). Likewise, the darkolivegreen module showed a significant up-regulation at T1 in the *Fom*-inoculated samples under both N-forms, but also at T1 and T2 in unfertilized samples. By contrast, at T2 an up-regulation was underlined only under NO_3^- supply in mock plants (Figure S3). The lightgreen module was induced at T0 in all the N-conditions (0 N, NH_4^+ and NO_3^-), and down-regulated at T1 under all fertilizations and regardless the *Fom* inoculation. Interestingly, at T2 the expression levels were significantly restored in *Fom* inoculated samples only under NO_3^- fertilization (Figure S3). Finally, the lightsteelblue1 module was induced at T0 in all three N-conditions and it was significantly downregulated in mock samples both at T1 and T2. Interestingly, *Fom* inoculated samples kept inducing the genes under all N fertilizations applied in the early response (T1), but with significantly higher levels under NO_3^- (Figure S3).

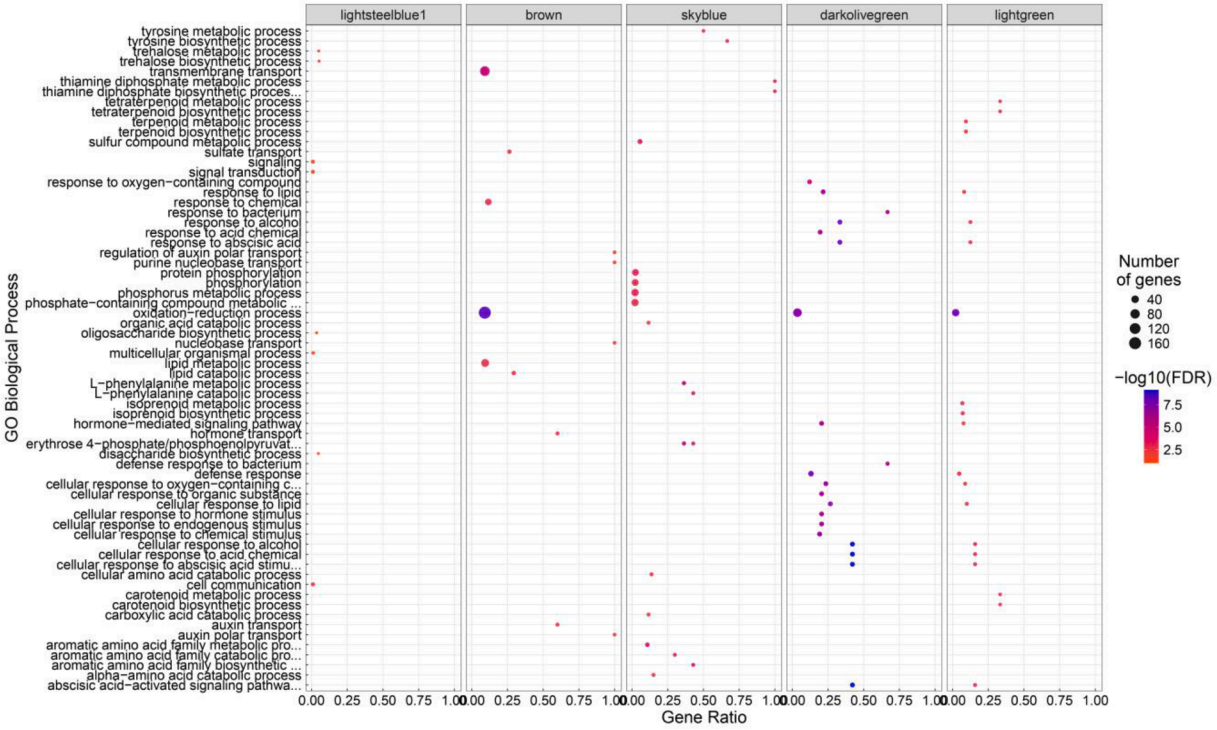

Fig. 4. GO terms enrichment analysis performed on best modules' gene set more informative. The dot plot shows significantly enriched GO terms (FDR < 0.05) identified using the Fisher's exact test. The size of the dots represents the number of genes in each GO biological process. The plot is divided in columns (modules). For each column the Gene Ratio of the number of genes for each GO term compared to the reference genome abundance, was reported.

The correlated modules were then characterized through a GO enrichment analysis to identify specific pathways or functions involved in the different responses to *Fom* under the two N-forms applied (Figure 4; Table S7).

The brown module showed a significant enrichment for several processes, including oxidation–reduction, transmembrane transport, response to chemical stimuli, lipid metabolism, and hormone transport (including auxin). This extensive upregulation of metabolism, transport, and signaling pathways highlights a comprehensive transcriptional reprogramming during the early plant response to *Fom*-infection. The darkolivegreen module exhibited a highly significant signature for cellular responses to several stimuli, including alcohol, lipids, oxygen, and defense against pathogens. Specific processes such as hormone-mediated signaling, response to bacterial infection, and oxidation–reduction were enriched. The skyblue module was mainly enriched in pathways related to aromatic amino acid metabolism, including L-phenylalanine biosynthesis and catabolism, as well as the related erythrose 4-phosphate/phosphoenolpyruvate pathway, suggesting a *Fom*-induced modulation in the primary metabolism for the biosynthesis of defense-related proteins. This module also showed an enrichment in protein phosphorylation and phosphorus metabolism, indicating a dynamic regulation of signaling cascades. The lightgreen module, upregulated in response to *Fom* under NO_3^- at long term (T2), showed enrichment for oxidation–reduction, isoprenoid/carotenoid biosynthesis, and hormone-related signaling pathways. The biological processes involved in transmembrane transport, including the nitrate transporters, response to wounding, and plant defense response were also enriched. This may suggest a stronger induction of genes related to *Fom* defense response at the later stages of infection by NO_3^- (Table S8).

Finally, the lightsteelblue1 module appeared enriched in signaling, signaling transduction, as well as cell communication, processes involved in the plant multilayered defensive response to pathogens. This module included genes involved in the early response to *Fom* infection activated in the AM199 tolerant genotype (Fig. 4).

To uncover the key mechanisms activated by the *Fom* infection under different N-forms, the highly connected (hub) genes within each module were identified and analyzed. Seventy-three (73), twenty-two (22), twenty-one (21), fifteen (15) and five (5) hub-genes in the brown, darkolivegreen, lightgreen, skyblue, and lightsteelblue1 modules were identified, respectively (Table S9; Figure S3; Figure S4).

In the brown module (Fig. 5), 9 out of 73 hubs are plant defense-related genes identified in several species, including tomato, potato and rice (Table S9). Two are included in the large family of the chitinases, *Cht6* and *EP3*, showing important biological effects, such as antifungal activities. The proteinase inhibitor type-2 (*CEVI57*) and the Kunitz trypsin inhibitor 5 (*KTI5*) are involved in plant defense against viroid and herbivores, respectively. Two Cytochrome P450 hub genes (*CYP82A3* and *CYP74D*), known for enhancing plant tolerance to both biotic and abiotic stresses, as well as two protein kinases (*XA21* and *CIPK6*), which are involved in immune responses to bacterial blight and *Pseudomonas*, respectively, within the same module were identified. Finally, a GDSL Esterase/Lipase like gene (*At1g28590*-like) that is required in several physiological and molecular functions in plants, such as the responses to biotic and abiotic stress, was also identified among the hub genes of the brown module (Table S9).

In the darkolivegreen module (Fig. 5), three defense-related STH-2 genes, three Pathogenesis Related Protein (*PR2, PR4*), an osmotin-like (*OSML13), a* Glycerol kinase *(GLPK)* and a PR5-like kinase *(PR5K)*

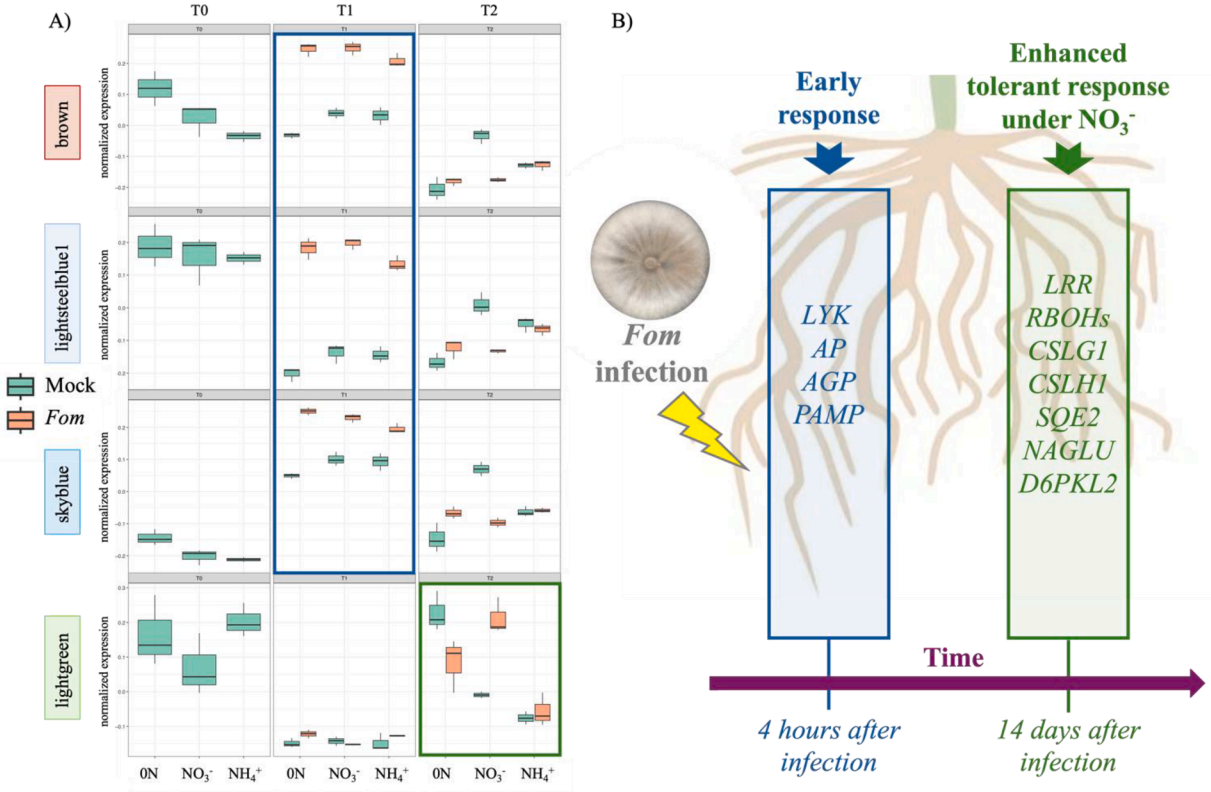

Fig. 5. A) Expression profile of the four most informative co-expression modules visualized using the Boxplots of Module Eigengenes. Brown, lighstelblue1, and skyblue grouped the key genes involved in the early response to *Fom* infection (at T1 – 4 h after infection; blue rectangle) of AM199 tolerant genotype; while the genes responsible of the enhanced response of the plants grown under NO_3^- supply at long time (T2 – 14 days after infection; green rectangle) under infection belonged to lightgreen module. **B)** An overview of the proposed model explaining the fast (T1) and long response (T2) to *Fom* in under NO_3^- supply in an eggplant tolerant genotype. The blue rectangle highlights the crucial genes/mechanisms (*LYK, AP, AGP, PAMP*) related to the tolerant early response to *Fom* activated by the AM199 tolerant genotype, while the green rectangle underlines the key genes involved in the long tolerant response enhanced by NO_3^-, triggered by the higher expression of the respiratory burst oxidases (*LRR, RBOHs*), resistance (R) related genes (*NAGLU, D6PKL2*), and the enhanced thickening of cell wall (*CSLG1, CSLH1, SQE2*).

were detected as hub genes (Table S9; Figure S3). The *STH-2* are members of a small gene family rapidly activated by pathogen infection, as well as the PRs induced as part of systemic acquired resistance in antimicrobial activity, useful to demolish molecules in the bacterial or fungal cell wall. The Osmotin-like protein (*OSML13*) is activated by both abiotic stimuli and fungal pathogen infection. The *Arabidopsis* homologue Glycerol kinase (*NOH1*) is able to promote resistance to nonhost pathogenic fungi, as well as the PR5-like receptor kinase (*PR5K*) *Arabidopsis* homologue and a basic endochitinase (*CHI14*) tomato homologue, both involved in the resistance to pathogens. The LysM domain receptor-like kinase 4 (*LYK4*) and the serine/threonine-protein kinase (*PBL3*), both involved in the plant responses to biotic stresses, were identified as hub genes in the skyblue module (Table S9; Figure S3).

In the lightsteelblue1 module three out of five hub genes identified were related to plant disease responses: an Aspartic proteinase (*ASP1*), an Arabinogalactan protein (*AGP16*), and a RING-H2 finger protein (*ATL51*). In rice *ASP1* was responsible for responses to fungal, bacterial and virus infection, while *ATL51* showed a pleiotropic effect on growth and defense response against biotic stresses (Liu et al., 2008; Prasad et al., 2010). The AGP gene family, whose encoded proteins are localized on the cell wall and in extracellular exudates, was implicated in plant-microbe interactions, providing disease resistance to fungal infection in several plant species, like apple, banana, and potato (Koroney et al., 2016; Wu et al., 2017; Leszczuk et al., 2019) (Table S9; Fig. S3).

Altogether, the lightsteelblue1, brown, and skyblue modules were highly enriched in hub-genes associated to the early tolerance response of eggplant to *Fom*. Several hub genes identified in the lightgreen module also showed a key role in the disease tolerance mechanisms in different plant species. A serine/threonine-protein kinase (*D6PKL2*) that conferred *Fusarium* resistance in tomato (Zhang et al., 2021), five carotenoid-related genes, which play a protective role against reactive oxygen species (ROS), were identified. Furthermore, a gene belonging to the alpha-N-acetylglucosaminidase family (NAGLU family), a putative responsible for resistance to *Stagonospora nodorum blotch* in spring wheat, and an *Ent-copalyl diphosphate synthase* (*CPS5*) involved in the biochemical defense response against pathogens through the gibberellin (GA) biosynthesis, were also isolated (Figure 4; Table S9). The expression of hub genes in the lightgreen module suggests a role in NO_3^--mediated defense responses, revealing a potential interplay between nitrate availability and plant immunity (Table S5; Table S9).

RNA-seq validation by qRT-PCR analysis

Seven genes were used to test the reliability of the transcriptome results (Table S10). The gene selection was based on the differential expression highlighted by RNAseq analysis and their involvement in disease resistance responses previously known. The expression levels showed a high correlation between the RNA-seq and qRT-PCR results, confirming the high reliability of RNA-seq results (Figure S5).

Discussion

Eggplant profiling associated to the Fom infection under alternative N-forms

N nutrition is very important for plant response to fungus disease (Y. Sun et al., 2020) which is affected by both fertilization management and N-form supplied. NO_3^- and NH_4^+ fertilization, the two primary N sources, significantly affect plant disease responses, with their effect, whether beneficial or detrimental, depending on the specific plant-pathogen interaction (Huber and Watson, 1974; Julian Maywald et al., 2023; Sun et al., 2021, 2020). Several studies have reported enhanced plant defence responses in NO_3^--fed plants compared to NH_4^+-fed ones, leading to contrasting disease indexes between N-treatments, with NH_4^+-fed plants usually displaying more severe symptoms and increased susceptibility to fungal diseases (Sun et al., 2021; Wang et al., 2019; Zhou et al., 2017). These different effects on plant disease responses are likely due to distinct metabolic pathways activated by each nitrogen source(Bolton and Thomma, 2008; Mur et al., 2016). NO_3^- supply increases hypersensitive response- (HR) mediated resistance, enhancing production of polyamines such as spermine and spermidine, both defense molecules (Mur et al., 2019). Otherwise, NH_4^+ nutrition can compromise defense, leading to increased γ-aminobutyric acid (GABA) levels, which is also a nutrient source for many pathogens (Huber and Haneklaus, 2007; Julian Maywald et al., 2023; Y. Sun et al., 2020).

Eggplant as well as many other crops significantly suffers from soil-borne diseases, such as *Fusarium* sp. and *Verticillium* sp. that cause the most devastating vascular wilt plant diseases and heavy yield loss (Brand-Daunay and Hazra, 2012). The adoption of tolerant/resistant cultivars is the most eco-friendly and cost-effective disease management practice. In the eggplant gene pool, partial resistances to most pathogens, including *Fusarium oxysporum* f. sp. *melongenae* (*Fom*), were found, although too scarce for an effective employment in breeding programs (L. Toppino et al., 2021). These partial resistance traits are of extreme interest for developing breeding lines that cumulate multiple R traits, avoiding that additional new pathogen strains may overcome the natural barriers of resistant cultivars, as well as able to enhance the expressivity of multiple independent resistance genes. An improved cropping system management, that includes a N-fertilization strategy, for controlling the *Fom* disease, may be considered a valid alternative/complement to plant genetic resistance. Nevertheless, the potential tolerance mechanisms related to *Fom* infection, and the fungal disease control influenced by N-fertilization are complex and not yet completely understood in eggplant. They depend on direct and indirect effects that specific N-forms may have on pathogen development and plant vigour, root exudates changes, soil N-content, and pH fluctuation, as well as plant resistance mechanisms (Tripathi et al., 2022; Walters and Bingham, 2007). To address this, the responses of two eggplant genotypes - AM199, which displays partial resistance to *Fom*, and the reference line 67/3 (Barchi et al., 2019), whose partial resistance has been well-documented (Barchi et al., 2018; Toppino et al., 2020) - were evaluated under NO_3^- and NH_4^+ fertilization during fungal infection (Barchi et al., 2018; Tassone et al., 2022).

The reference 67/3 pure line did not show any phenotypic differences in disease symptoms between NO_3^-- and NH_4^+-nutrition after *Fom*-inoculation. Conversely, the tolerant genotype AM199 showed contrasting responses to *Fom* inoculation between the two N-forms. At 14 DAI, NO_3^-- compared to NH_4^+-fed plants appeared significantly healthier-. In agreement, a susceptible cucumber genotype infected by *Fusarium* (*Fusarium oxysporum* f. *cucumerinum* - *Foc*) in hydroponic system showed similar results, with a decrease of disease index under NO_3^--supply, and an increase when NH_4^+ was adopted as N fertilizer (Zhou et al., 2017). The authors revealed that NO_3^--fed plants accumulated more organic acids compared to those grown under NH_4^+supply, being both the root *Foc* colonization and disease incidence negatively correlated to the root organic acid levels (Wang et al., 2019). Similarly, our results allowed to describe the potential tolerance mechanism induced against *Fom* by the AM199 eggplant tolerant genotype, characterizing the molecular plant response in the early reaction (4 h after infection), and how the alternative N-forms influence the reduction of disease incidence over a longer period (14 DAI).

The early molecular mechanism activated by the tolerant eggplant genotype under Fom infection

An early defense response of eggplant against *Fom* has been demonstrated, independent of the N-forms supplied. Several plant defence-related genes, previously identified in species such as tomato, potato, and rice, were detected. Notably, some of these genes were classified as hub genes within distinct co-expression modules, including brown, skyblue, and lightsteelblue1. The complex defence mechanism

activated in the AM199 genotype included chitinases, genes belonging to Cytochrome P450 (CYP) family, and other effectors associated with plant reaction to pathogens. *EP3* (homologous to *Arabidopsis*) and *Cht6* (homologous to rice), two chitinases from the brown module, have shown significant antifungal activities in many plant species. A recent transcriptomic analysis revealed a marked upregulation of *EP3* in response to *Colletotrichum nymphaeae* infection in olive tree, making the chitinase gene family a promising candidate for functional analysis (Inácio et al., 2024). A central role for chitinases in hypersensitive response (HR) to *Colletotrichum* spp. infection was previously reported in pepper (Ali et al., 2020). The chitinase *CaCHI7* was transcriptionally activated by *C. acutatum* infection and its knockdown conferred an increased hypersensitivity to the fungus in transgenic plants, determining its proliferation in infected leaves due to weakened defense responses (Ali et al., 2020). Furthermore, a phylogenetic analysis of the pathogenesis-related protein-3 chitinase gene family (including *Cht6*) in rice and their tissue-specific expression revealed their antifungal activity (Nakazaki et al., 2006).

Interestingly, the brown module contained hub genes encoding proteinase inhibitors (PI) such as *CEVI57* (PI type-2) and *KTI5* (Kunitz trypsin inhibitor 5), previously identified in tomato and *Arabidopsis*. Both genes are recognized as key components of plant defense response against both herbivores and pathogens (Ryan et al., 1990). A more recent genome-wide transcriptomic analysis showed that the expression of PI genes can be induced in tomato by both abiotic (drought and salt) and biotic (*Botrytis cinerea* and TSWV) stresses (Fan et al., 2020). Multiple wound-inducible members of the KTI gene family were involved in defense against herbivores in poplar (Major and Constabel, 2008).

The involvement of CYP gene family in the plant protection against biotic and abiotic stress is already well documented (Pandian et al., 2020; Sun et al., 2014; Yan et al., 2016). Recently, high *CYP82D47* transcript levels were associated to plant responses against powdery mildew (PM) and *Foc* in cucumber, strongly enhancing its resistance to both pathogens (Hong-yu Wang et al., 2024). *CYP82D47* overexpression determined increased expression levels in salicylic acid (SA) related genes (*PR1, PR2, PR4*, and *PR5*), involved in plant defence responses, and solavetivone biosynthesis, a potent antifungal phytoalexin, produced by plants *de novo* under pathogen infection (Ahuja et al., 2012; Song et al., 2019).

Interestingly, in the brown module *CYP82A3* and *CYP74D*, homologues of soybean and pepper, respectively, were identified as hub genes. The subfamily CYP82 were induced by *Phytophthora* infection, salinity and drought stress in soybean; more interestingly, transgenic *Nicotiana benthamiana* plants overexpressing *GmCYP82A3* exhibited high resistance to *Botrytis* and *Phytophthora* (Yan et al., 2016). In the same module, the protein kinases *XA21* and *CIPK6*, involved in plant tolerance to bacterial blight and *Pseudomonas* in rice and *Arabidopsis*, respectively, were identified as hub genes. More interestingly, a full-length cDNA encoding a putative receptor-like kinase (RLK) protein was recently isolated from maize (*ZmXa21*) and overexpressed in transgenic rice resulting in enhanced resistance to bacterial blight compared to wild-type plants (Gao et al., 2019). Finally, the hub gene GDSL Esterase/Lipase (*GELP*)-like gene (*At1g28590*) belonged to a gene family involved in several stress responses. In *Arabidopsis*, members of this family are required for the plant resistance to necrotrophic pathogen, such as *Alternaria* (Oh et al., 2005), while in rice, five GELP genes were strongly induced upon infection with *Fusarium verticillioides*, a response accompanied by impaired lipids mobilization (Dolui and Vijayaraj, 2020).

In agreement, a lysin motif receptor, a *LYK4 Arabidopsis* homologue, hub gene in the skyblue module, enhanced the tolerance to *Alternaria brassicicola* when overexpressed in transgenic Indian mustard (*Brassica juncea*) by increasing trichome density (De et al., 2021). The LYK family is part of the immune receptor complexes that recognize pathogen-associated molecular patterns (PAMPs) for a prompt response to pathogen infection (Böhm et al., 2014). More recently, the overexpression of *LYK4* tomato homologue conferred improved resistance to gray mold (*Botrytis cinerea*) infection (Ai et al., 2023). In the same module, a putative serine/threonine-protein kinase (*PBL3*), was also identified (Guy et al., 2013). As reported by (Guy et al., 2013), *PBL3* may play a role in plant defense signaling, similarly to other protein kinases. Among these, the Receptor-like cytoplasmic kinases (*RLCKs*) are considered key regulators of plant immunity, orchestrating major cellular communication networks in plants (Hailemariam et al., 2024).

Noteworthy, the hub genes detected in the lightsteelblue1 module are known to be involved in plant disease tolerance and were significantly up-regulated in the early phase after *Fom* infection. Among these, an Aspartic protease (*AP1*), was identified. Aspartic proteases constitute one of the four major protease superfamilies and play key roles in various biological processes, including resistance to biotic and abiotic stresses (Prasad et al., 2010). Two members of this family, *OsAP77* and *TaAP224*, were shown to be induced by blast and powdery mildew in rice and wheat, respectively, further supporting their key role in plant disease resistance mechanism (Alam et al., 2014; Yang and Feng, 2020). The hub-gene *AGP16* was also identified in the lightsteelblue1 module. *AGP16* belongs to the Arabinogalactan protein (AGP) family, a group of important cell wall components implicated in plant-pathogen interactions, mainly in root-associated defense responses (Leszczuk et al., 2019). The *AGPs* connections with xylogalacturonan in biofilms formed by infected roots, which serve as an anchorage for lignification, provide a mechanical barrier to fungal hyphae in apple trees (Driouich et al., 2010; Leszczuk et al., 2019). *AGPs* were significantly up-regulated by *Fusarium oxysporum* infection in banana roots, which is consistent with their role in the signaling to enhance the cell-to-cell communication in plants (Wu et al., 2017). *AGP* expression in root exudates was also up-regulated in potato in response to elicitors derived from *Pectobacterium atrosepticum*, the pathogen responsible for soft rot (Koroney et al., 2016). Interestingly, in our experiments, *AGP16* was significantly up-regulated in the *Fom*-infected plants supplied with NO_3^-. This finding aligns with the key role of nitrate assimilation in mitigating cell wall defects by providing amino donors for chitin precursors biosynthesis (Gong et al., 2024).

Cross-talk between Fom tolerance and nitrate supply in eggplant

The interplay between *Fom* tolerance and N availability is reflected in both molecular and morphological responses. Three Leucine-rich repeat (*LRR*) receptor-like protein kinases, an *RPM1* homologue, *PEPR1*, and two additional probable LRR receptor-like serine/threonine-protein kinase homologues of *At1g53440* were identified as differentially expressed (DE) in response to *Fom* infection and N supply. Their involvement in plant innate immunity highlights their potential role in defence mechanisms against pathogens in eggplant. Interestingly, the expression levels of genes belonging to LRR family increased significantly in the long-term response (T2) to *Fom* infection under NO_3^- supply, supporting its role in the modulation of disease tolerance mechanism. The leucine-rich repeat receptor kinases (*LRR-RKs*) constitute a larger subfamily of receptor-like kinases that regulate plant defence-related processes including host- and non-host-specific defence and wounding responses (Torii, 2004). Four *LRR-RKs* (SIF subfamily) were investigated in *Arabidopsis* where the overexpression of *SIF1* and *SIF4* in transgenic plants confirmed their involvement in plant pathogen defence, being rapidly induced by biotic or abiotic stress (Yuan et al., 2018). Interestingly, *PEPR1* seemed to regulate the plant immunity and growth in tomato, and its induction in response to insect herbivory and pathogen infections has been reported (Xu et al., 2018).

The role of CYP450 gene family in plant protection against biotic and abiotic stress, alongside other important functions, such as photosynthesis, has been previously discussed (Pandian et al., 2020). In our study, several Cytochrome P450 (*CYP*) and Ethylene-response factor (*ERF*) family members were identified among the differentially expressed genes (DEGs) in the *Fom*-NO_3^- interaction, with their expression mainly

observed at long-term (T2). Consistently, genes involved in nicotinamide nucleotides pathways (*NADH, NADPH*), which contribute to enhanced disease resistance and stress tolerance, were identified after *Fom*-inoculation, and they were mainly up-regulated under NO_3^- at 14 DAI. In agreement, nicotinamide mononucleotide (NMN), a precursor of nicotinamide adenine dinucleotide (NAD), was accumulated in barley cultivars resistant to phytopathogenic fungal *Fusarium graminearum* (Miwa et al., 2017). NMN, NAD, and nicotinamide adenine dinucleotide phosphate (NADP) have been associated to salicylic acid (SA) accumulation and the induction of pathogenesis-related (PR) genes, that boost the resistance to fungal pathogens (Miwa et al., 2017).

Many hub-genes of lightgreen module, upregulated at long-term (T2), appeared involved in the *Fom*-NO_3^- interaction, suggesting the role of this co-expression module in the distinct response in NO_3^--compared to NH_4^+-fed plantlets. At T2, biological processes related to respiratory burst oxidases, such as "oxidation–reduction process", were enriched, highlighting their role in redox homeostasis regulation under NO_3^- fertilization. Alongside, pathways involved in metabolism and transport, including "carotenoid biosynthetic process" and "transmembrane transport", were also upregulated. Notably, key processes related to plant defense and cell wall formation, such as "defense response", "response to wounding", "cellulose biosynthetic process", and "sterol biosynthetic process", were significantly enriched, reinforcing their contribution to cellulose synthesis and structural integrity in response to NO_3^- supply. These genes contributed to defense through ROS production (Camejo et al., 2016), mediated by CYP450 and NADP/NADPH family members (e.g., *CYP76A2, CYP711A1, CYP716B1*), as well as through cell wall reinforcement, triggered by genes involved in cellulose synthesis (*CSLG1, CSLH1*) and the biosynthesis of membrane sterol precursors and brassinosteroids (*SQE2*) (Engelsdorf et al., 2019).

Genes involved in pathogen defense response were also included as hub genes at long-term, induced by NO_3^- fertilization in the lightgreen module. Among them NO_3^- supply enhanced the expression level of a member of α-N-acetylglucosaminidase (*NAGLU*) family. In *Arabidopsis, NAGLU* was involved in the regulation of AGP metabolism (Ronceret et al., 2008), a class of extracellular matrix molecules, previously described, with a role in plant-development and pathogen interaction through cell-to cell communication. Consistently, an ent-copalyl diphosphate synthase (*CPS5*), which is involved in primary defense metabolism through GA (Harris et al., 2005), an important class of hormone stimulating plant innate defense responses (Moosavi, 2017), was up-regulated at T2 by the *Fom* under NO_3^-. A serine/threonine-protein kinase, *D6PKL2,* well known for its role in enhancing the lateral root formation, was activated by nitrate and may also contribute to disease resistance against *Fusarium* infection. This potential role is supported by its homolog in the oil tree (*Vernicia montana*), whose stress mitigation function were already demonstrated in tomato and *Arabidopsis* (Zhang et al., 2021). Interestingly, five carotenoid-related genes were also detected as hub-genes in this module. Carotenoids are essential for photosynthesis and photoprotection by scavenging free radicals and protecting plants against both abiotic and biotic stress (Ezquerro et al., 2023). Recent research verified that NO_3^- supply enhanced cucumber resistance to *Fusarium* by increasing photosynthesis and regulating photorespiration (Sun et al., 2021), which aligns with the up-regulation of nicotinamide nucleotide pathways (NADH, NADPH) highlighted in our results. Two NADPH-dependent 2-alkenal reductases (*AER* and *DBR*), which were classified as hub genes in the lightgreen module, are involved in plant oxidative defense during stresses. Transgenic tobacco overexpressing *AtAER* exhibited significantly reduced photooxidative damage in leaf cells (Mano et al., 2005). More recently, the ortholog gene (*ZmAER*) overexpressed in transgenic plants was able to alleviate the oxidative stress in maize (Wang et al., 2021).

Conclusions

Our study highlighted the mitigation mechanisms activated by the *Fom*-tolerant eggplant genotype after infection and a significant impact of different N-forms supply on disease management. Our results demonstrated a crosstalk between *Fom* attack and alternative N-forms supply in eggplant, as recently reported in cucumber (Sun et al., 2021; Wang et al., 2019; Zhou et al., 2017). In both species, NO_3^- supply triggered a stronger plant defence response by decreasing the disease index compared to NH_4^+ supply. However, the disease symptoms resulted mitigated by NO_3^- supply only at long term, 14 days after *Fom*-inoculation, but not earlier (T1), as confirmed by transcriptomic analysis.

Molecular evidence highlighted the defence mechanism activated by AM199 tolerant genotype at the early stage of infection by *Fom*, regardless N-form supplied. Four hours after *Fom*-infection (T1), the immune receptor complexes (*LYK*) implicated in the plant-pathogen recognition were activated, enhancing the signaling for cell-to-cell communication (driven by *AP, AGP*) linked to the pathogen associated molecular patterns (*PAMPs*) for a prompt response to infection (Fig. 5). The early response to *Fom* was followed by two possible defence mechanisms, responsible of the boosted reaction of the plants grown under NO_3^- compared to NH_4^+ (Fig. 5). A higher expression of the respiratory burst oxidases (*RBOHs*), encoding the reactive oxygen species (ROS) production in response to pathogen invasion, can be a first strategy to cope with *Fom*-infection. This system was significantly upregulated at long term (14 DAI - T2) in the NO_3^--fed and *Fom*-inoculated eggplant. A second strategy involved the enhanced thickening of cell wall, mainly in root, for contrasting tracheomycotic fungal disease infection. At T2, genes involved in the biosynthesis of cellulose (*CSLG1* and *CSLH1*) and membrane sterols (*SQE2*) resulted induced by *Fom* infection only under NO_3^- supply, in agreement to the key role of nitrate assimilation in rescuing cell wall deficiencies induced by pathogens (Gong et al., 2024). In agreement to this mechanism, the serine/threonine-protein kinase D6PKL2, an alpha-N-acetylglucosaminidase (*NAGLU*), and an ent-copalyl diphosphate synthase (*CPS5*) upregulated at T2, making the plant tolerant to *Fom*-infection, providing novel insight into *Fusarium* wilt resistance in plants (Kim et al., 2016; Zhang et al., 2021) (Fig. 5).

Our findings provide valuable new insights into the molecular mechanisms induced by *Fom* infection under different N-forms supply in eggplant, contributing to define the best fertilization practices for improving the disease control, in the framework of a more sustainable and low-impact agriculture.

Data availability

All sequenced data produced were deposited in the Sequence Read Archive (SRA) of NCBI (National Center for Biotechnology Information) with the identifier: PRJNA1226541.

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MR Tassone: Writing – review & editing, Writing – original draft, Resources, Investigation. **TM Sirangelo:** Investigation, Data curation. **G Puccio:** Writing – review & editing, Writing – original draft, Visualization, Software, Methodology. **K Gazzetti:** Validation, Investigation. **A Mauceri:** Investigation. **MR Abenavoli:** Writing – review & editing. **F Sunseri:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **GL Rotino:** Writing – review & editing, Supervision, Formal analysis, Conceptualization. **L Toppino:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation. **F Mercati:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare no competing interests

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.stress.2025.100960.

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