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An altered tocopherol composition in chloroplasts reduces plant resistance to *Botrytis cinerea*

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Running title: Tocopherols in plant response to biotic stress

Abstract

Tocopherols are lipid-soluble antioxidants that contribute to plant resistance to abiotic stresses. However, it is still unknown to what extent alterations in tocopherol composition can affect the plant response to biotic stresses. The response to bacterial and fungal attack of the *vte1* mutant of *Arabidopsis thaliana*, which lacks both α- and γ-tocopherol, was compared to that of the *vte4* mutant (which lacks α- but accumulates γ-tocopherol) and the wild type (with accumulates α-tocopherol in leaves). Both mutants exhibited similar kinetics of cell death and resistance in response to *Pseudomonas syringae*. In contrast, both mutants exhibited delayed resistance when infected with *Botrytis cinerea*. Lipid and hormonal profiling was employed with the aim of assessing the underlying cause of this differential phenotype. Although an altered tocopherol composition in both mutants strongly influenced fatty acid composition, and strongly altered jasmonic acid and cytokinin contents upon infection with *B. cinerea*, differences between genotypes in these phytohormones were observed during late stages of infection only. By contrast, genotype-related effects on lipid peroxidation, as indicated by malondialdehyde accumulation, were observed early upon infection with *B. cinerea*. We conclude that an altered tocopherol composition in chloroplasts may negatively influence the plant response to biotic stress in *Arabidopsis thaliana* through changes in the membrane fatty acid composition, enhanced lipid peroxidation and delayed defence activation when challenged with *B. cinerea*.

Keywords: biotic stress, hormonal balance, jasmonic acid, lipid profile, tocopherols

Introduction

Plants are continuously subjected to biotic stresses by a range of microbes, some of which are pathogens. Depending on the pathovar *Pseudomonas syringae* can infect several plant species where disease symptoms can range from leaf spots to stem cankers (Hirano & Upper, 2000). *P. syringae* pv. *tomato* strain (*Pst*) DC3000 is a well-characterized pathogen that causes hemibiotrophic symptoms in susceptible tomato (*Solanum lycopersicum*) and Arabidopsis plants (Preston, 2000). *Botrytis cinerea* is a necrotrophic fungus with a wide range of host plant species (Staats *et al.*, 2005), infecting important crops such as tomato, grapevine (*Vitis* spp.) and strawberry (*Fragaria×ananassa*) (Jarvis, 1977), as well as the model plant *Arabidopsis thaliana*.

In both plants and animals, glycerol-based membrane lipids such as phospho- and galactolipids play an important role in stress resistance. In animals, eicosanoids may be derived from the oxidation of arachidonic acid (C20:4) derived from the acyl chains of phospholipids. Subsequent differential processing leads to the generation of prostaglandins, leukotrienes and thromboxanes (Funk, 2001). These eicosanoid-derivatives have roles in inflammation, vasoconstriction or vasodilatation, coagulation, pain and fever. Similarly in plants, glycerol-based membrane lipid (predominantly galactolipid) processing, mostly through lipoxygenase activity on α-linolenic acid (C18:3) acyl chains, produce a range of oxylipin signals. These include jasmonates, which are potent mediators of plant defense against certain pathogens and insects (Koo & Howe, 2009). Jasmonates are thought to act with ethylene primarily against necrotrophic pathogens such as *B. cinerea* whilst salicylic acid is thought to influence defense against *Pst* (Pieterse & Van Loon, 2004).

Central to jasmonates formation is the regulation of the initial release of polyunsaturated fatty acid (PUFA) precursors (e.g. C18:3) as free fatty acids from the glycerol-based membrane galactolipid. By analogy with the animal systems it is assumed that α-linolenic acid release is mediated by lipases. However, it is clear that lipid peroxidation occurring as a result of oxidative stress additionally plays a key role in activating free fatty acid release from membrane bound glycerol-based lipids. Lipid peroxidation is an inevitable consequence of the generation of reactive oxygen species – the oxidative burst - that are characteristic of plant responses to pathogens. This is particularly the case with the hypersensitive response elicited by avirulent pathogens (Mur et al., 2008). Hydroxyl radicals

generated as part of oxidative burst will readily abstract a proton from, for example, phospholipids to initiate a lipid radical – lipid hydroperoxide chain reaction. The propagative nature of lipid radical generation necessitates its careful regulation to limit cell death to the site of infection and regulate the production of any derived signals.

Lipid peroxidation can be suppressed by non-polar antioxidants such as tocopherols. Tocopherols consists of four homologues (termed α, β, γ, and δ) that differ only in the number and position of methyl groups in the chromanol ring. In plants, the most important forms are α- and to a lesser extent γ-tocopherol in photosynthetic tissues. α-Tocopherol protects the photosynthetic membranes from the propagation of lipid peroxidation, helps to maintain the membrane stability and in a coordinated action with other antioxidants such as glutathione and ascorbic acid enables the correct functioning of the photosynthetic machinery under stress conditions (Munné-Bosch, 2005, Munné-Bosch *et al.*, 2013). γ-Tocopherol is the precursor of α-tocopherol and accumulates in small quantities in young or senescing tissues only (Munné-Bosch & Alegre, 2002; Szymanska & Kruk, 2008). There is some controversy about whether or not tocopherols can have more functions beyond their antioxidant role (Falk & Munné-Bosch, 2010). Some studies suggest that tocopherols can act in cell signaling which affect processes outside chloroplasts (Cela *et al.*, 2011). Mutants with an altered tocopherol biosynthetic pathway have suggested that altered antioxidant capacities led to an increase in anthocyanin accumulation in senescing leaves which may be linked to alterations in jasmonic acid contents (Munné-Bosch *et al.*, 2007). Also, tocopherol-deficient mutants of *A. thaliana* show alterations in photoassimilate transport at low temperatures through alterations in callose deposition in the phloem (Maeda *et al.*, 2006) and endoplasmic reticulum PUFA metabolism (Maeda *et al.*, 2008).

Given the centrality of lipid peroxidation in plant defense we hypothesized that lack of tocopherols or an altered tocopherol composition would influence interactions with pathogens. With the aim of evaluating the role of tocopherols in plant responses to biotic stress, we examined here the response of *vte1* and *vte4* mutants to *P. syringae* and *B. cinerea*. While the avirulent *P. syringae* used in this study is a well-characterized pathogen that causes hemibiotrophic symptoms and increases salicylic acid contents, *B. cinerea* is a necrotrophic fungus that elicits jasmonic acid-related resistance. Emphasis was put on evaluating the lipid and hormonal profiling of leaves to unravel possible mechanistic links between tocopherols

and defence signal production.

Materials and methods

Plant material and sampling

Seeds of *A. thaliana* Columbia ecotype (Col 0) and *vte1* and *vte4* mutants, which were provided by Kathleen Brückner (University of Kiel, Germany), were used in this study. The *vte1* mutant lacks both α- and γ-tocopherol, while the *vte4* mutant lacks α-tocopherol but accumulates instead γ-tocopherol (Porfirova *et al.*, 2002; Bergmüller *et al.*, 2003). The mutants have T-DNA insertions in the *VTE1* and *VTE4* genes, which encode tocopherol cyclase and γ-tocopherol methyltransferase, respectively.

Plants were grown in a constant environment chamber (8/16 light:dark photoperiod, 90–110 µmol quanta m^{-2} s^{-1}, air temperature 21-23ºC) in pots containing a mixture of peat:perlite:vermiculite (1:1:1, v/v/v) during 8 weeks. Then, plants were exposed to four treatments: either infection with *Botrytis cinerea*, *Pseudomonas syringae* or the respective untreated controls, one for each type of infection.

The infection by *B. cinerea* was carried out by applying a drop (~5 µL) of a suspension containing 10^5 spores/mL on the leaves surface. In controls the same treatment was applied but only with PDB medium. The infection with *P. syringae* pv DC3000, loci AVR (*Pst avrRpm1*), was carried out by infiltrating the leaves with a 10 mM $MgCl_2$ solution at different bacterial concentrations (10^6, 10^5, 10^4 bacterial cell/mL). For the control plants, the same treatment was applied but only with 10 mM $MgCl_2$.

For tocopherol and hormonal profiling, leaves were collected, immediately frozen in liquid nitrogen and stored at -80ºC until analysis. For *B. cinerea*, samples were collected before infection and after 24, 60 and 84h. For *P. syringae*, samples were collected at 0, 6, 9 and 24h. For lipidome (fatty acid content of lipid fractions) analyses, the samples were collected and lyophilized prior to analyses. Samples were collected at 24 and 84h of infection for *P. syringae* and *B. cinerea* infection, respectively. Experiments were repeated 4 times taking a pool of leaves from different plants for each experiment.

Infection symptoms

B. cinerea infection symptoms were assessed using a weighted scoring method where

negative scores were associated with resistance and positive scores with susceptibility (Lloyd et al. 2011). For the *P. syringae* infection symptoms, the bacterial numbers within lesion were counted.

Estimations of fungal biomass by RT-qPCR

DNA was extracted from eight leaf samples with single inoculations of *B. cinerea* using a DNeasy Mini Kit (Qiagen, UK) following manufacturer's instructions. DNA samples were diluted to 1 ng μL^{-1} ultra pure H_2O. Samples (25 µl) were prepared by mixing 10 µl DNA solution with 16 µl SYBR™ Green Mastermix (Applied Biosystems, UK) and primers (300 nM) for Arabidopsis (iASK1: CTTATCGGATTTCTCTATGTTTGGC; iASK2: GAGCTCCTGTTTATTTAACTTGTACATACC to generate an 131 bp amplicon [Gachon & Saindrenan 2004]) and *B. cinerea.* (CG11: AGCCTTATGTCCCTTCCCTTG; CG12: GAAGAGAAATGGAAAATGGTGAG to generate a 58 bp amplicon (Gachon & Saindrenan, 2004). PCR used a Bio-Rad ABI7300 thermocycler amplifying using the following conditions: 15 min at 95°C followed by 50 cycles of 95°C for 15 s, 58 °C for 30 s and 72°C for 1 min. Serial dilutions of pure genomic DNA from each species were used to trace a calibration curve, which was used to quantify plant and fungal DNA in each sample. Results were expressed as the CG11/iASK ratio of mock-inoculated samples.

Lipid peroxidation assay

Lipid peroxidation was determined by estimating the malondialdehyde (MDA) content following the method of Rosales *et al.* (2006). Infected leaves (~100 mg) were ground with a mortar and pestle with liquid nitrogen, homogenized in 50 mmol L^{-1} potassium phosphate buffer (pH 6.0) and centrifuged at 20 000 *g* for 25 min at 4ºC. To 50 µL aliquots of supernatant was added 200 µL of 200 gL^{-1} trichloroacetic acid containing 5 g L^{-1} thiobarbituric acid. The mixture was heated at 95 ºC for 30 min and then quickly cooled in an ice-bath. Subsequently, the samples were centrifuged at 10,000 *g* for 10 min at 4ºC and the absorbance of the supernatant was read at 532 nm. The value for the non-specific absorption at 600 nm was subtracted from the A_{532} reading. MDA content was expressed as a percentage of mock-inoculated controls.

Tocopherol analyses

The extraction and analyses of tocopherols were performed exactly as described by Amaral *et al.* (2005).

Hormonal profiling

The extraction and UHPLC-MS/MS analyses of phytohormones were performed exactly as described by Müller & Munné-Bosch (2011).

Fatty acid profiling

The extraction and analyses of total fatty acids were performed as described by Kramer & Zhou (2001) and Mossoba (2001). The separation of lipid fractions was performed as described by Nichols (1963) and Sukhija & Palmquist (1988). For the total lipid extraction, between 30-50 mg of freeze-dried material were ground and 100 µL of internal standard (C21, 15mg/mL) and 5 mL of chloroform:methanol (2:1, v/v) were added. The extracts were mixed for 5 min on an orbital shaker and were centrifuged for 5 min at 1500 rpm. The extraction procedure were repeated twice more and the supernatants were mixed. The final extract was divided in two halves and dried completely under a nitrogen stream at 50ºC. One part was methylated and used for the fatty acid profile by GC-MS; the other part was resuspended in 1 mL of chloroform/methanol (2:1, v/v) and used for lipid fractionation using thin-layer chromatography (Nichols, 1963).

Statistical analyses

Differences between treatments were evaluated using the analysis of variance (ANOVA) and were considered significant at a probability level of $P \leq 0.05$. Heat maps and dendrograms were generated using EPClust program (http://www.bioinf.ebc.ee/EP/EP/EPCLUST/).

Results

Characterising *Pseudomonas syringae* and *Botrytis cinerea* infection

Wild type plants and the *vte1* and *vte4* mutants of *A. thaliana* were challenged with either an avirulent strain of *P. syringae P.s.*pv. *tomato* DC3000 (*Pst*) *avrRpm1* or a virulent necrotrophic strain of *B. cinerea*. The possible impact on *vte1* or *vte4* on the symptoms of the

hypersensitive response elicited by *Pst avrRpm1* were assessed (Fig. 1). At 10^4 cell. mL^{-1} there was no apparent difference in symptoms at 72 h following challenge with *PstavrRpm1*; however, at $10^5$and 10^6 cell mL^{-1} there was clear evidence of increased lesion phenotype in *vte1,* and most particularly in *vte4*, compared to the wild type (Fig. 1A). Assessments of lipid peroxidation through the measurement of malondialdehyde concentrations within lesions at set time points indicated that lipid peroxidation was significantly elevated in *vte1* and *vte4* mutants compared to wild type at 72 h, but not at 24h, following challenge with 10^5 cell mL^{-1} *Pst* (Fig. 1B). Estimating bacterial numbers within lesions formed following inoculation with *Pst avrRpm1* 10^5 cell mL^{-1} at 72 h suggested no significant differences between the genotypes (Fig. 1C). These data suggest that symptom development rather than resistance was being affected in the *vte* mutants.

The infection with *B. cinerea* was assessed using a scoring method where positive scores were associated with susceptibility and negative scores with resistance (Lloyd *et al.*, 2011) (Fig. 2A). Based on this scoring system, the *vte1* mutant was more susceptible than wild type and the *vte4* mutant plants during the first 48 h of infection as shown by a significant mutant *x* time interaction (Fig. 2A). Symptoms appeared earlier in the mutants than the wild type, the *vte4* mutant being the most sensitive among genotypes tested. Malondialdehyde measurements indicated that lipid peroxidation was increased in both *vte* mutants, and most particularly in the *vte4* mutant (Fig. 2B). The extent of *in planta* fungal biomass was estimated by qPCR to indicate the relative degree of susceptibility. The *vte* mutants exhibited significantly increased fungal growth compared to infected wild type, indicating that resistance had been compromised in both mutants (Fig 2C).

The contents of tocopherols were measured in *vte1* and *vte4* mutants following pathogen challenge to allow these to be correlated with the altered symptom phenotypes (Fig. 3). α-Tocopherol levels in wild type plants did not change after challenge with *Pst avrRpm1* or *B. cinerea*. γ-Tocopherol contents remained constant in the *vte4* mutant but increased in wild type plants at the end of both type of infections. The *vte1* mutant lacked both α- and γ-tocopherol under all conditions.

Assessing lipid changes following challenge by *Pst avrRpm1* and *B. cinerea*

An extensive lipidomic screen was undertaken to identify the effects of tocopherols in Col-0,

vte1 and *vte4*. The sampling times reflected the times when cellular collapse linked to cell death were clearly initiated although not complete, i.e. at 24 h following the elicitation of a hypersensitive response by *Pst avrRpm1* and 84 h following necrotic lesion collapse with *B. cinerea*.

Initial experiments assessed total lipid levels in the wild type and the *vte1* and *vte4* mutants. Before infection, total lipid levels (sum of all fatty acids) were not significantly different in both wild type and mutant plants (data not shown). After challenge with *Pst avrRpm1* total lipid contents were significantly reduced compared to mock-inoculated controls and this did not significantly differ in either *vte* mutant compared to controls (Fig. 4). Following infection with *B. cinerea* there were not significant changes in total lipid content in wild type and *vte1* compared to the corresponding uninfected controls, however there was a significant increase in *vte4* (Fig. 4B).

The total glycerol-based and free fatty acid lipidome does not reveal the full subtlety of fatty acid processing. Thus, the total lipid samples were divided into four fractions: the polar fraction representing mostly membrane galacto- and phospholipids, the free fatty acid (FFA) fraction; the diacylglycerols and monoacylglycerols (DAG) fraction; and the triacylglcerols (TAG). Comparisons of the fatty acid totals within each lipid fraction indicated that lipids in the polar fraction were the most abundant (Fig. 5). Following challenge with *PstavrRpm1* the abundance of fatty acid in the polar fraction was significantly reduced whilst conversely, the fatty acid content in all other factions increased. With *B. cinerea* infections, distinctive genotype-specific effects in fatty acid content in the lipid fraction were observed. The fatty acid levels in the polar fraction in the *vte1* mutant were lower than in the wild type and the *vte4* mutant, and the infection caused a decrease in all genotypes, maintaining the differences. Interestingly, elevated TAG levels were observed in *vte4* prior to infection and did not change following infection with *B. cinerea*, however TAG levels increased in Col-0 and *vte1* to achieve the same level.

To provide further insights into the impact on the *vte* mutants on lipid processing between the different fractions, the concentrations of a panel of 76 fatty acids were measured. For clarity, these were sub-classified into major fatty acid groups which were always considered individually, a short chain intermediate (SCI) group (C6-C10) which was indicative of fatty acid anabolism, and a long chain (C20+) group which are “STORAGE”

fatty acid group.

To visualize the effects of the *vte1* and *vte4* on pathogen-induced changes, fold differences over mock-inoculated controls were displayed using heat maps (Figs. 6 and 7). Considering changes elicited by *Pst avrRpm1* there were no significant differences between the *vte* mutants and wild type plants with the majority of fatty acids in the polar fraction exhibiting a reduction on infection. Only C16:1 increased on infection (Fig. 6). The DAG fraction exhibited significant changes with increases in major fatty acids (C18:3, C18:2, C16:3, C18:1, C18:0) in wild type plants not being observed in either *vte* mutant which both exhibited significant reductions in fatty acids (Fig. 6). The TAG fraction did not exhibit any significant difference between *vte* and wild type plants. Some appeared to accumulate during the hypersensitive response (Fig. 6). Examining fatty acids in the free fatty acid fraction most of the main fatty acids were accumulated; consistent with losses seen in the polar fraction (Fig. 6). However, *vte1* exhibited a significant reduction in free C18:3, C18:2 and C18:0 compared to increases in both wild type and *vte4* plants.

Following infection with *B. cinerea* fatty acids in the polar fraction decreased (Fig. 7A). In the wild type plants this appeared to correlate with increases in the DAG (Fig. 7B) and TAG (Fig. 7C) fractions. There were no significant differences between the *vte* mutants and wild type plants in this fraction. Considering the free fatty acid fraction it was notable that many fatty acids did not show a change following infection (Fig. 7D). This presumably reflected a near equilibrium between release from the polar fraction to the free fatty acid fraction and further processing. An exception was C18:3 in the free fatty acid fraction, which was reduced on infection in wild type but was maintained or even increased in the *vte* mutants.

Hormonal changes during *Pseudomonas syringae* and *Botrytis cinerea* interaction with *Arabidopsis thaliana vte1* and *vte4* mutants

Defense-associated hormones are influenced by (per)oxidative events. Given the two pathogens under examination, the two most relevant defense hormones were salicylic acid and jasmonic acid. The infection with *Pst avrRpm1* resulted in increases in salicylic acid levels in wild type plants and both mutants but the increase was significantly delayed in both *vte1* and *vte4* mutants (Fig. 8). Jasmonic acid biosynthesis was also rapidly initiated

following inoculation with *Pst avrRpm1* but there were no significant differences between the plant genotypes (Fig. 8). Auxin can modulate defenses against *P. syringae* via non-salicylic acid dependent mechanisms (Mutka *et al.*,2013) but no significant difference in indole-3-acetic acid contents were observed between mock and *Pst avrRpm1* challenged plants of any genotype (data not shown). Considering other hormones, *trans*-zeatin contents were not significantly altered following challenge with *Pst avrRpm1* (Fig. 9).

Jasmonic acid content increased by about 240-fold after the infection with *B. cinerea*, and contents were ~2,5- and ~17-fold higher than in *vte1* and *vte4* mutants, respectively. However, these differences between genotypes appeared late upon infection only (Fig. 8). Examining the accumulation of salicylic acid following challenge with *B. cinerea,* a slight increase was observed at 60 h after infection but there was no significant difference between the genotypes (Fig. 8). ABA acts to suppress salicylic acid-mediated effects against *B. cinerea* (Audenaert et al.,2002), but the concentration of ABA was not significantly different in any genotype (data not shown). Infection with *B. cinerea* promoted, however, the increase of *trans*-zeatin after 84 h of infection but not in either *vte1* or *vte4* (Fig. 9).

Discussion

It is well known that tocopherols plays an important role in the defense against abiotic stress, increasing its contents in tolerant species (reviewed by Munné-Bosch & Alegre, 2002; Munné-Bosch, 2005). Previous studies using tocopherol-deficient *A. thaliana* mutants have shown that tocopherols can modulate extraplastidic PUFA metabolism, this effect strongly modulating low-temperature adaptation (Maeda *et al.*, 2008) and seedling germination (Sattler *et al.*, 2006). In addition, it has been suggested that tocopherols may be involved in cellular signaling by modulating the expression levels of some genes related with ethylene biosynthesis, perception and signaling (Cela *et al.*, 2011; Allu*et al.*, 2017). However, to our knowledge, nothing is known about the role of tocopherols in biotic stress responses.

Extensive characterization of plant interactions with pests and pathogens has allowed the major signaling networks governing biotic interactions to be elucidated (Pieterse & Dicke, 2007). The hypersensitive response form of cell death is effective mainly against (hemi)biotrophic pathogens such as *Pst* and this form of defense is often associated with salicylic acid (Mur *et al.*, 2008). Defenses against cell-death initiating necrotrophic

pathogens, such as *B. cinerea,* have been linked to jasmonic acid and ethylene signaling (Ton et al. 2002). Oxidative events play a key role in the hypersensitive response (Lamb & Dixon, 1997) and also necrotroph-elicited cell death (Muckenschnabel *et al.*, 2002). Salicylic acid production has been shown to be initiated by oxidative stress, which in turn could influence jasmonic acid production by modulating the relative availability of C18:3 acyl chains (Mur *et al.*, 2008). It is well known that a differential composition or mobilization of fatty acids can help the plant in the resistance to both abiotic and biotic stresses (Upchurch, 2008). The differences in lipid membrane composition influence membrane fluidity resulting in an appropriated environment for different membrane proteins, especially for photosynthetic machinery. Among others, increasing α-linolenic acid (C18:3) in chloroplast membranes of stress-tolerant plants has been shown to enhance tolerance to low temperature, salt, water and pathogen stresses (Iba, 2002; Zhang *et al.*, 2005).

B. cinerea infection led to important differences in the response of wild type plants and both mutants with a clear delay in resistance in the latter, as shown in more severe symptoms and increased fungal biomass. Moving to consider the mechanism underlying this loss of resistance in both mutants, and most particularly in the *vte4* mutant, it was observed an enhanced lipid peroxidation, as indicated by malondialdehyde accumulation already at early stages of infection, which was followed by a shift in the free fatty accumulation. C18:3 accumulated within the free fatty acid fraction, which occurred concomitantly with a major delay in increasing jasmonic acid contents in response to *B. cinerea* infection. However, this lack of C18:3 processing to produce jasmonic acid that might compromise resistance to fungal attack in both mutants occurred at late stages of infection only. Therefore, it appears that an altered tocopherol composition in chloroplasts may be associated with an increased susceptibility to *B. cinerea* and a delayed response to fungal attack by exposing the leaves to an increased chloroplast-related lipid peroxidation.

In previous studies, it has been shown that paraquat-sensitive tobacco plants showed severe symptoms after infection with *B. cinerea* (Barna *et al.*, 2003). This is consistent with the present study, in which alterations in the content and composition of tocopherols in chloroplasts compromises resistance to fungal infection. It appears therefore that enhanced oxidative stress in chloroplasts may lead to reduced resitance to *B. cinerea*. Furthermore, it has been shown that membrane damage caused by the non-specific fungal toxin fusaric acid

was reduced on tobacco leaves pretreated with anti-senescence plant hormones, such as the cytokinins, kinetin and benzyladenine (Barna *et al.*, 2003). In the present study, *trans*-zeatin contents were higher in wild type compared to both *vte* mutants after fungal infection, thus suggesting a link between chloroplastic antioxidants, anti-senescing hormones and plant resistance to *B. cinerea*.

Interestingly, the mutant still producing γ-tocopherol (*vte4*) showed a more severe phenotype after *B. cinerea* treatment than the mutant lacking both tocopherols (*vte1*). Instead of providing some protective effect, the presence of γ-tocopherol at high concentations made the *vte4* mutant more sensitive to fungal attack, which might appear counterintuitive at first sight. However, it is worthy to note that wild type plants increased γ-tocopherol contents upon fungal infection to some extent only, attaining maximum contents 70% lower than in the *vte4* mutant. This suggests that enhancing γ-tocopherol contents may provide some resistance to fungal attack in the wild type, but a constitutive accumulation of this compound at high concentrations will make the *vte4* mutant more sensitive to the pathogen. Interestingly, previous studies have shown that the *vte4* mutant is also more sensitive to abiotic stress than the *vte1* mutant and that the former generally responds with an enhanced stress sensitivity than the latter (Cela et al., 2011; Allu et al., 2017).

In conclusion, challenging with *PstavrRpm1* suggested that symptoms were more severe in *vte1* and *vte4* compared to wild type but this did not impact on resistance. By contrast, *B. cinerea* exhibited an increased susceptibility in the *vte* mutants as recorded by lesion scoring and fungal biomass accumulation and this was associated with an enhanced lipid peroxidation at early stages of infection and a lipidomic shift towards α-linolenic acid accumulation in the free fatty acid fraction at later stages of infection. The latter appears to be linked to a delay into linolenic acid processing towards jasmonic acid biosynthesis in both mutants. Furthermore, anti-senescing hormones (cytokinins) accumulated upon *B. cinerea* infection in the wild type but not in *vte* mutants. These data indicate that tocopherols play an important role in plant responses to biotic stress by modulating the extent of lipid peroxidation, membrane fatty acid composition and allowing the correct deployment of defences against fungal attack. Further research is however needed to better understand the molecular mechanisms underlying reduced resistance in *vte* mutants at earlier stages of fungal attack and whether or not camalexin may be involved.

Authors contributions

JC, LAJM and SMB designed experiments; JC, JKST, AS and MRFL performed experiments; JA, LAJM and SMB wrote the manuscript

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FIGURE LEGENDS

Fig. 1. Characterization of *Pseudomonas syringae* pathovar tomato (*Pst*) *avrRpm1* interactions with the *vte1* and *vte4 A. thaliana* mutants. **(A)** Lesion phenotypes in wild type plants and the *vte1* and *vte4* mutants at 72h following inoculation with 10^6, 10^5 or 10^4 cell/ mL of *PstavrRpm1*(bar = 1cm). **(B)** Lipid peroxidation at 24h, 48h and 72h following inoculation with *Pst avrRpm1* as estimated by MDA accumulation. **(C)** Bacterial numbers within lesion, obtained in dissected tissue, after 24h of infection in wild type plants and *vte1* and *vte4* mutants. Data are the mean ± SE of *n*=6. Significant differences between genotypes, time of infection and its interaction are given in the panels (ANOVA, $P \leq 0.05$). NS, not significant.

Fig. 2. Characterization of *Botrytis cinerea* interactions with the *vte1* and *vte4* mutants. (**A)** *B. cinerea* lesion phenotype scoring at 24h, 48h and 72h of infection following the approach of Lloyd *et al.* (2011) (bar = 1 cm). **(B)** Lipid peroxidation at 24h, 48h and 72h following infection, as estimated by malondialdehyde (MDA) accumulation**. (C)** Fungal growth quantification. The abundance of the *B. cinerea CG11* (cutinase A) gene was quantified in infected samples and normalized against the *A.thaliana ASK1* gene. The graph shows the *CG11/iASK1* expression ratio at 72h following infection with *B. cinerea.* Data are the mean S.E. of *n*=6. Significant differences between genotypes, time of infection, and its interaction are given in the panels (ANOVA, $P \leq 0.05$). NS, not significant.

Fig. 3. Contents of α- and γ-tocopherol in leaves of wild type and *vte1* and *vte4* mutants of *A. thaliana* after 0, 6, 9 and 24h of the infection with *Pseudomonas syringae* pathovar tomato (*Pst*) *avrRpm1* (Pseudomonas, top) and 0, 24, 60 and 84h of *Botrytis cinerea* (Botrytis, bottom). Data are the mean ± SE of *n*=4. Significant differences between genotypes, time of infection, treatment and their interactions are given in the panels (ANOVA, $P \leq 0.05$). NS, not significant.

Fig. 4. Total lipid contents in leaves of wild type and *vte1* and *vte4* mutants of *A. thaliana* at 24h infection with *Pseudomonas syringae* pathovar tomato (*Pst*) *avrRpm1*(Pseudomonas, top) and at 84h of infection with *B. cinerea* (Botrytis, bottom). Data are the mean ± SE of *n*=3. Significant differences between genotypes, time of infection, treatment and their interactions are given in the panels (ANOVA, $P \leq 0.05$). NS, not significant.

Fig. 5. Lipid composition per fractions in leaves of wild type and *vte1* and *vte4* mutants of *A.*

thaliana at 24h of infection with *Pseudomonas syringae* pathovar tomato (*Pst*) *avrRpm1*(Pseudomonas, top) and 84h of infection with *B. cinerea* (Botrytis, bottom). PF polar fraction, DAG diglycerides and monoglycerides, FFA free fatty acids, TAG triglycerides. Data are the mean ± SE of n=3. Significant differences between genotypes, time of infection, treatment and their interactions are given in the panels (ANOVA, $P \leq 0.05$). NS, not significant.

Fig. 6. Heat map comparing the changes in different fatty acid forms in wild type, *vte1* and *vte4* mutants of *A. thaliana* after 24h of the infection with *Pseudomonas syringae* pathovar tomato (*Pst*) *avrRpm1*. Fold differences in the concentrations of particular fatty acids following challenge with *P. syringae* are shown for each genotype. Results are grouped according to Euclidian clustering with the relative relationships shown by dendrograms. Fatty acids are grouped as **(A)** polar, **(B)** diacylglycerols, **(C)** triacylglycerols and **(D)** free fatty acid fractions. Fatty acids are sub-classified into a major fatty acid group; which were always considered individually, a short chain intermediate (SCI) group (C6-C10) which were indicative of fatty acid anabolism and a long chain (C20 +) group which are "STORAGE" fatty acid group. Fatty acids which displayed significantly different (P<0.05) content in either *vte* mutant compared to wild type are indicated with an asterisk.

Fig. 7. Heat map comparing the changes in different fatty acid forms in wild type, *vte1* and *vte4* mutants of *A. thaliana* after 84h of the infection with *Botrytis cinerea*. Fold differences in the concentrations of particular fatty acids following challenge with *B. cinerea* are shown for each genotype. Results are grouped according to Euclidian clustering with the relative relationships shown by dendrograms. Fatty acids are grouped as **(A)** polar, **(B)** diacylglycerols, **(C)** triacylglycerols and **(D)** free fatty acid fractions. Fatty acids are sub-classified into a major fatty acid group; which were always considered individually, a short chain intermediate (SCI) group (C6-C10) which were indicative of fatty acid anabolism and a long chain (C20 +) group which are "STORAGE" fatty acid group. Fatty acids which displayed significantly different (P<0.05) content in either *vte* mutant compared to wild type are indicated with an asterisk. **(E)** Contents of 18:3 in mock- and *B. cinerea*-inoculated leaves. Data are the mean ± SE of n=3. Significant differences between genotypes, time of infection, treatment and their interactions are given in the panels (ANOVA, $P \leq 0.05$). NS, not significant.

Fig. 8. Contents of salicylic acid (SA) and jasmonic acid (JA) in leaves of wild type and *vte1* and *vte4* mutants of *A. thaliana* at 0, 6, 9 and 24h of the infection with *P. syringae* (Pseudomonas, top) and at 0, 24, 60 and 84h of infection with *B. cinerea* (Botrytis, bottom). Data are the mean ± SE of n=4. Significant differences between genotypes, time of infection, treatment and their interactions are given in the panels (ANOVA, $P \leq 0.05$). NS, not significant.

Fig. 9. Contents of cytokinins, including *trans*-zeatin (Z) and *trans*-zeatin riboside (ZR) in leaves of wild type and *vte1* and *vte4* mutants of *A. thaliana* at 0, 6, 9 and 24h of the infection with *P. syringae* (Pseudomonas, top) and at 0, 24, 60 and 84h of infection with *B. cinerea* (Botrytis, bottom). Data are the mean ± SE of n=4. Significant differences between genotypes, time of infection, treatment and their interactions are given in the panels (ANOVA, $P \leq 0.05$). NS, not significant.

***Contribution**

An altered tocopherol composition in chloroplasts may negatively influence the plant response to biotic stress in *Arabidopsis thaliana* through changes in the membrane fatty acid composition and lack of defence activation when challenged with *Botrytis cinerea*.

Figure 1

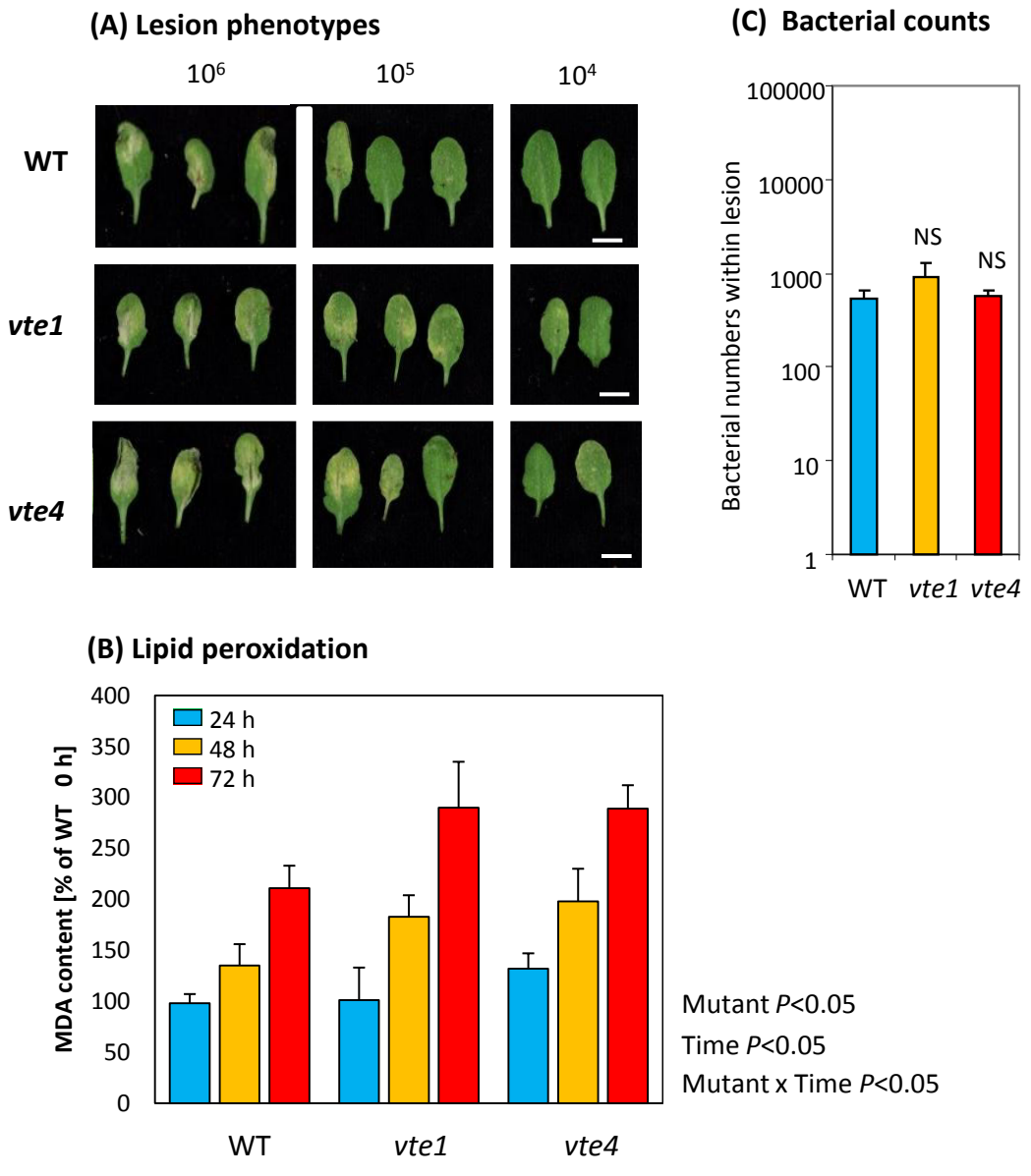
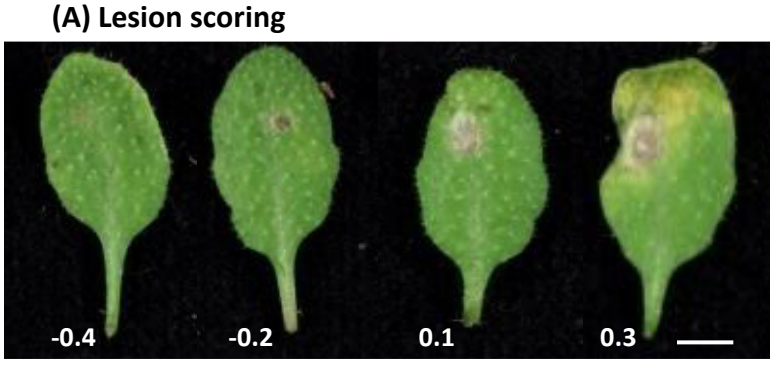
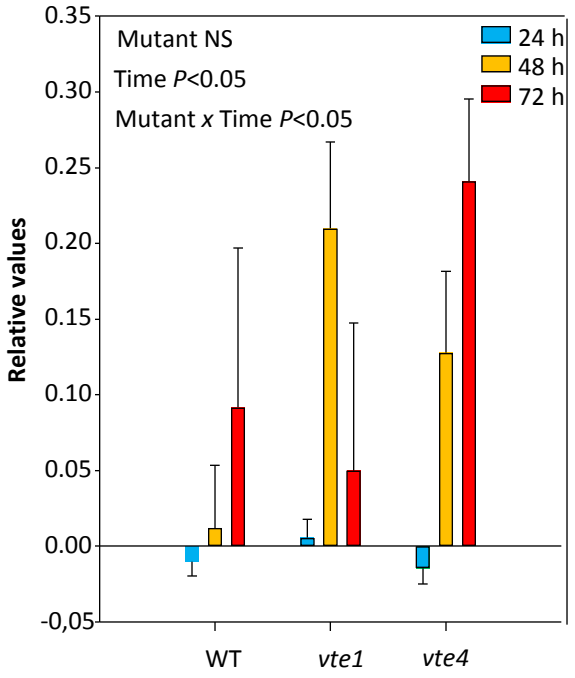
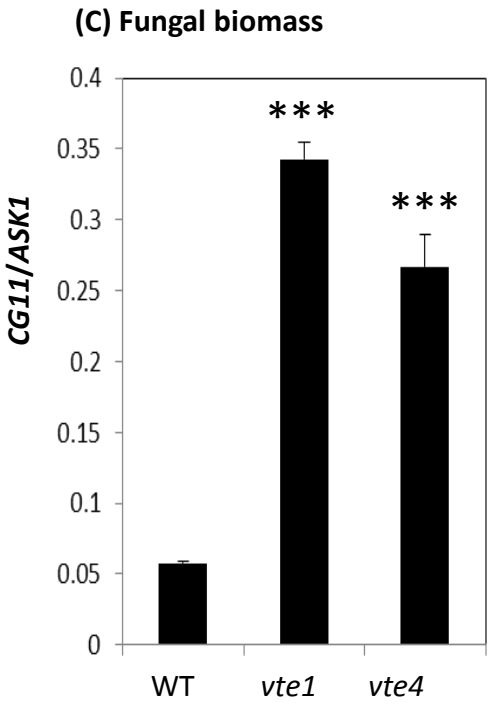
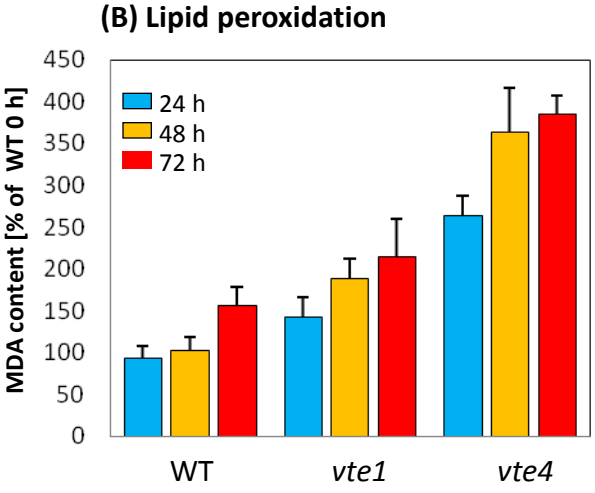

(A) Lesion phenotypes
10^6
10^5
10^4
WT
vte1
vte4
(C) Bacterial counts
Bacterial numbers within lesion
100000
10000
1000
100
10
1
NS
NS
WT
vte1
vte4
(B) Lipid peroxidation
MDA content [% of WT 0 h]
400
350
300
250
200
150
100
50
0
24 h
48 h
72 h
WT
vte1
vte4
Mutant P<0.05
Time P<0.05
Mutant x Time P<0.05

Figure 2



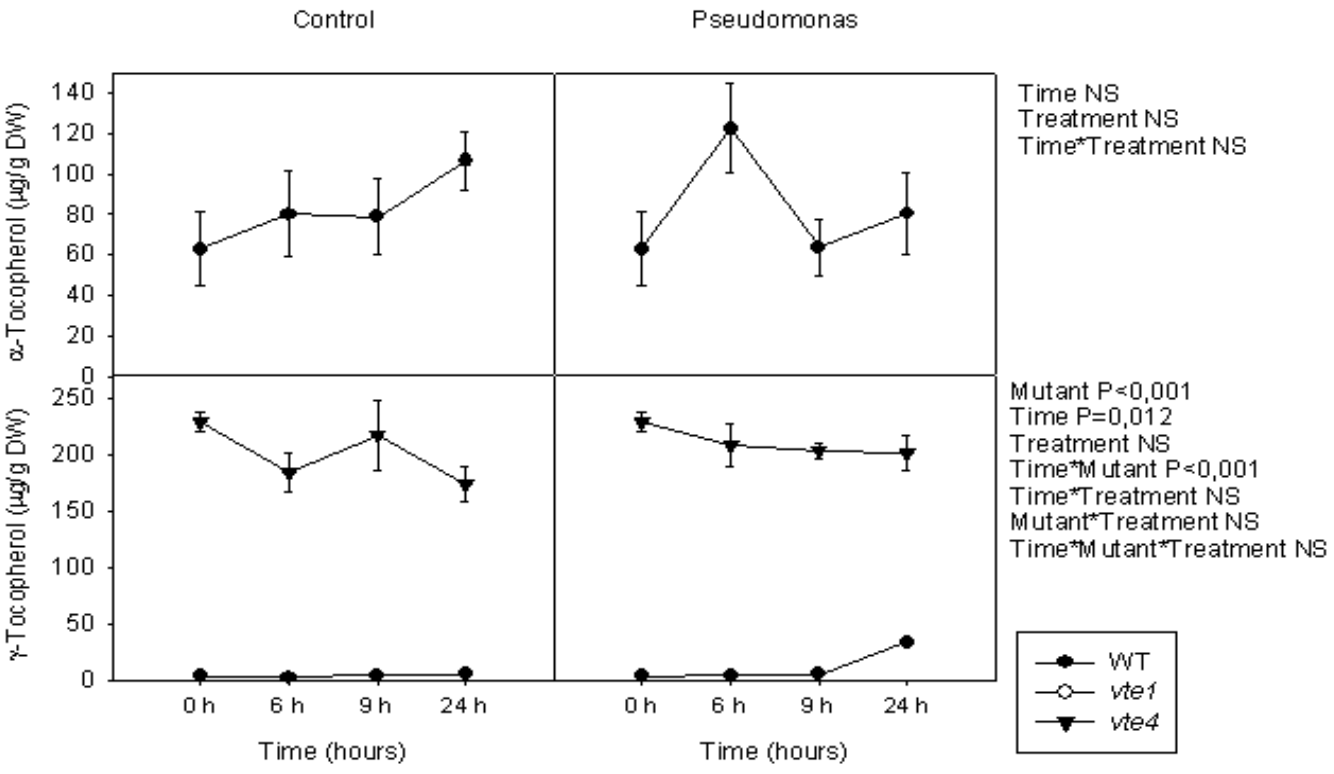






Mutant P<0.05
Time *P*<0.05
Mutant *x* Time *P*<0.05

Figure 3


Control
Pseudomonas
α-Tocopherol (µg/g DW)
γ-Tocopherol (µg/g DW)
Time NS
Treatment NS
Time*Treatment NS
Mutant P<0,001
Time P=0,012
Treatment NS
Time*Mutant P<0,001
Time*Treatment NS
Mutant*Treatment NS
Time*Mutant*Treatment NS
0 h
6 h
9 h
24 h
Time (hours)
WT
vte1
vte4

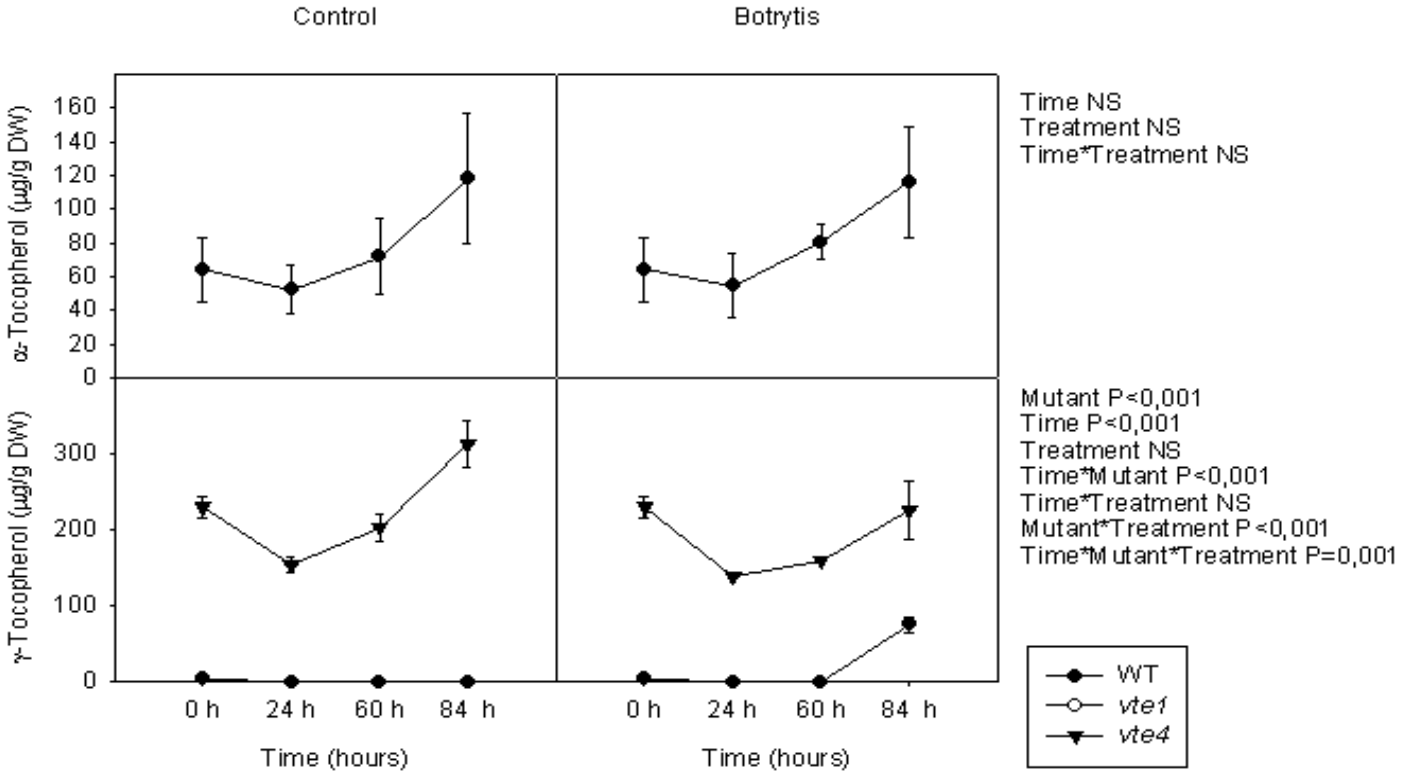
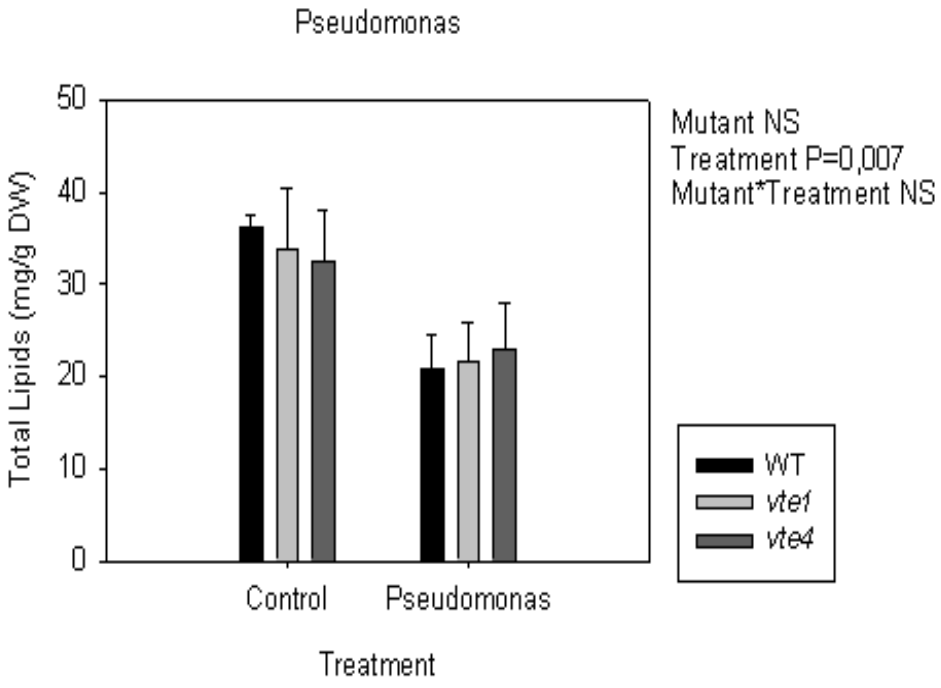

Control
Botrytis
α-Tocopherol (µg/g DW)
γ-Tocopherol (µg/g DW)
Time NS
Treatment NS
Time*Treatment NS
Mutant P<0,001
Time P<0,001
Treatment NS
Time*Mutant P<0,001
Time*Treatment NS
Mutant*Treatment P<0,001
Time*Mutant*Treatment P=0,001
0 h
24 h
60 h
84 h
Time (hours)
WT
vte1
vte4

Figure 4


Pseudomonas
Total Lipids (mg/g DW)
50
40
30
20
10
0
Control
Pseudomonas
Treatment
Mutant NS
Treatment P=0,007
Mutant*Treatment NS
WT
vte1
vte4

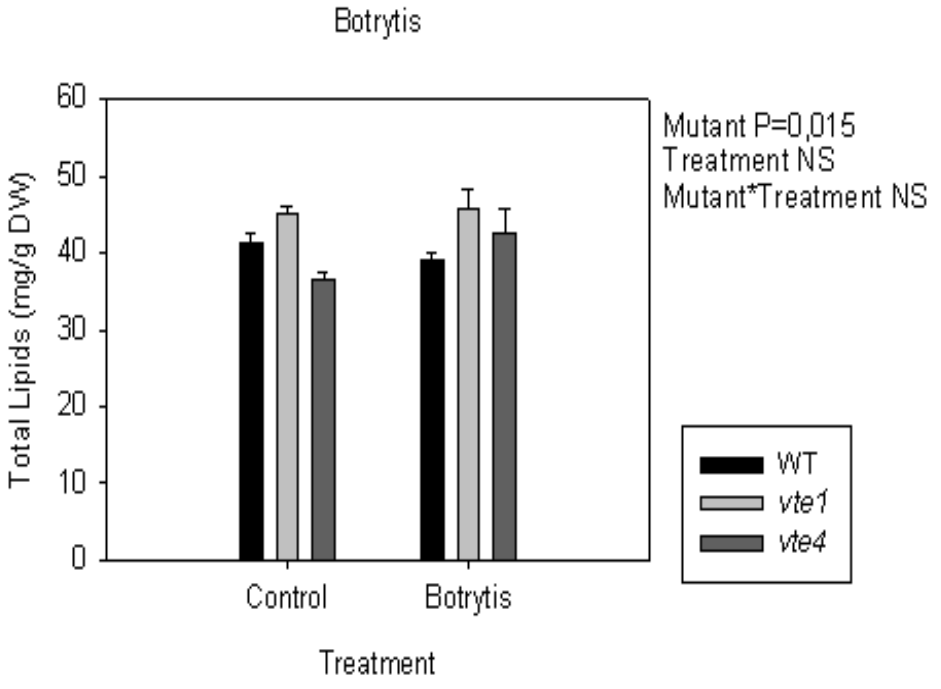

Botrytis
Total Lipids (mg/g DW)
60
50
40
30
20
10
0
Control
Botrytis
Treatment
Mutant P=0,015
Treatment NS
Mutant*Treatment NS
WT
vte1
vte4

Figure 5

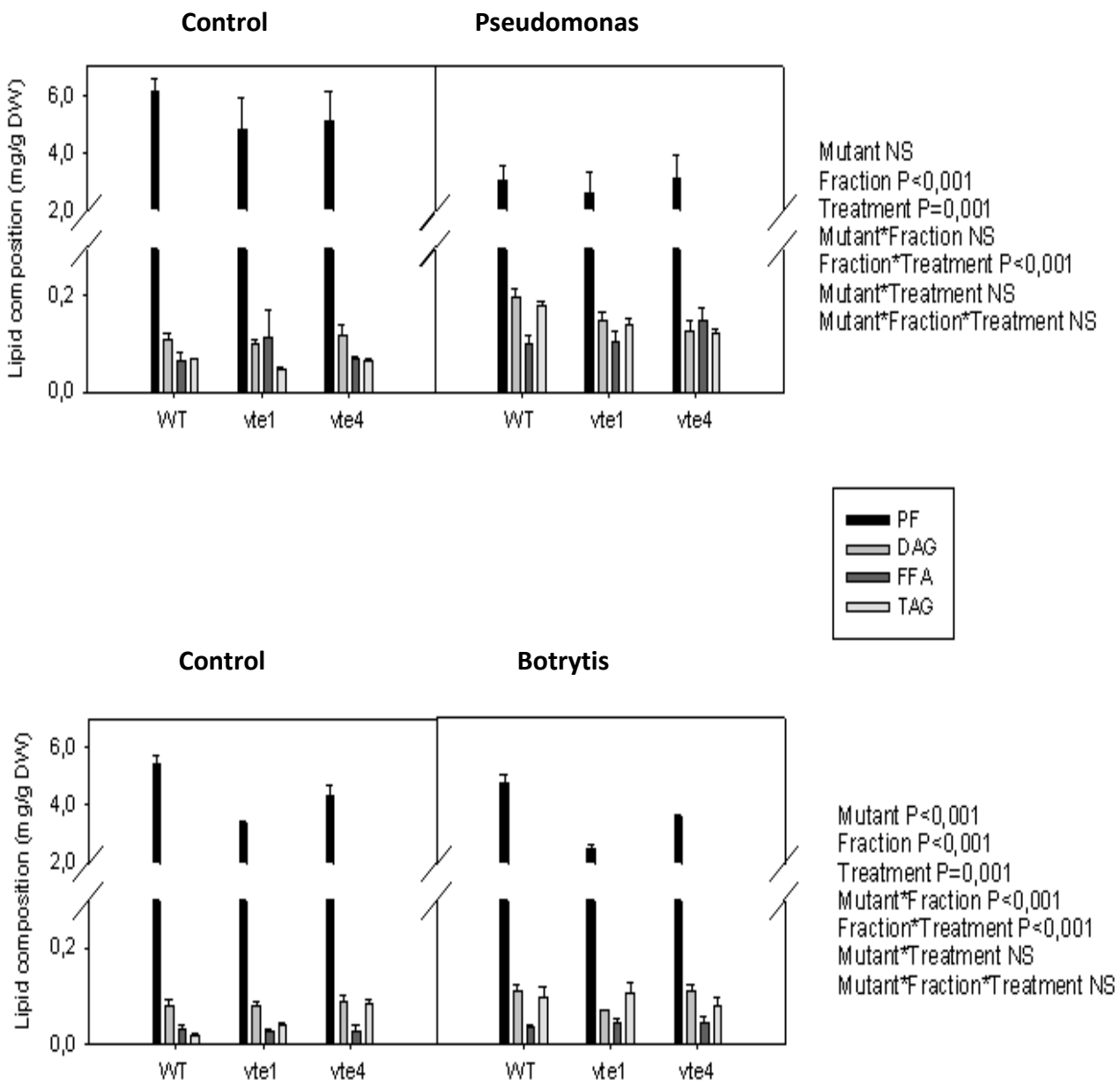

Control
Pseudomonas
Lipid composition (mg/g DW)
6,0
4,0
2,0
0,2
0,0
WT
vte1
vte4
Mutant NS
Fraction P<0,001
Treatment P=0,001
Mutant*Fraction NS
Fraction*Treatment P<0,001
Mutant*Treatment NS
Mutant*Fraction*Treatment NS
PF
DAG
FFA
TAG
Control
Botrytis
Mutant P<0,001
Fraction P<0,001
Treatment P=0,001
Mutant*Fraction P<0,001
Fraction*Treatment P<0,001
Mutant*Treatment NS
Mutant*Fraction*Treatment NS

Figure 6

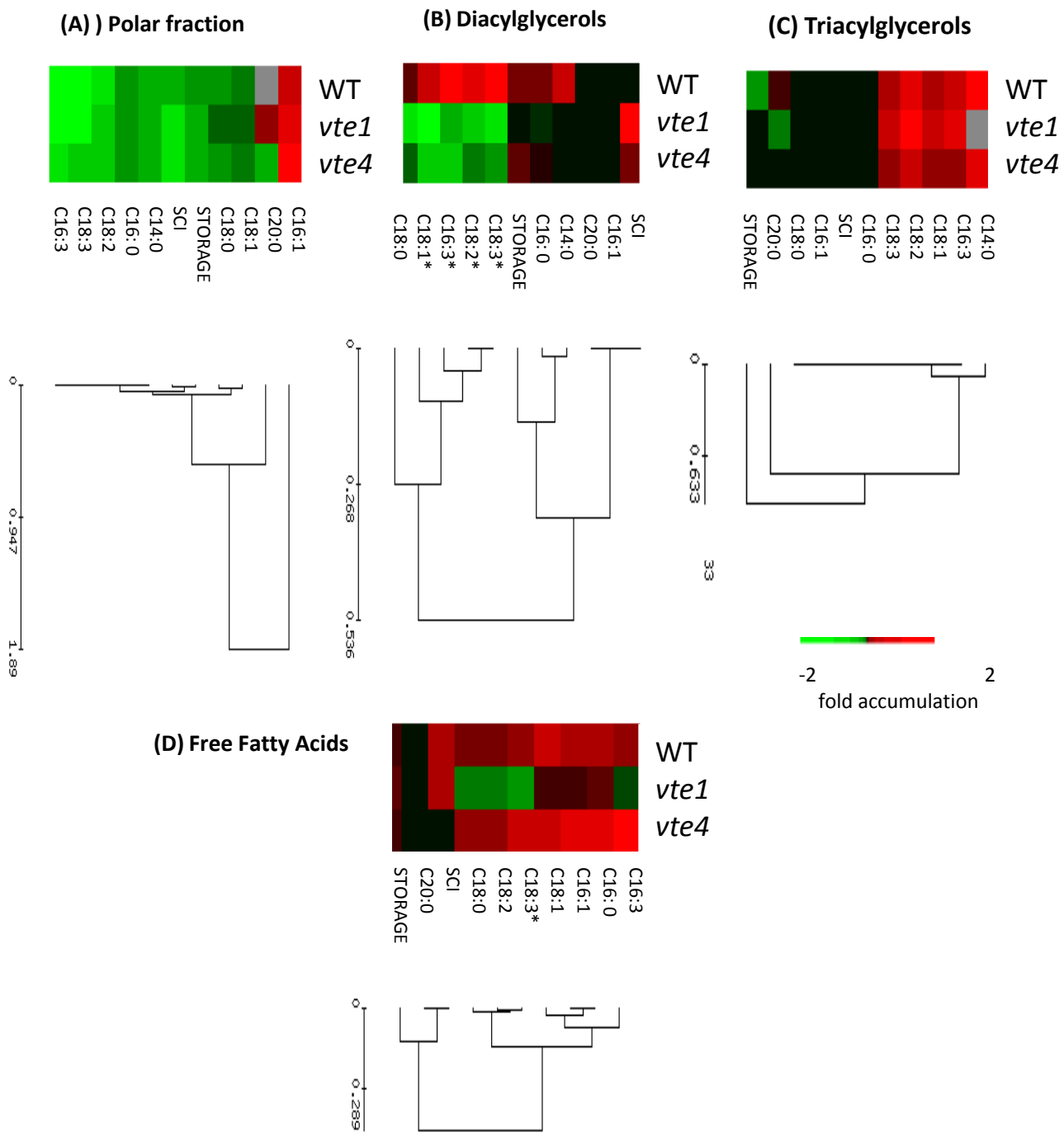
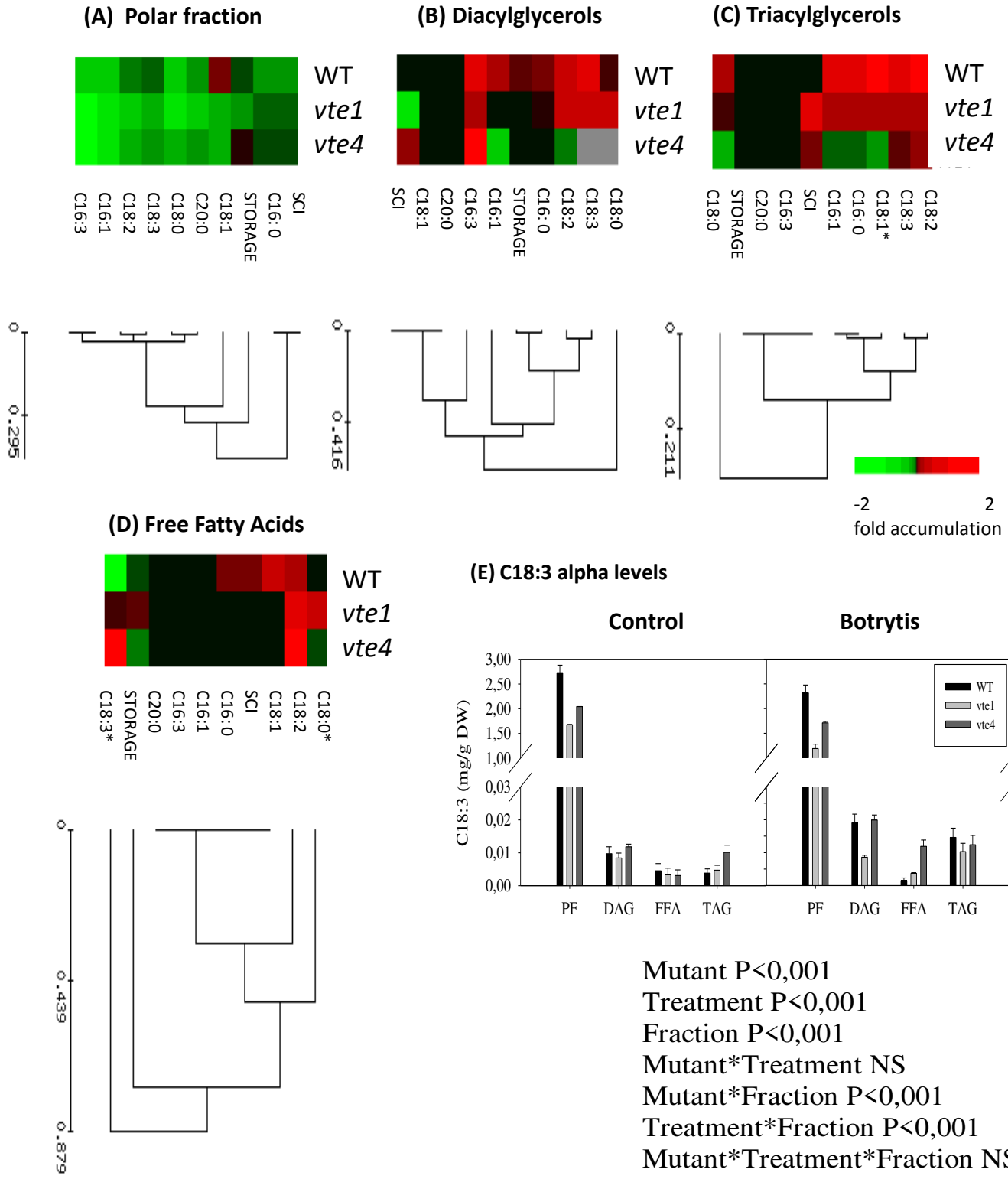
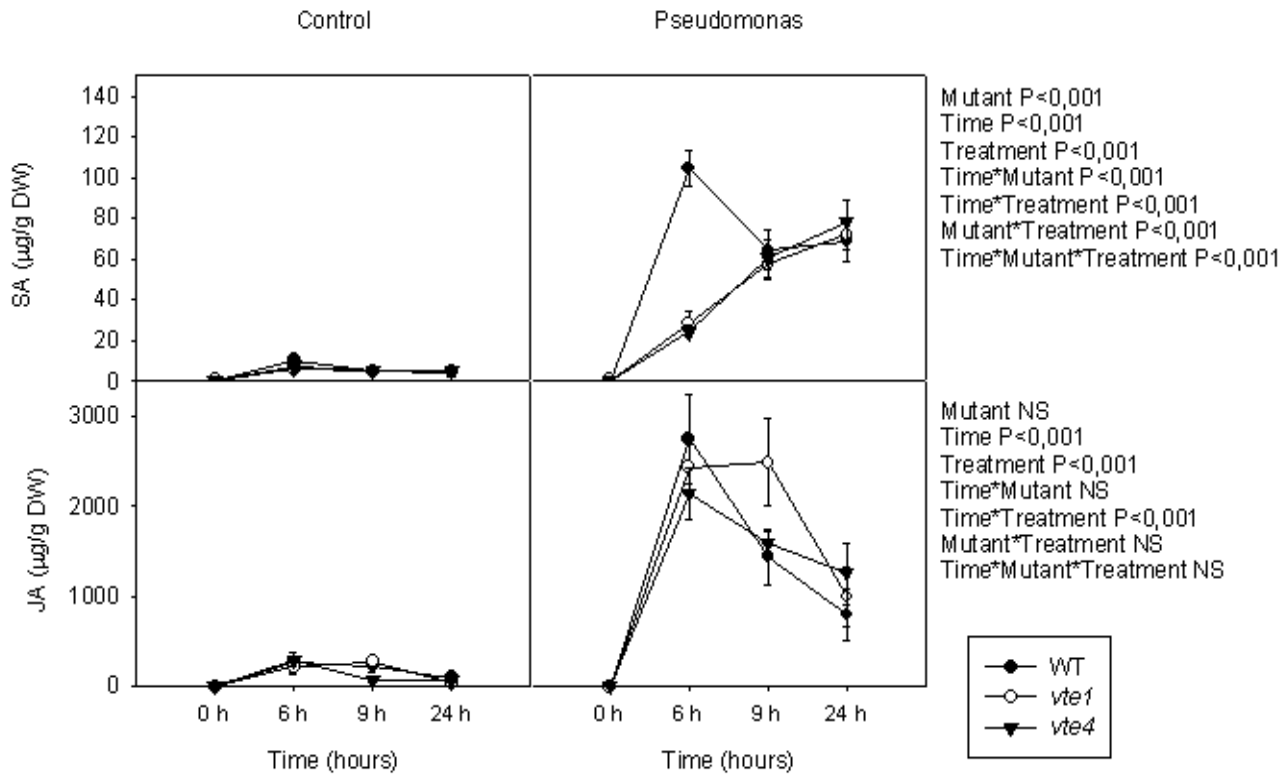

(A)) Polar fraction
WT
vte1
vte4
C16:1
C20:0
C18:1
C18:0
STORAGE
SCl
C14:0
C16:0
C18:2
C18:3
C16:3
0
0.947
1.89
(B) Diacylglycerols
WT
vte1
vte4
SCl
C16:1
C20:0
C14:0
C16:0
STORAGE
C18:3*
C18:2*
C16:3*
C18:1*
C18:0
0
0.268
0.536
(C) Triacylglycerols
WT
vte1
vte4
C14:0
C16:3
C18:1
C18:2
C18:3
C16:0
SCl
C16:1
C18:0
C20:0
STORAGE
0
0.633
33
-2
2
fold accumulation
(D) Free Fatty Acids
WT
vte1
vte4
C16:3
C16:0
C16:1
C18:1
C18:3*
C18:2
C18:0
SCl
C20:0
STORAGE
0
0.289

Figure 7



Mutant P<0,001
Treatment P<0,001
Fraction P<0,001
Mutant*Treatment NS
Mutant*Fraction P<0,001
Treatment*Fraction P<0,001
Mutant*Treatment*Fraction NS

Figure 8


Control
Pseudomonas
SA (µg/g DW)
JA (µg/g DW)
0 h
6 h
9 h
24 h
Time (hours)
Mutant P<0,001
Time P<0,001
Treatment P<0,001
Time*Mutant P<0,001
Time*Treatment P<0,001
Mutant*Treatment P<0,001
Time*Mutant*Treatment P<0,001
Mutant NS
Time P<0,001
Treatment P<0,001
Time*Mutant NS
Time*Treatment P<0,001
Mutant*Treatment NS
Time*Mutant*Treatment NS
WT
vte1
vte4

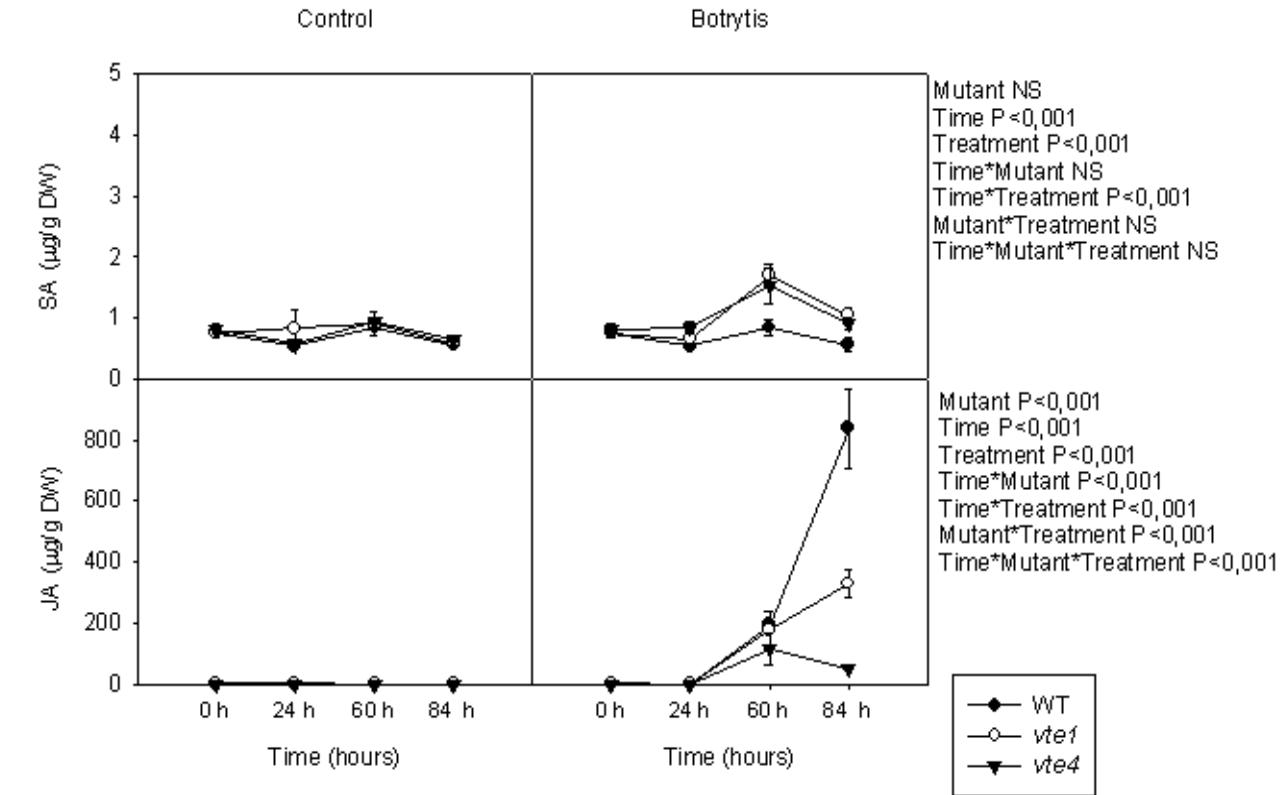
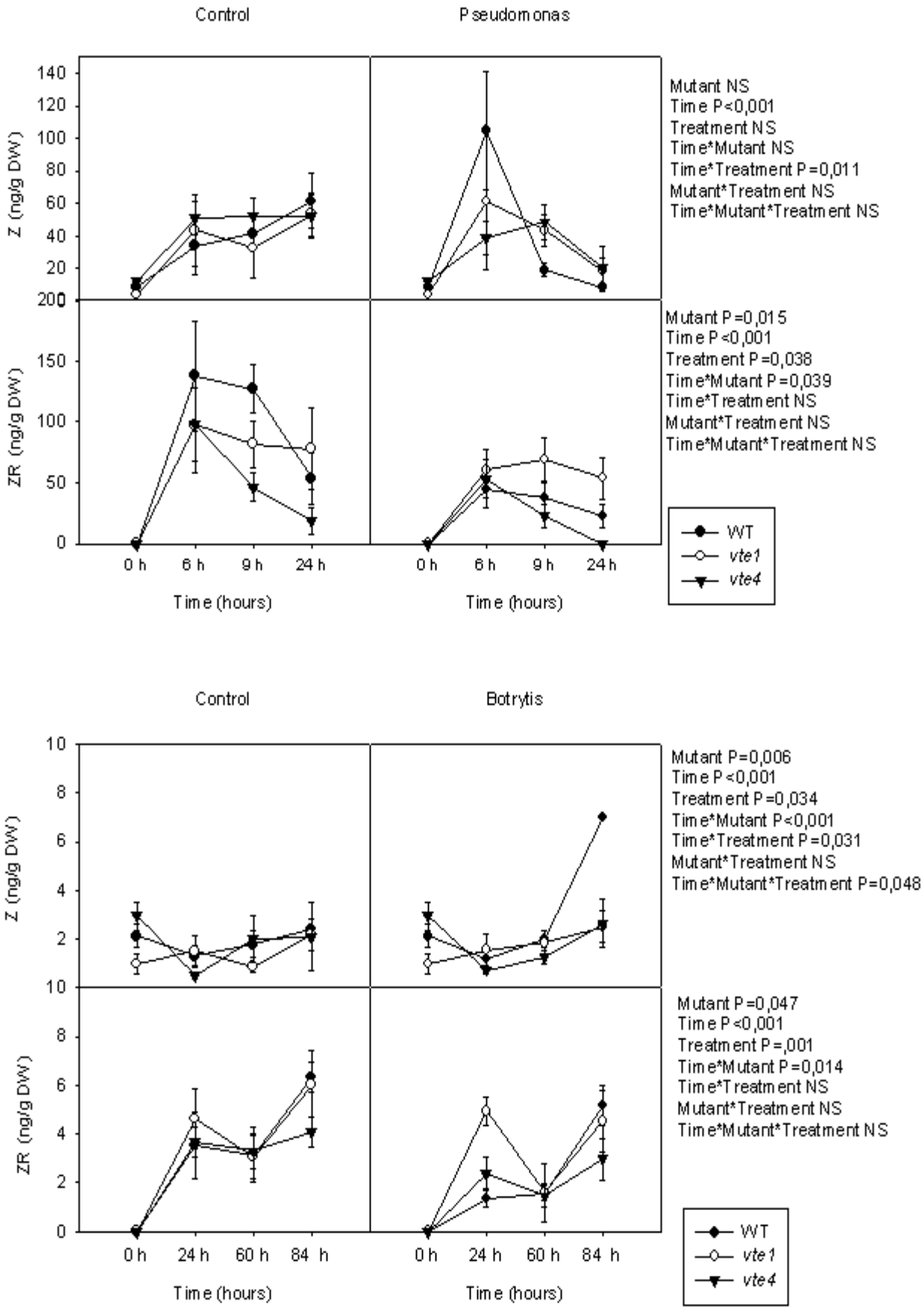

Control
Botrytis
SA (µg/g DW)
JA (µg/g DW)
0 h
24 h
60 h
84 h
Time (hours)
Mutant NS
Time P<0,001
Treatment P<0,001
Time*Mutant NS
Time*Treatment P<0,001
Mutant*Treatment NS
Time*Mutant*Treatment NS
Mutant P<0,001
Time P<0,001
Treatment P<0,001
Time*Mutant P<0,001
Time*Treatment P<0,001
Mutant*Treatment P<0,001
Time*Mutant*Treatment P<0,001
WT
vte1
vte4

Figure 9


Control
Pseudomonas
Z (ng/g DW)
ZR (ng/g DW)
Mutant NS
Time P<0,001
Treatment NS
Time*Mutant NS
Time*Treatment P=0,011
Mutant*Treatment NS
Time*Mutant*Treatment NS
Mutant P=0,015
Time P<0,001
Treatment P=0,038
Time*Mutant P=0,039
Time*Treatment NS
Mutant*Treatment NS
Time*Mutant*Treatment NS
0 h
6 h
9 h
24 h
Time (hours)
WT
vte1
vte4
Control
Botrytis
Mutant P=0,006
Time P<0,001
Treatment P=0,034
Time*Mutant P<0,001
Time*Treatment P=0,031
Mutant*Treatment NS
Time*Mutant*Treatment P=0,048
Mutant P=0,047
Time P<0,001
Treatment P=,001
Time*Mutant P=0,014
Time*Treatment NS
Mutant*Treatment NS
Time*Mutant*Treatment NS
0 h
24 h
60 h
84 h
Time (hours)
WT
vte1
vte4