DIUNSATURATED FATTY ACIDS (DUFAs) AND THEIR ROLES IN SEEDLING ESTABLISHMENT AND DEFENSE.

Dubey Olga

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DIUNSATURATED FATTY ACIDS (DUFAs) AND THEIR ROLES IN SEEDLING ESTABLISHMENT AND DEFENSE.

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Lausanne 2017
Cette thèse est dédiée à la mémoire de mon grand-père

Gennadiy Sodomov
Table of contents

SUMMARY .................................................................................................................. 5
RESUME EN FRANÇAIS .......................................................................................... 6
RESUME POUR UN LARGE PUBLIC .................................................................... 6
LIST OF ABBREVIATIONS ................................................................................ 8
LIST OF FIGURES .............................................................................................. 10
LIST OF TABLES ................................................................................................. 16

CHAPTER I: GENERAL INTRODUCTION ............................................................. 17
I.1. POLYUNSATURATED FATTY ACIDS IN PLANTS: STRUCTURES AND
    BIOSYNTHESIS ............................................................................................... 18
I.2. ROLES OF PUFAS IN PLANT DEVELOPMENT AND PHYSIOLOGY ........ 21
I.3. ROLES OF PUFAS IN DEFENCES ............................................................... 25
I.4. PUFAS AND FUNGAL PATHOGENS INCLUDING B. CINEREA .................... 27
I.5. MAIN GOALS OF THIS THESIS .................................................................. 30
I.6. LITERATURE ................................................................................................. 31

CHAPTER II: CHARACTERIZATION OF ARABIDOPSIS PUFA-
    DEFICIENT FAD MUTANTS .......................................................................... 41
II.1. GENERAL CHARACTERIZATION OF FAD MUTANTS ............................... 42
II.2. CHANGES IN FATTY ACID COMPOSITION DURING DEVELOPMENT .......... 47
II.3. PUFAS AS MAJOR PRECURSORS OF MALONDIALDEHYDE ................... 50
II.4. DISCUSSION ............................................................................................... 58
II.5. EXPERIMENTAL PROCEDURES ............................................................... 62
II.6. LITERATURE ............................................................................................... 65

CHAPTER III: THE EFFECT OF ARABIDOPSIS DUFA-DEFICIENT
    FAD2 MUTANTS ON SEEDLING GROWTH ..................................................... 71
III.1. CHARACTERIZATION OF ROOT GROWTH IN FAD2 MUTANTS .................... 72
III.2. DUFAS AND THEIR ROLE IN ROOT GROWTH UPON STRESS CONDITIONS .. 82
III.3. DISCUSSION ............................................................................................. 90
III.4. EXPERIMENTAL PROCEDURES ............................................................ 93
III.5. LITERATURE ............................................................................................ 97

CHAPTER IV: PUFAS AND THEIR ROLE IN SUSCEPTABILITY TO
    BOTRYTIS CINEREA .................................................................................. 106
IV.1. SCREENING FOR FAD MUTANT RESISTANT TO B. CINEREA IN 7 AND 14 DAY-
    OLD PLANTS ............................................................................................... 107
IV.2. DOES CUTICLE PERMEABILITY LEAD TO B. CINEREA RESISTANCE IN FAD
    MUTANTS? .................................................................................................... 113
IV.3. INDUCTION OF ROS AND DEFENSE HORMONES UPON B. CINEREA
    INFECTION IN FAD MUTANTS ................................................................. 121
IV.4. Changes in Topography of the Cotyledon Surface Can Lead to \textit{B. cinerea} Resistance ................................................................. 128
IV. 5. Surface Metabolites and Their Role in \textit{B. cinerea} Resistance. ... 138
IV. 6. What Is the \textit{B. cinerea} Entry Point in \textit{Arabidopsis} Cotyledons? 145
IV.7. Discussion.......................................................................................... 149
IV.8. Experimental Procedures ...................................................................... 154
IV.9. Literature.............................................................................................. 161

CHAPTER V: CONCLUDING REMARKS ............................................... 178
V.1. Characterization of \textit{Arabidopsis} PUFA-deficient \textit{fad} mutants.. 180
V.2. The effect of \textit{Arabidopsis} DUFA-deficient \textit{fad2} mutants on Seedling Growth................................................................. 181
V.3. PUFA\textsc{s} and their role in resistance to \textit{Botrytis cinerea}............. 183
V.4. Literature .............................................................................................. 184

ACKNOWLEDGMENTS.............................................................................. 186
Summary

Fatty acids (FA) are one of the major storage forms of carbon in living organisms, precursors for some plant hormones such as jasmonate (JA), and major building compounds of the membrane bilayer. In this thesis I address the question: what are the roles of diunsaturated fatty acids (DUFAs) in Arabidopsis seedling establishment and defence? In the first part of this work I found that fatty acid composition is different between roots and shoots of 7-day-old seedlings. The fatty acid composition of roots was constant during plant development, whereas in the shoots it changed within the first week of seedling establishment. Analysis of Arabidopsis mutants with low levels of polyunsaturated fatty acids (PUFAs) revealed chlorotic, dwarfish plants with delayed developmental phenotypes. Reduced levels of PUFAs in roots led to overaccumulation of reactive oxygen species (ROS) that correlated with reduced root growth and stronger susceptibility to abiotic stresses, such as salt treatment. Another interesting finding of this work was that Arabidopsis seedlings lacking DUFAs were highly resistant to the necrotrophic pathogen Botrytis cinerea. This work defines a first attempt to understand the role DUFAs in adaptation to some important abiotic and biotic stresses and their role in these processes. This research opened a lot of questions that could provide better understanding of plant development and adaptation to a changing environment.
Résumé en français

Les acides gras sont connus pour être l'une des principales formes de stockage du carbone dans les organismes vivants et les composés de construction majeurs des membranes cellulaires. Dans cette thèse, j'ai abordé la question suivante : quel est le rôle des acides gras di-insaturés (AGDI) dans l'établissement et la défense des plantules d'Arabidopsis? Premièrement, j'ai constaté que la composition des AG était différente entre les racines et les pousses. La composition en AG des racines était constante pendant le développement de la plante, alors que dans les pousses, elle a changé au cours de la première semaine de croissance. L'analyse de mutants avec de faibles niveaux d'acides gras polyinsaturés (AGPI) a révélé un phénotype végétal chlorotique avec un développement retardé. Les niveaux réduits d'AGPI dans les racines ont entraîné une accumulation excessive des espèces réactives de l’oxygène, ce qui corrèle avec la réduction de la croissance des racines et une forte susceptibilité aux contraintes abiotiques. Un autre résultat intéressant est que les plants dépourvus d’AGDI étaient aussi très résistants au pathogène nécrotrophique Botrytis cinerea. Ce travail a été une première tentative pour comprendre le rôle des AGDI dans l'adaptation à certains stress (a-) biotiques et leur rôle dans ces processus. Notre recherche ouvre beaucoup de questions concernant le développement des plantes et leur adaptation aux changements de l'environnement.
Résumé pour un large public

Acides gras di-insaturés (AGDI) et leur rôle dans l’établissement et la défense des plantules.

Olga Dubey, Département de Biologie Moléculaire Végétale (DBMV), Université de Lausanne.

Les plantes ont développé de multiples façons de s'adapter aux changements de l'environnement. Ce travail permet de mieux comprendre le rôle des acides gras di-insaturés (AGDI) et leur implication dans la résistance au stress. La première question de ce travail a été de comprendre le rôle des AGDI dans l'adaptation au stress salin. Nous avons constaté que les plantes avec des niveaux fortement diminués d'acides gras poly-insaturés (AGPI) possèdent des racines réduites. Lorsque ces plantes sont transférées sur un milieu de croissance avec une forte concentration en sel, la longueur des racines est diminuée encore plus fortement. Cette constatation indique un rôle important des AGDI pour la croissance des plantes dans un environnement salé. La deuxième question abordée dans cette recherche était de comprendre le rôle des AGDI dans la résistance au pathogène nécrotrophique Botrytis cinerea. Ces résultats indiquent un rôle important des AGDI pour le développement des plantes et leur adaptation au stress et ouvrent de nouvelles questions et de nombreuses possibilités d'approfondir les recherches en lien avec cette problématique.
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>TBA</td>
<td>2-thiobabituric acid</td>
</tr>
<tr>
<td>H2DCFDA</td>
<td>2,7-dichlorodihydrofluorescein diacetate</td>
</tr>
<tr>
<td>7MSOH</td>
<td>7-methylsulfinylheptyl glucosinolate</td>
</tr>
<tr>
<td>ABA</td>
<td>Abscisic acids</td>
</tr>
<tr>
<td>aos</td>
<td>Allene oxide synthase mutant</td>
</tr>
<tr>
<td>ABRC</td>
<td><em>Arabidopsis</em> Biological Resource Centre</td>
</tr>
<tr>
<td>Bcgfp1</td>
<td><em>Botrytis cinerea</em> enhanced GFP fluorescent B05 strain</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>DCA</td>
<td>Dicarboxylic acid</td>
</tr>
<tr>
<td>DUFA</td>
<td>Diunsaturated fatty acids</td>
</tr>
<tr>
<td>EMS</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ET</td>
<td>Ethylene</td>
</tr>
<tr>
<td>fad trip</td>
<td><em>fad3-2 fad7-2 fad8</em></td>
</tr>
<tr>
<td>FAD</td>
<td>Fatty acid desaturase</td>
</tr>
<tr>
<td>FAME</td>
<td>Fatty acid methyl ester</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty acids</td>
</tr>
<tr>
<td>GCMS</td>
<td>Gas chromatography - mass</td>
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<tr>
<td>Term</td>
<td>Abbreviation</td>
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<tr>
<td>----------------------------------------------------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>spectrometry</td>
<td></td>
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<tr>
<td>Glycerol-3-phosphate acyltransferase double insertion mutant</td>
<td>gpat4 gpat8</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>H$_2$O$_2$</td>
</tr>
<tr>
<td>Hydroxyl radical</td>
<td>·OH</td>
</tr>
<tr>
<td>Jasmonic acid</td>
<td>JA</td>
</tr>
<tr>
<td>Limit of quantification</td>
<td>LOQ</td>
</tr>
<tr>
<td>Malondialdehyde</td>
<td>MDA</td>
</tr>
<tr>
<td>Monogalactosyldiacylglycerol</td>
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</tr>
<tr>
<td>Non-enzymatic lipid oxidation</td>
<td>nLPO</td>
</tr>
<tr>
<td>Omega hydroxyl fatty acid</td>
<td>ω-OH</td>
</tr>
<tr>
<td>Pathogenesis-related protein 1</td>
<td>PR1</td>
</tr>
<tr>
<td>Phospholipase A</td>
<td>PLA</td>
</tr>
<tr>
<td>Polyunsaturated fatty acids</td>
<td>PUFAs</td>
</tr>
<tr>
<td>Potato dextrose broth</td>
<td>PDB</td>
</tr>
<tr>
<td>Principal component analysis</td>
<td>PCA</td>
</tr>
<tr>
<td>Rapid stress response elements</td>
<td>RSREs</td>
</tr>
<tr>
<td>Reactive electrophile species</td>
<td>RES</td>
</tr>
<tr>
<td>Reactive oxygen species</td>
<td>ROS</td>
</tr>
<tr>
<td>Respiratory burst oxidase homologs</td>
<td>RBOH</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>SA</td>
</tr>
<tr>
<td>Singlet oxygen</td>
<td>1O$_2$</td>
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<td>Term</td>
<td>Abbreviation</td>
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<td>-------------------------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Superoxide</td>
<td>O$_2^-$</td>
</tr>
<tr>
<td>Systematic acquired resistance</td>
<td>SAR</td>
</tr>
<tr>
<td>Transmission electron microscopy</td>
<td>TEM</td>
</tr>
<tr>
<td>Triunsaturated fatty acids</td>
<td>TUFAs</td>
</tr>
<tr>
<td>Ultra-performance liquid chromatography quadrapole time of flight mass spectrometry</td>
<td>UPLC–qTOF-MS/MS</td>
</tr>
<tr>
<td>Very long chain fatty acids</td>
<td>VLCFAs</td>
</tr>
<tr>
<td>Wild type</td>
<td>WT</td>
</tr>
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</table>
List of Figures

Figure 1.1. Structures of the major fatty acids and glycerolipids of plant cell membranes.............................................................................................................19

Figure 1.2. Fatty acid synthesis and glycerolipid assembly in *Arabidopsis* leaves ..................................................................................................................20

Figure 1.3. Triunsaturated fatty acid and their role in adaptation to temperature changes. ...........................................................................................................23

Figure 1.4. Jasmonic acid biosynthesis and metabolism in *Arabidopsis thaliana* .......................................................................................................................26

Figure 1.5. Cuticle biosynthesis in *Arabidopsis thaliana* plants. ..................28

Figure 2.1. *FAD2* and *FAD6* transcript expression in 3-day-old seedlings.....44

Figure 2.2. Developmental phenotype of diunsaturated fatty acid mutants .....45

Figure 2.3. Monounsaturated plants ...................................................................46

Figure 2.4. Relative expression of FAD2 and FAD6 genes in 7-day-old *fad* single mutants ...........................................................................................................47

Figure 2.5. Fatty acid composition in *fad* mutants .............................................48

Figure 2.6. Fatty acid composition during the first week of seedling establishment .........................................................................................................................49
Figure 2.8. *In situ* malondialdehyde (MDA) detection ........................................... 54

Figure 2.9. Total MDA levels in 3-day-old seedlings ........................................... 56

Figure 2.10 Total MDA levels in 7-day-old seedlings ........................................... 56

Figure 2.11. MDA pools in 3-day-old seedlings after 50 µM JA treatment, 20 hours post-application ................................................................. 57

Figure 2.12. Estimation of the contribution of different PUFAs to MDA generation in Arabidopsis seedlings.............................................................. 61

Figure 3.1. Organization of the Arabidopsis root............................................. 72

Figure 3.2. Reduction of oxygen to water ......................................................... 74

Figure 3.3. Root length analysis of 7-day-old Arabidopsis seedlings............ 76

Figure 3.4. Fatty acid composition of 7-day-old roots................................... 77

Figure 3.5. MDA levels in 7-day-old Arabidopsis roots................................. 78

Figure 3.6. ROS staining of 7-day-old roots ...................................................... 79

Figure 3.7. Morphological analysis of 7-day-old WT and fad2 roots ............ 81

Figure 3.8. Root length analysis of 7-day-old Arabidopsis seedlings growing in control and salt-rich media ......................................................... 84
Figure 3.9. Morphological analysis of 7-day-old roots of WT and \textit{fad2} seedlings grown in control and salt media were visualized with propidium iodide (PI) staining ......................................................................................................................... 86

Figure 3.10. ROS staining of 7-day-old \textit{Arabidopsis} roots ......................... 87

Figure 3.11. Relative expression of \textit{CYP81D11} and \textit{UGT73B3} genes in 7-day-old seedlings ............................................................................................................................................... 89

Figure 3.12. Germination of \textit{Arabidopsis} seeds (assessed 1 day post-germination) ......................................................................................................................................................... 93

Figure 4.1. \textit{B. cinerea} pathogenesis on \textit{Arabidopsis} seedlings ................ 109

Figure 4.2. \textit{B. cinerea} resistance bioassay on \textit{Arabidopsis} seedlings......... 110

Figure 4.3. Fatty acid composition of WT and \textit{fad2-3} mutant performed at 7- and 14-day-old stage................................................................................................................................. 112

Figure 4.4. Cuticle permeability assay in 14-day-old seedlings................. 116

Figure 4.5. Cuticle ultrastructure of 7-day-old \textit{Arabidopsis} cotyledons. ... 117

Figure 4.6. Quantification of aliphatic polyester monomers in 7-day-old \textit{Arabidopsis} shoots................................................................................................................................. 118

Figure 4.7. Scanning electron microscopy images of 7-day-old \textit{Arabidopsis} cotyledons................................................................................................................................. 120
Figure 4.8. ROS staining of 8-day-old cotyledons. ........................................... 123

Figure 4.9. Induction of defence marker genes upon B. cinerea infection in 8-day-old seedlings........................................................................................................ 126

Figure 4.10. Fatty acid composition in 7-day-old seedlings of WT and the gpat4 gpat8 double mutant............................................................................. 127

Figure 4.11. B. cinerea adhesion assay .......................................................... 129

Figure 4.12. B. cinerea development: a time course assay on 7-day-old cotyledons....................................................................................................... 130

Figure 4.13. B. cinerea hyphal length assay at two different time points ..... 132

Figure 4.14. Pavement cell morphology of 7-day-old cotyledons, stained with propidium iodide. ................................................................. 133

Figure 4.15. Example of a single pavement cell analyzed with Lobefinder_V1-4.0 software ........................................................................... 134

Figure 4.16. Quantitative analysis of pavement cell parameters in 7-day-old cotyledons............................................................................... 135

Figure 4.17. Atomic force microscopy images of 7-day-old Arabidopsis cotyledons................................................................................ 136

Figure 4.18. Quantitative analysis of atomic force microscopy results........ 137
Figure 4.19. PCA score plot from surface (S) and whole shoot (L) metabolites of 7-day-old WT and fad2-3 seedlings

Figure 4.20. Relative quantification of two metabolites differently present on the surface of WT and fad2-3 cotyledons and leaves.

Figure 4.21. Scanning electron micrographs of 7-day-old Arabidopsis cotyledons.

Figure 4.22. Transversal sections of WT cotyledons inoculated with B. cinerea for 24h.

Figure 4.23. Transversal section of cuticle deficient mutants.

Figure 4.24. B. cinerea entrance through the intracellular regions of Arabidopsis pavement cells.
List of Tables

Table 4.1. Characterized cuticle mutants and their responses to *B. cinerea*. 114

Table 4.2. List of metabolites differently abundant on the surface of cotyledons. 142
Chapter I: General introduction
I.1. Polyunsaturated fatty acids in plants: structures and biosynthesis.

Fatty acids (FAs) are hydrocarbons containing a long aliphatic chain that can be either saturated (no double bonds) or unsaturated (one or more double bonds). Chain lengths of C16 (∼ 30%) and C18 (∼ 70%) are the most abundant in higher plants (Tjellstroem et al., 2012). Depending on the level of saturation, FAs can be subdivided into the saturated (e.g. 16:0; 18:0), monounsaturated (e.g. 16:1; 18:1; MUFA), di- (e.g. 16:2; 18:2; DUFA) or tri-unsaturated (e.g. 16:3; 18:3; TUFA). Subgroups of 18:2 and 18:3 make up 55% of total fatty acids in Arabidopsis cell cultures (Tjellstroem et al., 2012). In leaves 18:2 and 18:3 represent, 18.5% and 50% respectively of total FAs (Maatta et al., 2012). In the roots the proportion of DUFAs and TUFAs is different from the shoots, 18:2 make up 37% of total FAs, whereas 18:3 only 28% (Beaudoin et al., 2009). Leaf and root fatty acids are, therefore, primarily polyunsaturated.

Most of the fatty acids found in cells are esterified to glycerol as glycerolipids. Their derivatives, glycolipids and phospholipids, form essential structural features of membrane bilayers. In many seeds, including Arabidopsis thaliana, triacylglycerols are the major energy source (Fig.1.1).
Figure 1.1. Structures of the major fatty acids and glycerolipids of plant cell membranes. From Ohlrogge and Browse (1995).

Fatty acid biosynthesis in plants is different from that in animals, fungi and some bacteria. More than 30 enzymatic reactions are required to synthesize C16 or C18 FAs from acetyl-coenzyme A (CoA) and malonyl-CoA in plants (Ohlrogge and Browse, 1995). Whereas in the other kingdoms this process requires only one multifunctional polypeptide complex located in the cytosol (Ohlrogge and Browse, 1995; Laliotis et al., 2010). Synthesis of the major glycerolipids in plants is carried out on the membranes of plastids and the endoplasmic reticulum (ER), called in short the “prokaryotic” and “eukaryotic” pathways (Browse and Somerville, 1991; Heinz, 1993). 16:0 and 18:1 acyl groups are the essential substrates for polyunsaturated fatty acid synthesis in
both pathways. Fatty acid desaturase (FAD) enzymes are necessary for insertion of double bonds in newly synthetized unsaturated fatty acids (Fig.1.2).

As shown in Figure 1.2, desaturation of 18:1 to 18:2 and 18:3 takes places in both pathways, but it requires different FAD enzymes. In the ER 18:1 desaturation takes place through the activity of FAD2 enzyme and 18:2 is converted to 18:3 by FAD3 (Miquel and Browse, 1992; Browse et al., 1993). In plastids, the synthesis of both 16:2 and 18:2 FAs is dependent on FAD6 enzyme (Browse et al., 1989) and 16:3/18:3 are produced by further desaturation catalysed by FAD7 and FAD8 (Browse et al., 1986; Gibson et al.,
Fatty acid synthesis is tightly regulated by the light/dark cycle in the photosynthetic organs of plants; malonyl-CoA is synthetized within seconds after light exposure (Sauer and Heise, 1983). This process is triggered by pH, Mg$^{2+}$ and redox cascade changes within the chloroplasts (Harwood, 1983; Sauer and Heiske, 1983; Eastwell and Stumpf, 1983; Sasaki et al., 1997). Other external factors that can control fatty acid biosynthesis will be described in detail within the following subchapters.

I.2. Roles of PUFAs in plant development and physiology.

Fatty acids biosynthesis is a primary metabolic pathway essential for plant growth and development. $fad2-2$ $fad6$ Arabidopsis mutants that lack both DUFAs and TUFAs were shown to be lethal on soil, due to the important role of these FAs in the assembly and maintenance of the photosynthetic complexes (McConn and Browse, 1998). Surprisingly, plants lacking only TUFAs ($fad3-2$ $fad7-2$ $fad8$) were shown not to have a strong impact on plant growth, but on male sterility caused by deficiency in jasmonic acid (JA; McConn and Browse, 1996). Based on recent studies, the composition of galactolipids is tissue specific: the major pools of PUFAs in leaves are synthetized by FAD7, FAD8 and FAD6 enzymes (Li-Beisson et al., 2013), whereas in meristematic zones,
FAD2 and FAD3 play the key role (Mei et al., 2015). In fast-growing tissues (calli) and highly dividing cells (cell suspension cultures), levels of phospholipids are significantly lower than in slow-growing cells. One of the current explanations for this phenomenon is associated with low activity of FAD2 and FAD3 enzymes in these tissues that leads to over-accumulation of 18:1 in membranes (Mei et al., 2015).

It has been long known that PUFAs have an important physiological role in plant adaptation to temperature changes (Pearcy, 1978; Raison et al., 1982; Iba, 2002). Under cold conditions, PUFA biosynthesis increases, whereas under hot conditions it decreases. For example, the average number of double bonds per glycerolipid (diacyl lipids) in the leaves of Arabidopsis drops from 4.8 at 17 °C to 2.9 at 36 °C. Mutants deficient in TUFAs (fad3-2 fad7-2 fad8) are strongly affected in long-term exposure to 4 °C due to inability to maintain high chlorophyll levels (Fig.1.3.A). Even if TUFAs are not essential for adaptation to high temperatures, low levels of these FAs need to be maintained to promote plant survival (Fig1.3. B).
Roles for DUFAs in plant physiology have been identified with the help of fad6 and fad2 mutants. No phenotypic differences were observed in these mutants compared to the WT at 22 °C, 4 weeks post-germination (Miquel et al., 1993; Maeda et al., 2008). However, dramatic chilling sensitivity was detected in both mutants leading to leaf chlorosis, reduced growth rate and changes in chloroplast morphology (Hugly et al., 1992; Miquel et al., 1993). In recent years, namely an additional role of DUFAs has been identified, adaptation to salinity stress. fad2 and fad6 mutant plants growing on 200 to 250
mM NaCl media were shown to be more sensitive to this treatment compared to WT seedlings (shorter roots and chlorotic cotyledons) due to an altered ion homeostasis in fad6 mutants (Zhang et al., 2009) and dysfunction of membrane-embedded Na\(^+\)/H\(^+\) exchangers (Zhang et al., 2012). These results suggest an essential role of PUFAs in plant development and physiology. Another more recently discovered role of PUFAs is in buffering of reactive oxygen species (Mene-Saffrane et al., 2009; Schmid-Siegert et al., 2016) that are the main mediators of non-enzymatic lipid oxidation (nLPO) (Weber et al., 2004). It was shown that a major by-product of lipid oxidation, malondialdehyde (MDA), is generated mainly from TUFAs (60-75%) in leaves and can promote gene expression in response to stresses (Weber et al., 2004; Schmid-Siegert et al., 2012). When the stress levels in plants go down, MDA is incorporated back into the chloroplast galactolipid 18:3-16:3-monogalactosyldiacylglycerol (MGDG), which is thought to act as a cell protectant (Schmid-Siegert et al., 2016). The other possible sources of MDA in plants can be mono- and di-unsaturated fatty acids (Lui et al., 1997), although this needs to be tested. This thesis will further investigate the role of DUFAs in plants defences.
I.3. Roles of PUFAs in defences.

PUFAs play important direct and indirect roles in defence. When plants are exposed to biotic stresses, PUFAs present in the membrane bilayer are oxidized leading to the formation of oxylipins and various reactive electrophile species (RES; Chehab et al., 2008). Oxylipins are oxygenated fatty acids that contain one or more oxygen atoms in addition to one in the carboxylic acid group. Oxygenation of 18:3 and 16:3 FAs generates oxylipins known as jasmonates (Wasternack and Kombrink, 2010; McDowell and Dangl, 2000). These molecules play important roles in plant defence against herbivores and necrotrophic pathogens (Walling, 2000; Kachroo and Kachroo, 2009). Plants subjected to herbivore attack were shown to produce more trichomes on the new emerging leaves and smaller petioles as response to this stress, features that are controlled by jasmonic acid pathway (Yan and Chen 2007; Zhang and Turner, 2008; Yoshida et al., 2009). Three cellular compartments (chloroplasts, peroxisomes and the cytosol) and multiple biosynthetic enzymes are required for JA biosynthesis (Fig.1.4).
Figure 1.4. Jasmonic acid biosynthesis and metabolism in *Arabidopsis thaliana*. From Acosta and Farmer (2010).

Another compound synthesized from PUFAs during nLPO is azelaic acid (one of the reactive electrophile species (RES)). This reactive electrophile species
(RES) was shown to induce systemic acquired resistance in *Arabidopsis* plants upon stress conditions (Jung *et al.*, 2009).

In the last years, a new role of PUFAs was identified, namely mediation of transcriptional responses. Phospholipase A (PLA) was shown to activate promoters containing rapid stress response elements (RSREs) *via* cleavage of 18:2 and 18:3 FAs from plant membranes upon pathogen attack. RSREs induce activation of defence genes upon multiple abiotic and biotic stresses providing plant protection on the early stages (Savchenko *et al.*, 2010). Another by-product of lipid oxidation involved in defence is phytoprostane (an oxylipin) that was shown to activate TGA motifs. TGA2, TGA5 and TGA6 factors induce expression of defence genes upon treatment with cyclopentenone oxylipins (Mueller *et al.*, 2008).

**I.4. PUFAs and fungal pathogens including *B. cinerea***

The cuticle is an extracellular hydrophobic layer that covers aerial parts of the plant epidermis and works as a barrier between internal parts of the plant and the environment (Yeats and Rose, 2013). It consists of highly water-repellent thin wax layer (procuticle) in young leaves that develops to cutin and cuticular wax in mature leaves (Jeffree, 1996). C16 and C18 diacids, ω- and mid-chain hydroxy FAs are the major structural components of cutin in *Arabidopsis*
leaves (Molina et al., 2006). Surprisingly, the main cutin monomer in leaf and stem cuticle is 18:2 dicarboxylic acid (Bonaventure et al., 2004; Franke et al., 2005). Very long chain fatty acids (VLCFAs) obtained by elongation of plastidial FAs are the major source for wax (Fig. 1.5; Jenks et al., 1995; Samuels et al., 2008).

![Figure 1.5. Cuticle biosynthesis in Arabidopsis thaliana plants. From Kachroo and Kachroo (2009).](image)

The cuticle is an important barrier that interacts with the surrounding environment. It provides the first layer of defence against pathogens and herbivores in addition to prevention of water, gas and solute loss (Eigenbroade
et al., 1995; Kolattukudy, 1996; Koteyeva, 2005). For cuticle-degrading pathogens, such as *Fusarium solani pisi*, *Botrytis cinerea*, *Magnaporthe oryzae* etc. cuticle and waxes play an important role in initiating pathogen development on the plant surface (Lin and Kolattukudy, 1978; Chassot et al., 2008; Gilbert et al., 1996). Changes in cuticle composition can affect fungal development and prevent penetration into the plant (Chassot et al., 2007). Additionally, FA levels can change during pathogenesis. Increased levels of 18:3 and reduced levels of 18:2 were seen 12 hours after *Phytophthora sojae* application to parsley cells. Surprisingly, no significant changes were detected in C16 fatty acids (Kirsch et al., 1997). Changes in the levels of linolenic acid (18:3) were also observed in other species, such as *Phaseolus vulgaris*, *Poa pratensis* and *Eschscholtza californica* upon attack by diverse rust species (*Puccinia spp.*; Hope and Heitefuss, 1974; Losel, 1978; Muller et al, 1993).

Jasmonate plays an important role in resistance to necrotrophic fungi; plants lacking TUFAs were shown to have strong susceptibility to these pathogens (Kachroo et al., 2001; Yaeno et al., 2004). The role of DUFAs in pathogenesis has been described only in a few studies. It was shown that 18:2 can be used as a precursor for antifungal dienes such as Z,Z-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene that is induced in avocado fruits upon *Collectotrichum gloeosporioides* infection (Madi et al., 2003). Another role of linoleic acid (18:2) is to promote morphological differentiation in *Aspergillus nidulans*, *A.*
flavus and A. parasiticus (Calvo et al., 1999), as well as sporogenesis in
Aspergillus tomato, Sclerotinia fructicola and Neurospora crassa (Katayama
and Marumo, 1978; Nukima et al., 1981). In summary, PUFAs have diverse
roles in pathogenesis. Additional discoveries in this area are likely.

I.5 Main goals of this thesis

The purpose of this research was to examine the role of DUFAs in defence and
development at the seedling stage, using Arabidopsis fad2 and fad6 mutants.
Early in this thesis, I characterized fad mutants at different developmental
stages from 3-day-old seedlings to 3-week-old rosettes. This was followed by
analysis of PUFAs on development in fad2 mutants and its involvement in the
adaptation to salt stress. In the last chapter of this thesis, I try to understand the
role of DUFAs in resistance to the necrotrophic pathogen Botrytis cinerea.
Application of this pathogen on wild type and fad seedlings showed resistance
of fad mutants compared to WT plants. The next and the biggest goal of this
work was to understand what leads to B. cinerea resistance. For this purpose
multiple experimental approaches were used, such as atomic force microscopy,
transmission electron microscopy, biochemical and molecular biological
approaches.


labeling of *Arabidopsis* cell suspension cultures: implications for models of lipid export from plastids. *Plant Physiology.* 158:01–611.


Chapter II: Characterization of *Arabidopsis* PUFA-deficient *fad* mutants
II.1. General characterization of fad mutants.

Fatty acids are one of the major storage forms of carbon in living organisms, precursors for some plant hormones such as jasmonate, and major components of membrane bilayers. Polyunsaturated fatty acids are highly abundant in plants: diunsaturated and triunsaturated fatty acids account for more than 70% of fatty acids in leaf cells and 50 to 70% in roots (Harwood, 1996). Fatty acid biosynthesis starts in the plastids with synthesis of 16:0 and 18:1 acyl groups. Higher unsaturated forms are made by fatty acid desaturases localized on the endoplasmic reticulum or in plastids (Browse et al., 1991; Heinz, 1993, Lou et al., 2014). Synthesis of linoleic acid (18:2) from oleic acid (18:1) requires two enzymes: FAD2 localized on the ER, and FAD6 in plastids (Miquel et al., 1993; Browse et al., 1989). At the seedling stage, fad2 and fad6 single mutants have a similar phenotype as wild type (WT).

The conversion of linoleic acid (18:2) to linolenic acid (18:3) requires 3 enzymes: FAD3 localized on the ER, FAD7 and FAD8 in plastids. The fad3-2 fad7-2 fad8 (fad trip) mutants have lighter leaf coloration and cannot produce seeds. These plants are able to grow in low-stress conditions but cannot be maintained at low temperatures (Routaboul et al., 2000). Remarkably, TUFAs do not play a crucial role in photosynthesis (McConn et al., 1996). Plant that is almost fully deficient in both di- and triunsaturated FA (fad2-2 fad6) cannot
grow on soil, but can be propagated on 2% sucrose media (McConn et al., 1998). The fad2-2 fad6 double mutants have a chlorotic and dwarfed phenotype, but are able to produce fertile seeds under these conditions. The aim of this chapter is to understand the role of DUFAs at different stages of plant development.

To obtain a mutant capable of growth on soil we investigated three mutant alleles of FAD2 and one allele of the FAD6 gene. The fad2-1 mutant was produced by ethyl methanesulfonate (EMS) mutagenesis and has a point mutation in the second exon, leading to an amino acid change from alanine to threonine (A->T). This is one amino acid before the first conserved His-Box (Zhang et al., 2012) that is involved in iron coordination complex at the active site together with two other His-Boxes (Shanklin et al, 1994). The fad2-2 allele (also obtained by EMS mutagenesis (James et al., 1990)) has a point mutation in the second exon, leading to an amino acid change from serine to phenyalanine (S->F), five amino acids before the second conserved region. This mutation was identified in this work. The fad2-3 mutant has a T-DNA insertion in the second exon, obtained from Arabidopsis Biological Resource Centre (SK18137). The fad6 mutant has a missense mutation in the fourth exon, leading to an amino acid change from glycine to alanine (G->A) in a membrane trans region (Zhang et al., 2009). All the mutations were confirmed by DNA sequencing and checked for homozygosity by PCR.
RT-PCR revealed similar to wild type levels of $FAD2$ and $FAD6$ gene expression in $fad2$-1, $fad2$-2 and $fad6$ mutants, whereas no expression of $FAD2$ was detected of the $fad2$-3 allele, consistent with T-DNA insertion. The $UBC21$ (At5g25760) transcript was used as a control (Fig. 2.1). Presence of three different $FAD2$ alleles allowed us to exclude the $fad2$-2 mutant from further work, due to its intermediate phenotype revealed in crosses of $fad2$-1 $fad6$; $fad2$-2 $fad6$ and $fad2$-3 $fad6$ double mutants and focus on $fad2$-1 and $fad2$-3 alleles of $FAD2$ gene.

![Figure 2.1. $FAD2$ and $FAD6$ transcript expression in 3-day-old seedlings. $UBC21$ was used as a loading control.](image)

PUFAs are one of the major components of membrane bilayers; deficiency in them might therefore strongly affect cell viability during plant development. To see if reduced levels of DUFAs affect plant morphology we compared 4-week-old mutant rosettes under two different light conditions. This revealed phenotypic difference between WT and $fad2$ mutants (Fig.2.2).
Figure 2.2. Developmental phenotype of diunsaturated fatty acid mutants. (A) 4-week-old rosettes grown in 9 h light / 15 h dark photoperiod. Scale bar 1 mm. (B) 6-week-old flowering plants growing in continuous light.

Figure 2.2, A shows that fad2 mutants grown under short day conditions were found to have shorter petioles than WT with darker and rounded leaves. fad6 mutants more resemble the WT phenotype with an exception of lighter leaf color and smaller rosette size. During flowering the main difference was observed in fad2-3 mutants that have a bushy phenotype, most probably due to lack of apical dominance. The remaining fad mutants were morphologically similar to the WT (Fig. 2.2, B).
To eliminate all detectable DUFAs and TUFAs from plants, two double mutants: \textit{fad2-1 fad6} and \textit{fad2-3 fad6} were generated in this work. We obtained similar results to \textit{fad2-2 fad6} plants (McConn \textit{et al.}, 1998). The \textit{fad2-3 fad6} double mutants had a dwarf phenotype, chlorotic leaves and did not produce seeds (Fig.2.3, A). The double mutants with the weaker allele (\textit{fad2-1}) were able to growth on soil, seed production was delayed to 2 extra months (Fig.2.3, B). The size of these plants was significantly smaller compared to the WT and the double mutant had chlorotic leaves. Under short day conditions the \textit{fad2-1 fad6} double mutants produced larger rosettes compared to those grown in 24 h light conditions.

![Figure 2.3. Monounsaturated plants. 4-week-old plants grown on (A) 2% sucrose media in Magenta boxes and (B) on soil. In both cases the same 14 h light, 10 h dark conditions were used.](image)

The \textit{fad2-2 fad6} and \textit{fad2-1 fad6} double mutants are strongly affected in development (McConn \textit{et al.}, 1998; this work), whereas the single mutants have morphologies similar to WT (Zhang \textit{et al.}, 2009; 2012; this work). That brought us to the idea of compensatory role between \textit{FAD2} and \textit{FAD6} enzymes. The aim was to understand if this mechanism was controlled at the
translational or transcriptional levels. For this purpose, we conducted qPCR analysis of *FAD2* and *FAD6* transcript levels on 7-day-old seedlings grown on agar media (Fig.2.4).

Figure 2.4. Relative expression of *FAD2* and *FAD6* genes in 7-day-old *fad* single mutants. *FAD2* and *FAD6* transcriptional levels were normalized to those of *UBC21* in WT seedlings. Error bars indicate a mean of ± SD. Bars represent the means of 4 biological replicates; each contains a pool of ~ 40 seedlings. Statistical significance in pair-wise comparison was evaluated by Student’s test, where * * * \( p \leq 0.05 \), *** \( p \leq 0.001 \).

The results obtained did not show significant upregulation of the *FAD6* gene in *fad2* mutants or of the *FAD2* gene in the *fad6* background. Suggesting that, compensatory mechanisms make take place at the post-transcriptional levels.

II.2. Changes in fatty acid composition during development.

Fatty acid profiling via gas chromatography - mass spectrometry (GCMS) was performed to monitor changes in the levels of PUFAs in *fad* mutants at early developmental stages. The time course from 3- to 7-day-old was chosen due to the known difference in the expression of *FAD2* and *FAD6* genes in this period
(Zhang et al., 2009; Zhang et al., 2012). Our results revealed similar FA profiling for seeds and 3-day-old seedlings (Fig.2.5) and further on changes during the first week of seedling establishment in the WT and mutants (Fig.2.6).

Figure 2.5. Fatty acid composition in fad mutants. *Arabidopsis* (A), seeds; (B), 3-day-old seedlings were used to perform fatty acid profiling. Error bars indicate a mean of ± SD. Bars represent the means of 3 - 6 biological replicates; each contains a pool of ~300 seeds or ~120 seedlings.
Figure 2.6. Fatty acid composition during the first week of seedling establishment. (A), WT; (B), fad6; (C), fad2-1; (D), fad2-3 seedlings were used for this analysis. Error bars indicate a mean of ± SD, bars represent the means of 4 - 6 biological replicates; each contained a pool of ≈ 40 seedlings.

Significant changes in FA composition occurred in the WT seedlings between the third and sixth day after germination. There was an increase in the levels of 16:3 from 2% to 13.5% and a decrease in 18:2 levels from 34% to 15% (Fig.2.6, A). The fad6 mutants showed similar changes in the levels of 18:2. However, fad6 plant reveals changes in C16 fatty acid composition, this mutant has no 16:2 FA, and it is associated with the over accumulation of 16:1 which increases from 1% to 10% of extracted fatty acids by the end of the 6th day (Fig.2.5, B). The fad2 single mutants have a similar to WT composition of
C16 FAs, but differ to WT C18 FA compositions due to strongly reduced levels of 18:2. This correlated with over-accumulation of 18:1 and 10% higher levels of 18:3 compared to the WT. Surprisingly, in fad2-3 mutants 18:3 levels stabilized on the 6th day after germination and in the fad2-1 on the 5th day (Fig.2.5, C-D). Unexpectedly, FA composition did not change strongly during the 7-day to 4-week stage in any of the genotypes analyzed (Fig 2.5, E). The fad2-1 fad6 double mutant had a consistent FA profile at all analyzed stages. The major FA components in fad2-1 fad6 mutants are 18:1 and 16:1 with an almost complete absence of 16:2, 18:2 and 16:3,18:3 FAs. Based on these results, fad2-1 fad6 can be considered as a monounsaturated plant.

II.3. PUFAs as major precursors of malondialdehyde.

Reactive oxygen species are the main source of non-enzymatic lipid oxidation, a process where polyunsaturated fatty acids (PUFAs) present in membrane lipid bilayers get oxidized and that leads to aldehyde formation (Esterbauer et al., 1991). By-products of lipid oxidation are of great interest, because of their association with mammalian diseases such as cancer, aging, arthritis, Alzheimer’s disease, atherosclerosis and alcoholic liver disease (Ramos et al., 1995; Esterbauer et al., 1991; Spiteller, 1996; Montine et al., 2002; Levonen et al., 2014; Han et al., 2016). According to a recent hypothesis, PUFAs work as
a buffering system for ROS and the by-products of lipid oxidation induce gene expression in response to stresses, leading to cell protection (Mene-Saffrane et al., 2007, Schmid-Siegert et al., 2016).

Malondialdehyde is one of the most studied products of lipid oxidation that is commonly believed to be a genotoxic and proteotoxic molecule (Marnett, 1999) generated from oxidative degradation of PUFAs with more than two methylene-interrupted double bonds (Pryor and Stanley, 1975). MDA can be present in two forms, depending on its pH: enolate, with a low chemical reactivity at cytosolic pH and a highly reactive protonated form at acidic pH that can lead to cell damage (Sawicki et al., 1963). MDA pools in Arabidopsis seedling can be visualized by 2-thiobarbituric acid (TBA) staining, which forms MDA(TBA)₂ adducts that can be detected by their fluorescence at 555±15 nm (Mène-Saffrané et al., 2007) and quantified via GC/MS using the cognate MDA internal standard D₂-MDA (Yeo et al., 1994; Liu et al., 1997, Weber et al., 2004).

The main sources of MDA in mammalian tissues are arachidonic and docosahexaenoic PUFAs, oleic and elaidic acids produce much lower amounts of MDA (Pryor and Stanley, 1975; Liu et al., 1997). In rodents the highest concentration of MDA was found in kidney and heart (Kelley et al., 2006; Kumasaka et al., 2007). This tightly correlates with the ratio of PUFAs in
kidney and heart (around 45-50%) compared to 25-30% in liver and brain (Spector, 2001).

Due to the availability of lipid biosynthesis mutants, *Arabidopsis* is a widely used model organism in lipid oxidation studies. The highest concentration of MDA in *Arabidopsis* was found in the leaves (Muckenschnabel et al., 2002), where the major pool (76% of total MDA) originates from $\alpha$-linolenic fatty acid (18:3; Weber *et al*., 2004). The sources of MDA in seedlings differ from those in adult plants and remain unknown (Schmid-Siegert *et al*., 2012). In this case di- and monounsaturated FAs could be the most likely source for MDA generation, since their abundance in shoots of *Arabidopsis* seedlings.

The main question of this part of the thesis was to find the major source of MDA in *Arabidopsis* seedlings, its localization and potential role in defense responses. To answer these questions we used 3- and 7-day-old *Arabidopsis* seedlings and tested how changes in fatty acid levels affect MDA pools.

According to earlier work (Schmid-Siegert *et al*., 2012), pools of MDA are localized in the meristematic zones of *Arabidopsis* seedlings. However, we found this tissue unsuitable, due to its small size, for biochemical analysis. To check this hypothesis, we used 3-day-old roots of *Brassica oleracea* (that is known to be closest relative to *Arabidopsis*) to conduct quantitative MDA
measurements of the tip and remaining part of the root (Fig.2.7, B). As a control we made fatty acid measurements from the root tip and remaining parts of the seedlings’ roots (Fig.2.7, A).

![Graph A](image1)

**A**

Figure 2.7. Biochemical characterization of *Brassica oleracea* roots. (A) Fatty acid composition of 3-day-old entire roots and separated root tips. This experiment includes 6 independent replicates. (B) Total MDA levels in 3-day-old tips and remaining part of the root. Error bars indicate a mean of ± SD, bars represent the means of 4-6 biological replicates; each containing a pool of ≈ 60 roots and 120 root rips. All tissue material was collected in liquid nitrogen to prevent oxidation of FAs.

The results obtained did not confirm the hypothesis of high MDA concentration in the meristematic regions. On the contrary, we found no difference in FA composition and MDA levels of the tip and entire *Brassica’s* root. These results suggest that high concentration of MDA detected in the
meristematic regions of *Arabidopsis* seedlings previously, could be caused by defects in root tip permeability.

*fad* mutants were used to estimate which polyunsaturated fatty acids can act as MDA precursors. To visualize MDA pools within the plant 2-thiobarbituric acid solution (35 mM TBA, for 60 min) was used (Mene-Saffrance *et al.*, 2007). Signal intensity was shown to correlate with MDA levels; the more MDA the higher fluorescence. TBA staining of the 3-day-old *Arabidopsis* roots revealed stronger fluorescence in the root tips of WT and *fad6* seedlings compared to *fad2* mutants (Fig. 2.8).

![Figure 2.8. In situ malondialdehyde (MDA) detection. 3-day-old *Arabidopsis* roots were incubated in 35 mM TBA solution for 60 min and visualized by confocal microscopy with excitation at 555 ± 15 nm.](image)
To confirm these results we conducted quantitative MDA measurements via GCMS. This revealed a decrease in MDA levels of \textit{fad2} mutants from 4.5 to 3 ng/mg dry weight (D.W.) in whole 3-day-old seedlings. We found no differences between \textit{fad6} and WT seedlings (Fig.2.9, A) and a strong MDA decrease in the \textit{fad2-1 fad6} double mutant – 1.19 ng/mg D.W. (below limit of quantification (LOQ)) relative to 4.5 ng/mg D.W. in the WT (Fig.2.9, B). MDA measurements in 3-day old roots showed stronger decreases in \textit{fad2} mutants that could be explained by overall lower levels of DUFAs, due to the absence of \textit{FAD6} activity in this tissue (Fig.2.9, C).
Figure 2.9. Total MDA levels in 3-day-old seedlings. MDA measurements were conducted on (A-B) whole seedlings and (C) roots of WT, fad2 and fad6 mutants. Each experiment was conducted 3 times with 4 independent replicates per each genotype. The limit of quantification (LOQ) is shown as the black line. Statistical significance in pair-wise comparison was evaluated by Student’s test, where ** $p \geq 0.01$; *** $p \leq 0.001$.

Due to massive changes in fatty acid composition during seedling establishment, we decided to make MDA measurements at the stage of 7-day-old seedlings (Fig. 2.10)

Figure 2.10. Total MDA levels in 7-day-old seedlings. Each experiment was conducted 3 times with 4 independent replicates per each genotype. The limit of quantification (LOQ) is shown as the black line. Statistical significance in pair-wise comparison of mutant vs WT was evaluated by Student’s test, where ** $p \geq 0.01$. 
Exogenous MDA is known to activate defence and stress-related genes upon stresses (Weber et al., 2004). MDA induction can be caused by jasmonic and salicylic acid (SA) treatment in Arabidopsis seedlings (Schmid-Siegert et al., 2012). JA was also shown to increase degradation of Rubisco that leads to the rapid chlorophyll loss and oxidative stress in barley leaves (Jung, 2004). In this work we extended the results of Schmid-Siegert et al. (2012) on MDA induction upon external hormonal treatment. JA application on 3-day-old seedlings leads to MDA induction in WT and mutants. There was a tight correlation between the amount of PUFAs and MDA increase upon JA treatment (Fig. 2.11).

![Figure 2.11: MDA pools in 3-day-old seedlings after 50 µM JA treatment, 20 hours post-application. Error bars indicate a mean of ± SD. Each experiment was conducted 3 times with 4 independent replicates per each genotype. One biological replicate consisted of a pool of ≈ 300 -400 seedlings. The limit of quantification is shown as the balck line. Statistical significance in pair-wise comparison was evaluated by Student’s test, where * $p \geq 0.05$; ** $p \geq 0.01$; *** $p \leq 0.001$.](image-url)
II.4. Discussion.

PUFAs are major components of lipid bilayers and, in plants; the lack of both di- and triunsaturated FAs is lethal (McConn et al., 1998). However, plants with only one type of PUFAs (di- or triunsaturated FAs) can grow under laboratory conditions. This leads to the conclusion that DUFAs and TUFAs may have similar overall roles in development. Nevertheless, TUFAs have at least one unique role that cannot be fulfilled by DUFAs. TUFAS are the only precursors for JA synthesis (Schaller et al., 2009). One of the questions addressed in this work was to look for specific roles of DUFAs in plant development by analysing single and double mutants of FAD2 and FAD6 genes.

The rosette phenotypes of 4-week old fad2 and fad6 mutants differed from that of the WT. The fad2 mutants were slightly smaller, with shorter petioles and rounded leaves, whereas fad6 mutants are similar in size to the WT, but with lighter leaf coloration, due to the dysfunction of photoinhibitory machinery (Perumal et al., 2002). The fad2-3 mutants had bushy morphology during flowering period. Generation of fad2-1 fad6 double mutants, confirmed the overall importance of DUFA and TUFAs in plant development. However, since these plants remained fertile they must produce very low levels of TUFAs that are involved in promotion of male fertility in Arabidopsis plants.
That is why we assume that the \textit{fad2-1} allele is likely to be hypomorphic. Even a small amount of PUFA can allow the plant to survive under low stress conditions. Chlorotic leaves are the result of strong inhibition of chlorophyll accumulation. This was already described in the \textit{fad2-2 fad6} mutant, where the leaf chlorophyll content was 11% of WT levels. Surprisingly, such a strong reduction did not affect the entire photosynthetic complex, but mainly the PSII quantum yield - parameter that best characterizes photosynthetic efficiency due to its link with downstream process (PSI and CO$_2$ assimilation), was 50% less efficient in the \textit{fad2-2 fad6} mutant compared to the WT (McConn \textit{et al.}, 1998). Such a strong defect in photosynthetic activity of \textit{fad2-2 fad6} mutant could be explained by the deficiency in PUFAs that work as buffers for ROS, molecules that are generated during the photosynthesis process and lead to oxidative stress.

\textbf{Fatty acid composition changes during the early steps of plant development.} Strong changes in fatty acid profiles were observed during the transition phase from germination to the appearance of the first true leaves. Prior to this, during the first 3-days after germination FA composition remained relatively stable but changed within the following days. To summarize, the 3-day-old \textit{fad2} seedlings display an over accumulation of 18:1 FA whereas at the 7-day old stage levels of 18:1 decreased from approximately 80 to 5%, but the levels of 18:3 increased approximately from 10 to 65%.
Changes in FA composition could be explained by seedling transition from semi-autotrophic growth at 3-day-old stage to autotrophic that takes place at the end of the first week. Due to the absence of fully established photosynthetic machinery and the use of reserves in the form of oil bodies plants obtain major source of the energy, such as triacyl glycerols from the seed. However, at the end of the first week, seedlings have fully expended cotyledons that can perform photosynthesis and generate needed amount of energy to promote plant growth and \textit{de novo} fatty acid synthesis (Frey-Wyssling \textit{et al.}, 1963; Murphy, 1990).

**Diunsaturated FAs are second major source of MDA in \textit{Arabidopsis} seedlings.** \textit{In vitro} studies suggest that polyunsaturated fatty acids are the major source of MDA and recent discoveries revealed role of TUFAs as a major MDA source in the shoots of 3-day-old seedlings, but not in the roots (Schmid-Siegert \textit{et al.}, 2012). This can be explained by abundance of PUFAs in different \textit{Arabidopsis} tissues: in roots DUFAs and TUFAs are equally abundant, however in shoots TUFAs are the dominating FAs. Quantitative MDA measurements in seedlings support correlation in the amount of di- and triunsaturated fatty acids and MDA levels (Fig.2.6; 2.9; 2.10).

To estimate the sensitivity to oxidation \textit{in vivo} of different PUFAs in 3-day-old seedlings, we combined data obtained from \textit{fad2} and \textit{fad2-1 fa6} mutants with
data from the fad3 fad7-2 fad8 mutant that was published previously (Mene-Saffrane et al., 2007; Schmid-Siegert et al., 2012). Involvment of different PUFAs in MDA generation was estimated by the following principle: percentage of MDA generated in seedlings of fad2-1 fad6 and roots of fad2-3 mutants minus percentage of MDA generated in roots and whole seedlings of fad trip mutants (Schmid-Siegert et al., 2012) (Fig.2.12).

Figure 2.12. Estimation of the contribution of different PUFAs to MDA generation in Arabidopsis seedlings.

According to our results, PUFAs are the major source of MDA in Arabidopsis seedlings. Surprisingly the effect of different PUFAs on MDA generation appears to be tissue specific. Despite the fact that shoots and roots contain similarly high levels of 18:2 and 18:3 the main source of MDA in the shoots are triunsaturated fatty acids (16:3, 18:3), whereas in roots it is diunsaturated fatty acids (16:2, 18:2).
MDA induction in seedlings can be caused by stresses such as JA, SA treatment and wounding (Schmid-Siegert et al., 2012; 2016). To investigate if the basal and induced MDA pools originate from the same source, oleate desaturase mutants were treated with jasmonic acid. This treatment is known to stimulate MDA production in roots (Schmid-Siegert et al., 2012). The results obtained confirmed that PUFAs are the major source of basal and induced MDA; the lower amount of PUFAs the less MDA can be generated under control and stress conditions. Reduced levels of PUFAs in plant can lead to over accumulation of ROS and permanent oxidative stress that will diminish plant growth.

II.5. Experimental procedures.

Plant growth conditions, genotypes and chemicals - Wild-type Arabidopsis (WT Columbia background) was used. The following alleles in the Columbia background were received from the Arabidopsis Biological Resource Centre (ABRC): fad2-1 (CS8041), fad2-3 (SK18137), fad6 (CS207), J. Browse kindly provided fad2-2 and fad2-2 fad6 alleles and fad2-1 fad6 and fad2-3 fad6 double mutants obtained in this work, Brassica oleracea var. Mezzo Nano was from Samen Mauser (Winterthur, Switzerland). Seeds were stratified for 2 days at 4 °C and then grown at 21 °C under 100 µE m⁻² s⁻¹ of light with photoperiod depending on the application (seedlings: 14 h light, 10 h dark (long days); on
soil for seed propagation: 24 h light (continuous days) or experiments with adult plants at: 9 h light, 15 h dark (short day)). Seedlings were grown on half-strength Murashige and Skoog solid media (half-strength MS, 2.15 g/L, pH 5.7; Duhefa Biochemie, Haarlem, The Nederlands) supplemented with 0.5 g/L of MES hydrate (Sigma, Buchs, Switzerland) and 0.7% agar.

Gene expression in fad2-1, fad2-2, fad2-3- and fad6 mutants – Each biological replicate consisted of ≈ 40 seedlings. Collected tissue was immediately frozen in liquid N₂. RNA isolation and RT-PCR of UBC21 (At5g25760) were conducted as described (Park et al., 2002). For the FAD2 gene the following primer pair was used: forward - GCCATTCCCCCATCTGACCACC and reverse - CCAACAAAAACAGCGATGAGA for FAD6: forward - GAGGTGAGGGCTCTTTCACAG and reverse - AGAAGGCTCATCGCTTGGAAA. RT-PCR was performed with GoTaq DNA polymerase (Promega, Dubendorf, Switzerland) as indicated by the manufacturer. For the FAD2 gene the following qPCR primer pairs were used: forward - GCTCCATCTCCAGAAACATGG and reverse - TTGAGCGTTTGAAACAATGC for FAD6: forward - GAGGTGAGGGCTCTTTCACAG and reverse - TCACAAAGAACCCTGTAATTG.

Fatty acid composition analysis – 3-day-old seedlings and roots were collected
(≈ 30 mg) and placed in 15 ml Pyrex SVL capped test tubes (Milian, Switzerland). 1ml of 2.5 % H₂SO₄ solution, in methanol was added to the samples. Samples were incubated for 90 min at 80 °C, 1.5 ml 0.9% NaCl and 1 ml of hexane (≥97.0%, Sigma-Aldrich, Buchs, Switzerland) were added. The solution was vortexed briefly (2 sec) then centrifuged for 5 min at 1000 rpm. 250 µl of the upper phase containing fatty acid methyl esters were collected. 1µl of a 1:5 dilution was analyzed by GCMS (initial T = 150°C for 3 min, with a following increase from 150°C to 200°C).

Gas chromatography/Mass Spectrometry – 3-day-old seedlings or roots (≈ 150 mg) were harvested in liquid nitrogen and ground for 15 sec at 3000 rpm/sec in TissueLyser II (Quiagen, Hombrechtikon, Switzerland) MDA levels were measured by a gas chromatography/ mass spectrometry with D₂-MDA internal standard generated from (2D₂)-1,1,3,3-tetraethoxypropan as described (Weber et al., 2004)

2-Thiobarbituric acid staining – 3-day-old seedlings were transferred in 35 mM TBA or in 35 mM trichloroacetic acid solution as a negative control and incubated for 60 min at 37 °C. MDA-TBA adducts were detected by excitation 515nm and emission: 555±15 nm.

Jasmonic acid seedling treatment – At the end of day three, seedlings were
transferred in 2 different plates with: control (half-strength Murashige and Skoog solid media with 0.5 g/L of MES hydrate dissolved in double distillated H₂O) and jasmonic acid (50 µM) dissolved in control solution. Plates were incubated for 18 hours (long days conditions) before the tissue collection and following GC/MS analysis.

Statistical analysis - Statistical significance in pair-wise comparison was evaluated by Student’s test, where * p ≤ 0.05; ** p ≤ 0.01; ***p ≤ 0.001.


Chapter III: The effect of *Arabidopsis* DUFA-deficient *fad2* mutants on seedling growth

An effect of \textit{fad2} mutants on \textit{Arabidopsis} root growth in the presence of sodium chloride has already been reported (Zhang \textit{et al.}, 2012). Here we focussed further on the effect of \textit{fad2} mutation on root development. We were able to extend the results of Zhang \textit{et al.} (2012) and to provide insights on the growth differences of wild type and \textit{fad2} roots.

Roots are important not only for providing nutrients and water to shoots, but also for detecting and adapting to environmental changes (Tsukagoshi, 2016). The \textit{Arabidopsis} root (which is typical for dicotyledonous plants) can be divided longitudinally into four regions: apical and basal meristem, elongation zone and differentiation zone (Fig. 3.1).

![Figure 3.1. Organization of the \textit{Arabidopsis} root. \textbf{Left}: Longitudinal cross section. \textbf{Right}: Transversal section. Adapted from De Smet \textit{et al.} (2015).]
Apical and basal meristematic zones undergo active division, whereas cellular elongation and partial differentiation strictly take place in the elongation zone (Beemster et al., 1998). The final step of root tissue establishment together with lateral root formation takes place in the differentiation zone (Mendrinna and Persson, 2015; Cano-Delgado et al., 2010).

Root development is tightly regulated by plant hormones such as auxin, cytokinin, ethylene, gibberellins, jasmonate, brassinosteroids and abscisic acid that can induce changes in the reactive oxygen species (ROS) levels (Vanstraelen and Benkova, 2012; Yoshimitsu et al., 2011; Bu et al., 2008; Guzman et al., 1990; Wang et al., 2011; Claeys et al., 2013; Laplaze et al., 2007; De Smet et al., 2015). ROS that are strongly abundant in plants were also shown to play an important role in regulation of the root growth (O’Brien et al., 2012). Hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (OH) and superoxide (O$_2^-$) are the major ROS in plant that are known to be by-products of oxygen metabolism (Wojciechowski, 2014; Fridovich, 1986). During photosynthesis, respiration and pathogen attack oxygen undergoes a series of univalent reductions that requires acceptance of one electron at a time by the oxygen atom. The outcome of four univalent reductions is synthesis of two water molecules and multiple ROS (Fig.3.2; Triantaphylides and Havaux, 2009; Triantaphylides et al., 2008, Halliwell, 2006; Krieger-Liszkay, 2005, Fukai and Ushio-Fukai, 2011).
In general, ROS in low concentration work as regulatory compounds, whereas at high concentration they lead to arrested growth and become toxic for the plant (Swanson et al., 2010; Choudhury et al., 2013). In Arabidopsis roots, O$_2^-$ is generally required for cellular proliferation, whereas H$_2$O$_2$ plays role in cell differentiation and in the lignification process (Tsukagoshi et al., 2010). \textit{\'OH} is thought to be involved in cell wall loosening and cell expansion (Liszkay et al., 2004).

At the molecular level, ROS such as \textit{\'OH} or $^1$O$_2$ (singlet oxygen) are the main mediators of non-enzymatic lipid oxidation that leads to generation of fatty acid–derived aldehydes (Esterbauer et al., 1991). The end-products of lipid oxidation are of great interest due to their association with diseases such as cancer, arthritis, Alzheimer’s disease and atherosclerosis (Spiteller, 1996; Montine et al., 2002). Recent studies on plants have led to the hypothesis that

Figure 3.2. Reduction of oxygen to water. Adapted from Wojciechowski (2014). This scheme does not include singlet oxygen (O$_2$).
polyunsaturated fatty acids may work as a buffering system for ROS (Mene-Saffrane et al., 2009, Farmer and Mueller, 2013; Schmid-Siegert, et al., 2016). Another role of fatty acid fragments produced as by-products of lipid oxidation is modulation of gene expression in response to stresses (Weber et al., 2004). The current findings are mainly based on the aerial tissues and so far, only a small amount of work has been conducted on the role of PUFAs in root stress responses. For example, there is evidence for the involvement of diunsaturated fatty acids in adaptation to salinity stress (Zhang et al., 2009; Zhang et al., 2012). External PUFAs application was shown to decrease ROS production in stimulated 264.7 human macrophages that leads to protection of innate lymphoid cells (Ambrozova et al., 2010). These examples confirm the potentially important role of DUFAs in stress responses. The purpose of this work was to understand the role of DUFAs in Arabidopsis roots during control growth conditions and under stresses.

In order to address these questions we used Arabidopsis WT, oleate desaturase mutants and seedlings deficient in triunsaturated fatty acids (fad3-2 fad7-2 fad8). Root growth was the first parameter investigated. The length of Arabidopsis roots was measured at 7-days post germination (Fig.3.3).
Figure 3.3. Root length analysis of 7-day-old *Arabidopsis* seedlings. (A) Image of the WT and *fad* mutants growing vertically on half MS plates. (B) Quantitative analysis of root length. Error bars indicate a mean of ± SD, bars represent the means of three independent experiments; each contains a pool of ~ 60 seedlings. Letters indicate statistically significant differences between pairs as determined by Tukey’s HSD test (p < 0.05).

Both *fad2* mutants caused significant reduction in root length (Fig. 3.3). This phenotype is not caused by deficiency in triunsaturated fatty acids or jasmonate, since no significant difference was observed between WT and *fad trip* roots.
To understand what affects development of *fad2* mutants, we conducted fatty acid analysis on 7-day-old seedling roots (Fig. 3.4).

![Figure 3.4. Fatty acid composition of 7-day-old roots. Bars represent the means of six biological replicates; each contains a pool of ~120 seedlings roots. Statistical significance in pair-wise comparison WT vs mutant was evaluated by Student’s test, where ** \( p \leq 0.01 \); *** \( p \leq 0.001 \). ND – not detected.](image)

These results showed changes in the levels of C18 FAs in *fad2* mutants, revealing strong reduction in the levels of 18:2 and 18:3. This led to the idea that *fad2* plants may be under constant oxidative stress, due to the absence of PUFAs (Mene-Saffrane *et al.*, 2009, Schmid-Siegert, *et al.*, 2016). To test this hypothesis, we performed malondialdehyde measurements using GCMS (Fig. 3.5). MDA is a marker of singlet oxygen or free radical-catalysed peroxidation in *Arabidopsis* that is synthetized mainly from PUFAs (Janero *et al.*, 1990, Schmid-Siegert, *et al.*, 2012; 2016). Earlier studies showed that reduced levels of MDA in *fad trip* mutant leaves correlated with higher levels of ROS in these
tissues (Mene-Saffrane et al., 2007; Schmid-Siegert, et al., 2012). Based on these studies we can predict that reduced levels of PUFAs can lead to ROS accumulation.

Figure 3.5. MDA levels in 7-day-old Arabidopsis roots. Bars represent the means of 4 biological replicates; each contains a pool of ~ 480 seedlings roots. The limit of quantification (LOQ) is shown as a black line. Statistical significance in pair-wise comparison was evaluated by Student’s test, where *** \( p \leq 0.001 \).

In 7-day old roots of WT plants, MDA levels are almost three times higher than in fad2 mutants. These results are consistent with the hypothesis that PUFAs (and in this case, DUFAs) are the major source of MDA in the root. For the next step, we tested ROS levels in the roots, hypothesizing that reduced MDA levels lead to a more oxidative environment in the cells (Fig.3.6). The ROS-ID total ROS/Superoxide detection kit from Enzo (Lausanne, Switzerland; Fig.3.6, A) and 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA) overall ROS detection solutions (Fig.3.6, B) were used to perform root staining.
Figure 3.6. ROS staining of 7-day-old roots. (A) Seedlings treated with ROS-ID total ROS/Superoxide detection kit were vacuum infiltrated for 15 min. Hydrogen peroxide, peroxynitrite and hydroxyl radicals were visualized at wavelengths 490 nm /525 nm excitation/emission (Ex/Em) and labelled ‘green stain’, superoxide ‘O2’ at 550 nm /620 nm, ‘red stain’ (Ex/Em). Each photo is representative of the six biological replicates. Scale bar 1 mm. (B) 15 µM of H2DCFDA ROS detection solution was used in living roots of Arabidopsis seedlings visualized at wavelengths 495 nm /527 nm (Ex/Em). Arrows indicate differences in fluorescence between WT and fad2 mutants.

Figure 3.6, A. highlights the difference between ROS fluorescence in WT and fad2 mutants. Strong green fluorescence was observed in meristematic and differentiation zones of fad2 mutants, but a difference in superoxide (red staining) was only observed in case of fad2-3 mutants relative to the WT. The
total ROS-ID solution also indicated increased fluorescence in differentiation zone of both fad2 mutants’ roots (Fig.3.6, B).

The next aim of this work was to identify which cell types were affected by low DUFA levels in the roots of fad2 mutants. For this purpose, we measured root meristem cell number (cells from quiescent center to the elongational zone) and cortex cells (Fig.3.7).
Figure 3.7. Morphological analysis of 7-day-old WT and fad2 roots. (A-B) Primary root meristem visualization and cell number quantification. (C) Cortex cell visualization, the arrow indicates cells of interest. (D) Measurements of cortex cell lengths. Bars represent the means of 10 roots (± SD). Letters indicate statistically significant differences between pairs as determined by Tukey’s HSD test (p < 0.05).

Analysis of the meristematic and elongation zones of WT and fad2 mutants revealed significant changes in both regions. Interestingly, stronger differences were observed in meristematic zone where cell number was reduced by 20% in fad2-1 and 39% in fad2-3 mutants. The elongation zone was not significantly affected in fad2-1 mutants compared to the WT plants, but showed 18% reduction in fad2-3 seedlings. In conclusion, reduced root length in fad2-1...
mutants is caused primarily by a reduced number of meristematic cells. The $fad2\text{-}3$ mutants had stronger decrease in root length compared to WT and $fad2\text{-}1$ seedlings. This phenotype could be explained by reduction in both the number of meristematic cells and a reduction of cortex cell length that is caused by deficiency in PUFAs.

III.2. DUFAs and their role in root growth in stress conditions.

Arid and semi-arid territories are increasing yearly and amount to more than 40% of the land surface (Shanon, 1986). Fertilizers and climate change are two of the main factors leading to such modifications. Aridification is often associated to an over-accumulation of salt and minerals in the soil (Jiang et al., 2016), which can be even more challenging for plants. However, some plants are able to partly cope with increased salinity. For example, it was shown, that $Arabidopsis$ seedlings can grow at relatively mild salt levels ($<100$ mM; Jiang et al., 2016), but once the salinity levels increase the plant cannot function normally. For example, high salt levels lead to reduced $Arabidopsis$ root lengths through decreases in cell elongation (Potters et al., 2007; Bernstein, 2013), reduced number of meristematic cells (West et al., 2004), as well as changes in root architecture (Julkowska et al., 2014) and gravity responses (Sun et al., 2008).
Root development during salt treatment depends on multiple factors including hormonal changes, such as upregulation of abscisic acid (ABA) and down regulation auxin (IAA) (Hernandez et al., 2010), but one general mechanism has been described for all of them – induction of ROS (Bernstein, 2010; Hernandez et al., 2010). Due to the high potential toxicity of ROS, plants have developed defence mechanisms, e. g. the production of ROS-detoxifying enzymes (Dat et al., 2000; Apel and Hirt, 2004). Recently, it has been shown that salt treatment down-regulates auxin signalling in roots by stabilization of Aux/IAA repressors (Iglesias et al., 2014). The aim of this part was to deduce the role of DUFAs in root adaptation to salinity stress, by growing WT and fad2 seedlings in 100 mM NaCl media or exposing them to salt for brief periods (1 hour).

For the first part of this research we grew WT, fad2-1 and fad2-3 Arabidopsis seedlings on 100 mM NaCl media and then analysed their root length (Fig.3.8).
Figure 3.8. Root length analysis of 7-day-old *Arabidopsis* seedlings growing in control and salt-rich media. Bars represent the means of 3 independent experiments; each contains a pool of ~ 60 seedlings. Letters indicate statistically significant differences between pairs as determined by Tukey’s HSD test (p < 0.05).

Constant exposure to salt led to a strong reduction in the growth of WT and *fad2* roots. In the case of WT plants, salt treatment led to a 45% reduced root length compared to the control conditions. In *fad2* mutants this effect was even stronger, leading to respectively 86 and 94% reduced root length in *fad2-1* and *fad2-3* mutants, hence supporting the earlier findings on strong susceptibility to salt treatment in *fad2* mutants (Zhang *et al.*, 2012).

The next part of this project was to localize the root zone associated with length reduction upon salt treatment. For this purpose plants were grown for 7-days on media containing 100 mM NaCl. Meristematic and elongation zones were analysed (Fig. 3.9).
Root meristem cell number

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>A</td>
<td>C</td>
</tr>
<tr>
<td>fad2-1</td>
<td>B</td>
<td>D</td>
</tr>
<tr>
<td>fad2-3</td>
<td>C</td>
<td>E</td>
</tr>
</tbody>
</table>

A: Images of WT, fad2-1, and fad2-3 root meristems.
B: Bar graph showing root meristem cell number for WT, fad2-1, and fad2-3 under control and NaCl conditions.
C: Additional images of WT, fad2-1, and fad2-3 root meristems.
Figure 3.9. Morphological analysis of 7-day-old roots of WT and \textit{fad2} seedlings grown in control and salt media were visualized with propidium iodide (PI) staining. (A-B) Primary root meristem visualization and cell number quantification and comparison with control conditions. (C) Cortex cell visualization, the arrows indicate cells of interest. (A, C) Scale bar 100 µm. (D) Measurements of the cortex cells grown in salt media. Bars represent the means of 10 roots (±SD). Letters indicate statistically significant differences between pairs as determined by Tukey’s HSD test (p < 0.05).

The results obtained revealed that root length in the WT plants was reduced upon salt treatment, due to a 37% reduction in the number of meristematic cells. Changes in cell sizes in elongation zones were not significant. In \textit{fad2} mutants, root growth was affected in both meristematic and elongation zones. Relative to the WT, \textit{fad2-1} mutants showed decreases in the number of meristematic cells from 79% in control to 29% in salt conditions and in cortex cell length from 91 to 53%. Similarly to the changes observed in the \textit{fad2-1} mutant, in \textit{fad2-3} roots the number of meristematic cells was reduced from 60% in control to 16% in salt - treated conditions. The strongest reduction in
cortex cell length was observed in fad2-3 mutants, showing a decrease from 81 to 36% in treated conditions relative to the WT.

For the next step of this work, we decided to use the ROS-ID total ROS/Superoxide detection kit to compare WT and fad2-1 seedlings in three different conditions: control, transfer to salt media for 1 h followed by visualization, and growth on salt media (Fig. 3.10). We performed a time course assay to find highest ROS induction in WT seedlings upon salt treatment. 1 h treatment showed higher fluorescence in roots. Hence, it was used to detect early root responses to salt stress in combination with other treatments.

Figure 3.10. ROS staining of 7-day old Arabidopsis roots. WT and fad2-1 roots were grown in 3 different conditions, followed by ROS staining. Hydrogen peroxide, peroxynitrite and hydroxyl radicals were visualized at wavelengths 490 nm/525 nm (Ex/Em) and labelled ‘green stain’, superoxide ‘red stain’ was
The difference between ROS-associated fluorescence in WT control and salt treatment is shown in Figure 3.9. In the control, green associated fluorescence is mainly localized in elongation zone and superoxide is mildly induced all along the root. ROS-associated fluorescence in the WT plants grown on salt media was equally spread along the root with stronger fluorescence than in the control conditions. A one-hour salt treatment lead to the strongest ROS induction compared to the other treatments in both WT and fad2-1 mutant, with no differences in ROS localization. Green fluorescence was induced over the whole root, whereas red was mainly induced in the elongation root zone. MDA measurements and ROS staining showed that roots of fad2-1 mutants have higher overall ROS levels in control conditions, compare to the WT plants. The fad2-1 seedlings grown on salt-rich media displayed no difference to the control conditions for green stain, but the fad2-1 mutant had higher superoxide levels. These results led us to the idea to test the expression of the detoxification genes. For this reason, we used genes that were induced upon 75 µM phytoprostane A₁ treatment that is reported to inhibit root growth and cell division (Mueller et al., 2008). Two detoxification genes that showed more than 100 fold induction in that publication were chosen for this work: cytochrome P450 family protein (CYP81D11) and UDP-glucoronosyl/ UDP-
glucosyl transferase family (*UGT73B3*). Their expression was checked in three different conditions in WT and *fad2* mutants (Figure 3.11).

![Graph](image1.png)

![Graph](image2.png)

Figure 3.11. Relative expression of *CYP81D11* and *UGT73B3* genes in 7-day-old seedlings. Transcriptional levels of detoxification genes were normalized to those of *UBC21*. Bars represent the means of 4 biological replicates; each contains a pool of ≈ 30 (control and 1h salt treatment) to 120 (salt grown) seedlings. Letters indicate statistically significant differences between pairs as determined by Tukey’s HSD test (p < 0.05).

These results indicate induction of both detoxification genes upon 1h salt treatment in WT and *fad2* mutants. Surprisingly, they showed different expression rates: *CYP81D11* had higher levels upon 1h salt treatment in WT,
whereas \textit{UGT73B3} was expressed most highly in \textit{fad2-1} after one hour exposure to salt. Interestingly, expression of the \textit{UGT73B3} gene under control conditions was lower in \textit{fad2-3} mutants compared to the WT seedlings. No difference in gene expression was found between WT and \textit{fad2} mutants grown on salt for 7-days. These findings suggest that adaptation to salt stress is a very complicated process that requires multiple levels of plant adaptation.

\textbf{III.3. Discussion.}

PUFAs play an important role in plant adaptation to stresses, by forming membrane bilayers and working as substrates for hormone production and buffering ROS levels (Vick and Zimmerman, 1984; Routaboul \textit{et al.}, 2000; Falcone \textit{et al.}, 2004, Zhang \textit{et al.}, 2009; Zhang \textit{et al.}, 2012; Schmid-Siegert \textit{et al.}, 2012; 2016). Plants deficient in TUFAs showed no major changes in growth and development, other than a male sterility phenotype due to the absence of jasmonic acid (McConn and Browse, 1996). Currently, there is almost no information available on the role of DUFAs in plant development. This is was why we decided to base our research on the root growth of \textit{fad2} mutants.

\textbf{fad2 mutants have shorter root lengths than the WT in control conditions.}

Plants lacking both di- and tri-unsaturated fatty acids (\textit{fad2-2 fad6} double
mutants) were shown to be unviable on soil, whereas if only one PUFA is missing they can grow normally (McConn and Browse, 1998). In this work we analysed the fatty acid composition analyses of \textit{fad2} roots. This revealed significant differences to the WT plants. 2% of PUFAs and 68% of MUFAs from the overall extracted fatty acids were detected in both \textit{fad2} roots, compared to 55% of PUFAs and no detected levels of MUFAs in WT plants. These findings lead us to the assumption that overall ROS levels might be higher in \textit{fad2} mutants, due to their deficiency in PUFAs, which may prevent them from buffering ROS (Mene-Saffrane \textit{et al}., 2009, Schmid-Siegert, \textit{et al}., 2016). ROS staining assays and malondialdehyde measurements provided some support for this hypothesis. Our results are also consistent with the hypothesis that high levels of H$_2$O$_2$ and O$_2^-$ can lead to reduced root growth in \textit{Arabidopsis} (Swanson and Gilroy, 2010; Choudhury \textit{et al}., 2013).

\textbf{\textit{fad2} mutants are more susceptible to salt treatment compared to WT plants.} An earlier study has shown an important role of the \textit{FAD2} enzyme in the function of Na$^+$/H$^+$ exchangers. Roots of \textit{fad2} mutants were susceptible to salt treatment and had high cytoplasmic levels of Na$^+$ (Zhang \textit{et al}., 2012). Similarly, our study confirmed that \textit{fad2} mutants are sensitive to salt and provided further insights into the effect of salt and growth.
Based on our findings, the short root length of fad2 mutants growing on salt media was caused by a reduced number of meristematic cells and reduced cortical cell lengths. This effect could be related to changes in redox status and hormone signalling.

Salt treatment induces ROS accumulation in Arabidopsis roots, leading to a more oxidized redox status, which in turn may cause the reduction in meristem cell number (Jiang et al., 2016). Based on the results of root staining, fad2 mutants grown on salt media have overall higher ROS levels and a stronger reduction in the number of meristematic cells than in WT plants.

Reduced root cortex cell length is observed in Arabidopsis plants exposed to salt treatment (Jiang et al., 2016). This correlated with high levels of abscisic acid (ABA) in the elongation zone (Duan et al., 2013; Ondzighi-Assoume et al., 2016). Unfortunately, we did not perform a quantitative analysis of ABA levels. However, our germination assay on WT and fad2 mutants (Fig.3.12) is consistent with the fact that plants with upregulated levels of ABA are known to have delayed germination rates compared to the WT (Kermode, 2005; Bentsink and Soppe, 2008).
Indeed, we found that seed germination rate was reduced respectively by 24% and 11% in fad2-1 and fad2-3 mutants relative to WT. These results might be related to changes in ABA levels of fad2 seedlings. However, this hypothesis would require further testing.

### III.4. Experimental procedures.

*Plant growth conditions, genotypes and chemicals* - Wild-type *Arabidopsis* (WT *Columbia* background) was used. The following alleles in the *Columbia* background were received from the *Arabidopsis* Biological Resource Centre: *fad2-1* (CS8041), *fad2-3* (SK18137) and *fad3-2 fad7-2 fad8* mutant seeds from McConn and Browse (1996). Seeds were stratified for 2 days at 4 °C and then
grown at 21 °C under 100 µE m⁻² s⁻¹ of light with photoperiod depending on the application (seedlings: 14 h light, 10 h dark (long days); on soil for seed propagation: 24 h light (continuous days) or experiments with adult plants at: 9 h light, 15 h dark (short day)). Seedlings were grown on half-strength Murashige and Skoog solid media (½ MS, 2.15 g/L, pH 5.7; Duhefa Biochemie, Haarlem, The Nederlands) supplemented with 0.5 g/L of MES hydrate (Sigma, Buchs, Switzerland) and 0.7 % agar.

Root phenotypic analysis – plants were grown vertically for 7-days on half-strength Murashige and Skoog solid media (½ MS) or 100 mM NaCl in ½ MS media. Photos were taken and analysed by ImageJ (https://imagej.nih.gov), 60 seedlings were used for one biological replicate. Root cell measurements were performed as described in Acosta et al. (2013).

Fatty acid composition analysis – roots of 7-day-old Arabidopsis seedlings (≈ 30 mg) were placed in 15ml Pyrex SVL capped test tubes (Milian, Switzerland). 1ml of 2.5% (v/v) H₂SO₄ in methanol was added to the samples. Samples were incubated for 90 min at 80 °C, 1.5 ml 0.9 % NaCl and 1 ml of hexane (≥97.0% (GC), Sigma-Aldrich, Buchs, Switzerland) were added. The solution was vortexed briefly (2 sec) then centrifuged for 5 min at 1000 rpm. 250 µl of the upper phase containing fatty acid methyl esters were collected. 1 µl of a 1:5 dilution in hexane was analyzed by GCMS (initial T = 150 °C for 3
min, followed by a ramp 150 °C to 200 °C at 10 °C per minute).

Gas chromatography/Mass Spectrometry – 7-day-old roots (≈ 150 mg) were harvested in liquid nitrogen and ground for 15 sec at 3000 rpm/sec in TissueLyser II (Quiagen, Hombrechtikon, Switzerland). MDA levels were measured by a gas chromatography/ mass spectrometry with D₂-MDA internal standard generated from (2D₂)-1,1,3,3-tetraethoxypropan as described (Weber et al., 2004).

Salt assay – 7-day-old Arabidopsis seedlings were subjected to salt stress. In one case, seedlings were permanently grown in ½ MS media complimented with 100 mM NaCl (Shi et al., 2000). In the second case, to detect early changes upon salt stresses, plants were grown for 7-days on ½ MS followed by transfer and 1h incubation on ½ MS media containing 100 mM NaCl.

Germination assay – Arabidopsis seeds were planted on ½ MS media and left in a long day conditions for 24 h, followed by quantification of the emerged roots.

Gene expression analysis – 7-day-old Arabidopsis roots were collected after 3 different treatments: control (Growing on half-strength MS plates), 1 h 100 mM NaCl treatment and permanent growth on ½ MS plates containing 100
mM salt. Finally, seedlings were collected and stored in liquid nitrogen. 30 to 50 seedlings were used per one biological replicate. RNA isolation and quantitative RT-PCR was performed as described earlier (Gfeller et al., 2011). CYP81D11 and UGT73B3 transcripts were quantified as described earlier (Köster et al., 2012; Langlois-Meurinne et al., 2005).

**ROS detection assay** – 7-day-old Arabidopsis roots were stained with two different ROS indicators: ROS-ID™ Total ROS/Superoxide Detection Kit (Enzo Life Sciences AG, Lausanne, Switzerland) and 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA; Sigma-Aldrich, Buchs, Switzerland). For the first solution, roots were vacuum infiltrated with ROS-ID™ Total ROS/Superoxide Detection Kit for 15 min under dark conditions followed by visualization with Leica MZ16A stereomicroscope. Signals from hydrogen peroxide, peroxynitrite and/or hydroxyl radicals were visualized at wavelengths 490 nm /525 nm (Ex/Em) and labelled ‘green stain’. Superoxide ‘red stain’ was detected at 550 nm /620 nm (Ex/Em). Second solution - 15 µM H2DCFDA in water was used to visualize living roots (Oyama et al., 1994) at wavelengths 495 nm /527 nm (Ex/Em) using Zeiss 200 BIOP confocal microscope.

**Statistical analysis** - Statistical significance in pair-wise comparison was evaluated by Student’s test, where * $p \leq 0.05$; ** $p \leq 0.01$; ***$p \leq 0.001$, 

96
multiple comparison analysis of variances (ANOVA) followed by Turkey’s HSD test was performed using JMP – statistical analysis software.

**III.5. Literature.**


Halliwell B. 2006. Reactive species and antioxidants. Redox biology is a


Chapter IV: PUFAs and their role in susceptibility to *Botrytis cinerea*. 
IV.1. Screening for *fad* mutant resistant to *B. cinerea* in 7 and 14 day-old plants.

Plant-fungal interactions are considered to be as old as the terrestrial vascular plants. According to evolutionary studies, the colonization of the land by plants happened with the help of fungi 400 - 460 million years ago (Redecker et al., 2000; Remy et al., 1994). Most plant-fungal interactions are considered to be symbiotic, promoting growth and stress tolerance in plants and providing carbohydrates to the fungus (Buscot et al., 2000). However, some fungal species became plant pathogens and these have led to fast evolution of resistance mechanisms in the plant kingdom (Jones and Dangl, 2006).

*B. cinerea* is one of the most studied and abundant necrotrophic pathogens that affects more than 200 plant species and causes serious agricultural loss each year (Jarvis, 1977). The efficiency of this fungus is caused by its rapid attachment on the host surface and fast germ tube development that promotes penetration (Epstein et al., 1997). The asexual spores of *B. cinerea* (conidia) are produced on conidiophores and are easily dispersed in nature. Conidial attachment occurs in two phases: immediate adhesion – caused by hydration with the help of weak adhesive forces (Doss et al., 1993) and delayed adhesion – induced by secretion of extracellular matrix-degrading enzymes a couple of hours after (Doss et al., 1995; Doss et al., 1999). Development of the germ tubes depends on multiple factors, such as: surface hardness, hydrophobicity,
topography and structural components. If some of these factors are missing, germ tube growth is arrested, if not, the fungus forms an appressorium as a next step of its development (De Zwaan et al., 1999; Staples et al., 1987; Talbot et al., 1996; Wessels et al., 1991; Tucker et al., 2001).

Plants, on the other hand, have developed several defense mechanisms against necrotrophic fungi such as B. cinerea: a) prevention of pathogen penetration (Fleishman et al, 1995); b) increased levels of reactive oxygen species (ROS; Malolepsza and Urbanek, 2002); c) induction of defense hormones, such as jasmonate (Thomma et al., 1998), ethylene (ET; Feys et al., 2000), salicylic (Audenaert et al., 2002) and abscisic acids (ABA; Pandey et al., 2005).

This study aimed to uncover other possible sources of defense against B. cinerea. Diunsaturated fatty acids were chosen as possible candidates due to their strong involvement in cuticle synthesis, that forms the first layer of defense against many pathogens. We chose 7 and 14 day-old Arabidopsis seedlings to study the role of DUFAs in B. cinerea resistance.

To address these issues, three Arabidopsis oleate desaturase mutants were used: two alleles of FAD2 (fad2-1, fad2-3) unable to synthetize 18:2 FAs in the endoplasmic reticulum, and one allele of FAD6 (fad6) that prevents the generation of 16:2 and 18:2 fatty acids in plastids. In addition, we used as
positive controls mutants deficient in production of triunsaturated fatty acids (fad3-2 fad7-2 fad8), jasmonate (the allene oxide synthase mutant: aos) and for cutin biosynthesis (a glycerol-3-phosphate acyltransferase double insertion mutant: gpat4 gpat8). B. cinerea application was conducted on 7-day-old cotyledons and 14-day-old first true leaves; plants were analysed 48 hours post-inoculation by counting the number of organs with lesions (Fig.4.1).

Figure 4.1. B. cinerea pathogenesis on Arabidopsis seedlings. Lesions were detected visually 48 hours post treatment.

Wild type (Columbia) seedlings including mutants described earlier were tested for B. cinerea susceptibility when they were 9- and 16-days-old. Results are presented in Figure 4.2.
Figure 4.2. *B. cinerea* resistance bioassay on *Arabidopsis* seedlings. (A) 9-day- and (B) 16-day-old seedlings were used in this assay. 2 µL of *B. cinerea* spore suspension (5×10⁵ s/ml) was applied on one cotyledon or first true leaf of each seedling and incubated for 48 h. The number of organs with lesions was scored. (A) Error bars represent the means of four biological replicates (± SD), each from a pool of ≈ 120 seedlings. Letters indicate statistically significant differences between pairs as determined by Tukey’s HSD test (p < 0.05). (B) Error bars represent the means of four biological replicates (± SD), each from a pool of 70 seedlings. Statistical significance in pair-wise comparison control vs treatment was evaluated by Student’s test, where **p ≤ 0.01.
The results obtained revealed strong increase in resistance of \textit{fad2} and \textit{gpat4 gpat8} mutants to \textit{B. cinerea} in relation to the WT at 7-day-old stage, whereas the \textit{fad6} mutant showed no significant difference to WT. Surprisingly, \textit{fad trip} and \textit{fad2-1 fad6} mutants also showed resistance to this pathogen. This was unexpected due to the absence of JA in these mutants. Jasmonates are very important hormones for defences against necrotrophic pathogens (Thomma \textit{et al.}, 1998). The \textit{aos} mutant showed enhanced susceptibility upon \textit{B. cinerea} treatment. These results indicate that resistance of \textit{fad trip} and \textit{fad2-1 fad6} mutants was caused by changes in fatty acid composition and was not related to jasmonate responses.

\textit{B. cinerea} application on the WT revealed a 20\% increase in susceptibility of leaves compared to cotyledons. Similarly to WT, \textit{fad2-3} and \textit{fad trip} mutants showed an average of 60\% increase in susceptibility in leaves, showing the same levels as WT. Finally, the \textit{gpat4 gpat8} double mutant’s leaves remained resistant to \textit{B. cinerea}. To estimate roles of fatty acids in increased susceptibility on the first true leaves, we conducted fatty acid profiling of WT and \textit{fad2-3} mutant at both the 7-day- and 14-day-old stages (Fig.4.3).
Figure 4.3. Fatty acid composition of WT and fad2-3 mutant performed at 7- and 14-day-old stage. Error bars represent the means of 4 - 6 biological replicates (± SD); each pool contained approximately 40 seedlings.

The major difference was detected in the levels of 18:1 and 18:3 fatty acids. At the stage of 7-days fad2-3 mutants had 4% of oleic acid, whereas at the stage of 14 days, these levels were below limit of quantification. Increased levels of 18:3 FAs at the stage of 14-days-old seedlings could not explain *B. cinerea* resistance. No correlation was found between increase in the levels of 18:3 FAs and higher levels of jasmonic acid – a major defence hormones involved in resistance to necrotrophic pathogens. This leads to the conclusion that 18:1 fatty acid could play an important role in resistance to *B. cinerea*. 
IV.2. Does cuticle permeability lead to *B. cinerea* resistance in *fad* mutants?

*B. cinerea* enters within the plant by using cutinase, an enzyme that degrades the cuticle. To avoid activation of defence response genes in host, *B. cinerea* produces oxalate that prevents generation of reactive oxygen species (Commenil *et al.*, 1998; Liu *et al.*, 1998). In Table 4.1, we summarize current knowledge concerning cuticle mutants and their resistance or susceptibility to this necrotrophic pathogen using earlier published data from Serrano *et al.* (2014).

<table>
<thead>
<tr>
<th>Mutant</th>
<th><em>B. cinerea</em> susceptibility</th>
<th>Resistance factor</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>lcr</em> (lacerata)</td>
<td>-</td>
<td>Fungitoxic diffusate, ROS</td>
<td>Voisin <em>et al</em>., 2009</td>
</tr>
<tr>
<td><em>lacs2</em> -long-chain acyl-CoA synthetase</td>
<td>-</td>
<td>Fungitoxic diffusate; SAR; 6-week old</td>
<td>Bessire, <em>et al</em>., 2007; Schnurr <em>et al</em>., 2004</td>
</tr>
<tr>
<td><em>ace/hth</em> – adhesion of calyx edges /hothead</td>
<td>+</td>
<td></td>
<td>Celine Chassot thesis, 2006, University of Freiburg</td>
</tr>
<tr>
<td><em>bdg</em> (bodyguard)</td>
<td>-</td>
<td>Fungitoxic diffusate; ROS</td>
<td>Chassot <em>et al</em>., 2008</td>
</tr>
<tr>
<td><em>fdh</em> (fiddlehead)</td>
<td>-</td>
<td></td>
<td>Voisin <em>et al</em>., 2009</td>
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<tr>
<td>Gene</td>
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<td>---------</td>
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<tr>
<td>acbbp1</td>
<td>+</td>
<td></td>
<td>Xue et al., 2014</td>
</tr>
<tr>
<td>pec1</td>
<td>-</td>
<td>Fungitoxic diffusate; ROS</td>
<td>Chassot et al., 2008</td>
</tr>
<tr>
<td>acp4</td>
<td>-</td>
<td>ROS, SAR</td>
<td>Bessire, et al., 2007; Schnurr et al., 2004</td>
</tr>
<tr>
<td>gly1</td>
<td>+</td>
<td>SAR</td>
<td>Xia et al., 2010</td>
</tr>
<tr>
<td>cdk8</td>
<td>-</td>
<td>PDF1.2</td>
<td>Zhu et al., 2014</td>
</tr>
<tr>
<td>aba2</td>
<td>-</td>
<td>ROS, ABA down regulation</td>
<td>L’Haridon et al., 2011</td>
</tr>
<tr>
<td>aba3</td>
<td>-</td>
<td>ROS, ABA down regulation</td>
<td>L’Haridon et al., 2011</td>
</tr>
</tbody>
</table>

Symbols indicate: susceptibility (+) and resistance (-) to *B. cinerea*; SAR: systematic acquire resistance.

As shown in Table 4.1, most cuticle mutants are resistant to *B. cinerea*. This could be explained by fast induction of antifungal compounds (Bessire *et al.*, 2007) or constitutive overexpression of genes involved in lipid transfer (LPT), peroxidase (PER) and proteinase inhibitors (PI) (Chassot *et al.*, 2007).
Unfortunately, these results do not explain why some cutin biosynthesis mutants do not show resistance to this pathogen.

Plant waxes were also shown to play a role in fungal development on the host; for example, terpenoids found in the wax layer of avocado fruits induced appressorium formation in *Collettrichum gloeosporioides* (Podila et al., 1993; Kolattukudy et al., 1995). C22 FAs, fatty alcohols or alkanes, present in the wax layer of rice leaves promoted development of *Magnaporthe grisea* (Hegde and Kolattukudy, 1997). In grapes, wax composition was shown to change during maturation and this correlated with increased *B. cinerea* susceptibility in mature grapes. This effect was linked to high levels of primary alcohols and oleanic acid in young berries and their diminution in mature grapes (Commenil et al., 1996). *B. cinerea* resistance is a complicated process that requires overlap of different defence pathways that are strongly variable depending on the plant genotypes.

Hence, the purpose of this part was to disengage the role of the cuticle in resistance to *B. cinerea* in fad mutants. We performed cuticle permeability assays to describe the effect of deficiency of di- or triunsaturated fatty acids on cuticle formation (Fig. 4.4).
Toluidine blue staining of 14-day-old seedlings permits the visualization of cuticle permeability in cotyledons and fully expanded true leaves. WT, fad6 and aos did not stain, meaning that cuticle function is not affected in these plants. fad2-3, fad2-1, fad2-1 fad6 and gpat4 gpat8 double mutants have cuticle permeability defects in both cotyledons and first true leaves, whereas fad trip permeability effected only in the cotyledons.

Transmission electron microscopy (TEM) was performed to describe how the cuticle ultrastructure is affected in fad2-3, fad trip and gpat4 gpat8 mutants. (Fig.4.5)
Figure 4.5. Cuticle ultrastructure of 7-day-old *Arabidopsis* cotyledons. C – cuticle, CW- cell wall; EC- epidermal cell. Small boxes represent zoomed in part with the cuticle; scale bars 50 µm. Arrows indicate the cuticle layer. This experiment was repeated three times with similar results in all cases, this graph is representative of one of these experiments; scale bars 200 µm.

Cuticle of the WT plants is seen as dark electron dense layers, whereas in the *gpat4 gpat8* double mutant these are absent and instead there is a light grey layer that is not tightly attached to the cell wall. Concerning *fad trip* and *fad2-3* mutants, they both have thin electron dense layers of the cuticle with less electron dense patches.
To identify which cuticle monomers were affected in \textit{fad} mutants, we performed a quantification of aliphatic polyester monomers from the shoots of 7-day-old seedlings (Fig. 4.6).

![Figure 4.6](image)

Figure 4.6. Quantification of aliphatic polyester monomers in 7-day-old \textit{Arabidopsis} shoots. Error bars represent the means of six biological replicates (± SD), each containing a pool of ≈ 1920 seedlings. FAME - fatty acid methyl ester; DCA - dicarboxylic acid; ω-OH – omega hydroxyl fatty acid. This experiment was repeated three times with similar results in all cases, this graph is representation of one of these experiments.

Polyester monomer profiles revealed a strong overall reduction of cutin monomers in the \textit{gpat4 gpat8} double mutant, highlighting a very strong permeability in cotyledon and leaves of these plants. \textit{fad2-3} and the \textit{fad2-1 fad6} double mutant displayed an 2.5 times lower levels of DCA 18:2 and 8 times higher levels of DCA 18:1 compared to the WT. Surprisingly, \textit{fad2-3}
mutants showed an over accumulation of C18:2 FAME, that was not observed in fad2-1 fad6 double mutant. Both mutants had almost no detectable levels of 18:1 FAME.

The fad trip mutant that showed a permeable cuticle phenotype at the cotyledon stage displayed an over-accumulation of 18:2 DCA, similar to fad6 mutant, i.e. three times higher levels of 18:1 FAME and two times reduction in 18:0 FAME. The fad6 mutant showed almost no difference in its polyester profile compared to the WT, except as mentioned earlier, a 1.5 times higher level of 18:2 DCA. This change did not appear to affect cuticle permeability in this plant.

Due to the involvement of waxes in B. cinerea resistance, we performed scanning electron microscopy to visualize wax layers in WT and fad mutants (Fig. 4.7).
Figure 4.7. Scanning electron microscopy images of 7-day-old *Arabidopsis* cotyledons. Arrows indicate wax crystals. Five cotyledons were analyzed for each genotype; the images shown are representative.

These images clearly show that there is almost no wax layer on *Arabidopsis* cotyledons and small patches of crystals chaotically organized on the surface, similar to the situation for *Arabidopsis* leaves (Jenks *et al.*, 1995; Lee and Suh *et al.*, 2014).
In summary, aliphatic polyester monomer profiling revealed strong changes in the cuticle composition of fad mutants. Monomer compositions differed in fad2-3, fad2-1 fad6, fad trip and gpat4 gpat8 mutants. No changes in wax composition were observed. As a conclusion, no polyester monomers could explain resistance to B. cinerea. Visualization of waxes on the surface of cotyledons also did not explain resistance to this pathogen. One more time, our results confirm that host-pathogen interaction is a very complicated mechanism that still has to be uncovered.

IV.3. Induction of ROS and defense hormones upon B. cinerea infection in fad mutants.

ROS are known to play an important role in the control of plant development (Gapper and Dolan, 2006) and in responses to abiotic and biotic stresses (Apel and Hirt, 2004; Mittler et al., 2004), by inducing defense response genes (Levine et al., 1994).

The role of ROS in B. cinerea resistance remains unknown. According to one hypothesis B. cinerea, like other necrotrophic pathogens, can suppress ROS production, preventing induction of antifungal compounds and defence response genes at the early steps of fungal penetration (Germeier et al., 1994;
Pezet et al., 2004). However, some cuticle-deficient mutants have high basal ROS levels that lead to the constant upregulation of major defense genes (Bessire et al., 2007; Chassot et al., 2007; L'Haridon et al., 2011; Liu et al., 2015) and hence induce B. cinerea resistance. The aim of this part was to analyse ROS levels in fad mutants upon B. cinerea infection and to compare it with the expression of common hormonal marker genes induced upon B. cinerea application.

We used 7-day-old Arabidopsis cotyledons either with mock treatment (24h treatment with potato dextrose broth (PDB)), or infected with B. cinerea in PDB for 24h. To detect changes in the ROS levels, we used ROS-ID™ Total ROS/Superoxide Detection Kit (Enzo Life Sciences AG, Switzerland) as described by Marschal and Tudzynski (2014). It is important to note that this technique has not, to our knowledge, been used before on plants, however it provided promising results in the present study (Fig.4.8). Due to the previously published results showing stronger ROS fluorescence with later infection time points (L’Haridon et al., 2011), we performed our assay 24 h after the treatment.
Figure 4.8. ROS staining of 8-day-old cotyledons. (A), 24 h post PDB application and (B) 24 h post *B. cinerea* application in PDB. Treated seedlings were vacuum infiltrated in ROS staining solution for 30 min. Hydrogen peroxide, peroxynitrite and hydroxyl radicals were visualized via wavelengths 490 nm/525 nm (Ex/Em) and called as ‘green stain’, superoxide via 550 nm/620 nm (Ex/Em), ‘red stain’. Each photo is representative of ten analysed cotyledons.

No visual difference was found between the WT, *fad trip* and *fad2-3* mutant upon PDB treatment. Cotyledons infected with *B. cinerea* showed stronger induction of analyzed ROS species. Patchy fluorescent spots on the WT cotyledons revealed areas of fungal penetration. In this case the detected fluorescence is likely to come from both plant and pathogen, since both of them induce ROS during the pathogenesis process. The *fad2-3* mutants showed
less fluorescence with both dyes, a result that could be explained by a lower ratio of fungal penetration (not tested in the present study). The *fad trip* mutant displayed the strongest induction of ROS compared to WT and *fad2-3* mutant. This could be explained by the absence of TUFAs in this plant and its inability to conduct ROS buffering due to absence of these fatty acids (Mene-Saffrane *et al.*, 2007; Schmid-Siegert *et al.*, 2016).

In summary, WT, *fad trip* and *fad2-3* mutants showed no difference in ROS fluorescence after the mock treatment. 24 h post *B. cinerea* application induces stronger fluorescence of ROS in *Arabidopsis* cotyledon. We noticed a gradient of increased green and red staining in WT, *fad2-3* and *fad trip* mutants.

The next step was to correlate increased ROS levels with hormonal changes (Fig.4.9). In order to detect changes in the levels of JA and ET – the (ethylene- and jasmonate-responsive plant defensin) PDF1.2 marker gene was used (Penninckx *et al.*, 1996,1998). PDF1.2 expression was seen at the site of infection and at the distal regions leading to the similar kinetics at both parts (Penninckx *et al.*, 1996, Ferrari *et al.*, 2003). To analyse the potential role of the SA pathway in *B. cinerea* resistance, we used the *PR1* (pathogenesis-related protein 1) marker gene. This gene is activated upon pathogen infection downstream of systematic acquired resistance (SAR; Von Loon *et al.*, 1994). In contrast to PDF1.2, *PR1* is expressed only in the region of pathogen
penetration (Ferrari et al., 2003). Finally, to test the involvement of ABA, we used the *ABA1* (encoding an zeaxanthin epoxidase involved in ABA biosynthesis), *aba1* mutants in *Arabidopsis* show *B. cinerea* susceptibility that might be caused by JA and ET changes this mutant (Korolev et al, 2004).
Figure 4.9. Induction of defence marker genes upon *B. cinerea* infection in 8-day-old seedlings. qRT-PCR of *PDF1.2* (A), *PR1* (B) and *ABA1* (C) expression in 7-day-old *Arabidopsis* seedlings 24 h after *B. cinerea* application. Transcripts were normalized to SAND and displayed relative to the expression in WT mock. Error bars represent the means of six biological replicates (±SD), each containing a pool of ≈30 seedlings. The dashed line indicates the limit of quantification (LOQ) is indicated. (A-B) Letters indicate statistically significant differences between pairs, as determined by Tukey’s HSD test (p < 0.05). (C) No significant interaction between treatment and genotypes was revealed. Tukey’s HSD test was performed on genotypes, revealed significant effect of infection (p < 0.0008).

According to the qPCR results, no similar pattern of hormonal response was revealed between mutants showing resistance to *B. cinerea*. Expression of the *PDF1.2* marker gene was similar between WT and *fad2-3* mutants, whereas *fad2-1 fad6, fad6* and *fad trip* showed much lower *PDF1.2* gene expression compare to the WT. This result could be explained by potentially lower levels of JA in these mutants, due to the complete absence or strong reduction of triunsaturated fatty acids. Significantly lower levels of *PDF1.2* in the *gpat4 gpat8* double mutant require further investigation. Indeed, according to fatty acid profiling, no difference was found between WT and *gpat4 gpat8* double mutant (Fig.4.10), leading to the idea that ET levels might be affected in this mutant.
Figure 4.10. Fatty acid composition in 7-day-old seedlings of WT and the gpat4 gpat8 double mutant. Error bars represent the means of six biological replicates (± SD); each contained a pool of ≈ 50 seedlings.

According to previously published results, SA was not strongly induced upon *B. cinerea* infection in WT plants (Birkenbihl et al., 2012). We confirmed this result with 7-day-old WT seedlings by conducting qPCR with the SA-responsive the *PR1* marker gene (a similar pattern upon *B. cinerea* infection was observed in *fad6* and *fad2-1 fad6* double mutants). Surprisingly, *fad2-3* and *gpat4 gpat8* double mutants showed more than 10-fold *PR1* induction upon *B. cinerea* infection. Concerning *fad trip*, it revealed higher basal levels of *PR1* expression and 10-fold induction upon treatment.

During *B. cinerea* infection, ABA signalling is strongly down regulated (Windram et al., 2012), due to its antagonistic relations with ET, which is induced upon infection (Yang et al., 2005). A similar pattern of down regulation of the *ABA1* gene upon *B. cinerea* infection was observed on WT,
fad2-3, gapt4 gpat8 and fad2-1 fad6 double mutants, with the exception of a 2-fold higher level of ABA1 in fad2-1 fad6 plants. Surprisingly, the fad6 mutant displayed no down regulation of the ABA1 gene upon B. cinerea infection, leading to assumption that ethylene levels in this mutant are lower than in WT plants during infection process. As shown above (Fig.4.8), mild changes in ROS levels in fad2-3 mutants and strong basal levels of green stain in fad trip did not lead to upregulation of genes such as a PDF1.2 or ABA1, but did lead to strong induction of PR1. Interestingly, SA is involved in the local defence to B. cinerea, by decreasing lesion size, but not the overall survival rate of infected seedlings (Ferrari et al., 2003).

IV.4. Changes in topography of the cotyledon surface can lead to B. cinerea resistance.

Early pathogenesis is a crucial step that requires spore attachment to the host surface, spore germination, formation of appressoria and further penetration (Kuo and Hoch, 1996). Development of the germ tube depends on multiple factors, such as surface hardness, hydrophobicity, topography and structural components. It is important to note that if one or more of these factors are perturbed, germ tube growth is reduced or arrested. If all the required conditions are present, the fungus forms an appressorium as a next step of its development (DeZwaan et al., 1999; Staples et al., 1987; Talbot et al., 1996;
Wessels et al., 1991; Tucker et al., 2001). For these various reasons, we examined cotyledon topography in relation to \textit{B. cinerea} penetration in WT plants and fatty acid desaturase mutants.

To examine immediate adhesion and hyphal growth on \textit{fad} mutants, we performed adhesion assays (Fig.4.11). For this purpose, cotyledons were incubated with \textit{B. cinerea} spore suspension for 4 h after which seedlings were washed in 100\% ethanol for 1 h to remove unattached spores from the cotyledon surface.

![Figure 4.11. \textit{B. cinerea} adhesion assay. WT and \textit{fad2-3} cotyledons were infected with spores of \textit{B. cinerea}, the amount of attached spores per cotyledon was quantified 4 h after spore application. Error bars represent the means of ten biological replicates (± SD), each initially containing a pool of ≈ 500 spors of \textit{B. cinerea}; Statistical significance in pair-wise comparison was evaluated by Student’s test, where * \( p < 0.05 \).

The initial concentration of applied \textit{B. cinerea} spores was 500 spores/µl. The number of attached spores on WT cotyledons equalled approximately 350
spores, whereas on *fad2-3* seedlings these were approximately 250 spores (*p* < 0.05; Fig. 4.11). This result could explain a partial resistance to *B. cinerea* in the *fad2-3* mutant. It led us to the idea that a modified topography in *fad2-3* cotyledons might be responsible for this difference.

The second step was to test the effect of delayed adhesion by analysing development of germ tubes several hours post infection. First, we conducted a time course assay to estimate the speed of *B. cinerea* development on WT and *fad* mutants (Fig. 4.12).

Figure 4.12. *B. cinerea* development: a time course assay on 7-day-old cotyledons. Pictures were taken at three different time points: 4, 8 and 24 hours post inoculation. For 4 and 8 hours a 40X standard objective was used and for 24 hours a 20X DIN standard objective. Hyphae were stained with Trypan blue. The experiment was repeated 3 times. Scale bar 50 µm.
No difference was found between WT and \textit{fad} infected cotyledons 4 h post infection, whereas infection at later time points (8 and 24 h) revealed increased hyphal growth on WT cotyledons compared to the \textit{fad} mutants. To be able to estimate the difference between at these time points we performed hyphae length measurements at 4 and 8 h post infection (Fig. 4.13). The 24 h time point was excluded from the experiment, due to the difficulty of measuring hyphae lengths on and in WT plants caused strong overlay of hyphae.
Figure 4.13. *B. cinerea* hyphal length assay at two different time points. 2µL suspension of $5 \times 10^5$ spores was applied to single cotyledons of 7-day-old seedlings (A) 4 h and (B) 8 h post application. Error bars represent the means of 100 *B. cinerea* hyphae measured on 10 cotyledons (± SD). Letters indicate statistically significant differences between pairs as determined by Tukey’s HSD test ($p < 0.05$).

According to our results, the average length of hyphal 4 h post infection was 25 µm on WT cotyledons and 20 µm on the *fad* mutants. After 8 h, hyphal length increased two times on WT, whereas on *fad* mutants, hyphal growth was arrested at the stage of 4 h post infection. We concluded that delayed adhesion reduces *B. cinerea* development on *fad* mutants.

Earlier papers considered leaf topography as an important factor promoting pathogen development (DeZwaan *et al.*, 1999; Staples *et al.*, 1987; Talbot *et al.*, 1996). To examine if cell morphology in *fad* mutants was affected, we used confocal microscopy to visualize cotyledon epidermal cells of WT and cuticle mutants at the stage of 7-day-old seedlings (Fig.4.14).
Figure 4.14. Pavement cell morphology of 7-day-old cotyledons, stained with propidium iodide. Pavement cells have different morphology depending on their location, for this reason, only the region near to cotyledon tip was chosen for the analysis (n = 10 cotyledons). Scale bar 50 µm.

Visually, pavement cells of cuticle mutants have a different morphology to those of the WT: smaller cells with fewer lobes. To obtain quantitative results, we used Lobefinder_V1-4.0 software (Wu et al., 2016). Single pavement cells were selected in ImageJ and converted to roi files, further analyses were carried out in Lobefinder (Fig.4.15).
Figure 4.15. Example of a single pavement cell analyzed with Lobefinder_V1-4.0 software.

The numeric data were saved in xlsx format and analyzed as described in Materials and Methods. Quantified parameters of pavement cells are shown below (Fig.4.16).
Figure 4.16. Quantitative analysis of pavement cell parameters in 7-day-old cotyledons. (A) Lobe number, (B) perimeter and (C) circularity were measured. Error bars represent the means of 45-50 pavement cells measured on 10 cotyledons (±SD). Letters indicate statistically significant differences between pairs as determined by Tukey’s HSD test (p < 0.05).

*fad* mutants had reduced lobe numbers, cellular perimeters and increased circularity compared to the WT cells, no significant difference was found between WT and *gpat4 gpat8* cotyledons. This could be caused by morphological and turgor changes of the cell (Hamant *et al.*, 2008, Routier-Kierzkowska *et al.*, 2012, Sampathkumar *et al.*, 2014). For this reason we decided to use atomic force microscopy to examine the spatial distribution of mechanical properties such as height, elasticity and adhesion of *Arabidopsis thaliana* cotyledons (Fig.4.17).
Figure 4.17. Atomic force microscopy images of 7-day-old *Arabidopsis* cotyledons. (A) Height, (B) Young’s modulus (stiffness) and (C) adhesion maps were obtained. Five cotyledons were analysed in this work, representative images were chosen for each map. Arrows indicate stomata. Experiment performed by Petar Stupar, Laboratory of the Physics of Living Matter, EPFL, Lausanne.

These images show the difference in elastic modulus (Young’s modulus), a measure of stiffness in *fad2-3* mutants compared to WT cotyledons. However, only slight changes were noticed in the adhesion and no difference in the height maps. Due to the observed difference in adhesion and stiffness maps we performed quantitative analysis of these features (Fig.4.18).
Figure 4.18. Quantitative analysis of atomic force microscopy results. (A) Young’s modulus, (B) adhesion and (C) height were measured. Error bars represent the means of 15 to 20 pavement cells parameters obtained from five cotyledons (±SD). Statistical significance in pair-wise comparison control vs treatment was evaluated by Student’s test, where ** \( p \leq 0.01; *** \ p \leq 0.001. \) Measurements performed by Petar Stupar, Laboratory of the Physics of Living Matter, EPFL, Lausanne.

Figure 4.18 show that mean Young’s modulus is different between WT and \( fad2-3 \) mutants and was 0.7 and 0.4 MPa respectively. Adhesion parameters were also affected and were 0.3- and 0.4 nN in WT and mutants respectively. Cell height was the only feature that did not change in WT and mutants cotyledons and was 4 µm in both cases. These results revealed multiple non-uniformities that could be explained by heterogeneous composition of the cell surface in the WT and the \( fad2-3 \) mutant. Unfortunately, such a slight change
in morphology of the pavement cells cannot explain resistance to \textit{B. cinerea} in \textit{fad2-3} mutant, but might explain slight changes in adhesion of \textit{B. cinerea} spores on cotyledons of \textit{fad2-3} mutants relative to the WT (Fig.4.11).

\textbf{IV. 5. Surface metabolites and their role in \textit{B. cinerea} resistance.}

Early stages of \textit{B. cinerea} development are crucial for the successful process of pathogenesis (Perryman \textit{et al.}, 2002). Plants have developed multiple defense mechanisms to prevent fungal spreading: release of volatiles, induction of ROS and defence genes. For example, (E)-2-hexanal and \(\beta\)-phellandrene volatiles identified from \textit{Solanum lycopersicum} were shown to have an inhibitory effect on the germination and hyphal growth of \textit{B. cinerea} (He \textit{et al.}, 2005). On another hand, \textit{B. cinerea} has developed multiple layers of adaptation to parasitize on the host that makes it very efficient necrotrophic pathogen. However, when plant undergoes developmental changes such as, impaired secondary cell wall function in \textit{Arabidopsis} mutants, \textit{B. cinerea} development is strongly supressed in these plants (Hernandez-Blanco \textit{et al.}, 2007). The aims of this subchapter were to identify compounds found on the surface of \textit{Arabidopsis} cotyledons and relate it to \textit{B. cinerea} resistance in \textit{fad} seedlings.
The first step of this work was to extract metabolites localized on the surface of WT and fad2-3 plants. We applied a drop of isopropanol (2 µl) on each cotyledon, collecting it 30 sec after application. 784 cotyledons were used for the surface analyses. Further samples (196) were used to analyse the metabolite composition of the entire seedling. The extracts were analysed using ultra-performance liquid chromatography quadrupole time of flight mass spectrometry (UPLC–qTOF-MS/MS) in collaboration with Gaétan Glauser at Platform of Analytical Chemistry, University of Neuchâtel. For the positive control, we used fresh weight powder obtained from whole WT, fad2-3- and fad trip seedlings shoots and compared it to surface metabolites extracted with gently placed isopropanol drops. To visualize the similarities between WT and fad2-3 samples we used a principal component analysis (PCA; Fig 4.19).

![Figure 4.19. PCA score plot from surface (S) and whole shoot (L) metabolites of 7-day-old WT and fad2-3 seedlings. t[1] and t[2] are first and second principle components, with percentage of explained variance.](image-url)
The score plot analysis revealed 51% difference between metabolites located on the cotyledon surface and within the whole shoot. The difference between genotypes based on t2 score was 14%. Based on the results we were able to generate a list of metabolites that were more abundant on the surface of WT (Tab.4.2, A) or fad2-3 cotyledons (Tab.4.2, B).

Table 4.2. List of metabolites differently abundant on the surface of cotyledons.

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(A) WT and (B) *fad2-3* seedlings were analysed in this experiment. List was created automatically based on principal component plots: p1 and p2 vectors. These vectors indicate relation of different variables to each another.

To select compounds potentially involved in *B. cinerea* resistance we performed metabolomics analysis from the surface of true leaves of WT and *fad2-3* two-week old seedlings. This approach was chosen due to the loss of resistance in the first true leaves of *fad2-3* mutants. Metabolites obtained from the surface of first true leaves were compared to the ones obtained from the cotyledons. Only compounds that show more than two fold increase were chosen for further analysis (Fig 4.20).
Figure 4.20. Relative quantification of two metabolites differently present on the surface of WT and fad2-3 cotyledons and leaves. Error bars represent the means of 3 - 4 biological replicates; one biological replicate consisted of isopropanol drops collected from 784 cotyledons and 196 leaves (± SD). Statistical significance in pair-wise comparison was evaluated by Student’s test, where *** $p \leq 0.001$.

With the help of a surface metabolomics approach we were able to identify two compounds that could be involved in B. cinerea resistance in fad2-3 mutant. Glutathione is one of the most important sulfur-containing antioxidants and redox buffers in plants (Bergmann and Rennenberg, 1993). It is a tripeptide thiol ($\gamma$-glutamyl-cysteiy1-glycine) that was shown to play multiple roles in the plant such as: uptake, transport and storage of reduced sulfur, detoxification of ROS, xenobiotics, heavy metals and other substances that can damage cell functioning (Noctor et al., 1998; Tausz 2001, Blokhina et al., 2003; Kopriva and Rennenberg, 2004; Tausz et al., 2004).
The other possible candidate may be 7-methylsulfinylheptyl glucosinolate (7MSOH). The mass of this compound is 494.083 in negative mode and its fragmentation products are consistent with such a structure. However, this is a tentative identification that needs conformation by, for example, nuclear magnetic resonance. This compound belongs to the group of secondary metabolites containing sulfur and nitrogen, common in order of Capparales and involved in herbivore and pathogen resistance (Rask et al., 2000; Redovnikovic et al., 2008). Levels of glucosinolates are differently distributed in Arabidopsis leaves. The highest concentration of glucosinolates was found in the midvein and the outer lamina, whereas one of the lowest on the surface of Arabidopsis leaves (Shroff et al., 2008; Soenderby et al., 2010; Shroff et al., 2015). 7MSOH is an aliphatic glucosinolate that is synthetized from pentahomomethionine, that is not strongly abundant in Arabidopsis shoot and roots making in average 15 to 20 pmol mg⁻¹ of FW (Hogge et al., 1988; Maruyama-Nakashita et al., 2008). 7MSOH is not strongly abundant in Arabidopsis leaves and no biological role for this glucosinolate in nature was found so far (Maruyama-Nakashita et al., 2006). Once the cell is damaged glucosinolates are hydrolyzed by myrosinases (thioglucosidase) to form isothiocyanates, thiocyanates or nitriles (Rask et al., 2000). 7MSOH might play a role in preventing B. cinerea development on the surface of Arabidopsis seedlings.
IV. 6. What is the *B. cinerea* entry point in *Arabidopsis* cotyledons?

*B. cinerea* is an opportunistic fungus that can infect host organisms through wounded regions, sites previously infected by other pathogens, intact epidermal cells or, by direct penetration through cuticle into the cell wall (McKeen, 1974; van Kan, 2006). The purpose of this subchapter was to identify the entrance points of *B. cinerea* on the cotyledons of the WT and cuticle mutants at the 7-day-old seedling stage.

We used scanning electron microscopy to follow development and appressorium formation of *B. cinerea*. A 6 h post-inoculation time point was chosen to follow germ tube growth (considering previous results, it is an optimal time point). Fig. 4.21 shows a 7-day-old WT cotyledon with developing *B. cinerea* hyphae on it.
Figure 4.21. Scanning electron micrographs of 7-day-old *Arabidopsis* cotyledons. (A) Control, central part of cotyledon, (B) 6 h post *B. cinerea* inoculation, (C) hyphal growth on the surface of cotyledon avoiding stomata as an entrance point, (D) Appressorium formation at the boundary of two pavement cells.

*B. cinerea* enters the plant through intercellular spaces. The same process has been observed on broad bean leaves (Mansfield and Richardson, 1981), onion (Gourgues *et al.*, 2004) and Chinese wild *Vitis* (Wan *et al.*, 2015). To further examine this we made transversal sections of cotyledons inoculated with *B. cinerea* (Fig.4.22).
Figure 4.22. Transversal sections of WT cotyledons inoculated with *B. cinerea* for 24h. (A), appressorium formation in the intercellular region of two epidermal cells. (B), *B. cinerea* entrance in *Arabidopsis* cotyledon. B.c.- *Botrytis cinerea*, EP – epidermal cell, red rows indicate intercellular space. Scale bar 5µm.

This clearly showed that *B. cinerea* penetration started in intercellular spaces.

To identify the entrance point of *B. cinerea* in cuticle mutants, cotyledons of *fad2-3* and *gpat4 gpat8* mutants were inoculated with *B. cinerea* for 24 h with further preparation of transversal sections and visualization under a transmission electron microscope (Fig.4.23).
On both images *B. cinerea* formed mucilage (light grey layer of undefined shape, surrounding spores) - an early step of germ tube development (Hawker and Hendy, 1963). These results support the hypothesis that *B. cinerea* enters *Arabidopsis* cotyledons through the intercellular spaces of epidermal cells.

The next question addressed in this study was to identify preferable cell type for *B. cinerea* entrance. For this purpose we used *Botrytis cinerea* enhanced GFP fluorescent B05 strain (*Bcgfp1*), kindly provided by Prof. Matthias Hahn (Technical University of Kaiserslautern). The fluorescent *B. cinerea* strain was applied to fad2-3 and WT cotyledons and incubated for 6 h followed by visualization on a Zeiss LSM700 confocal microscope (Zeiss, Feldbach, Switzerland; Fig. 4.24).
Figure 4.24. *B. cinerea* entrance through the intracellular regions of *Arabidopsis* pavement cells. Cellular borders were stained with propidium iodide. *Bcgfp1* strain was visualized 6h post application. Arrows indicate entrance points of *B. cinerea*.

This image clearly shows that *B. cinerea* enters through the intercellular spaces of *Arabidopsis* pavement cells. We analysed 10 cotyledons of WT and *fad2-3* mutant. No difference in *B. cinerea* entrance on these genotypes was observed.

**IV.7. Discussion.**

Triunsaturated fatty acids were shown to have an important role in resistance to necrotrophic fungi, as precursors of jasmonic acid (Thomma *et al.*, 1998), whereas the function of diunsaturated fatty acids in this process remained unknown. However, there is only some evidence concerning their involvement in the conidiation process in *Aspergillus parasiticus* (Wilson *et al.*, 2014). DUFAs are major components of the surface defense layer, i.e. in the cuticle.
(Bonaventure et al., 2004). Our study further investigated the roles of DUFAs resistance to the necrotrophic fungus *Botrytis cinerea*.

**Cuticle deficiency and its role in *B. cinerea* resistance.** We showed that cotyledons of mutants deficient in 18:2 (*fad2-3*); 16:3 and 18:3 (*fad trip*) or both DUFAs and TUFAs (*fad2-1 fad6*) have permeable cuticle. Increased permeability of *fad* cotyledons could be explained by changes in composition of major structural polyester components. However, no common cutin monomer defect, compared to WT, was detected in the mutants analysed. Moreover, the *gpat4 gpat8* double mutant showed no differences in FA composition compared to WT, but this mutant displayed the highest cuticle permeability in cotyledons and the first true leaves, together with the most reduced levels of cutin monomers. Toluidine blue staining of the first true leaves showed permeability defect only in *fad2-3* and *fad2-1 fad6* mutants, showing a specific role of TUFAs in the polyester composition of *Arabidopsis* cotyledons, but not true leaves. If only cuticle permeability would be involved in *B. cinerea* resistance, *fad trip* plants would be the only ones to show susceptibility to this pathogen, however leaves of *fad2-3* mutants also show this phenotype. These results lead to conclusion that cuticle permeability might be one of the factors to promote *B. cinerea* resistance, but additional mechanisms should be activated to fight this pathogen.
The role of ROS in *B. cinerea* resistance at the stage of 7-days old seedlings. Another question that we addressed in this research was to understand what leads to *B. cinerea* resistance in *fad* mutants at the stage of 7-day-old seedlings. To address this problem, we analysed internal ROS levels in our mutants. Their upregulation was shown to play a crucial role in the resistance to *B. cinerea* by the activation of defence response genes (Malolepsza and Urbanek, 2002). We used malondialdehyde as a marker for nonenzymatic lipid oxidation. Surprisingly, no difference was found in MDA levels in the WT and the *fad2-3* mutant in non-stressed conditions, but the *fad2-1 fad6* double mutant had MDA levels close to the limit of quantification and *fad trip* levels were lower than in the WT, consistent with previously published results (Schmid-Siegert et. al., 2012). These finding indicate that *fad2-1 fad6* and *fad trip* mutants are present under constant oxidative stress, that might lead to the higher internal levels of the defence hormones, such as SA or JA. However, no difference in the hormonal levels should be found between WT and *fad2-3* seedlings. We assume that *fad2-3* can have different mechanism of *B. cinerea* resistance to *fad2-1 fad6* and *fad trip* seedlings.

Defense hormones and their induction upon *B. cinerea* treatment. Most of the defence marker genes examined did not show a difference upon *B. cinerea* infection at cotyledon stage between WT and mutants. The exception was the *PRI1* gene that showed strong basal and induced resistance in *fad trip* mutants,
as well as 5-fold induction in \textit{fad2-3} seedlings. These differences might be explained by possibly higher levels of ROS such as, for example, H$_2$O$_2$ in \textit{fad trip} seedlings (Schmid-Siegert \textit{et. al.}, 2012), leading to the synthesis of salicylic acid (Nawrath and Metraux, 1999; Yalpani \textit{et al.}, 1991). Salicylic acid is an important component of plant defence against \textit{B. cinerea} that inhibits fungal entrance at the site of infection (Ferrari \textit{et al.}, 2003), but does not promote resistance to this fungus at the later stages of infection. Consequently, we concluded that \textit{B. cinerea} resistance in \textit{fad} mutants is not simply due to increase in the levels of defence hormones upon \textit{B. cinerea} infection.

**Changes in morphology of epidermal cells correlated with \textit{B. cinerea} resistance** Previously it was shown that some necrotrophic fungi cannot penetrate into the plant due to surface changes or cell topography (DeZwaan \textit{et al.}, 1999; Staples \textit{et al.}, 1987; Talbot \textit{et al.}, 1996). In this work we showed that \textit{fad} mutants have a different pavement cell morphology compared to the WT. Using atomic force microscopy to analyse pavement cells revealed differences in adhesion and stiffness parameters of \textit{fad2-3} mutants compared to the WT. These results did not explain the arrested growth of \textit{B. cinerea} hyphae in the \textit{fad2-3} mutant, but may relate to the partial spore adhesion defect in this mutant.
Different to the WT metabolites on the surface of fad2-3 cotyledons. To understand *B. cinerea* resistance in the fad2-3 mutant surface metabolomic analysis was performed. This technique allowed us to identify metabolites that were abundant on the surface of fad2-3 cotyledons compared to the WT. Of these 7-methylsulfinylheptyl glucosinolate is of strong interest. Surprisingly, previous works on aliphatic glucosinolates did not show involvement of these compounds in *B. cinerea* resistance (Tierens *et al.*, 2001). The inability to identify the potential involvement of compounds such as 7-methylsulfinylheptyl glucosinolate in fungal resistance previously, could be explained by a low abundance of this glucosinolate in WT plants. Over-accumulation of this compound on the surface of fad2-3 cotyledons may be promoted by the permeable cuticle that permits chemical transport onto the cotyledon surface. The might also be an indirect effect of DUFA deficiency that causes over-accumulation of methyl-derived glucosinolates.

*B. cinerea* entrance in *Arabidopsis* cotyledons. The last question of this work was to identify the entrance point of *B. cinerea* in WT and *fad* mutants. According to results of scanning electron microscopy, *B. cinerea* enters into the plant through the intercellular spaces of pavement cells. This is consistent with images from previous publications (Mansfield *et al.*, 1981, Gourgues *et al.*, 2004, Wan *et al.*, 2015). To estimate if there was cell preference for penetration, we applied Bcgfp on cotyledons of WT and fad2-3 mutants. No
cell preference was identified. However, we confirmed that *B. cinerea* indeed enters through the intercellular spaces. Surprisingly, developing hyphae did not grow directly into the mesophyll layer. Instead, they sometimes re-emerged from the cotyledon after the first entrance through not only the intercellular spaces but also through the epidermal cells directly.

**IV.8. Experimental procedures.**

*Plant growth conditions, genotypes and chemicals -* Wild-type *Arabidopsis* (WT Columbia background) was used. The following alleles in the Columbia background were received from the ABRC: *fad2-1* (CS8041), *fad2-3* (SK18137), *fad6* (CS207), *fad2-1 fad6* and *fad2-3 fad6* double mutants were obtained by crosses in this work, *gpat4 gpat8* double mutant seeds were kindly provided by Frederic Beisson (Li *et al.*, 2007, Li-Beisson *et al.*, 2009), *fad3-2 fad7-2 fad8* mutant seeds from McConn and Browse (1996) and *aos* was from Park *et al.* (2002). Seeds were stratified for 2 days at 4 °C and then grown at 21 °C under 100 µE m⁻² s⁻¹ of light with photoperiods depending on the application (seedlings: 14 h light, 10 h dark (long days); on soil for seed propagation: 24 h light (continuous days). Seedlings were grown on half-strength Murashige and Skoog solid media (1/2 X MS, 2.15 g/L, pH 5.7; Duchefa Biochemie, Haarlem, The Nederlands) supplemented with 0.5 g/L of MES hydrate (Sigma, Buchs,
Switzerland) and 0.7 %. No sucrose was added to the media.

*Infection with Botrytis cinerea* – two strains of *B. cinerea* were used in this work: BMM, kindly provided by C. Nawrath and B 05.10 fused to the GFP (Bcgfp1) provided by M. Hahn. The fungus was grown on potato dextrose for 10 days. Conidia were filtered and diluted to 5x10⁵ spores per µl in half PDB. 2 µl of this suspension was applied on cotyledons of 7 day old *Arabidopsis* grown in petri dishes with ≈ 100% humidity. After infection Petri dishes were incubated for 48 hours in long day conditions. Infection rate was scored visually by counting the number of seedlings (cotyledons or first true leaves) with lesions. All the chemicals were obtained from Sigma-Aldrich (unless mentioned below).

*Fatty acid composition analysis* – 7- and 14-day-old seedlings were collected (≈30 mg) and placed in 15 ml Pyrex SVL capped test tubes (Milian, Switzerland). 1 ml of 2.5% H₂SO₄ solution, in methanol was added to the samples. Then, samples were incubated for 90 min at 80 °C, 1.5 ml 0.9% NaCl and 1 ml of hexane (≥ 97.0% (GC), Sigma-Aldrich, Buchs, Switzerland) were added. The solution was vortexed briefly (2 sec) then centrifuged for 5 min at 1000 rpm. 250 µl of the upper phase containing fatty acid methyl esters were collected. 1 µl of a 1:5 dilution was analysed by GCMS (initial T = 150 °C for 3 min, with a following increase from 150 °C to 200 °C).
Cuticle permeability assay – 14-day old seedlings were placed in 6-well plates with 1 ml of Toluidine blue solution (500 mg TB dissolved in 1 L of water and 0.001% of Tween 20), incubated for 15 min and gently rinsed with water (Tanaka et al., 2004).

Transmission electron microscopy – 7-day-old Arabidopsis cotyledon discs (1.5 mm in diameter) were fixed and sectioned as indicated earlier (Fabre et al., 2015). Micrographs were taken with a Philips CM100 TEM (FEI, Eindhoven, the Netherlands) at an acceleration voltage of 80 kV with a TVIPS TemCam-F416 digital camera (TVIPS GmbH, Gauting, Germany).

Polyester analysis – aliphatic polyester composition was performed on 7-day-old seedlings (only the areal part of seedling was used for this analysis). 2000 shoots were collected and pooled in liquid nitrogen to obtain one biological replicate. Six replicates were made for each genotype. Then, the polyester composition was analysed after trans esterification by base-catalysis and following acetylation as described previously (Li-Beisson et al., 2013).

ROS detection assay – 7-day-old seedlings were divided into two groups. For the first group, B. cinerea suspension (2 µl) in PDB media was applied to cotyledons. For the second group (used as a control) only PDB (2 µl) was applied to cotyledons. Both groups were then incubated for 24 prior to staining
by ROS-ID™ Total ROS/Superoxide Detection Kit (Enzo Life Sciences AG, Switzerland) as described in the manual. Seedlings were vacuum infiltrated for 30 min under dark conditions. ROS species were visualized via wavelengths 490 / 525 (Ex / Em), superoxide via 550 / 620 (Ex / Em).

Gene expression analysis – 7-day-old seedlings were either infected with *B. cinerea* or only PDB solution was applied (as a control) with 24 h incubation. Finally, seedlings were collected and stored in liquid nitrogen. 30 to 50 seedlings were used per one biological replicate. RNA isolation and quantitative RT-PCR was performed as described earlier (Gfeller et al., 2011). *PDF1.2, PRI* and *ABA1* transcripts were quantified as described earlier (Solano et al., 1998, Hilfiker et al., 2014, Chen et al., 2013).

*B. cinerea adhesion assay* – 1 µL drops of *B. cinerea* spore suspension (5x10⁵ spores/ml) were applied onto 10 cotyledons of WT and fad mutants, incubated for 4 h, then seedlings were washed with 100% ethanol with gentle shaking for 1 h. Cotyledons were stained by dipping 7-days-old cotyledons in preheated solution of Trypan blue (500 mg/L Trypan blue with 1 ml/L of Tween 20 dissolved in water) for 15 sec and directly rinsed in H₂O and visualized under Leica 5000 microscope. Modified and adapted from Doss (1995).
B. cinerea development assay – Spores were applied on 7-day-old seedlings and visualized with Trypan blue at: 4, 8 and 24 h post inoculation. To obtain stain solution, 20 mg of Trypan blue was dissolved in 10 ml of lactic acid, 10 ml glycerol, followed by addition of 10 ml warm water-saturated phenol. To perform staining, seedlings were dipped for 15 to 25 seconds in the warm solution, followed by further 30 min wash in 70 % ethanol to remove excessive stain and to remove chlorophyll. Cotyledons were visualized under a bright field microscope. Hyphal length was measured using the ImageJ freehand tool option.

Cryo-scanning electron microscopy – 7-day-old shoots were fixed with a mixture of 50 % colloidal graphite (Scientific, Essex, United Kingdom) and 50 % Tissue Tek OCTTM (Sakura Finetek, Torrance, USA) at room temperature and then cryofixed. The observations were performed as described previously (Mazurek et al., 2013).

Atomic force microscopy – 7-day-old cotyledons of Arabidopsis thaliana (WT and fad2-3) were dissected in a moist environment. Each cotyledon was fixed with double-sided tape in a small Petri-dish and quickly covered with ultra-pure water. Measurements were done on NanoWizard III (JPK Instruments, Berlin, Germany) mounted on an Axiovert inverted optical microscope (Zeiss, Jena, Germany). Quantitative Imaging (QI) mode and DNP-10 (Bruker Probes,
Camarillo, USA) cantilevers with spring constants of 0.06 N/m were used. Properly calibrated (glass indentation, thermal tuning) cantilevers indented the samples with 2.5 nN force at 40 µm/s with Z length of 2 µm. Resolution of 0.7 µm per pixel was kept constant throughout imaging. All measurements were conducted within 1h of cotyledon attachment. JPK SPM Data Processing software (JPK Instruments, Berlin, Germany) was used for force-curve fitting, while Matlab was used for data presentation. The Hertz model was used for force-curve fitting, assuming the Poisson ratio of 0.5, and pyramidal geometry of the tip (front angle of 15°). For histogram representations of pavement cells only a masking parameter was applied on the height, stiffness and adhesion maps.

Metabolomic analysis - was carried out by UHPLC-QTOF-MS on an Acquity UPLC coupled to a Synapt G2 QTOF mass spectrometer (Waters). An Acquity UPLC BEH C18 column (50 x 2.1 mm, 1.7 µm; Waters) was employed at a flow rate of 400 µL/min and maintained at a temperature of 25°C. The following gradient was used with 0.05 % formic acid in water as mobile phase A and 0.05% formic acid in acetonitrile as mobile phase B: 0.0 - 7.0 min 2-100 % B, 7.0 - 9.0 min 100% B, 9.0 - 11.0 min 2% B. The injection volume was 2.5 µL. The QTOF was operated in electrospray negative mode using the MS<sup>E</sup> mode. Mass spectrometric parameters were as follows: mass range 85 - 1200 Da, scan time 0.2 s, source temperature 120°C, capillary voltage -2.0 kV, cone
voltage -25V, extraction cone -4.5 V, desolvation gas flow and temperature 800 L/h and 400 °C, respectively, cone gas flow 20 L/h, collision energy 4 eV (low energy acquisition function) and 10 - 35 eV (high energy acquisition function), collision gas (argon) 7 x 10^{-3} mbars. A 400 ng/mL solution of the synthetic peptide leucine-enkephaline in water: acetonitrile: formic acid (50 : 50 : 0.1) was infused constantly into the mass spectrometer as internal reference to ensure accurate mass measurements. Data were recorded by Masslynx v.4.1. Marker detection was performed using Markerlynx XS (Waters) with the following parameters: initial and final retention time 0.0 and 9.0 min, mass range 85 - 1200 Da, mass window 0.02 Da, retention time window 0.06 min, intensity threshold 500 counts, automatic peak width and peak-to-peak baseline noise calculation, deisotoping function applied. Data were mean - centred and Pareto - scaled before applying principal component analysis. Markers of interest were tentatively identified based on their molecular formula determination and fragments obtained by collision-induced dissociation.

Statistical analysis - Statistical significance in pair-wise comparison was evaluated by Student’s test, where * p ≤ 0.05; ** p ≤ 0.01; ***p ≤ 0.001, multiple comparison analysis of variances (ANOVA) followed by Turkey’s HSD test was performed using JMP – statistical analysis software.
IV.9. Literature.


Hara K, Yokoo T, Kajita R, Onishi T, Yahata S, Peterson KM, Torii KU, Kakimoto T. 2009. Epidermal cell density is auto-regulated via a secretory


Chapter V: Concluding remarks
The focus of this work was to understand the roles of diunsaturated fatty acids (DUFAs) in seedling establishment and defence. Previous work revealed that plants lacking DUFAs could not survive on soil (McConn et al., 1998). In this thesis we generated plants with extremely low levels of DUFAs (< 1% total FAs) that had dwarfish and yellowish phenotypes but were viable on soil and able to produce seeds. So far no specific morphological phenotypes have been reported for *Arabidopsis fad2* mutants during their first weeks of development. In this work we found that *fad2* mutants show reduced root growth and that this correlated with high internal ROS levels. The most surprising finding of this research concerned aerial tissues. Both *fad2-3* and *fad trip* seedlings were strongly resistant to the necrotrophic pathogen *Botrytis cinerea*. We focused our research on cotyledons and found high levels of a glucosinolate (potentially 7-methylsulfinylheptyl-glucosinolate) on the surface of *fad2-3* cotyledons. This could potentially have a fungitoxic affect on *B. cinerea*. The thesis covered a broad range of questions related to polyunsaturated fatty acids and their involvement in plant development and adaptation to stresses. However, even more questions need to be addressed to better understand roles of DUFAs in plant. This chapter contains perspectives for further work on DUFAs.

In the chapter II of this thesis I compared phenotypic and biochemical characteristics of fad2 and fad6 mutants to the WT. Fatty acid analysis of the WT identified changes in fatty acid composition during the 3- to 7-day-old stage. These changes probably reflect semi-autotrophic growth during the first days, followed by switch to fully autonomous growth on the 5th to 6th day of seedling development. One of the open questions from this part was what are the mechanisms used by the plant to control fatty acid changes? Are these processes controlled at the translational or transcriptional level? To separate these processes qPCR can be performed to analyse gene expression and western blot could be performed to visualize changes in protein levels. However, this was not addressed and the thesis instead concentrated on the questions more related to the roles of DUFAs in plants.

An interesting finding was the bushy phenotype of fad2-3 mutants observed during the flowering period. Notably, this was not observed in fad2-1 mutant. The absence of this phenotype in fad2-1 mutants could be caused by a weaker (hypomorphic) allele in this plant that leads to slightly higher levels of PUFAs in fad2-1 plant compared to fad2-3, which appears to be a null mutant. Quantitative FA analysis using C18 and C16 standards will be required to test this hypothesis and this may be challenging since DUFA levels are low in
fad2-1 mutant. The bushy phenotype could be related to changes in the levels of auxin, strigolactone or cytokinin in this mutant. These hormones control rosette morphology (Fellner, 1996; Prochazka and Truksa, 1999; Nordström et al., 2004; Brewer et al., 2009 Mordhorst et al., 1998; Nogue et al., 2000). All of these hypotheses require further analysis, for example, by performing hormonal measurements at the flowering stage to identify which hormone causes to the bushy morphology of fad2-3 mutants. This could be followed by detailed analysis of the biosynthesis of this hormone to identify the stage that could potentially involve FAs. Performance of these experiments could possibly lead to a better understanding of plant development and role of DUFAs in this process.

V.2. The effect of Arabidopsis DUFA-deficient fad2 mutants on seedling growth.

In the Chapter III of this thesis I identified a role of PUFAs in root development using fad2 mutants. The reduced root growth in these seedlings was explained by almost complete absence of PUFAs that are known to be ROS scavengers (Mène-Saffrané et al., 2007; Schmid-Siegert et al., 2016). High internal ROS levels in fad2 mutants were suggested by ROS staining. These high ROS levels would lead to constant oxidative stress. The most obvious hypothesis is that once another external stress is added, for example, salt stress, it leads to stronger suppression of the root growth in fad2 mutants.
compared to WT seedlings (Zhang et al., 2012; this work). Analysis of root growth characteristics in this thesis showed that this effect is mainly caused by a reduction in the number of meristem cells and reduced cortex cell lengths in the mutants, due to over accumulation of ROS that leads to oxidative stress followed by decrease in the levels of growth hormones. This work can be continued in multiple ways. First of all, the FAD2 gene could be expressed in different cell types of fad2-3 roots to attempt to rescue root length. Then fad2-1 or/ and fad2-3 could be crossed with respiratory burst oxidase homologs (rboh) mutants to obtain plants with reduced ROS levels in the meristem and elongation zones (Lee et al., 2013).

The almost complete absence of PUFAs and their replacement by 18:1 FAs could lead to the changes in root polyester composition in fad2-1 and fad2-3 mutants. This might affect root suberinization and casparian strip maturation that can be investigated by fluorol yellow and propidium iodide staining. If changes in suberin levels are detected, it would be interesting to apply pathogens to WT and fad2 roots to examine the role of monounsaturated fatty acids in root defence.
V.3. PUFAs and their role in resistance to *Botrytis cinerea*.

In the last experimental chapter (IV) of this thesis I identified resistance of *fad2-3, fad2-1 fad6* and *fad trip* cotyledons to the necrotrophic pathogen *Botrytis cinerea*. These mutants were found to have permeable cotyledon cuticles, but no detectable changes in the internal ROS levels or upregulation of common hormonal marker genes in aerial tissues were found. The increased resistance of *fad2* mutants activated at the early stages of *B. cinerea* infection. Changes in pavement cell topography of *fad2-3* mutants can explain a partial adhesion defect of *B. cinerea* on *Arabidopsis* cotyledons. However, the major resistance of *fad2-3* to *B. cinerea* may be associated with surface metabolites. In this work one such metabolite (tentatively identified as the glucosinolate 7MSOH) was found. The structure of this compound needs to be established. To test whether this compound has a fungitoxic activity additional experiments need to be done. For example, incorporation of the glucosinolate into *B. cinerea* growth media followed by application on WT cotyledons and quantifying number of seedlings showing resistance. Another possible experiment to extend this research could be to perform surface metabolite analysis on *fad trip* and *fad2-1 fad6* cotyledons and first true leaves. This should identify similar or different compounds involved in *B. cinerea* resistance in these mutants. Beyond this, it would be interesting to identify *B. cinerea* mutants that show resistance to a 7MSOH, followed by full
transcriptomic analysis compared to control *B. cinerea* strain. These results will help us to better understand internal mechanisms that are involved in fungal susceptibility to this glucosinolate.

**V.4. Literature.**


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