Exploring Suberin's Functionality in Roots, Shoots and Tubers

A Comparative Analysis

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Summary

Suberization is a key physiological process in plants that forms protective barriers at interfaces to control water loss and regulate the exchange of substances with their environment. Besides suberin, plants developed further specialized apoplastic barriers made of hydrophobic biopolymers like cutin, lignin and often combined with waxes, especially in above-ground organs. The introductory review section of this thesis traces the evolution of our understanding of cutin and suberin from early microscopic studies to the latest discoveries about their chemical composition, biosynthesis and the transport mechanisms behind their assembly. It also highlights the unique properties that make these polymers such effective barriers in plant tissues. Mainly, it highlights the gradient of water potential difference in soil and the atmosphere. The functionality of suberin might vary completely in roots, shoot and tubers due to the more negative water potential experienced by the shoots compared to roots. The efficiency of suberized plant/environment interfaces as transpiration barriers was studied in five different plant species. Results from the cross-species study demonstrated that neither the number of suberized cell layers nor the amount of suberin correlated with their effective water loss prevention. Instead, the presence of waxes proved critical, as their removal led to a ten-fold increase in permeance, highlighting their dominant role in transpiration resistance. Furthermore, barley roots grown in soil were studied to explore how barley they respond to drought. By comparing a modern cultivar with a wild barley accession, the study shows that as soil water potential decreases, suberin and lignin begin to accumulate much earlier towards the young root tip. This shift is not just physiological, it is also accompanied at the molecular level, by the increased expression of stress-responsive genes responsible for suberin and lignin biosynthesis. It was also obvious that the wild accession had longer roots than the modern cultivar pointing to the value of wild genetic resources in breeding for drought resilience. Interestingly, changes to drought were more pronounced in soil-grown roots than in hydroponically grown ones, highlighting the importance of studying plants in their natural environment. Soil-grown plants had an earlier onset of endodermal suberization occurring closer to the root tip and total suberin amounts were doubled compared to hydroponic conditions. Overall, this thesis shows how plants build and optimize their protective barriers in response to environmental stress. These insights have important implications for improving crop resilience, postharvest storage and drought adaptation strategies.

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1. Introduction

The introduction provides an overview of the evolutionary transition of plants from water to land and all the secondary modifications followed by it. It briefly introduces suberin's history, role in different plant tissues, study methods, composition, structures, biosynthesis and genes involved. Additionally, it explains the role of suberin in plant survival and adaptation under biotic and abiotic stress and its synergistic role with lignin and wax. Soil-atmosphere water potential difference in relation with functionality of apoplastic barriers is briefly introduced. Overall, this section is the cumulative introduction of all three publications, which intends to advance the understanding of suberin's function as an apoplastic barrier.

1.1 Evolution and secondary cell wall modifications

The plant-environment interfaces are dynamic and continuously evolve due to external and internal influences to ensure survival and reproduction. These interface changes are based on environmental conditions, growth media, water availability, biotic interactions and genetic factors. The transition of life from aquatic to terrestrial environments marks a significant event in Earth's history, initiated around 450 million years ago when plants began colonizing land during the Ordovician period (Delwiche & Cooper, 2015). This process featured numerous essential adaptations that permitted land plants to survive this harsh land environment (Kumar *et al.*, 2022). Although the direct ancestors of modern plants appeared about 500 million years ago, members of a group closely similar to the charophyte green algae, with which they are thought to share a common ancestor, have been preserved as fossils from Lower Ordovician rocks formed 470 million years ago (Strother & Foster, 2021). These algae were aquatic photosynthetic organisms. Algae and early plants shared many characteristics, including the possession of chlorophyll a and b, cellulose cell walls and the storage of carbohydrates in the form of starch. Plants upon transitioning into land, they had to face many challenges like dehydration, maintenance of stability and reproduction and the development of strategies of acquiring nutrients from the soil (Mischler, 2001).

The nonvascular plants (liverworts, hornworts and mosses) were the first plant species to colonize land around 470 million years ago in the Ordovician period (Field *et al.*, 2015). These plants developed a waxy cuticle reducing water loss, the evolution of gametangia (specialized

organs to protect gametes from drying out) and sporopollenin, a rigid polymer protecting spores from harsh conditions (Graham *et al.*, 2000; Raven & Edwards, 2001). Vascular plants like ferns and club mosses evolved around 420 million years ago in the Silurian period (Harrison *et al.*, 2005). Overtime, these had the capacity and ability to develop specialized tissues (xylem and phloem) for water and nutrient transport, allowing them to grow taller and more prominently (Delwiche & Cooper, 2015). The emergence of lignin in cell walls also provided more structural support, enabling plants to stand taller and transport water around the plant body with network of xylem vessels. Roots anchor the plant and take up water and nutrients from the soil, while leaves evolved to capture energy from sunlight more efficiently than other shoot organs. Xerophytes evolved for dry conditions and hydrophytes for aquatic habitats (Friis *et al.*, 2011; Pennington, 2002).

During this process of evolution, plants also changed their structure depending on the surrounding conditions. These secondary modifications refer to changes in the cell walls or other cellular components following the primary structure. Cell wall post-modifications are the changes required for the development, growth and environmental response of plants and these often follow certain biosynthetic pathways. Some of the key secondary modifications are:

a. **Suberization:** Suberin, a hydrophobic polymer, creates a barrier in the roots (rhizo-, hypo- and endodermis), periderm (bark), tuber and seed coats. It mainly comprises long-chain fatty acids (aliphatic pathway), phenolic chemicals and glycerol. The fatty acid precursors are hydroxylated following the chain elongation and modification. These elongated fatty acids are esterified, transported and polymerized across the plasma membrane. The formation of suberized layers improves plant defense, reduces water loss and protects against environmental stresses (Ranathunge *et al.*, 2011; Schreiber, 2010).
b. **Lignification:** The lignin polymer provides rigidity and structural support, especially in capsular tissues like xylem vessels. The monolignols (p-coumaric, coniferyl and sinapyl alcohols) are polymerized to form a lignin structure and the biosynthesis of these monomers happens via the phenylpropanoid pathway. The amino acid phenylalanine is deaminated, hydroxylated, methylated and finally it is reduced to form monolignols and transported to cell walls. Lignification also aids pathogen resistance and wound healing (Bonawitz & Chapple, 2010; Vanholme *et al.*, 2010).

c. **Cutinization:** Fatty acid-derived cutin is one of the protective polymers deposited on the walls of epidermal cells along with epicuticular waxes. The fatty acids synthesized are modified by mid-chain hydroxylation and epoxidation. These cutin monomers are transported to plasma membranes and are assembled. This polyester layer along with waxes is a water-impervious protective layer against mechanical damage, UV radiation and plant pathogens (Nawrath, 2006; Yeats & Rose, 2013).
d. **Cell wall polysaccharide modifications:** Precursor sugars and galacturonic acid esters are assembled to form hemicellulose and pectin, respectively in the Golgi apparatus. Enzymes like peroxidases and laccases form the linkages between cell wall hemicellulose and pectin. These cross-linked polysaccharide chains toughen the cell walls. Modifying pectin by methylation and demethylation changes the wall properties like stiffness, porosity and interactions with other components. (Cosgrove, 2005; Mohnen, 2008).
e. **Cell wall protein modifications:** Hydroxyproline-rich glycoproteins (HRGPs), extensins and arabinogalactan proteins (AGPs) are modified and cross-linked in the cell wall, contributing to its structural integrity and flexibility. Enzymes like peroxidases, glycosyltransferases and transglutaminases modify cell wall proteins, influencing cell wall properties and interactions with other cells (Ellis *et al.*, 2010; Kieliszewski & Lamport, 1994).
f. **Formation of secondary cell walls:** In specialized cells, such as tracheary elements, fibers and sclerenchymatous cells, a secondary cell wall forms inside the primary cell wall. These secondary walls are rich in cellulose, hemicelluloses (xylan and glucomannan) and lignin. These layers with special orientation of the microfibrils provide support, rigidity, an efficient transport system for water and are involved in defense mechanisms (Meents *et al.*, 2018; Zhong & Ye, 2015).
g. **Mineralization:** Cell walls in grasses have silica (SiO_2) deposition that provides protection against herbivory and rigidity to leaf blades. Similarly, calcium carbonate deposition contributes to structural integrity and mechanical strength. These deposits form crystals known as cystoliths and can be found in certain groups of plants such as Urticaceae and Acanthaceae (Currie & Perry, 2007; Franceschi & Nakata, 2005; Hodson *et al.*, 2005).

Altogether, these secondary modifications are crucial for plant survival, growth and adaptation to their environment. They provide structural support, defend against biotic/abiotic stresses and facilitate the transport of water and nutrients.

1.2 Suberin

1.2.1 History of suberin

Suberin was first described in the early 19th century. It was identified as a substance that made plant tissues impervious to water. The term "suberin" was derived from the Latin word "suber," meaning cork. It was recognized as a key component of cork cells in tree bark, especially cork oak (*Quercus suber*). Charles-Frédéric Gerhardt, a French chemist, is often credited with some of the earliest research into cork substances, particularly identifying its unique properties in 1840. Already early chemical studies aimed to understand its composition. Initially, suberin was thought to be similar to cutin, another plant polymer, due to its lipid nature and water-repellent properties (Graça & Pereira, 2000a; Kolattukudy, 1981).

Research in the mid-20th century began to reveal the complex nature of suberin. It was found to be a polyester composed of long-chain fatty acids and glycerol. Unlike cutin, suberin also contains aromatic compounds and longer chain length monomers. Studies indicated that suberin is not just a simple barrier but has a complex structure with both aliphatic (fatty acids) and aromatic (phenolics) domains (Holloway, 1983; Kolattukudy, 1981). This dual nature contributes to its function as a protective and structural component in plant tissues. Suberin's role in reducing water loss, especially in cork cells and root endodermal cells, was understood during this period. It was seen as crucial for the formation of Casparian strip in roots, which regulate the movement of water and solutes into the vascular system.

In the late 20th century, scientists uncovered the biochemical pathways of suberin synthesis, which involves the production of monomers like fatty acids, glycerol and phenolic compounds that are later polymerized. Advances in molecular biology also identified the genes responsible for suberin biosynthesis. Key enzymes such as fatty acid elongases, acyltransferases and peroxidases were crucial for the synthesis and assembly of suberin (Beisson *et al.*, 2012; Schreiber & Franke, 2011; Vishwanath *et al.*, 2015). Studies on plant mutants deficient in suberin production helped to elucidate their functions. Mutants with reduced suberin synthesis experienced increased water loss and greater sensitivity to salt stress, emphasizing suberin's protective role. Advanced techniques like solid-state NMR, mass spectrometry and electron microscopy have revealed that suberin is a heterogeneous polymer with a complex, layered

structure. Recent research has highlighted the importance of suberin in plant responses to environmental stresses, such as drought, salinity and pathogen attack (Grünhofer *et al.*, 2022b; Grünhofer & Schreiber, 2023). It is involved in wound healing and the formation of barriers that protect plants from biotic and abiotic stressors. There is growing interest in how suberin contributes to carbon sequestration in soils and how plants with enhanced suberin production can be developed to better cope with climate change-related stresses.

1.2.2 Suberin in different plant tissues

Suberin is a hydrophobic biopolymer found in various plant tissues. It acts as a protective barrier against environmental stress, water loss and pathogen invasion. The composition and function of suberin vary depending on the tissue type and the plant species.

- **Root endodermis:** Suberin forms part of the Casparian strip, a crucial barrier that regulates the selective uptake of water and nutrients while preventing the uncontrolled movement of solutes between the soil and the vascular tissue. The suberin in the root endodermis consists of polyaliphatic domains and aromatic phenolic components. Suberin deposition in the endodermis increases with age and under environmental stress, such as drought or salinity (Enstone *et al.*, 2003).
- **Root hypo-, exodermis:** The exodermis, located near the root surface, provides an additional barrier to protect against pathogen attack, excessive water loss and toxic compounds in the soil. The exodermal suberin has a lipid-rich composition, similar to the endodermis but often less extensive. Suberin accumulation in the exodermis is influenced by external factors such as water availability and soil composition (Cantó-Pastor *et al.*, 2024; Schreiber *et al.*, 2005b).
- **Bark (Periderm):** Suberin is a significant component of the bark's periderm layer, forming a physical barrier that prevents water loss and protects the plant from mechanical injury and pathogen invasion. Bark suberin contains long-chain fatty acids, ω-hydroxy acids and dicarboxylic acids, often linked with phenolic compounds. Suberin biosynthesis is upregulated in response to wounding, leading to the formation of wound periderm to seal off injured tissues (Graça, 2015; Rains *et al.*, 2018).
- **Tuber periderm:** In tubers (potato, sweet potato, cassava and yam), suberin in the periderm plays a key role in protecting against water loss and pathogen entry, especially

during storage and wounding. The post-harvest storage quality of potato tuber is better than others because of suberin, which is rich in fatty acids, glycerol and phenolic compounds (Schreiber *et al.*, 2005a; Suresh *et al.*, 2022).

- **Fruit periderm:** In fruits, suberin along with cutin and wax acts as a barrier to protect against environmental stress, moisture loss and microbial infection, particularly in species like apples, avocados and citrus. The suberization of the fruit periderm increases with ripening and senescence, enhancing the fruit's defense mechanisms. Russeting in fruits is caused by epidermal damage, subsequent wound healing and deposition of suberin and lignin (Straube *et al.*, 2021).
- **Seeds and seed coats:** Suberin acts as a barrier in seeds to prevent desiccation, protect against microbial invasion and regulate water and gas exchange. Seed coats contain suberin in their outer layers, rich in long-chain aliphatic molecules. The presence of suberin in seed coats influences germination by regulating water uptake and acts as a barrier against toxicity (de Silva *et al.*, 2021a; Molina *et al.*, 2008).
- **Wound-healing tissues:** Suberin forms a crucial part of the wound-healing response in plants, especially in tissues like tubers, roots, leaves and bark. After injury, suberin is deposited at the wound site to form a barrier that prevents infection and dehydration. The suberin in wound-healing tissues consists of aliphatic and aromatic domains, with a high content of ω-hydroxy fatty acids and phenolics. Wound-induced suberization involves rapid upregulation of biosynthetic genes for suberin formation (Chen *et al.*, 2022; Schreiber *et al.*, 2005a).

1.2.3 Methods for studying suberin

Suberin studies involve biochemical, analytical and molecular techniques to understand its composition, structure, biosynthesis and regulation.

- **Microscopy techniques:**
  a. **Histochemical staining:** Specific dyes or fluorochromes are used to localize and visualize suberin in plant tissues. Fluorol Yellow 088, a fluorescent dye that binds suberin, is used for fluorescence microscopy. Sudan IV and Sudan Black B detect suberin's lipid-rich components. These stains help identify suberized tissues in the

root hypo-, rhizo, endodermis, periderm (bark) and wound tissues (Brundrett *et al.*, 1991; Lulai & Morgan, 1992; Ursache *et al.*, 2018).

b. **Transmission Electron Microscopy (TEM):** TEM is an effective method for identifying suberin in plant tissues. Suberin is an electron-dense lamellar structure commonly found in the endodermis, periderm, or wound-healing zones. The sample preparation process includes fixation with agents such as glutaraldehyde and osmium tetroxide, dehydration and resin embedding for ultrathin sectioning. Specific stains, including osmium tetroxide, potassium permanganate and ruthenium tetroxide, can be used to enhance the contrast of suberin (Franke *et al.*, 2005; Li-Beisson, 2011; Sitte, 1962).

c. **Scanning Electron Microscopy (SEM)**: SEM is widely used to visualize suberin deposition, cell wall structure and tissue morphology. It reveals suberin's polygonal structures or layered granular texture and helps assess barrier integrity, environmental responses and stress-induced changes. SEM is often paired with EDS/EDX for elemental analysis or combined with techniques like FTIR for enhanced visualization. Proper sample preparation, including fixation, dehydration and gold coating, is crucial for clear imaging. Key studies highlight SEM's role in understanding suberin's protective functions in plants (Correia *et al.*, 2020; Ferreira *et al.*, 2012).

- **Mass spectrometry techniques:**

a. **Gas Chromatography-Mass Spectrometry (GC-MS):** Suberin is depolymerized using alkaline methanolysis or transesterification and the resulting monomers (e.g., ω-hydroxy acids, α,ω-dicarboxylic acids and fatty acids) are analyzed by GC-MS (Marques & Pereira, 1987). GC-MS provides detailed chemical profiling of suberin, allowing for the qualification and identification of more than 20 individual monomers (Graça & Pereira, 2000a; Marques & Pereira, 2013). The amount is quantified using the GC-FID (Flame Ionization Detector) and the peaks are identified from the GC-MS chromatogram.

b. **Liquid Chromatography-Mass Spectrometry (LC-MS):** LC-MS excels in detecting non-volatile, polar and high-molecular-weight components. It can identify both monomers and oligomers that result from partial depolymerization. Characterizing larger suberin fragments or assessing the complexity of suberin networks can be done using LC-MS (Thiombiano *et al.*, 2020).

c. **Pyrolysis-MS (Py-MS):** Thermal degradation products is used for chemical fingerprinting in screening phenolic and lipid profiles, especially in thermochemical conversion studies for biofuel applications. Trace plant-derived suberin markers in soils are used to understand the contribution of different plant species to soil organic matter and track changes in soil composition over time (Kiersch *et al.*, 2012; Melnitchouck *et al.*, 2006).

d. **Matrix-Assisted Laser Desorption/Ionization (MALDI-MS):** MALDI-MS is excellent for analyzing large suberin fragments, including ester-linked oligomers that are challenging to detect with traditional GC-MS or LC-MS. Comprehensive suberin profiling of complex mixtures of monomers and oligomers provides a broad view of suberin architecture, especially for studying polymeric structures and cross-linking patterns (Veličković *et al.*, 2014).

- **Fourier-Transform Infrared Spectroscopy (FTIR):** The functional groups in suberin, namely ester, alkyl and aromatic group, have a characteristic IR absorption spectrum that can be measured. This method is beneficial in studying intact suberin polymers and comparing the chemical composition across species and different stress conditions (Mattinen *et al.*, 2009).
- **Nuclear Magnetic Resonance (NMR) spectroscopy:** NMR spectroscopy, with its unparalleled precision, unveils the exact molecular structure and the linkages of suberin monomers. The structure of depolymerized suberin is meticulously characterized using both ^{1}H NMR and ^{13}C NMR (Bernards & Razem, 2001; Serra *et al.*, 2012). NMR identifies the nature and proportions of different chemical groups in suberin and provides profound insights into how these groups are linked in the polymer (Garbow *et al.*, 1989). Other methods in NMR like solution-state and solid-state, are essential for analyzing suberin's structure and composition. Solution-state NMR is used to identify soluble components such as aliphatics, phenolics, glycerol, hydroxycinnamates like ferulic acid and lignin-like subunits. Solid-state NMR, ideal for studying suberin in its native polymeric form, reveals distinct methylene environments, with more rigid acylglycerols in the core and dynamic alkanols and alkanoic acids present on its periphery. Gel-state NMR further helps in understanding the primary structure of suberin, showing how monomeric units link to form oligomeric and polymeric networks. Together, these NMR methods provide a comprehensive view of suberin’s complex molecular structure and dynamics (Harman-Ware *et al.*, 2021).

- **CRISPR/Cas9 genetic manipulation:** CRISPR/Cas9 gene editing is a powerful tool in suberin research. It can knock out or modify genes involved in suberin biosynthesis, such as those encoding fatty acid elongases, cytochrome P450 enzymes and transcription factors (Grünhofer *et al.*, 2024). By targeting these genes, researchers can investigate the impact of specific genetic changes on suberin formation, composition and regulation in plant models such as *Arabidopsis thaliana* (Shukla *et al.*, 2021; Vishwanath *et al.*, 2015). The insights gained from this work can be applied to crop species and the function of genes can be further validated.
- **RNA sequencing (RNA-seq) and qRT-PCR:** For a comprehensive examination of suberin biosynthesis-related genes, RNA-seq and qRT-PCR are the go-to methods. RNA-seq thoroughly explains transcript levels and other transcriptomic changes linked to the suberin pathway. Meanwhile, qRT-PCR allows to validate RNA-seq data and measures the expression of certain suberin biosynthetic genes (Kreszies *et al.*, 2019; Lashbrooke *et al.*, 2016).

Studying suberin is not a simple task. It requires a multidisciplinary approach, combining histochemical techniques, chromatographic analysis, spectroscopy, molecular biology and genetic engineering. Other methods like chromatography-based metabolite profiling, X-ray Diffraction (XRD) and electron microscopy are also used to study the ultrastructure of suberin in plant tissues. This multidisciplinary approach underscores the complexity and depth of suberin research.

An indirect approach to evaluate suberin efficiency in plants usually involves examining physiological or biochemical responses associated with suberin's protective function rather than directly measuring the compound itself.

1. **Drought Tolerance Assay:** This method evaluates how suberin helps plants retain water during drought (Cantó-Pastor *et al.*, 2024). As a protective barrier, suberin reduces water loss, leading to higher Relative Water Content (RWC) and improved plant stomatal control (Yan *et al.*, 2018). Kosma *et al.* (2014) indicated that *Arabidopsis* mutants with impaired suberin formation show decreased drought resistance.
2. **Radial Oxygen Loss (ROL):** The effectiveness of the suberin layer in plant roots such as rice can be measured by quantifying the oxygen lost across the root surface. Plants

with well-developed suberin typically show reduced Radial Oxygen Loss (ROL), especially in waterlogged conditions. This approach is commonly used in wetland plant studies, where controlling ROL is vital for root aeration and stress response (Colmer, 2003; Ejiri & Shiono, 2019).

3. **Ion Leakage Test (Electrolyte Leakage):** This technique indirectly assesses suberin's role in maintaining membrane integrity (Puthmee *et al.*, 2013). Suberin-deficient plants experience more electrolyte leakage under osmotic stress or freezing conditions. Researchers can evaluate suberin efficiency by measuring ion concentration in the surrounding solution. Baxter *et al.* (2009) found that suberin-deficient *Arabidopsis* mutants showed increased ion leakage, indicating a weakened barrier function.
4. **Salt Stress Assay:** Suberin regulates ion uptake in saline environments (Shao *et al.*, 2021b). This method evaluates its efficiency by exposing plants to salt stress and measuring sodium (Na^+) and potassium (K^+) levels in roots and shoots. Practical suberin barriers limit Na^+ uptake, improving salt tolerance. According to Barberon *et al.* (2016), suberin deficiency is linked to higher sodium levels in *Arabidopsis* mutants.
5. **Water Loss Measurement (Gravimetric Method):** This method assesses suberin efficiency by measuring water loss from excised plant tissues. Since suberin reduces water permeability, tissues with more effective suberin layers lose water more slowly. Li *et al.* (2007) and de Silva *et al.* (2021b) showed that *Arabidopsis* mutants with increased suberin production had lower water loss in controlled conditions.
6. **Pathogen Resistance Assay:** Suberin is a barrier that prevents pathogen entry into plant tissues. Researchers assess suberin's effectiveness by inoculating plants with soil-borne pathogens like *Phytophthora* spp. and monitoring infection rates (Ranathunge *et al.*, 2008). Plants with higher suberin levels typically show greater resistance to root-infecting pathogens. Thomas *et al.* (2007) found that suberin-deficient mutants were more susceptible to infection.
7. **Hydraulic Conductivity Measurement:** This method examines how suberin regulates water flow in root tissues by measuring the hydraulic conductivity of plant roots using a pressure chamber and root pressure probe. Lower conductivity indicates greater suberin efficiency, as it restricts water movement. Enstone *et al.* (2003), Ranathunge & Schreiber, (2011) and Kreszies *et al.* (2019) effectively demonstrated this in roots, showing suberin's role in reducing water permeability and forming apoplastic barrier formation.

1.2.4 Suberin composition and structure

Suberin is known as a bio-polyester made of a polyaliphatic and a polyaromatic domain with glycerol as its backbone (Graça, 2015; Graça & Pereira, 2000a; Schreiber & Franke, 2011). The polyaliphatic domain of suberin consists primarily of long-chain mono- or bi-functional fatty acids and their derivatives. These compounds contribute to suberin's hydrophobic nature, making it impermeable to water. Key components of the aliphatic domain include: saturated and unsaturated long-chain fatty acids (typically C_{16} to C_{26}) in varying relative amounts (Holloway, 1983; Kolattukudy, 2005). These are the primary building blocks of the suberin aliphatic domain. Hydroxylated fatty acids, such as ω-hydroxy fatty acids and α,ω-dicarboxylic acids, represent most of the aliphatic monomers**.** Hydroxylation increases the cross-linking potential, strengthening the suberin structure. Long-chain alcohols add to the hydrophobic nature of the aliphatic domain (Ballal & Chapman, 2013). The detailed monomers reported, along with their related substance classes and corresponding plant materials, are summarized and referenced in Table S1.

The aliphatic domain forms hydrophobic lamellar layers, creating a waterproof barrier that limits water and solute movement in suberized tissue. The phenolic domain adds structural rigidity and resistance to microbial degradation (Bernards, 2002; Kligman *et al.*, 2022). The phenolic domain is covalently bound to the cell wall and interacts with lignin, reinforcing the cell wall structure. The most prominent phenolic compound in suberin is ferulic acid that is cross-linked by ester and ether bonds, forming a robust, resistant network (Graça, 2015). Suberin also contains other phenolic monomers, such as p-coumaric acid and sinapyl alcohol, contributing to its rigidity and antimicrobial properties (Reyes *et al.*, 2024). Suberin's phenolic chemicals bear similarities to lignin, another important biopolymer found in plant secondary walls. The phenolic domain can interact with lignin and enhance the mechanical strength of the cell wall (Bernards & Razem, 2001). Suberin lamellae are usually deposited directly between the plasma membrane and the primary cell wall. The aliphatic domain in suberin layer aligns towards outside, whereas the phenolic domain forms the inner portion of the layer next to the principal cell wall. Because of this configuration, the phenolic domain can anchor suberin to the cell wall, while the aliphatic domain can act as a barrier (Serra & Geldner, 2022). The above-described linkages and orientations are, to a large degree, still model hypotheses and up to now, the organized three-dimensional suberin polymer structure in its unaltered native form has not been studied.

1.2.5 Biosynthesis of suberin and the genes involved

Suberin biosynthesis is a highly regulated and complex process that involves multiple pathways and enzymes. The production of monomer precursors of fatty acids, glycerol and phenolics are crucial steps. The polyaliphatic domain of suberin comprises Long-Chain Fatty Acids (LCFAs), ω-hydroxy fatty acids, α,ω-Dicarboxylic Acids (DCAs) and primary alcohols. The Fatty Acid Synthase (FAS) complex in the plastids synthesizes C16:0, C18:0 and C18:1 fatty acids. Key enzymes include fatty acid elongases, such as Ketoacyl-CoA synthetase (KCS) proteins, fatty acyl-CoA synthetases and various hydroxylases that generate Very Long-Chain Fatty Acids (VLCFAs). VLCFAs are transported into the Endoplasmic Reticulum (ER) after CoA groups are added and elongated. Initially, the biosynthesis of VLCFAs involves acetyl-CoA conversion into malonyl-CoA. Fatty acid elongases (FAEs) elongate malonyl-CoA by adding C_2 units and the condensation of C_2 units involves KCS (Beisson *et al.*, 2012; Franke *et al.*, 2009; Lee *et al.*, 2009; Serra *et al.*, 2009a). Subsequently, VLCFAs can be oxygenated by the members of the cytochrome P450 oxidase protein family (Höfer *et al.*, 2008). The addition of hydroxy- and carboxy groups results in the most prominent suberin monomers, ω-hydroxy fatty acids and DCAs (Compagnon *et al.*, 2009). Primary alcohol formation is achieved by reducing the carboxyl groups with the help of Fatty acyl-CoA Reductase (FAR) enzymes (Domergue *et al.*, 2010; Liu *et al.*, 2019). These modified fatty acids are esterified to glycerol by BAHD-type acyltransferases, which form the glycerolipid backbone of the polyaliphatic domain (Beisson *et al.*, 2007). The polyaromatic domain is derived from monomers of the phenylpropanoid pathway. Deamination of phenylalanine by Phenylalanine Ammonia-Lyase (PAL) leads to the formation of cinnamic acid (Bernards, 2002; Bernards & Razem, 2001). It is further hydroxylated and methylated to produce other phenolic acids that are found in the polyaromatic domain. These phenolic acids are polymerized and cross-linked to form a rigid network by peroxidases and laccases. The integration of both domains into the cell wall occurs via ester and ether linkages (Pollard *et al.*, 2008).

The studies of different genes and associated enzymes helped to identify their role in suberin biosynthesis. Studies related to KCS2/DAISY and KCS20 showed that they encode FAE-condensing enzymes for aliphatic suberin production. Mutants showed reduced growth rate and suberin amount; C_{22} was depleted but had enhanced C_{16}-C_{20} derivatives (Lee *et al.*, 2009). KCS6 in potatoes is involved in the formation of suberin monomers with $>C_{28}$ chain length (Serra *et al.*, 2009a). A reverse genetics approach characterized the CYP86A1/HORST gene

as a key enzyme in synthesizing ω-hydroxyacids. The mutants displayed reduced suberin accumulation, particularly of chain lengths shorter than C_{20} (Höfer *et al.*, 2008). CYP86A33-RNAi lines producing potato tubers showed reduced $C_{18:1}$ ω-hydroxy and α,ω-diacid levels of suberin with thinner suberin lamellae (Serra *et al.*, 2009b). A similar approach was used to identify CYP86B1/RALPH as a key oxidase in the biosynthesis of suberin ω-hydroxyacids and α,ω-dicarboxylic acids. Chemical analysis showed that the level of C_{22} and C_{24} ω-hydroxy acids and α,ω-dicarboxylic acids were reduced in mutants (Compagnon *et al.*, 2009). CYP94B1 from *Avicennia officinalis* and its putative ortholog in *Arabidopsis* are shown to control root suberin biosynthesis (Krishnamurthy *et al.*, 2020) and also the mutants of CYP94B3 showed decreased suberin in *Arabidopsis* (Krishnamurthy *et al.*, 2021). The FAR1, FAR4 and FAR5 triad are expressed in root endodermal cells and it was shown that they are involved in reducing C_{22}, C_{20} and C_{18} primary alcohols, respectively. The triple mutant had 70-80 % less suberin than the wildtype (Domergue *et al.*, 2010). GPAT5 acts on a wide range of long-chain ω-oxidized and/or unsubstituted acyl-CoA components; mutants showed >50 % aliphatic suberin reduction in root endodermis and seed coats (Beisson *et al.*, 2007). GPAT4, GPAT 6 and GPAT 8, which are associated with cutin biosynthesis, showed strict preference for C_{16} and $C_{18:1}$ ω-oxidized acyl-CoA (Wendel *et al.*, 2009). ASFT (Aliphatic Suberin Feruloyl Transferase) and BAHD (Benzyl alcohol acetyltransferase, Hydroxycinnamoyl-CoA: Hydroxyacid acyltransferase, p-Hydroxybenzoate: CoA ligase and Deacetylvindoline 4-O-acetyltransferase) family of acyltransferases is expressed in the seed coat and root endodermis and mutants were devoid of ferulic acid in their polyphenolic domain (Gou *et al.*, 2009; Molina *et al.*, 2009). Other enzymes like ESB1, GELPs, HHT, FHT, FACT, LPT, ABC transporters peroxidases and laccases are involved in the crucial step of suberin biosynthesis (Grünhofer *et al.*, 2021b; Nomberg *et al.*, 2022a).

1.2.6 Suberin transport and regulation of the suberin pathway

Exporting suberin monomers from their biosynthesis sites to deposition sites is a complex process and the transport mechanisms are still poorly understood. *In-vivo* and in-silico assays showed that many suberin-associated enzymes are located in the endoplasmic reticulum (ER). The synthesis and modification of aliphatic monomers occur in the ER, whereas aromatic monomers synthesis occurs in the cytosol. Some of the hypothesized mechanisms are: ER domains attached to the plasma membrane (PM); cytosolic carriers carry soluble proteins; oleophilic droplets bud from ER and travel through the cytosol to the PM; specialized vesicles

from Golgi-mediated sections transport suberin components (Pollard *et al.*, 2008). In rice, ER-derived ribosome-bearing vesicles were reported to transport aliphatic suberin (Oparka & Gates, 1982). Transporters such as ATP-Binding Cassette (ABCG) and Lipid Transfer Proteins (LTP) superfamilies are speculated to participate in suberin transport. DSO/ABCG11 plays a role in suberin monomer export (Panikashvili *et al.*, 2010). ABCG2, ABCG 6 and ABCG 20 were associated with suberin metabolism and mutants had lower suberin deposition in seeds of *Arabidopsis* (Yadav *et al.*, 2014). The ABCG1-RNAi-silenced potato had lower VLCFAs, primary alcohols, dicarboxylic acids levels and distorted suberin layers (Landgraf *et al.*, 2014). The RCN1/ABCG5 rice mutant line had lower C_{28} and C_{30} fatty acids and ω-hydroxyacids (Shiono *et al.*, 2014). LTPI4 mutants had lower C_{18} VLCFAs and a significant rise in C_{24} and C_{26} VLCFAs (Deeken *et al.*, 2016). Furthermore, a Glycosylphosphatidylinositol (GPI)-Anchored LTP mutant had lower levels of C_{20}-C_{24} fatty acids, C_{20} and C_{22} primary alcohols, C_{22} and C_{24} ω-hydroxyacids and C_{20} and C_{22} dicarboxylic acids in *Arabidopsis* seeds (Lee & Suh, 2018).

Several suberin related transcription factors and regulators have been identified in the last decade, but only limited knowledge remains. Early studies in potato tubers with supplementation of ABA increased suberin amount and diffusion resistance (Lulai *et al.*, 2008). Similar results were obtained for the roots of maize and *Arabidopsis* (Hose *et al.*, 2000; Wang *et al.*, 2020). Co-expression studies showed that MYB, NAC and WRKY transcription factor gene families also play a role in the suberization process. MYB41 in *Arabidopsis* is required for aliphatic suberin deposition of cell-wall-associated-suberin-like lamellae (Kosma *et al.*, 2014). MYB107 and MYB9 transcription factors coordinate the transcriptional induction, biosynthesis and transport of aromatic and aliphatic monomers (Lashbrooke *et al.*, 2016). MYB107 interacts with regulatory elements in their promoters for the expression of suberin genes (Gou *et al.*, 2017). MYB107 and MYB41 in kiwi fruit interact with the CYP86A1 promoter and regulate aliphatic suberin gene expression (Wei *et al.*, 2020). MYB78 is a regulator of suberin in sugarcane internodes during culm development and wound healing. MYB78 in *Nicotiana benthamiana* leaves induced suberin deposition by activating suberin synthesis genes (Figueiredo *et al.*, 2020). MYB41, MYB53, MYB92 and MYB93 all promote endodermal suberization in *Arabidopsis* roots (Shukla *et al.*, 2021) and *Nicotiana benthamiana* (To *et al.*, 2020). ANAC103 negatively regulates suberin synthesis in potato tubers (Verdaguer *et al.*, 2016). MYB1 in cork oak is involved in transporting and assembling both suberin and lignin biosynthesis and is associated with secondary growth and cork development (Capote *et*

al., 2018). MYB93 in apple fruit regulates suberin formation during the russeting of the fruit periderm during its growth (Legay *et al.*, 2016). MYB102 and MYB74 in potatoes regulate wound suberization (Wahrenburg *et al.*, 2021). MYB70 in *Arabidopsis* is the root negative regulator of suberin biosynthesis (Wan *et al.*, 2021). *Arabidopsis* ANAC058, an ortholog of potato ANAC103, positively regulated endodermal suberization (Markus, 2018). ANAC046 is a transcriptional activator of suberin in *Arabidopsis* (Mahmood *et al.*, 2019). WRKY33 in *Arabidopsis* is the upstream regulator of CYP94B1 oxidase; mutants showed lower suberin amounts and were more sensitive to salt exposure (Krishnamurthy *et al.*, 2020). WRKY9 in *Arabidopsis* (Capote *et al.*, 2018) is the upstream regulator of CYP94B3 and CYP86B1 oxidases (Krishnamurthy *et al.*, 2021). Most biosynthesis and regulation research is still conducted in *Arabidopsis* primarily and most of these results still need to be validated in other crop species.

1.2.7 Key factors influencing suberin deposition

The formation of suberin is influenced by several factors such as drought, salinity, nutrient availability and pathogen attack. Genetic regulation involves transcription factors, along with hormonal signals (abscisic and jasmonic acid) which modulate suberization under stress conditions (Lashbrooke *et al.*, 2016; Shukla *et al.*, 2021). Additionally, developmental cues, such as tissue-specific expression, plant age and cell differentiation regulate suberin deposition (Ranathunge *et al.*, 2011; Ursache *et al.*, 2018).

1.2.7.1 Biotic stress

Suberin is reported as a barrier in stopping nematodes and other pathogens from penetrating into the root. The suberized endodermis acts as a line of defense for pathogens entering the vascular tissues and spreading throughout the plants (Holbein *et al.*, 2019; Kashyap *et al.*, 2022; Ranathunge *et al.*, 2008). In soybeans, the amount of suberin and fungal resistance is correlated (Thomas *et al.*, 2007) and also some cultivars with high suberin amounts experienced slower hyphae growth (Ranathunge *et al.*, 2008). Suberin plays a key role in facilitating beneficial biotic interactions and maintaining a balanced plant ionome through modulation by the root microbiome, thereby contributing to enhanced plant adaptation to various stress conditions (Salas-González *et al.*, 2021). The ligno-suberin deposition in the endodermis and lignification of the secondary cell wall structures are also reported as an immune response due to biotic

stress (Floerl *et al.*, 2012; Kashyap *et al.*, 2022; Reusche *et al.*, 2012; Zhou *et al.*, 2020). A detailed list of biotic stresses is given with references in Table S2.

1.2.7.2 Abiotic stress

Environmental conditions can also negatively impact the plant growth and development. Such abiotic stress may include, Osmotic stress, water deficit/drought, flooding/water-logging, hypoxia, salinity, nutrient excess/deficiency, heavy metal exposure etc. Suberin, particularly in the root endodermis and periderm plays a crucial role by regulating water and solute movement in response to stress. Controlled water deficit stress experiments are conducted in hydroponics systems using polyethylene glycol (PEG), which induces non-toxic osmotic stress, allowing the precise adjustment of the medium's water potential. Decreasing the water potential for maize and barley plants caused a decreased root length and increased the suberin amounts (Kreszies *et al.*, 2019, 2020a; Zeier & Schreiber, 1998). NaCl stress has the potential to cause root organs to experience significant ion toxicity in addition to the osmotic stress that already PEG alone can cause (Grünhofer *et al.*, 2022b). Suberin genes are expressed upon salt treatment and the amount of aliphatic and aromatic suberin increased when compared to control (Knipfer *et al.*, 2021; Krishnamurthy *et al.*, 2011). In rice, during salt stress, the extent of suberin deposition in primary roots negatively correlated with Na^+ ion uptake in the shoot.

Other nutrient deficiencies such as Fe, Mn and Zn were shown to decrease suberization in *Arabidopsis*, while a lower amount of S and K resulted in increased suberin contents (Barberon *et al.*, 2016). Ethylene was found to mediate the reduction in suberin deposition, whereas abscisic acid (ABA) promoted suberization. By adjusting the Fe, Mn and Zn intake and retaining K and S in the stele, plant roots may have adapted to manage changing nutrient availability (Barberon, 2017; Chen *et al.*, 2019; Doblas *et al.*, 2017). Deficits in phosphorus (P) and nitrogen (N) significantly impact plant suberization patterns and root morphology. In barley, a nitrogen shortage led to longer roots and increased deposition of aliphatic suberin, which enhanced root suberization near the tip (Armand *et al.*, 2019; Melino *et al.*, 2021). In contrast, ammonium limitation decreased suberin levels in rice (Ranathunge *et al.*, 2016). On the other hand, barley exposed to phosphorus deficiency had longer roots but lower levels of aliphatic suberin (Armand *et al.*, 2019). The quantified suberin amounts contradict the microscopic observations, which suggested that phosphorus deficiency enhances suberization. These variations emphasize integrating chemical and histochemical analyses and species-

specific reactions. Silicon (Si) supplementation influences suberin deposition in maize and rice differently; while some studies report enhanced suberization, others indicate no effect or even a reduction (Fleck *et al.*, 2011, 2015; Vaculík *et al.*, 2012). Chemical tests showed that there was occasionally a drop in aromatic suberin but no effect on aliphatic suberin (Fleck *et al.*, 2015; Hinrichs *et al.*, 2017). In barley, silicon supplementation did not alter suberin levels but resulted in a slight increase in root length (Kreszies *et al.*, 2020b). By increasing the expression of the aquaporin gene, Si mitigated the decreases in root hydraulic conductivity in sorghum caused by osmotic and salt stress (Liu *et al.*, 2014, 2015). Crop plants exposed to heavy metals, especially cadmium (Cd), have shorter roots and more suberin lamellae deposits (Líška *et al.*, 2016; Lukačová *et al.*, 2013; Vaculík *et al.*, 2009). A complex sensing mechanism was demonstrated by the unilateral suberization of maize roots in response to Cd (Líška *et al.*, 2016). Compared to aeroponics, hydroponic growth increased Cd uptake but decreased suberin deposition (Redjala *et al.*, 2011). When exposed to Cd, the maize endodermis and exodermis had higher levels of suberin, according to a quantitative study (Zeier, 1998).

In rice, oxygen deprivation caused a decrease in radial oxygen loss but not water transfer due to enhanced suberin deposition in the exodermis and endodermis (Kotula *et al.*, 2009). The exodermal barrier's function in limiting NaCl permeability was validated by genetic research (Shiono *et al.*, 2014). According to similar findings, hypoxia-induced exodermal suberization decreased oxygen loss in *Hordeum marinum* (Kotula *et al.*, 2017). Suberin genes were activated by small organic acids such as ABA in low-oxygen environments, improving barrier qualities (Colmer *et al.*, 2019). A more comprehensive review validated suberin reinforcement in rice, wheat and maize under waterlogging conditions (Pedersen *et al.*, 2021), but this could not be confirmed for poplar (Grünhofer *et al.*, 2023).

1.3 Suberin’s synergistic roles with wax

It is not justifiable to assume that a suberized cell wall creates a watertight transport barrier. The degree of effectiveness of the suberized tissues as water barriers might vary significantly depending on the organs and tissues of the plant (Schreiber *et al.*, 2005a; Grünhofer & Schreiber, 2023). The water and dissolved solutes permeability of the cuticle often rise by 100–1000 times once the cuticular waxes are extracted (Schönherr, 1976; Schreiber, 2010). The cuticular associated wax function might vary from the suberized surface waxes. The complex

connection between suberin and waxes in plant tissues reflects their complementary functions in plant physiological processes as well as their shared biosynthesis routes. The results of Soler *et al.* (2020) underline this shared biosynthesis by showing that salt stress affects suberin-associated waxes, indicating a regulatory overlap between abscisic acid pathways for wax and suberin biosynthesis. The thorough investigation by Huang *et al.* (2017) demonstrates that the potato periderm tissues consistently contained notable amounts of fatty acids, alkanes and alcohols—the building blocks of suberin and waxes. A comprehensive biosynthetic approach in plants is suggested by Kosma *et al.* (2015), who show that several enzymes involved in the synthesis of suberin polymers are also essential for the production of waxes.

Suberin and related waxes have physiological purposes that go beyond their structural ones. Waxes significantly increase the periderm's impermeability, even while suberin might serve as the barrier to prevent water loss (Schreiber *et al.*, 2005a; Serra *et al.*, 2010). The work of Chemelewski *et al.* (2023), further supports the protective functions by observing that wax buildup is crucial to the permeability and integrity of plant organs, offering an extra line of defense against environmental challenges. These lipid molecules also play an important role in interactions between plants and pathogens. By strengthening the plant cell wall, suberin and waxes both help to increase resistance to infections where these substances function as a first line of defense (Wang *et al.*, 2020). Waxes offer a physical barrier against microbial invasion, while suberin may have both structural and signaling functions during defense reactions. According to Zhou *et al.* (2022), suberin and waxes are essential elements in the larger context of lipid metabolism and are necessary for the development of plant resilience mechanisms. Overall, the association of suberin and waxes in plant defense mechanisms illustrates a sophisticated synergy between these two lipid classes, rooted in their biosynthetic interconnectedness and their shared roles in enhancing plant resilience against various biotic and abiotic stresses.

1.4 Suberin’s synergistic roles with lignin

Suberin and lignin are essential plant polymers that work synergistically to improve plant resilience to environmental challenges while contributing to structural integrity. Both apoplastic barriers are essential to producing the secondary plant cell wall, particularly in

specialized tissues like the endodermis and exodermis, where they regulate water and nutrient transport while acting as a barrier to pathogens and harmful chemical substances.

Suberin is deposited in between the cell walls and plasma membrane forming suberin lamellae, which prevent water and other solutes from moving through the apoplast (Sexauer *et al.*, 2021). Maintaining nutrient balance and shielding the soil from dangerous contaminants depend on this deposition (Li *et al.*, 2017). MYB proteins, among other transcription factors, control the production of suberin by coordinating the expression of genes involved in suberin biosynthesis (Capote *et al.*, 2018; Shukla *et al.*, 2021). For example, it has been demonstrated that the gene MYB52 promotes the manufacture of lignin in apples during the suberization process, suggesting a complicated interaction between these two polymers (Xu *et al.*, 2022). On the other hand, the phenolic polymer lignin gives plant tissues mechanical support and helps maintain their structural integrity. It has a crucial role in water conduction and resistance to biotic and abiotic stressors and is present mainly in the secondary cell walls of vascular plants (Liu et al., 2018). Specific transcription factors, such as the R2R3-MYB family, tightly regulate the production of lignin, which is also impacted by various environmental. Along with suberin, lignin is deposited in the Casparian strips of the endodermis and exodermis, forming a strong barrier that restricts the passive movement of solutes and water (Lee et al., 2013; Li et al., 2017).

The relationship between lignin and suberin is significant when considering plant defense mechanisms. Both polymers have a role in the plant's defense reaction to wounding and pathogen attack and phytohormones, including salicylic acid and abscisic acid, can cause their deposition (Boher *et al.*, 2013). Suberin and lignin in cell walls improve the plants' structural integrity and reduce the permeability to harmful agents, enhancing the resistance to environmental stresses. Recent research also shows that manipulating their biosynthesis pathways can further boost the plants resilience and adaptability. For instance, discovering specific genes that control these polymers creates opportunities for biotechnological uses to improve agricultural stress tolerance (Lashbrooke *et al.*, 2016; Lin *et al.*, 2019).

Altogether, lignin and suberin are crucial elements of plant cell walls that complement each other to improve structural integrity and offer protection from environmental stimuli. They are crucial to plant physiology and have potential uses in agriculture since their production is complexly controlled by a network of transcription factors and impacted by outside stimuli.

1.5 Water potential gradient acting on plants

Knowing the significance of the soil and atmospheric water potentials for plants is essential to comprehend plant physiology, especially regarding stress reactions and water availability. Water flow and availability are governed by the Soil-Plant-Atmosphere Continuum (SPAC), including but not limited to the atmospheric and soil water potentials. The water potential describes the water status of a sphere (e.g., the atmosphere, a plant tissue or even a single cell) and can range from a high water-saturation (e.g., water potential close to 0 MPa) to extreme water-deficiency (e.g., water potential as low as -100 MPa). Ultimately, it is the water potential gradient between two adjacent spheres (e.g., rhizosphere to root or leave to atmosphere) that orchestrates the direction and intensity of water flow (Grünhofer & Schreiber, 2023).

A plant's capacity to absorb water through its roots is directly impacted by the soil water potential, representing the energy state of the water in the soil. Monteiro *et al.* (2018) state that plants can absorb water effectively when the soil's water potential is higher (less negative) than that of the roots. This is necessary to sustain turgor pressure, promote nutrient transport and assist with photosynthesis. In contrast, drought conditions lower the soil's water potential (more negative), reducing water intake and potentially subjecting plants to physiological stress. Water stress can affect photosynthesis and the plant's general health by causing a drop in the water potential of the leaves (Lewar *et al.*, 2022; López-Serrano *et al.*, 2019). According to previous studies, plants exposed to low soil water potential, for example, have lower stomatal conductance, which restricts transpiration and CO_2 absorption, ultimately affecting growth and yield (Gisbert-Mullor *et al.*, 2023; Spinelli *et al.*, 2017). Conversely, temperature and humidity also impact on the atmospheric water potential, affecting transpiration rates. Water moves more easily from the soil to the leaves when the transpiration (stomatal) generates a negative pressure inside the plant (Yan *et al.*, 2015). During dry conditions, plants can reduce water stress by absorbing atmospheric moisture through foliar uptake (Gong *et al.*, 2019; McHugh *et al.*, 2015).

Understanding the interaction between soil and atmospheric water potentials is crucial for comprehending how plants respond to environmental stressors. For instance, the soil water potential might worsen during drought conditions because the water from soil moves to atmospheric with much lower humidity (Shackel, 2007; Spinelli *et al.*, 2017). According to

Yan *et al.* (2015) and Xue & He (2008), gradients in water potential between the soil, plant and atmosphere play a crucial role in regulating water balance, which is essential for maintaining plant physiological functions.

To achieve effective irrigation management, it is essential to integrate soil and atmospheric water potentials. By monitoring the plant water potential, farmers can schedule irrigation in a way that maximizes water use efficiency and minimizes crop stress. This approach is particularly beneficial in regions with limited water resources, where improving agricultural practices and enhancing crop resilience can result from a better understanding of water potential dynamics (Gisbert-Mullor *et al.*, 2023; Spinelli *et al.*, 2017). Suberin, lignin and water potentials are important physiological research areas in plants, highlighting the roles of these biopolymers in plant stress responses and water management. Suberin and lignin are critical components of the plant cell wall, particularly in root tissues, where they regulate water permeability and provide structural support. In conclusion, the atmospheric and rhizospheric water potential significantly influence plant-water relations. These factors affect stress responses, physiological functions and water uptake. Understanding these relationships is crucial for optimizing agricultural practices and managing plant health in various environmental conditions.

1.5 Aims of this thesis

- Is there a correlation between the barrier properties of the suberized interfaces and the number of suberized layers, the amount of soluble wax and the amount of suberin?

Suberized cell walls function as protective barriers at the interfaces between plants and soil or the atmosphere in different plant organs (soil-grown roots, aerial roots, tubers and bark). Does the number of suberized layers show relative barrier properties? Does suberin alone form an effective apoplastic barrier, or do the properties of these interfaces change upon wax extraction?

- How does the response of root apoplastic barrier formation change according to soil water potential in wild barley and a modern cultivar, how does this differ in hydroponic versus soil-based media?

Hydroponic plant cultivation is a commonly used method for root research and soil experiments are rare. Previous results in hydroponics have shown that suberin deposition patterns along the root and amounts vary with differing water potential and cultivars. We hypothesised that the root response to drought stress of wild barley (Pakistan) will differ from the modern cultivar (Scarlett) and differ between soil and hydroponic conditions.

2. Results

The results section contains summaries of three peer-reviewed articles published in Taylor & Francis Group, Springer Nature and John Wiley & Sons. This thesis does not include Materials and Methods section, since each publication provides a detailed description of methodologies on its own.

The introductory Chapter 1 explains how plant cell walls are rendered hydrophobic through the presence of the apoplastic barriers cutin, suberin and waxes, which all are essential for the survival of terrestrial plants. The chapter also discusses the technological applications and future research directions in the study of plant hydrophobic barriers. Chapter 2 examines the suberized interfaces between plants and their environments in five different plant species in more detail. It focuses on their anatomy, chemical composition (including suberin and wax) and water permeability. Chapter 3 explores the impact of water stress on the formation of apoplastic barriers in soil-grown barley roots, highlighting differences compared to hydroponically cultivated barley plants subjected under osmotic stress.

The original publications are attached in the appendix (Chapter 1-3 ≙ Appendix 1-3, respectively), or copyable Portable Document Format (PDF) versions can be found online by using the Digital Object Identifier (DOI) hyperlinks.

The Project DEAL agreement ensures that all publications are open access.

Chapter 1

Hydrophobization of cell walls by cutin, suberin and associated waxes

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"Hydrophobization of cell walls by cutin, suberin and associated waxes", was published in 2023 as one of the book chapters in Plant Cell Walls: Research Milestones and Conceptual Insights (1st ed.) by CRC Press, Taylor & Francis Group. The original publication is attached in the appendix of this dissertation. The following overview is intended to highlight my contribution and outcome. As an abstract that primarily focuses on the most important elements for conciseness and clarity, relevant references to some claims have been excluded.

I am the first author of this book chapter, which reviews the following elements: (i) a brief recapitulation of the main steps in the history of research into cutin and suberin; (ii) a summary of the current state of knowledge regarding the chemical composition, biosynthesis and function of hydrophobized plant cell walls; (iii) the selected relevant methodological and applied aspects of the problems associated with plant interfaces made of cutin and suberin; and finally, (iv) pointing out the critical future questions and approaches further improving our knowledge about hydrophobic plant interfaces. The key discussion in the book chapter is as follows:

- **Historical perspective**: The article demonstrates the evolutionary importance of plant cell wall modifications, including cutin and suberin, which protect plants against water loss, pathogen invasion and environmental stresses. It shows how scientific findings,

like microscopy, have clarified the nature of these polymers and their roles in adapting plants to terrestrial life.

- **Hydrophobic barriers and water permeability**: It emphasizes how cutin and suberin decrease uncontrolled water loss in plants. These polymers form hydrophobic barriers and especially wax plays a significant role in water retention. The comparison of species and tissues reveals that wax accumulation largely determines the water permeability of cutinized and suberized layers.
- **Chemical nature of cutin and suberin**: It explains the chemical composition of these biopolymers, focusing on fatty acid derivatives, waxes and aromatic compounds like lignin and certain suberin monomers. It highlights the role of glycerol as a cross-linker in forming polyesters and enzymes such as cytochrome P450 monooxygenases involved in fatty acid elongation and hydroxylation. Meanwhile, cutin and suberin primarily act as barriers to water, nutrients, microorganisms and gases. The discussion in this chapter includes the functions of these polymers in forming essential barrier coatings, preventing pathogens from entering plants and reducing nutrient loss from plant tissues.
- **Transport barriers in roots and leaves**: The publication explores suberin's role in roots, preventing harmful materials like sodium chloride from entering while allowing water and mineral absorption. This study examines the differences in transport mechanisms within roots and leaves, with a particular focus on the role of suberin in regulating two key pathways: apoplastic (between cells) and symplastic (through cells). The findings also have practical implications for agriculture. The wax or suberin amount changes may help the plant adjust to different environmental stresses, for example, drought and salinity. Transport barrier studies will aid in selecting or developing the properties required to thrive under various challenging conditions.
- **Technological and future applications**: The derived cutin and suberin polymers can also be used as bio-based polymers, offering biodegradable alternatives to petroleum-based plastics for packaging and contributing to sustainability. This publication identifies current methodological limitations in fully understanding the hydrophobic barriers' molecular structure and function. It calls for further research on the ultrastructural arrangement of these biopolymers and their precise roles in plant-environment interactions.

Apoplastic barriers, mainly those formed by Casparian strips and suberin lamellae, are significant in controlling the inflow of water and nutrients into the roots while excluding the entry of toxic ions. The chapter talks about the differences between the hydrophobic barriers in roots and leaves: leaves have very effective cuticular barriers, while the roots have to achieve an equilibrium of water and nutrient absorption while preventing the flux of specific ions. The apoplastic barrier is essential in the roots to balance water uptake and protection against environmental stress, with two components that carry out this function: suberin and lignin. The effectiveness of transport barriers in plants are discussed with regarding the surrounding water potential both in soil and air.

The open questions related to the molecular and ultrastructural basis of the cuticular barrier properties, their relationship to species-specific chemical compositions and the chemical differences between plant cuticles are further discussed. The upcoming two chapters will try to answer some of the questions related to the chemical composition, water permeability and water stress acting on apoplastic barriers.

I carried out all the literature research and made a draft manuscript with its contents. The subtopics were further refined with the guidance of Lukas Schreiber and Viktoria Zeisler-Diehl. Already published results from several publications were referred to compile the graph related to the correlation of permeances of isolated cuticles along with average wax coverage. I made the microscopy and SEM picture for the book chapter. The final design of figures and graphs was carried out along with both co-authors. Pro-create software was used to design the schematic diagram of water potential gradients between soil, plant and air.

The structure and content of the manuscript were conceived and written after a prior discussion with Lukas Schreiber regarding the book chapter preparation. After writing the first manuscript, Viktoria Zeisler-Diehl helped me revise its content. The final version of the manuscript was corrected and approved by Lukas Schreiber before being submitted to the corresponding editors.

Chapter 2

Comparing anatomy, chemical composition and water permeability of various suberized tissues

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"Comparing anatomy, chemical composition and water permeability of various suberized tissues" was published in 2022 as an online article in Planta by Springer Nature. The original publication is attached in the appendix of this dissertation. The following overview is intended to highlight my personal contribution. As an abstract that primarily focuses on the most important elements for conciseness and clarity, relevant references to some claims have been excluded; nevertheless, they can be found in the complete publication linked (Appendix 2).

I am the first author of this study, in which suberized cell walls were enzymatically isolated and collected from five different plant species including *Clivia miniata*, *Monstera deliciosa*, *Solanum tuberosum*, *Manihot esculenta* and *Malus domestica*. The structural barriers at the plant/soil or plant/atmosphere interface in various plant organs including soil-grown roots, aerial roots, tubers and bark were examined. Anatomy, chemical composition (suberin and wax) and efficiency as transpiration barriers (water loss in m s^{-1}) of the different suberized cell wall samples were quantified. Diverse plant materials were selected for the experiment and two distinct types within the same plant organ were chosen for comparison (young vs matured root, soil-grown v/s exposed roots above soil and fresh v/s 3-week stored tubers). For *Clivia miniata* the soil-grown and roots exposed to the atmosphere were used and in *Monstera deliciosa* young growing and matured aerial roots were examined. Tubers of *Solanum tuberosum* and *Manihot esculenta* (fresh periderm and 3-week-old stored root tubers periderm)

were compared with each other. The bark of *Malus domestica*, which is mainly made up of secondary cell wall modification accumulating over time, was used as a reference.

The overall key findings are as follows:

- **Water permeability and suberized layers**: The effectiveness of suberized tissues as a water barrier does not depend solely on the number of suberized cell layers or the total amount of suberin. Although various suberized tissues differ in both the number of layers and their chemical composition, not all are equally effective in preventing transpiration.
- **Wax plays a key role**: Wax molecules impregnated into the suberin polymer play a crucial role in forming an effective water barrier. This is most easily illustrated in species like *Monstera deliciosa* and *Solanum tuberosum*, which showed a dramatic increase in permeability to water upon wax extraction. For the tissues of *Clivia miniata* and *Manihot esculenta* with lower amounts of wax, weak or no barriers to water were formed even though they were partially suberized.
- **Plant organ differences**: The potential for the formation of water barriers by different plant organs, i.e., roots, tubers and bark vary greatly. *Solanum tuberosum* and *Monstera deliciosa* (aerial roots) form a strong barrier, while the roots of cassava and the bark of the apple form a weak barrier. In fact, it has been postulated that the wide variability in barrier efficiency in cassava could be a contributing factor in post-harvest loss since the periderm with root developmental origin in this plant fails to provide an effective barrier against water loss. The functionality of different apoplastic barriers varies depending on the location, maturity and environmental factors.
- **Transpiration rates**: The transpiration rates varied within suberized tissues and those of *Solanum tuberosum* periderms were the lowest in terms of water loss, showing the most efficient water barrier. On the contrary, the root tuber periderm of *Manihot esculenta* indicated that it was highly permeable to water, which accounts for why cassava tubers deteriorate quickly after being harvested.
- **Chemical composition**: Detailed analyses of the wax and suberin compositions revealed variations in chain lengths across different substance classes. For instance, apple bark may have a higher total wax content than other tissues but still performs poorly in water retention. Similarly, suberin amounts varied among tissues, yet did not consistently correlate with water permeability. A direct relationship was observed

between wax content in the suberized periderm and transpiration rates, particularly in potato and cassava.

- **Physiological implications**: These findings have implications for agricultural practices, particularly in postharvest storage and reduction of losses. Some suberized tissues, such as those in cassava, do not provide effective water barriers. Improving the wax biosynthesis of the plant could be one of the targets towards improving the water retention properties of the plant's suberized tissues.

Overall, this study demonstrates that there is no linear correlation between the number of suberized cell layers and the effectiveness of water barrier properties. Instead, it highlights the critical role of wax deposition within suberized tissues in significantly reducing the water permeability.

I conducted all the experiments related to this chapter after discussing them with Lukas Schreiber. At first, I carefully excised different tissues by measuring dimensions to conduct quantitative chemical analysis and transpiration experiments. The samples were microscopically investigated by staining and then the further experiment was designed based on the number of suberized layers, location and function of the plant material. For the transpiration experiments, each set was further divided as intact suberized tissue with wax and wax-extracted suberized tissues. Wax and suberin amounts were calculated after identifying the individual monomers on GC-MS and the transpiration experiments were repeated with several replicates for the data reproducibility. Viktoria Zeisler-Diehl assisted me in gas chromatography to verify the identified monomers. Tobias Wojciechowski provided cassava tubers for investigation and he was actively part of the discussion of data and provided helpful insights in writing the manuscript along with Lukas Schreiber.

Before writing the publication, all co-authors discussed the data collected during this investigation. The main figures and structure of the publication were performed by me and closely discussed with Lukas Schreiber before writing the draft. After I finished the first draft of the paper, all co-authors helped me refine its content. The final version of the manuscript was then read and approved by all co-authors before being submitted to the relevant journal.

Chapter 3

Effects of water stress on apoplastic barrier formation in soil grown roots differ from hydroponically grown roots: histochemical, biochemical and molecular evidence

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"Effects of water stress on apoplastic barrier formation in soil grown roots differ from hydroponically grown roots: histochemical, biochemical and molecular evidence" was published in 2024 as an original research article in Plant, Cell & Environment by John Wiley & Sons. The original publication is attached in the appendix of this dissertation. The following overview is intended to highlight my personal contribution and summarized results. As an abstract that primarily focuses on the most important elements for conciseness and clarity, relevant references to some claims have been excluded; nevertheless, they can be found in the complete publication linked (Appendix 3).

I am the first author of this publication, in which I investigated the response of 12-day-old barley roots grown in soil-filled rhizotrons to different soil water potentials (SWP). A modern cultivar (cv. Scarlett) was compared with a wild accession (ICB181243) from Pakistan. Soil water potentials were measured across different relative water contents. Root anatomy was

analyzed using histochemistry and microscopy, while suberin and lignin levels were quantified through analytical chemistry. Transcriptomic changes were assessed via RNA sequencing.

The results section of the study on the effects of water stress on apoplastic barrier formation in soil-grown barley roots highlights several key findings:

- **Soil Water Content (SWC) and Soil Water Potential (SWP)**: The SWC and SWP were systematically measured in soil samples across different depths in the rhizotrons. The results showed that well-watered soil had an SWC of about 72.6%, while mild and strong water stress treatments resulted in lower SWCs of 54.3% and 41.3%, respectively. The SWP ranged from -0.09 MPa for well-watered soil to -1.04 MPa for strong water stress. The nonlinear relationship between SWC and SWP explains how a small change in a relative quantity leads to a major change in another parameter.
- **Root and shoot morphology**: The length of barley roots decreased with increasing water stress. In control conditions, the barley accession from Pakistan (wild) exhibited longer roots than the modern cultivar Scarlett. Root lengths under mild and strong water stress also showed similar trends, with Pakistan roots being consistently longer. Strong water stress significantly decreased the total root and shoot length in both cultivars, but the reduction was more pronounced in Scarlett. Root/shoot ratios increased under water stress conditions, indicating a shift in resource allocation favoring root growth to cope with limited water availability.
- **Histochemical analysis of suberin and lignin**: Histochemical staining revealed that under control conditions, suberization of endodermal cells occurred between 5% and 10% of the root length (root tip defined as 0%, root base as 100%), while in water-stressed plants the suberization occurred earlier and was more extensive. Under both mild and severe water stress, suberization increased significantly in both barley cultivars, with Scarlett exhibiting more pronounced suberization than Pakistan. Lignification followed a similar trend, intensifying with increasing water stress, particularly in the metaxylem and endodermal cells. The outer tangential walls of endodermal cells near the shoot showed the strongest suberin and lignin deposition in response to stress.
- **Chemical analysis of suberin and lignin**: Additional quantitative analysis confirmed that the total amount of aliphatic suberin monomers increased over the root length in both genotypes, especially under strong water stress. Pakistan roots showed enhanced

suberin accumulation in the basal root zones under mild and strong stress. Lignin analysis revealed that lignin accumulation also increased over the length of the roots, with strong water stress significantly boosting lignification, especially in Scarlett. The lignin monomers predominantly included guaiacyl, syringyl and p-hydroxyphenyl units.

- **Transcriptomic analysis**: RNA sequencing of root tips from both cultivars under strong water stress revealed differential gene expression associated with suberin and lignin biosynthesis pathways. Genes related to fatty acid elongation, alcohol synthesis and polymerization were upregulated in both cultivars, supporting the observed increase in suberin and lignin deposition. Pakistan showed more upregulation of genes related to stress responses, including those involved in reactive oxygen species (ROS) detoxification, glutathione metabolism and nutrient transport. The analysis showed that nutrient transporter genes, particularly those associated with nitrate, potassium and phosphate uptake, were upregulated under water stress conditions. The expression of aquaporin genes, which facilitate water transport across membranes, also increased in response to stress, particularly in the root tips.

Overall, water stress induced higher suberization and lignification in the soil-grown roots, which are important for protecting the plant under drought conditions. Suberization for the soil-grown roots started way closer toward the root tip compared to the hydroponic growth conditions. Roots grown in soil had approximately twice the amount of suberin compared with hydroponically grown roots, which indicated that the apoplastic barrier of roots was stronger for soil conditions. The root lengths decreased with water stress treatment and the gene expression related to suberin and lignin synthesis significantly increased. Soil-grown roots reacted differently to water stress, when compared to hydroponically grown roots, with earlier and stronger suberization responses, which should be crucial for the survival of plants under drought stress.

The experimental setup was developed over the years to establish a suitable method for obtaining reliable results. Initial pot experiments were conducted alongside water potential measurements and moisture content analysis to understand the dynamics of various growth media and their differing soil compositions. Before defining the experiment's objective, preliminary root samples were examined microscopically and suberin contents were quantified through chemical analysis. This idea was further co-ordinated with Tobias Wojciechowski and

Lukas Schreiber in a collaboration using a rhizotron root growth system from Jülich (Germany) and a final experimental design was formed. I quantified water potentials in soils with varying relative water contents. This was used as a reference to conduct water stress experiments in soil. The plants were regularly monitored throughout the growth period for physiological parameters, including the total root length, maximum root depth, maximum root width, lateral root length and convex hull area. I measured the soil moisture content and determined the water potential at various depths. With the harvested roots, anatomy was studied using histochemistry and microscopy. The roots were further divided into different zones to determine the amounts of suberin and lignin through analytical chemistry. I developed a micro-analysis of lignin using a thioacidolysis protocol and further GC analysis of suberin and lignin was carried out. The observed transcriptomic changes were studied using RNA sequencing. With the guidance of Sabarna Bhattacharyya, I analyzed the transcriptomic data to understand the activities of the genes of interest. The data was further sorted and the representative figures were designed to support the results from microscopy and histochemistry. I designed the schematic diagram using digital tools to show the different root zones that were harvested for gas chromatography analysis and RNA-sequencing analysis.

The data obtained from this study were primarily discussed with Lukas Schreiber and the co-authors involved in the preparation of the manuscript. After drafting the initial version, I collected written feedback from all co-authors, with Lukas Schreiber providing the most substantial input. Upon completion of the final version, all authors reviewed and approved the manuscript prior to its submission to the respective journal.

3. Discussion

The discussion section will try to link all three individual articles and establish a more comprehensive interrelation. All of the publications contribute to our understanding of how plant organs physiologically and biochemically respond to water loss and water stress, with a particular emphasis on the processes of suberization. This process is crucial for plants to adapt to varying levels of water availability and environmental conditions surrounding the plants.

The introductory book chapter (Chapter 1; Suresh *et al.*, 2023): describes the history of suberin research, the current state of knowledge, discusses methodological and applied aspects related to plant interfaces and finally points out important future questions. In the field of suberin research, several open questions remained unanswered. These include the molecular organization of dark and light lamellae, the structural and molecular nature of polar transport pathways and the challenge of synthetically mimicking suberin. Additionally, the detailed pathway of suberin biosynthesis is still not fully understood. Other questions include whether an apoplastic barrier in roots can be as efficient as in leaves, whether barrier properties are species-specific and how the chemical composition of Casparian strips varies among species. Furthermore, it remains unclear whether suberin alone or in combination with wax and lignin forms an effective barrier.

The schematic diagram in Chapter 1, representing the water potential between soil, plant and air, is key to identifying the aim of this thesis. In contrast, the atmosphere has a mean relative humidity of 99% which corresponds to -1.36 MPa and well-watered plant tissue has a water potential up to -1.0 MPa. Thus, the gradient between the leaf and the atmosphere is really steep and wax extraction from cuticles increases the water loss by between 10 to 1000-fold, which substantiates the role of wax as being the key determinant in controlling water loss. Other suberized plant interfaces in the shoot-root axis are believed to be equally efficient in preventing water loss and this needs to be verified. The plant interfaces where transpirational water loss can be measured were carefully selected from five different species and organs (roots, tubers and stems) (Chapter 2; Suresh *et al.*, 2022).

The second chapter explores the anatomy and chemical composition of suberized organs in various plant species, highlighting the role of waxes in regulating water permeability. This

paper notes that while cuticles are highly efficient in preventing water loss, suberized cell walls can vary significantly in their impermeability depending on the species, plant organ and environmental conditions. The study highlights that the presence of waxes, which are often associated with cutin and suberin, plays a pivotal role in enhancing the hydrophobicity of these barriers. This is particularly important in maintaining water retention during periods of drought, as wax layers maintain reduced leaf transpiration rates as soon as stomata are closed. Additionally, the comparative analysis of suberization among five plant species highlights the differences in structural adaptations to varying water potentials in the soil/root region, indicating that evolutionary pressures have influenced these traits to enhance survival in various environments.

Referring back to the question outlined in this thesis's aim, the response can now be formulated as follows:

- Is there a correlation between the barrier properties of the suberized interfaces and the number of suberized layers, the amount of soluble wax and the amount of suberin?

No, there is no significant correlation between the barrier properties of the suberized interfaces and the number of suberized cell layers, the content of soluble waxes, or the total suberin content. Based on all results, there is no straightforward explanation for why certain suberized tissues act as efficient transpiration barriers. Upon wax extraction, the permeance increased, underscoring the importance of wax in establishing a transpiration barrier (*e.g.*, *Monstera* aerial root and potato). In the case of tuber crops periderm (potato and cassava), differences in ontogenic origin and biochemical machinery helped to explain the observed contrasting barrier properties (potato highly efficient after storage and cassava lose moisture).

Post-Harvest Losses (PHL) pose a major challenge in agriculture, especially for perishable crops like fruits and vegetables, which are highly susceptible to damage and quality decline after harvest (Krishna *et al.*, 2022). These losses are often driven by poor handling, environmental stress and natural spoilage processes. To address this, researchers have explored the role of natural plant biopolymers such as suberin, lignin and cuticular waxes in boosting the resilience of plant tissues. For instance, increased lignin levels can improve a plant's resistance to fungal infections, while cuticular waxes help retain moisture and shield against pathogens (Lee *et al.*, 2021; Wang *et al.*, 2020; Xu *et al.*, 2011). Our research revealed a striking difference between *Manihot esculenta* and *Solanum tuberosum*: while the latter develops a periderm with wax that acts as an effective barrier against water loss, *M. esculenta*

fails to do so, leading to rapid dehydration and quality decline. Why *M. esculenta* cannot form a functional transpiration barrier remains an intriguing and unresolved question.

Water stress in plants, which resembles drought-like conditions, triggers many physiological and molecular responses that affect plant growth and survival. One such response includes the development of apoplastic barriers, especially in roots, impacting water and nutrient uptake. These barriers limit the passive movement of water and ions, forcing water uptake through more regulated pathways like the symplastic route. This adaptation enables plants to maintain water balance and avoid leaching out essential nutrients under drought conditions. The last chapter (Chapter 3; Suresh *et al.*, 2024), explores how water stress affects the formation of apoplastic barriers in roots, emphasizing the distinct responses observed in a modern and a wild cultivar grown in soil. Pakistan, a wild barely genotype, developed longer roots than Scarlett, its cultivated counterpart. This was observed both in well-watered and also in decreasing soil water potential conditions. As correlated with the surface area available for water volume and dissolved nutrients (Grünhofer et al., 2024), Pakistan's longer roots may provide a competitive advantage. In both wild and modern genotypes, the histochemical and chemical analysis results were largely similar. In general, wild accessions, naturally adapted to harsher environments, might exhibit stronger drought resistance with more efficient water retention mechanisms. Their robust root structures and apoplastic barriers potentially helps to mitigate water loss. In contrast, modern cultivars, often bred for higher yield rather than stress tolerance, might tend to have weaker barriers, making them more vulnerable to drought. However, recent breeding efforts aim to reintroduce drought-resistant traits from wild relatives to enhance the resilience of modern crops (Baldoni *et al.*, 2021; Khoury *et al.*, 2015; Prohens *et al.*, 2017). The soil-grown versus hydroponically grown roots show differences than shoots when both are grown under controlled conditions. The histochemical and biochemical analyses demonstrate that suberization, a process where suberin, a hydrophobic biopolymer, is deposited in cell walls, is significantly more pronounced in soil-grown roots under water stress conditions compared to well-watered conditions and in hydroponics. This is particularly evident in the endodermal cells, where nearly complete suberization occurs at 25% from the root tip in both control and stress conditions for soil-grown plants, contrasting sharply with the limited suberization observed in hydroponically grown roots. The study further reveals that the chemical composition of suberin in soil-grown roots contains higher levels of aliphatic and aromatic monomers along with lignin, which are crucial for enhancing the hydrophobic properties of the cell walls, thereby presumably reducing water permeability and preventing

backflow of water from the xylem. This research examines the molecular mechanisms that lead to the upregulation of genes associated with suberization and lignification in response to water deficits. Several transcription factors, including WRKY, MYB, NAC and ERF, regulate these processes, indicating a complex network of genetic responses that help plants acclimate to abiotic stress. Histochemical analyses reveal enhanced suberization and lignification in the cell walls of the endodermis and xylem vessels. These findings support the conclusion that these structural modifications are crucial for maintaining plant integrity and function during stress.

Referring back to the question outlined in this thesis's aim, the response can now be formulated as follows:

- How does the response of root apoplastic barrier formation with the change with soil water potential in wild barley and a modern cultivar, how does this differ in hydroponic versus soil-based media?

Comparing the wild accession Pakistan and the modern cultivar Scarlett, Pakistan developed longer roots. However, the differences in histochemical and chemical analyses between the two were not significant. As soil water potential decreased, root lengths declined, while the amounts of aliphatic suberin and lignin increased and genes involved in their biosynthesis were upregulated. Compared to hydroponic cultivation, soil-grown roots were much longer and the degree of suberization was much closer to the root tip. The soil environment even under well-watered (control) conditions had much higher and more rapid suberization and lignification compared with hydroponic cultivation.

The complex interactions between environmental conditions, anatomical adaptations and molecular responses in plant roots under water stress are highlighted in the above discussion. Roots grown in soil develop more suberin and lignin than those in hydroponics, presumably helping retain water and prevent backflow. Additionally, specific transcription factors and cell wall modifications play key roles in stress responses, offering insights for breeding more resilient crops to combat climate change. The degree of suberization varies across root developmental stages, species and growth conditions, underscoring the need for standardized analytical methods. Genetic variability further complicates this process, necessitating genetic mapping and targeted studies on individual plant responses to environmental cues. An integrated approach will help us better understand and enhance suberin’s role in agriculture. Future approaches and experiments should be planned accordingly, considering all the points

listed below. Since suberin is a complex apoplastic barrier, a multi-diagnostic approach will help us understand its functionality in roots, shoots and tubers:

1. Lab experiments: Plants grown in labs and those in the field often look and react very differently, especially under water stress. This calls for a closer look at research methods on stress resilience. The seedling stage also matters when young plants may respond in ways that don't reflect mature ones (Villani *et al.*, 2024). Previous results show that as plants grow, their heat tolerance, leaf structure and photosynthesis can change significantly (Lampayan et al., 2019; Grünhofer *et al.*, 2022a; Xu et al., 2024) and even the root architecture can also change. To truly understand stress resilience, studies must consider the plant's full lifecycle and environment.

2. Lignin: Lignin often gets overlooked when discussing plant stress responses, but it plays a crucial role in maintaining cellular integrity, especially under drought (Yan *et al.*, 2021). Drought-tolerant maize lines produce more lignin than sensitive ones, helping them withstand dry spells (Hu *et al.*, 2009). In rice, overexpression of lignin-related genes not only increases lignin production but also improves drought resistance by optimizing water transport (Bang *et al.*, 2019; Lee *et al.*, 2016). Enhanced lignification strengthens plant structures, reduces water loss and helps them cope with both biotic and abiotic stress (Moura *et al.*, 2010). The biosynthesis of suberin and lignin in plants is closely linked, with phenolic compounds serving as precursors with MYB transcription factors playing a key role in regulating both (Kosma *et al.*, 2014; Lashbrooke *et al.*, 2016; Xu *et al.*, 2022). With further research, we may be able to fine-tune these pathways, paving the way for stronger, more adaptable plants that can thrive in challenging conditions.

3. Lateral roots: Lateral roots play a crucial role in helping barley absorb water, making up about two-thirds of its root surface and supplying as much as 92% of the water young plants take up (Knipfer *et al.*, 2011). When water or nutrients are scarce, these roots get suberized, which helps with water absorption but might also slow down transport when roots are densely packed (Zaiats *et al.*, 2020; Zexer & Elbaum, 2021). This balance suggests that the chemical makeup of roots, including suberin and lignin, plays a big part in their effectiveness. The study approaches using X-ray CT scan, MRI, Rhizotubes combined with computational tools for data analysis gives us a non-invasive approach

to studying both seminal and lateral roots while still being intact (Jeudy *et al.*, 2016; Metzner *et al.*, 2015; Shao *et al.*, 2021a). Most studies are done in hydroponics and the main seminal roots and the influence of nodal and lateral roots is ignored. As seen from the soil root studies, the response of root growth is different than from hydroponics and developing a better system to study the roots will be useful.

4. Soil curve establishment: In soil-related drought experiments, a common approach involves regulating watering or using the pot-drying method by withholding water for a specific duration (Amin *et al.*, 2021). One research study simulated drought conditions, showing that at 100% field capacity, $\Psi w = -2.89$ MPa and at 40% field capacity, $\Psi w = -3.9$ MPa (Gupta & Senthil-Kumar, 2017). However, these results cannot be directly compared with other pot experiments or osmotic stress experiments (such as hydroponics) without a proper soil curve. Soil curves and moisture gradients vary depending on the soil mixture. Establishing an exact calibration curve that correlates soil water potential with soil water content is essential for obtaining comparable results across different experiments.

5. Other crop species: We all know that *Arabidopsis* serves as a powerful model organism species, but its limitations in agricultural relevance must be acknowledged. *Arabidopsis* research doesn't always translate well to crops due to key differences in physiology, growth and nutrient needs (Yaschenko *et al.*, 2024). Traits like yield and biomass, crucial for crops like corn and rice, are often poorly represented (Spannagl *et al.*, 2011). Real crops also have complex traits shaped by polyploidy and environmental interactions that *Arabidopsis* cannot fully mimic, especially for drought tolerance. Additionally, *Arabidopsis* is adapted to temperate climates, making it less relevant for tropical crops. Its small size and simple structure further limit its usefulness in studying traits like root architecture and biomass allocation, which are critical for crop performance (Stephenson *et al.*, 2019).

6. Holistic molecular approach: Gene interactions are complex, meaning a mutation in one gene can significantly impact others, especially those linked to suberin, lignin and drought resistance. This highlights the need for multi-tissue studies. Research has identified co-expression networks (Shahriari *et al.*, 2022), key hub genes and related microRNAs that show activity in both roots and shoots (Arjmand *et al.*, 2021). Drought

tolerance is regulated by various mechanisms, including specific transcription factors (Sun *et al.*, 2022) and long non-coding RNAs (Chen *et al.*, 2021), which help fine-tune gene expression. This broader perspective will provide valuable insights regarding the complex inter-linked network.

7. Mutants: Studying suberin mutants provides valuable insights into plant water regulation, nutrient transport and stress responses (Cantó-Pastor *et al.*, 2024; Fedi *et al.*, 2017; Shukla *et al.*, 2021; Vestenaa *et al.*, 2024). These mutants help researchers understand suberin's role in drought tolerance, pathogen resistance and interactions with soil microbiomes. *Arabidopsis*, with its simple genome, serves as an ideal model for dissecting suberin biosynthesis and identifying key regulatory genes. Controlled experiments allow precise investigation of how suberin influences plant resilience, offering applications for improving drought-resistant crops. Additionally, research on suberin mutants supports advancements in breeding, genetic engineering and collaborative efforts using technologies like CRISPR to enhance agricultural sustainability. However, mutant studies in crop species are complex and time consuming.

The proposed multi-diagnostic approach provides a comprehensive framework for studying suberin functionality within roots, shoots and tubers. Employing a combination of histochemical techniques, analytical chemistry, molecular biology, physiological assays and field studies will yield significant insights into the diverse roles of suberin in plant biology. Future research should prioritize interdisciplinary collaboration to effectively address the complexity of suberin and its implications in plant health and adaptation.

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Appendix

Appendix 1

Chapter 1

Hydrophobization of cell walls by cutin, suberin and associated waxes

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Appendix 2

Chapter 2

Comparing anatomy, chemical composition and water permeability of various suberized tissues

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ORIGINAL ARTICLE

Comparing anatomy, chemical composition, and water permeability of suberized organs in five plant species: wax makes the difference

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Abstract

Main conclusion **The efficiency of suberized plant/environment interfaces as transpiration barriers is not established by the suberin polymer but by the wax molecules sorbed to the suberin polymer.**

Abstract Suberized cell walls formed as barriers at the plant/soil or plant/atmosphere interface in various plant organs (soil-grown roots, aerial roots, tubers, and bark) were enzymatically isolated from five different plant species (*Clivia miniata*, *Monstera deliciosa*, *Solanum tuberosum*, *Manihot esculenta*, and *Malus domestica*). Anatomy, chemical composition and efficiency as transpiration barriers (water loss in m s^{-1}) of the different suberized cell wall samples were quantified. Results clearly indicated that there was no correlation between barrier properties of the suberized interfaces and the number of suberized cell layers, the amount of soluble wax and the amounts of suberin. Suberized interfaces of *C. miniata* roots, *M. esculenta* roots, and *M. domestica* bark periderms formed poor or hardly any transpiration barrier. Permeances varying between 1.1 and 5.1 × 10^{-8} m s^{-1} were very close to the permeance of water (7.4 × 10^{-8} m s^{-1}) evaporating from a water/atmosphere interface. Suberized interfaces of aerial roots of *M. deliciosa* and tubers of *S. tuberosum* formed reasonable transpiration barriers with permeances varying between 7.4 × 10^{-10} and 4.2 × 10^{-9} m s^{-1}, which were similar to the upper range of permeances measured with isolated cuticles (about 10^{-9} m s^{-1}). Upon wax extraction, permeances of *M. deliciosa* and *S. tuberosum* increased nearly tenfold, which proves the importance of wax establishing a transpiration barrier. Finally, highly opposite results obtained with *M. esculenta* and *S. tuberosum* periderms are discussed in relation to their agronomical importance for postharvest losses and tuber storage.

Keywords Bark · Diffusion barrier · Periderm · Suberization · Storage root · Transpiration · Tuber · Water loss · Wax

Introduction

Plant environment interfaces are formed by hydrophobized cell walls which are additionally modified by cutin and suberin polymers (Pollard et al. 2008). Outer epidermal cell walls of leaves facing the atmosphere are modified by the deposition of the plant cuticle (Riederer and Müller 2006). It is highly impermeable for water and it protects leaves from uncontrolled water loss when stomata are closed due to water limitation (Schreiber 2010). Root soil interfaces are characterized by the apoplastic deposition of suberin (Schreiber et al. 1999). Best known is the endodermis forming a root internal apoplastic barrier separating the central cylinder of the primary root from the cortex. However, the actual interface of roots directly facing the soil environment is formed by the rhizodermis and the hypodermis. The primary cell walls of these cell layers, especially the single or multi-layered hypodermis, are also characterized by the deposition of suberin (Hose et al. 2001; Serra et al. 2022). Tubers as storage organs of plants are characterized by a multi-layered periderm, which is suberized (Lulai and Corsini 1998). Finally, roots and shoots in their secondary developmental stages with a radial growth of thickness develop multi-layered suberized tissues as plant/environment interfaces.

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Both, cutin and suberin are composed of oxygenated fatty acids, with varying chain lengths, which are polymerized (Kolattukudy 1980). Major cutin and suberin monomers are ω-hydroxy fatty acids and α,ω-diacids. The cutin polymer is always modified by the deposition of cuticular wax (e.g., linear, long-chain fatty acids, alcohols, aldehydes, alkanes, and esters) sealing the polymer and thus rendering it highly impermeable for water and dissolved substances (Kunst and Samuels 2003). The suberin polymer may contain wax molecules (Schreiber et al. 2005) or not (Schreiber et al. 1999; Teixeira and Pereira 2010). Cutin, suberin, and wax biosynthesis have intensively been investigated in the last 2 decades and many genes and enzymes involved have successfully been identified and characterized (Nawrath 2002; Samuels et al. 2008; Vishwanath et al. 2015; Fich et al. 2016).

Whereas there is no doubt that plant cuticles are highly efficient and impermeable polymer membranes, perfectly protecting plants from uncontrolled desiccation (Riederer and Schreiber 2001; Yeats and Rose 2013), this must not necessarily be the case with suberized cell walls. With the suberized periderm of potato, it was shown that the native periderm already formed a very efficient transpiration barrier directly after digging out from soil (Schreiber et al. 2005). Upon storage up to 4 weeks in the dark at ambient temperature and humidity, even a strong induction of suberin and wax biosynthesis was induced. The final water permeability of native potato periderm was further decreased by a factor of ten and was not different from leaf cuticles. However, water permeability of the wound periderm of potato, although it contained comparable amounts of suberin and wax, failed to form a transpiration barrier, since its water permeability was 100–1000 times higher compared to the native periderm.

Based on these contradictory results obtained with potatoes in the past, we investigated a series of further suberized tissues isolated from different plant organs (roots, tubers, and stems) from 5 different species in order to cover a broad spectrum of suberized tissue investigating their barrier properties. The anatomy (number of suberized cell layers) and the chemical composition (suberin and wax) of the various suberized samples was characterized and related to its properties as a water barrier, quantified by measuring transpiration kinetics. This larger set of data on the structure and function of suberized plant cell walls should help to estimate to what extent suberized plant/environment interfaces form transpiration barriers as efficient as plant cuticles or not.

Materials and methods

Selection and preparation of suberized tissues

Roots of *Clivia miniata* (Lindl.) Regel and *Monstera deliciosa* Liebm. were harvested from plants growing in the IZMB in Bonn. Soil-grown roots of *C. miniata* were carefully washed to remove adhering soil particles, whereas the air-exposed roots, which were slightly green due to photosynthetic pigments, were collected from the surface of the soil. Aerial roots of *M. deliciosa* were separated into young aerial roots (root tips with a smooth surface) and mature aerial roots (with a rough surface). Tubers of *Solanum tuberosum* L. var. WEGA were purchased from a local supermarket. Tubers of *Manihot esculenta* Crantz cultivated in a greenhouse at IGB-2 at Forschungszentrum Jülich GmbH (Jülich, Germany) were used for periderm isolation directly after harvest (fresh tubers) and after 3-week storage (stored tubers) at ambient temperature and humidity in the dark. Bark from *Malus domestica* Borkh. cv. Pinova was collected from 21-year-old trees growing in an orchard of the Institute of Horticultural Production Systems at the Hannover University.

The cylindrical roots of *C. miniata* and *M. deliciosa* were cut into 1.5–2 cm sections and the diameter was measured using a vernier caliper. Disks were punched out from *M. esculenta* and *S. tuberosum* skins with a cork borer (1.0 cm diameter) carefully avoiding any regions with lenticels. Bark samples from *M. domestica* were cut into sections of 1 cm^2 with a razor blade. Suberized tissues from all samples were enzymatically isolated using 2% cellulase (Novozymes) and 2% pectinase (Novozymes) dissolved 0.01 M citric buffer (Carl Roth) with the pH adjusted to 3.0 (Vogt et al. 1983; Schönherr and Riederer 1986). The enzyme solution contained 1 mM of NaN_3 (Fluka) to prevent microbial growth. The solution was changed once in 2 days until all suberized tissues were free from cellular debris. Isolated tissues were washed first with 0.01 M borate buffer (Carl Roth), adjusted to pH 9.0, and finally washed with deionized water. The cylindrical tissues of roots were carefully cut over the length in longitudinal sections and were fixed using paper clips on Teflon strips to carefully flatten them. A gentle air stream was used to air-dry isolated suberized tissues, which were stored in Petri dishes for 2–3 months at room temperature until the experiments were performed. In addition to the suberized samples, transpiration measurements were conducted with open transpiration chambers representing no barrier at all. Measurements with pure cellulose filter mimicking a primary carbohydrate cell wall without any further lipophilic modification were performed as well.

Fluorescence microscopy

Freehand cross-sections were made for *C. miniata* and *M. deliciosa* roots with a razor blade. For other species, fresh samples were cut to a size of 1 cm × 0.2 cm (length × width) and semi-thin sections of about 20 µm thickness were made using a cryostat microtome (Microm HM 500 M, Microm International, Walldorf, Germany). Suberized cell

walls were stained with 0.01% (w/v) Fluorol Yellow 088 (Sigma Aldrich) for 1 h and samples were rinsed with water before microscopic investigation (Brundrett et al. 1991). Cross-sections were analyzed by fluorescence microscopy using an ultraviolet (UV) filter set (excitation filter BP 365, dichroic mirror FT 395, barrier filter LP 397; Zeiss). Images were made with a Canon EOS 600D camera at ISO 100–400.

Chemical analysis of wax and suberin composition of suberized tissues

Wax analysis of the suberized tissues was performed as described in Baales et al. (2021). Wax was extracted by immersing isolated suberized tissues in chloroform (5 ml) overnight in glass vials kept on a rolling bench. Before extraction, chloroform was spiked with 20 µg tetracosane (100 µl of a solution of 10 mg tetracosane in 50 ml chloroform; Fluka) as an internal standard for wax quantification. The total solvent volume of wax extracts was reduced under a gentle stream of nitrogen gas to a final volume of 200 µl. For suberin analysis, wax-extracted samples were depolymerized using boron trifluoride/methanol (BF_3/MeOH, Fluka) as described by Baales et al. (2021). Before extraction of the released monomers with chloroform, transesterified samples were spiked with 20 µg of dotriacontane (100 µl of a solution of 10 mg dotriacontane in 50 ml chloroform; Fluka) as internal standard. The final chloroform volume was reduced to 200 µl using a gentle stream of nitrogen.

Both wax and suberin samples were derivatized for 45 min at 70 °C using 20 µl each of pyridine (Sigma Aldrich) and BSTFA (N, N-bis-trimethylsilyl-tri-fluoroacetamide, Machery-Nagel). This converts free functional groups of alcohols and acids to trimethylsilyl-ethers and -esters. Wax and suberin samples (1 µl) were quantified by GC-FID (CG-Hewlett Packard 5890 series H, Agilent) analysis and individual wax and suberin compounds were identified by GC–MS (quadrupole mass selective detector HP 5971, Hewlett Packard, Agilent) analysis. 1 µl of the wax samples were on-column injected, whereas analysis of suberin samples was done using split/splitless injection. Identification of the compounds was done using a homemade MS library.

Transpiration measurements of suberized tissues

Transpiration was measured by gravimetry (Schönherr and Lendzian 1981). Prior to the measurement, dry and brittle suberized tissues were equilibrated overnight in an atmosphere with 100% humidity, making them soft and flexible, which allowed to handle them carefully without the danger of breaking. Suberized samples were carefully mounted on water-filled stainless-steel transpiration chambers with an open circular area of 0.28×10^{-4} m^2 across which transpiration was possible. Before starting the transpiration experiment a 10 µl drop of ethanol was applied to the outer surface. This allows detecting micro-defects invisible to the eye since suberized samples immediately turn dark with ethanol penetrating defect suberized tissues. The chambers were closed with a lid (inner opening 0.28×10^{-4} m^2).

Transpiration chambers were placed upside-down in an air-tight polyethylene box containing freshly activated silica at 25 °C, resulting in 2% humidity. Water loss across the suberized tissues was measured every hour up to 6 h using an analytical balance (Sartorius) with a resolution of 0.1 mg. As references, the transpiration of water from an open chamber (upright chamber) and across a pure cellulose filter (thickness: 140 µm) mounted to transpiration chambers were measured. The amount of water lost from individual suberized tissues or control samples were plotted as a function of time, and the slopes of the linear regression lines (in g s^{-1}) fitted to the transport kinetics were used to calculate permeances P (m s^{-1}) using the formula: $P = \text{slope}/A \times \Delta C$ (Schreiber and Schönherr 2009), where A (m^2) corresponds to the exposed area across which transpiration took place (0.28×10^{-4} m^2) and ΔC (g m^{-3}) represents the driving force for the transpiration given by the density of water (10^6 g m^{-3}).

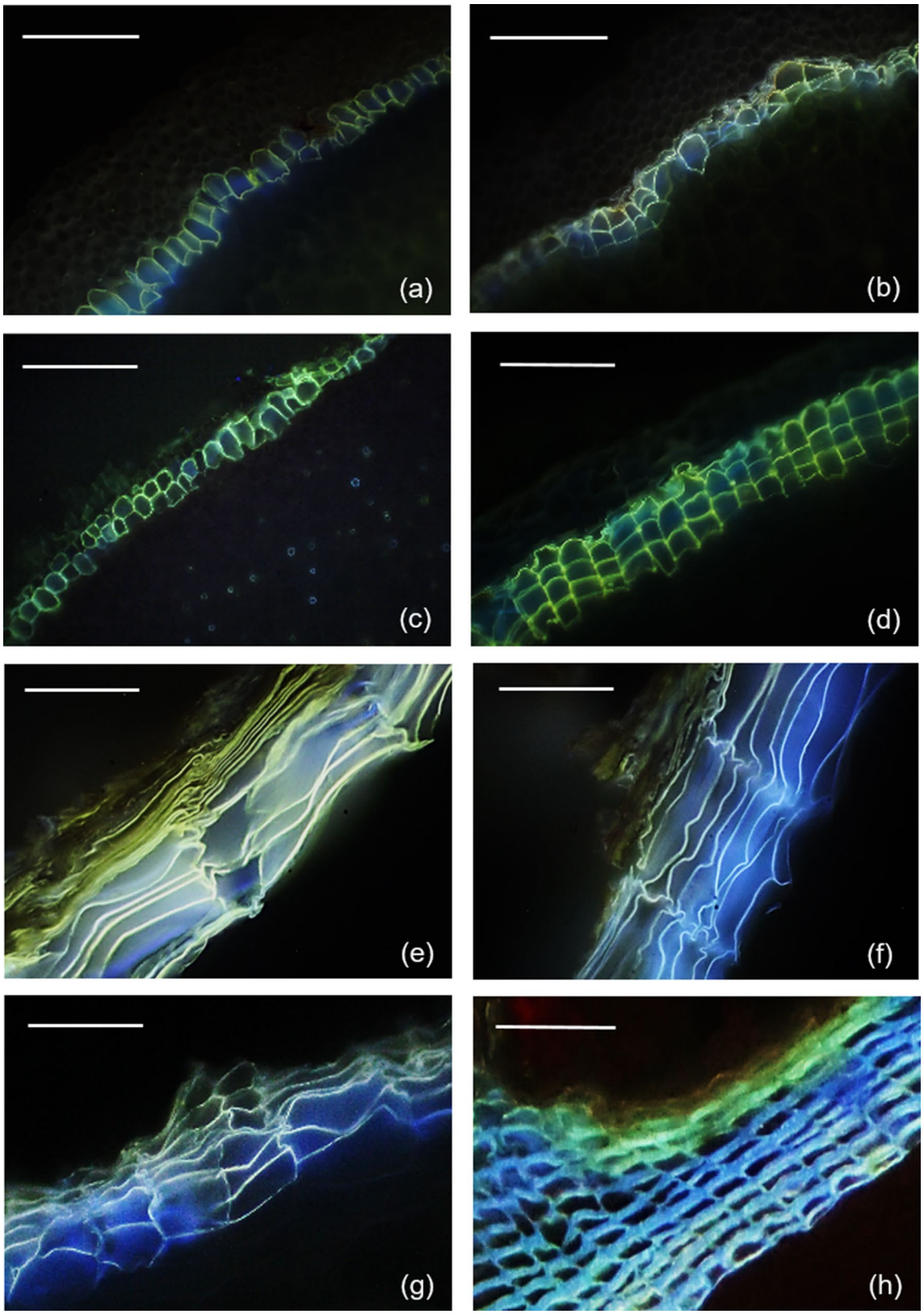
Statistical analysis

The number of suberized cell layers in the different samples was determined with 6–10 representative microscopic pictures for each species (Fig. 2). Wax and suberin analyses were done using 3 replicates (Figs. 3, 4, 5, 6). The transpiration kinetics were measured with 5–10 isolated samples before and after wax extraction (Fig. 7). Results are given as means with standard deviations or box plots. *t*-Tests were conducted and the levels of significance are indicated in the figures as 95% (**) or 99% (***), respectively.

Results

Microscopic investigation of suberized tissues

Cross-sections of the isolated suberized tissues were observed using fluorescence microscopy (Fig. 1a–h). Suberized cell walls appeared greenish/yellow or sometimes bluish/yellow after Fluorol Yellow 088 staining under UV light (395 nm). In soil-grown roots of *C. miniata* and air-exposed roots, the number of outer suberized cell layers varied between one and three (Figs. 1a, b, 2). Outer suberized tissues of young and mature aerial roots of *M. deliciosa* had two to four suberized cell layers (Figs. 1c, d, 2). Outer suberized periderms of tubers of *M. esculenta* had between 15 and 12 cell layers (Figs. 1e, f, 2). Suberized periderms from freshly harvested tubers (Fig. 1e) had several highly


(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)

◂**Fig. 1** Fluorescence microscopic cross-sections of suberized tissues stained with Fluorol Yellow 088 and viewed at 365 nm. The presence of suberin is indicated by the greenish-yellow fluorescence. Suberized hypodermis isolated from **a** soil-grown roots of clivia (*Clivia miniata*) and from **b** air-exposed roots. Suberized hypodermis isolated from **c** young aerial roots of monstera (*Monstera deliciosa*) and from **d** mature aerial roots. Suberized periderm isolated from **e** freshly harvested cassava (*Manihot esculenta*) tubers, from **f** stored cassava tubers, and from **g** potato (*Solanum tuberosum*) tubers. Suberized shoot periderm isolated from **h** apple (*Malus domestica* cv. Pinova) bark. Bars = 100 µm

compressed suberized cells on the outer surface above the lately formed young suberized cells. In suberized periderms isolated from *M. esculenta* after storage for 3 weeks, these highly compressed layers were not intact anymore and to some extent thus missing (Fig. 1f). Periderms of *S. tuberosum* had 7 to 9 cell layers (Figs. 1g, 2). Suberized bark isolated from *M. domestica* shoots had between 8 and 10 suberized cell layers often only faintly stained (Figs. 1h, 2).

Amounts and composition of wax extracted from suberized tissues

Wax amounts extracted from suberized tissues varied among different species both in amount and composition (Fig. 3). The detected wax monomers are separated into aliphatic wax amounts (linear, long-chain aliphatic wax monomers), sterols, and terpenoids. Sterols (stigmasterol and β-sitosterol) were detected only in minor and comparable amounts in nearly all samples except for potato. Fairly high amounts (84.7 ± 8.7 µg cm^{-2}) of terpenoids were detected only in bark samples isolated from *M. domestica* (Figs. 3 and 4d). The highest amount of aliphatic waxes was found in suberized tissues isolated from mature aerial roots of *M. deliciosa* (28.5 ± 2.5 µg cm^{-2}) and suberized bark of *M. domestica* (27.2 ± 0.7 µg cm^{-2}), respectively (Fig. 3). In suberized tissues isolated from soil-grown *C. miniata* roots, aliphatic wax amounts were only 0.9 ± 0.1 µg cm^{-2}, whereas higher wax amounts of 2.5 ± 0.2 µg cm^{-2} were detected in air-exposed roots (Fig. 3). The average amount of total wax in suberized tissue of young aerial roots of *M. deliciosa* was 17.4 ± 1.9 µg cm^{-2} (Fig. 3). Freshly isolated periderms of *M. esculenta* had only 2.6 ± 0.4 µg cm^{-2} aliphatic wax and amounts increased after 3 weeks storage to 6.0 ± 1.3 µg cm^{-2} (Fig. 3). The amounts of wax extracted from *S. tuberosum* were 13.0 ± 1.3 µg cm^{-2} (Fig. 3).

The fraction of the aliphatic wax molecules was composed of the substance classes alcohols, acids, alkanes, esters, and aldehydes (Fig. 4) of varying chain lengths (C_{16}–C_{34}). In *C. miniata*, the substance classes alcohols, acids, alkanes, and aldehydes were detected and amounts of all substance classes were significantly higher in air-exposed roots (Fig. 4a). In *M. deliciosa* alcohols, acids, alkanes, and esters were detected and amounts of all substance classes increased in mature aerial roots with the alkanes showing the highest increase (Fig. 4b). In *M. esculenta* and *S. tuberosum*, only three substance classes, namely alcohol, acids, and esters, were present and the amount of alcohols and esters increased two- to threefold in stored *M. esculenta* periderms (Fig. 4c). In *S. tuberosum*, the fraction of esters was essentially composed of ferulic acid esters of the chain lengths C_{20} to C_{32}. In *M. domestica*, alcohols and acids were the dominating substance classes of wax whereas alkanes were only present in minor amounts (Fig. 4d).

Amounts and composition of suberin monomers depolymerized from suberized tissues

Suberin content of the different suberized tissues varied between 100 and 1000 µg cm^{-2} (Fig. 5). Monomers obtained after suberin depolymerization were classified into aliphatic (linear, long-chain oxygenated fatty acids) and aromatic suberin (essentially coumaric and ferulic acids). Except for soil-grown roots of *C. miniata*, the aromatic fraction of the suberin polymer in the other samples amounted only to a few percent of the total suberin amounts (Fig. 5). In *C. miniata* roots, the aliphatic suberin amount in hypodermis was about twofold higher in air-exposed roots (52.4 ± 1.5 µg cm^{-2}) when compared to soil-grown roots (29.1 ± 2.4 µg cm^{-2}) (Fig. 5). In *M. deliciosa*, the total aliphatic suberin content was 120.70 ± 1.7 µg cm^{-2} in young aerial roots and it increased to 417.9 ± 18.2 µg cm^{-2} (Fig. 5). Upon 3 weeks of storage, the amount of aliphatic suberin in *M. esculenta* decreased from 268.2 ± 40.8 to 202.1 ± 20 µg cm^{-2} (Fig. 5). In *S. tuberosum*, the total aliphatic suberin content was 120.7 ± 7.9 µg cm^{-2} and the highest aliphatic suberin content of 891.2 ± 109.4 µg cm^{-2} was measured with the periderm isolated from *M. domestica* bark (Fig. 5). Dominating substance classes of the aliphatic suberin monomers detected in all samples after depolymerization were ω-hydroxy acids and α,ω-diacids (Fig. 6). In addition, varying amounts of linear long-chain alcohols and fatty acids were also released by transesterification (Fig. 6). The chain length of the aliphatic suberin monomers ranged from C_{16} to C_{30} (data not shown). Substance classes of suberin tissues did not change when comparing soil-grown with air-exposed *C. miniata* roots (Fig. 6a), young with mature aerial *M. deliciosa* roots (Fig. 6b), and fresh with 3-week-stored *M. esculenta* tubers (Fig. 6c).

Rates of water loss (transpiration) from suberized tissues

Linear transpiration kinetics were obtained plotting the amounts of water lost from the transpiration chambers vs. time (Fig. 7). The highest transpiration rates were measured

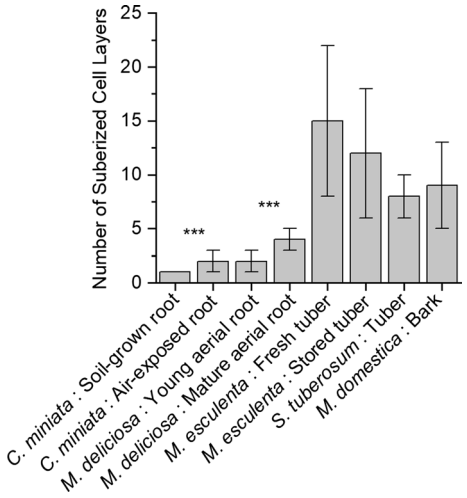


Fig. 2 Number of cell layers in the different suberized tissues isolated from five different plant species (*Clivia miniata*, *Monstera deliciosa*, *Solanum tuberosum*, *Manihot esculenta,* and *Malus domestica*). The average number of cell layers given as means ± standard deviations was obtained by investigating at least 5 or more independent microscopic cross-sections of each sample. Asterisks indicate a significant difference between the number of cell layers of soil-grown and air-grown *Clivia* roots and of young and mature aerial root of *Monstera*, respectively (*** = 99%)

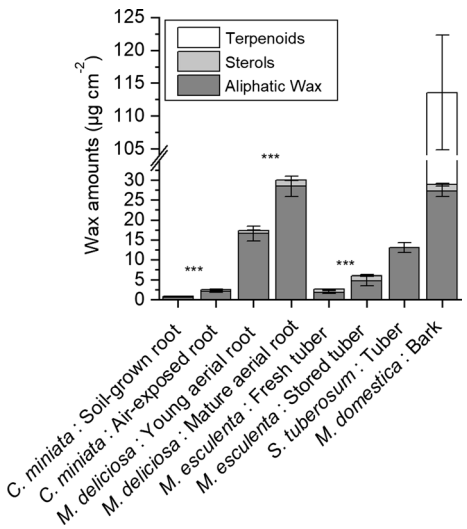


Fig. 3 Total amounts (µg cm^{-2}) of soluble wax extracted with chloroform from the different suberized tissues isolated from five different plant species (*Clivia miniata*, *Monstera deliciosa*, *Solanum tuberosum*, *Manihot esculenta,* and *Malus domestica*). Waxes are classified into 3 groups: aliphatic wax (linear long-chain aliphatic wax molecules), sterols (cyclic sterols), and terpenoids (triterpenoids and sesquiterpenoids). Data points represent means with standard deviations ($n = 3$). Asterisks indicate a significant difference between aliphatic wax amounts of soil-grown and air-exposed *Clivia* roots, of young and mature aerial root of *Monstera* and of fresh and stored Cassava tubers, respectively (*** = 99%)

with open chambers and cellulose filters (Fig. 7h) and slopes of the transpiration kinetics were not significantly different between the two samples. The lowest rates of transpiration were measured with periderms isolated from *S. tuberosum* (Fig. 7f). The transpiration kinetics of all other samples were between these upper and lower value ranges (Fig. 7b–e, g). Water permeability of the suberized samples increased by factors of 1.7 ± 1.7 (*M. domestica*), 2.8 ± 1.8 (the exposed root of *C. miniata*), 5.6 ± 3.8 (young roots of *M. deliciosa*), 9.2 ± 7.7 (mature roots of *M. deliciosa* and 9.6 ± 2.1 (*S. tuberosum*) after solvent extraction of waxes with chloroform (Fig. 8). With the suberized hypodermis isolated from *C. miniata* soil-grown roots and the periderm isolated from *M. esculenta* fresh tubers, the rates of water loss were slightly decreased after wax extraction (Fig. 8).

From the slopes of the regression lines fitted to transpiration kinetics, permeances P (m s^{-1}) were calculated for suberized intact periderm, wax-extracted periderm, the open transpiration chamber, and the cellulose filter (Fig. 9). Permeances obtained for intact suberized samples varied between the lowest value of 6.5×10^{-10} ($\pm 6.2 \times 10^{-10}$) m s^{-1} measured with matured aerial roots of *M. deliciosa* and the highest value of 5.1×10^{-08} ($\pm 3.0 \times 10^{-08}$) m s^{-1} measured for stored tubers of *M. esculenta* (Fig. 9). Permeances obtained for wax-free suberized samples varied between the lowest value of 5.8×10^{-09} ($\pm 5 \times 10^{-09}$) m s^{-1} measured with matured aerial roots of *M. deliciosa* and the highest values of 4.4×10^{-8} ($\pm 5.4 \times 10^{-08}$) m s^{-1} measured for air-exposed roots of *C. miniata* (Fig. 9). Permeances obtained for the open chamber and the cellulose filter were 7.4×10^{-08} ($\pm 5.6 \times 10^{-09}$) m s^{-1} and 6.4×10^{-08} ($\pm 5.0 \times 10^{-09}$) m s^{-1} (Fig. 9).

Discussion

Extremely steep gradients with water potentials of about – 160 MPa (about 30% relative humidity) in the atmosphere, strongly driving foliar transpiration, can be followed within hours by very flat gradients with water potentials higher than – 1.5 MPa (about 99–100% relative humidity), hardly causing any gradient for an efficient transpiration of water from the leaf (Milburn 1979; Pickard 1981; Chen et al. 1999). Due to these rapid changes in water vapor gradients between the inside (nearly 100% relative humidity) and the outside of leaves, throughout their life period leaves need constant and nearly perfect protection from uncontrolled water loss. There is no doubt, that this protection is successfully provided by the plant cuticle sealed with waxes (Schreiber and Schönherr 2009), which is highly impermeable for water, especially when compared to stomatal transpiration (Grünhofer et al. 2022). Permeances of plant cuticles, efficiently protecting leaves and fruits from uncontrolled water loss,

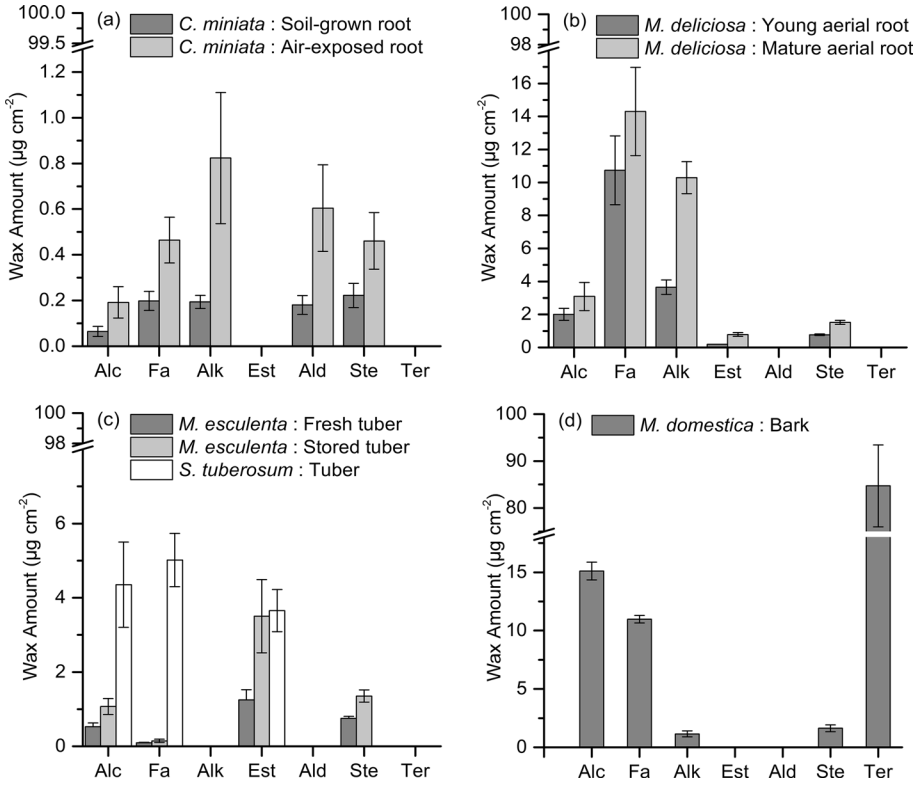


Fig. 4 Substance classes of soluble waxes (µg cm^{-2}) extracted with chloroform from the different suberized tissues isolated from five different plant species (*Clivia miniata*, *Monstera deliciosa*, *Solanum tuberosum*, *Manihot esculenta,* and *Malus domestica*). Besides sterols and terpenoids, amounts of aliphatic waxes are separated into alcohols (Alc), fatty acids (Fa), alkanes (Alk), esters (Est), aldehydes (Ald), sterols (Ste) and, terpenoids (Ter). **a** Wax composition of the suberized hypodermis isolated from soil-grown roots and air-exposed roots of *Clivia miniata*. **b** Wax composition of the suberized hypodermis isolated from young aerial roots and mature aerial of *Monstera deliciosa*. **c** Wax composition of the suberized periderms isolated from freshly harvested tubers and 3 weeks stored tubers *Manihot esculenta* and from *Solanum tuberosum*. **d** Wax composition of the suberized bark isolated from shoots of *Malus domestica*. Data points represent means with standard deviations ($n = 3$)

cover a range between 10^{-10} and 10^{-9} m•s^{-1} (Schreiber and Riederer 1996). As examples for representative permeances for cuticles, P of *Hedera helix* leaf cuticle and of *Solanum lycopersicon* fruit cuticle can be given here (Fig. 9), having a value of 0.9×10^{-11} m s^{-1} and 1.9×10^{-09} m s^{-1} respectively (Schönherr and Lendzian 1981). Upon wax extraction, permeances of wax-free cuticles of *Solanum lycopersicon* were 3.7×10^{-08} m s^{-1} and permeances of *Hedera helix* were 2.6×10^{-08} m s^{-1} (Schönherr and Lendzian 1981). Thus, wax extraction resulted in 20- (*Solanum lycopersicon*) and 265-fold (*Hedera helix*) increased permeances.

The highest possible transpiration rate of water, which can theoretically be measured with the system used here, is given by the permeance of an open transpiration chamber without any membrane mounted. This measurement resulted in a permeance of 7.4×10^{-08} m s^{-1} (Fig. 9). The fact that a primary carbohydrate cell wall, without any further aromatic (lignin) or aliphatic (suberin or cutin) modification, does not represent an efficient transpiration barrier is shown for the filter membrane made of pure cellulose, which was mounted to the transpiration chamber. Although the cellulose filter investigated here had a thickness of 140 µm, which is by far thicker than a regular primary carbohydrate cell wall of a leaf, varying about 1–2 µm (Moghaddam and Wilman 1998), the permeance was 6.4×10^{-08} ($\pm 5 \times 10^{-09}$) m s^{-1} which is statistically not different from the value measured for an open chamber (Fig. 9). It is remarkable that permeances of wax-free cuticles were only about two- to threefold lower than the permeance of the open transpiration chamber (Fig. 9). All further permeances measured here with the different suberized cell wall samples isolated from roots, tubers or shoots, are located between the low values of cuticles and the high values of cellulose filter and the open transpiration chamber (Fig. 9).

In comparison to the atmosphere surrounding the leaves, which is characterized by wide variation in water potentials reaching very low values, the range and the temporal variation in soil by far less pronounced. Field capacity is defined as the maximum amount of water absorbed by the soil, water potentials are very close to 0 (– 0.03 MPa), whereas

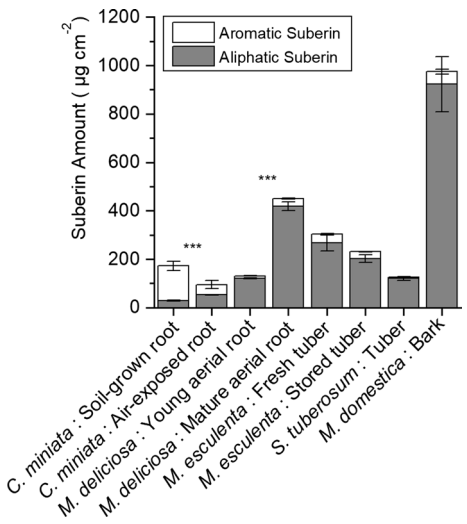


Fig. 5 Total amounts (µg cm^{-2}) of aromatic (ferulic and coumaric acids) and aliphatic suberin (linear long-chain aliphatic suberin monomers) obtained after depolymerization of the different wax-extracted suberized tissues isolated from five different plant species (*Clivia miniata*, *Monstera deliciosa*, *Solanum tuberosum*, *Manihot esculenta,* and *Malus domestica*). Data points represent means with standard deviations ($n = 3$). Asterisks indicate a significant difference between aliphatic suberin amounts of soil-grown and air-exposed *Clivia* roots, of young and mature aerial root of *Monstera* and of fresh and stored Cassava tubers, respectively (*** = 99%)

a potential of – 1.5 MPa is already defined as the permanent wilting point of plants (Kramer and Boyer 1995). At soil water potential of – 1.5 MPa, corresponding to a relative humidity of nearly 99% (Milburn 1979), most herbaceous plants and crops cannot take up water anymore and will start to wilt. This permanent wilting point of – 1.5 MPa leads to the conclusion that the main problem for plants, when dealing with water shortage, is not related to the fact that they do not have an efficient transport barrier on the root surface, protecting them from desiccation, but obviously plants are not able to decrease their internal water potentials to values significantly more negative than – 1.5 MPa. Consequently, at a soil water potential of – 1.5 MPa or lower, the driving force for the passive water diffusion usually directed inwards is inversed leading to wilting of plants already at 99% soil humidity.

Thus, it is not too surprising that the permeance measured with the suberized hypodermal cell layers, isolated from soil-grown roots of *C. miniata*, was 5×10^{-08} ($\pm 3.2 \times 10^{-09}$) m s^{-1}, which is nearly as high as the cellulose membrane and the open transpiration chamber (Fig. 9). Upon wax extraction, permeance was statistically not significantly different from the intact hypodermis (Fig. 8), indicating that there is hardly any diffusional barrier for water developed with the outer suberized cells of soil-grown *C. miniata* roots. The suberized hypodermis isolated from *C. miniata* roots exposed to the atmosphere had fivefold lower permeance of 1.6×10^{-08} ($\pm 1.2 \times 10^{-08}$) m s^{-1} compared to the soil-grown roots. Upon wax extraction the permeance increased on an average threefold, indicating that the wax in the suberin polymer of the air-exposed roots established this, albeit very weak, diffusional barrier for water and not the slightly increased amounts of aliphatic suberin (Fig. 5).

Very different from *C. miniata*, the suberized hypodermis isolated from aerial roots of *M. deliciosa* had fairly low permeances between 4.2×10^{-09} ($\pm 3 \times 10^{-09}$) m s^{-1} measured for the young still developing aerial root tip and 6.5×10^{-10} ($\pm 6.2 \times 10^{-10}$) m s^{-1} measured for the mature aerial root zone (Fig. 9). These values nearly match permeances located in the upper range of isolated cuticular membranes (Schreiber and Schönherr 2009). Thus, aerial roots of *M. deliciosa*, facing the steep gradient in water potential between the roots and the atmosphere, obviously need an efficient transpiration barrier for survival. The intensity of suberization (Fig. 5), and the amounts of wax (Fig. 3), being significantly higher in *M. deliciosa* compared to *C. miniata*, established this pronounced transpiration barrier (Fig. 3), which is again largely lost upon wax extraction (Fig. 8). This emphasizes the significance of the wax in establishing a transpiration barrier, as it is also the case with leaf cuticles. In addition, it is worth pointing out that the number of suberized cell layers is on average only twofold higher with *M. deliciosa* (2–4 cell layers) compared to *C. miniata* (1–2 cell layers), whereas the permeance of the suberized tissue of *M. deliciosa* compared to *C. miniata* is on average 1–2 orders of magnitude lower (Fig. 9). Thus, it is not so much an increase in the number of suberized cell layers and in suberin amounts reducing water permeability, but in wax deposition establishing the transpiration barrier of aerial roots of *M. deliciosa*.

Tuber and storage roots as subterranean storage organs of plants are growing all their life span in soil and they develop fairly thick outer periderms as interfaces towards the soil environment. This is also the case here with *M. esculenta*, characterized by 12 to 15 suberized cell layers, and with *S. tuberosum* having a slightly lower number of about 10 suberized cell layers (Fig. 1 and 2). Therefore, it is very surprising that the periderm of *M. esculenta* nearly completely failed to establish reasonable transpiration barriers, whereas it was exactly the opposite with the periderm of *S. tuberosum*, forming a highly efficient transpiration barrier (Fig. 9). Permeances measured with the periderms of *M. esculenta* varied between 2.3×10^{-08} m s^{-1} and 5.1×10^{-08} m s^{-1}, which is comparable to the values obtained with soil-grown *C. miniata* roots and already very close to the values obtained with the cellulose filter and the open transpiration chamber (Fig. 9). However, permeances obtained for *S. tuberosum* were 7.4×10^{-10} ($\pm 2.7 \times 10^{-10}$) m s^{-1}, which is 1 to 2 orders of magnitude lower compared to the permeances of *M. esculenta*. The permeances measured here for

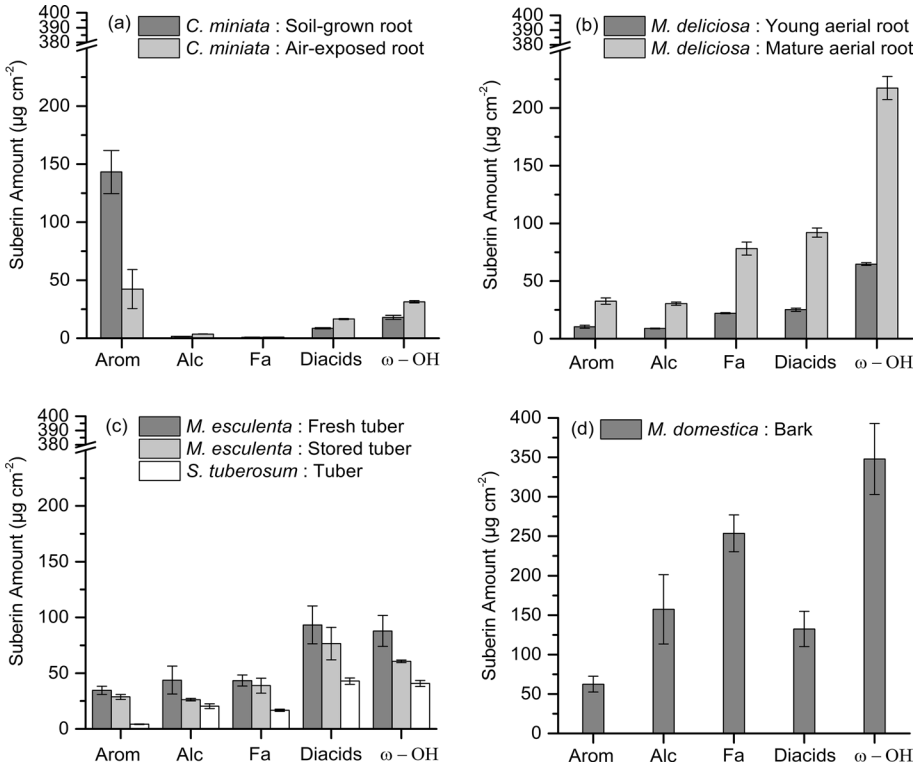


Fig. 6 Amounts of the different substance classes of suberin monomers (µg cm^{-2}) obtained after depolymerization of the different suberized tissues isolated from five different plant species (*Clivia miniata*, *Monstera deliciosa*, *Solanum tuberosum*, *Manihot esculenta,* and *Malus domestica*). Substance lasses are separated into aromatics (Arom), primary alcohols (Alc), fatty acids (Fa), α,ω-diacids, and ω-hydroxy acids (ω-OH). **a** Substance classes of the suberized hypodermis isolated from soil-grown roots and air-exposed roots of *Clivia miniata*. **b** Substance classes of the suberized hypodermis isolated from young aerial roots and mature aerial of *Monstera deliciosa*. **c** Substance classes of the suberized periderms isolated from freshly harvested tubers and 3-week-stored tubers *Manihot esculenta* and from *Solanum tuberosum*. **d** Substance classes of the suberized bark isolated from shoots of *Malus domestica*. Data points represent means with standard deviations ($n = 3$)

S. tuberosum also fit values published for *S. tuberosum* in the past (Schreiber et al. 2005) and they are in the range of permeances (10^{-11}–10^{-09} m s^{-1}) published for highly water-impermeable cuticular membranes (Schreiber and Riederer 1996).

Different from *S. tuberosum*, where it was shown that upon 4-week-storage permeances of periderms decreased by 1 order of magnitude (Schreiber et al. 2005), permeances of *M. esculenta* periderms isolated after 3 weeks of storage were statistically not different from freshly isolated periderms (Fig. 9), although aliphatic wax amounts of *M. esculenta* periderms increased by about twofold during 3-week storage (Fig. 3). This completely opposing behavior between the periderm of *S. tuberosum*, forming a very efficient transpiration barrier, and the periderm of *M. esculenta*, completely lacking the ability to form an efficient water barrier (Fig. 9), protecting tubers against water loss, fits the well-known difference in shelf-life between both tubers. Whereas *S. tuberosum* tubers ideally can be stored for several months (Alamar et al. 2017), *M. esculenta* tubers rapidly start to deteriorate within 24 h after harvest (Saravanan et al. 2016). Besides many other physiological and enzymatic processes leading to rapid deterioration and loss of nutritional quality of harvested *M. esculenta* tubers, this total failure of the periderm protecting the tubers from rapid dehydration represents another significant factor for the pronounced postharvest losses of *M. esculenta*. Covering the tubers with paraffin wax, which will reduce tuber dehydration, can delay the postharvest deterioration by a couple of weeks (Uchechukwu-Agua et al. 2015). At the moment, it remains an interesting and unsolved question, why *M. esculenta* completely fails to establish an efficient transpiration barrier protecting the tubers, whereas *S. tuberosum* is highly successful?

One could speculate that this difference between *M. esculenta* and *S. tuberosum* establishing a transpiration barrier could be related to additional yet unknown differences in the polyphenolic cell wall modifications of both periderms, which, however, would need to be investigated in the future. Another reasonable explanations could be the completely different ontogenetic origin of both types of tubers. The tuber of *M. esculenta* develops from the root,

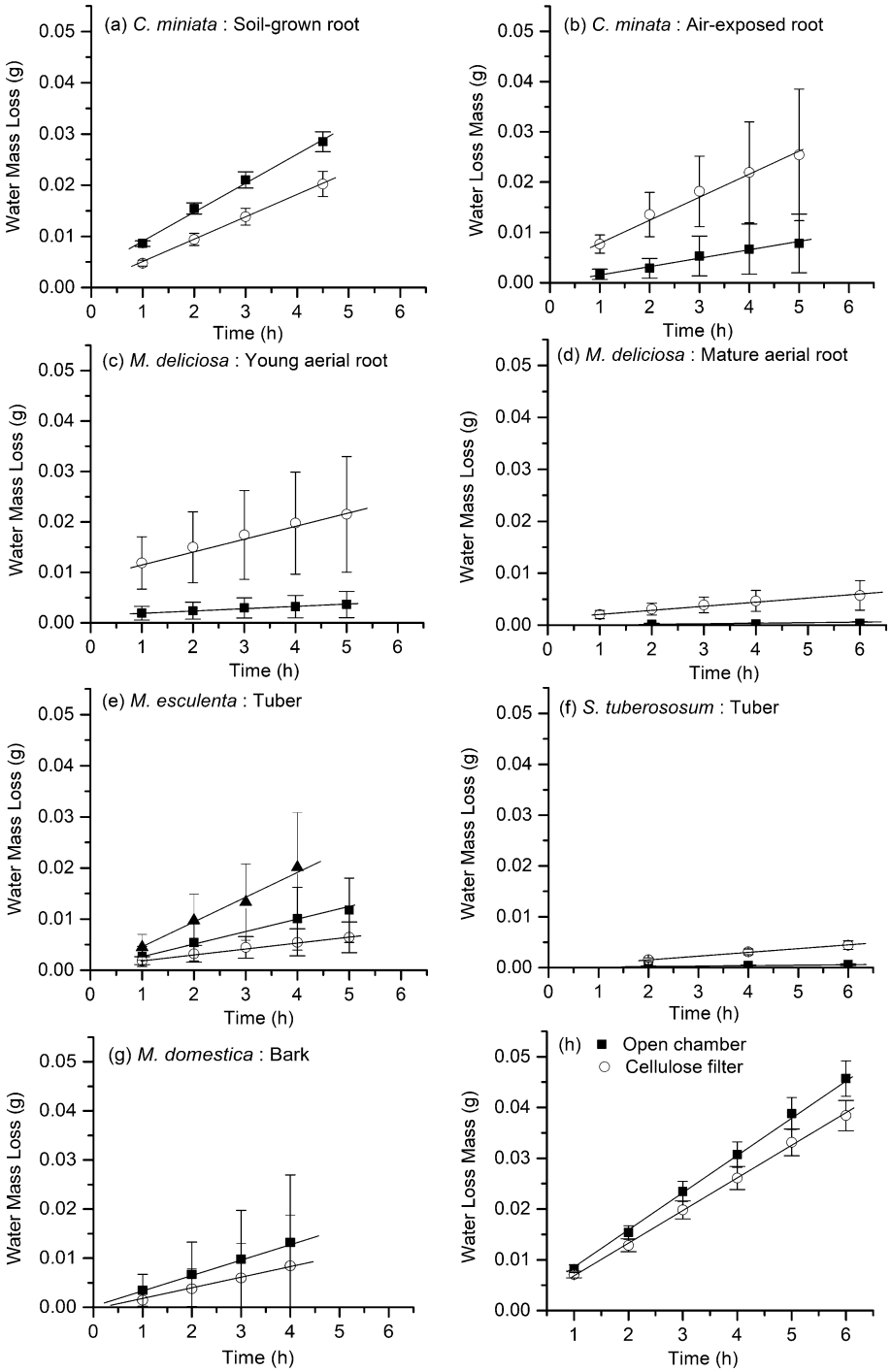


Fig. 7 Transpiration kinetics (g h^{-1}) measured for the different suberized tissues isolated from five different plant species (*Clivia miniata*, *Monstera deliciosa*, *Solanum tuberosum*, *Manihot esculenta,* and *Malus domestica*). **a–g** Intact suberized tissues (black squares) and wax-extracted suberized tissues (white circles) were compared. **e** Intact periderms isolated from 3-week-stored tubers of *Manihot esculenta* (black triangles) are shown in comparison to intact periderms isolated from freshly harvested tubers of *M. esculenta* (black squares). **h** Transpiration kinetics were measured with cellulose filters (white circles) and with open transpiration chambers (black square). Data points represent means with standard deviations of ($n \geq 10$)

whereas the tuber of *S. tuberosum* originates from a shoot growing horizontally belowground. Potentially the genetic and biochemical machinery, leading to a pronounced suberin and wax biosynthesis, is activated a lot more in a tuber being homologous to a plant shoot, naturally facing the atmosphere, instead of a tuber originating from a root, normally facing the soil environment. A further explanation could be the functions of the tuber of *S. tuberosum* and storage root of *M. esculenta*. Potato tubers allow re-growth after dormancy protecting the apical and lateral axillary buds and the resources for re-growth from abiotic and biotic conditions (Suttle 2004), while the storage roots of *M. esculenta* do not experience times of dormancy but facilitate growth of the perennial shrub serving as a carbon sink and source tissue for growth (El-Sharkawy 2004). *Solanum* species show a huge variation in dormancy, and it is affected by pre- and postharvest environmental conditions (Sonnewald 2001; Suttle 2004). For example, the Chilean and European

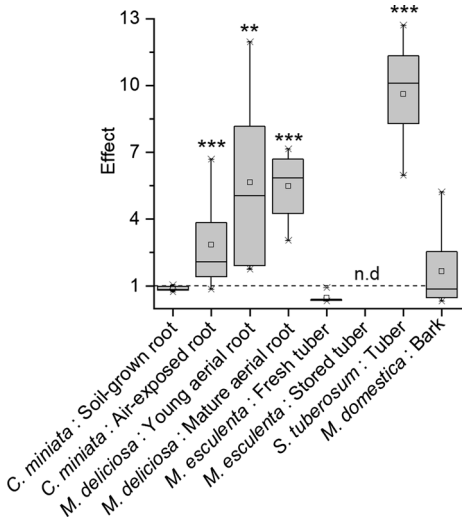


Fig. 8 Effects of wax extraction on transpiration measured with suberized tissues isolated from five different plant species (*Clivia miniata*, *Monstera deliciosa*, *Solanum tuberosum*, *Manihot esculenta,* and *Malus domestica*). Effects were calculated by dividing the slopes of transpiration kinetics of wax-extracted suberized tissues by the slopes of transpiration kinetics measured with intact suberized tissues. As a reference (dotted line), the effect of 1 is shown, indicating that there was no change in transpiration after the extraction of wax from the isolated suberized tissue. Means ($n \geq 5$) with standard deviations are shown. n.d. = not determined. Asterisks indicate a significant different effect from 1 (** = 95%; *** = 99%)

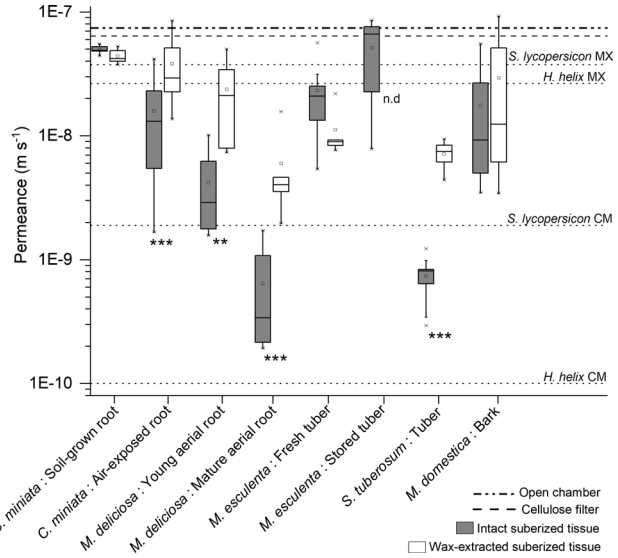


Fig. 9 Box plots of permeances P (m s^{-1}) calculated from the regression lines fitted to the transpiration kinetics measured with suberized tissues isolated from five different plant species (*Clivia miniata*, *Monstera deliciosa*, *Solanum tuberosum*, *Manihot esculenta*, and *Malus domestica*). Intact suberized tissues (grey box plots) and wax-extracted suberized tissues (white box plots) were compared. *P* (dash dot line) of the open transpiration chamber (7.4×10^{-08} m s^{-1}) and *P* (dashed line) of the cellulose filter (6.4×10^{-08} m s^{-1}) is given as "upper" reference lines. *P* (dotted line) of the leaf cuticle isolated from *Hedera helix* 9.9×10^{-11} m s^{-1} and fruit cuticle from *Solanum lycopersicon* 1.9×10^{-9} m s^{-1}; Schönherr and Lendzian (1981) is given as a "lower" reference line. Box plots with medians (line in the box), means (square in the box), whiskers (10–90% of the values), and outliers (crosses) are given ($n \geq 5$; n.d. = not determined. Asterisks indicate a significant difference between permeances of intact periderms and wax-extracted periderms (** = 95%; *** = 99%)

potatoes are believed to derived from a domestication event that took place in Peru at an altitude of 3000–4000 m. As a consequence, tubers of potatoes would need suberin barriers to withstand the abiotic and biotic conditions guaranteeing re-growth after the dormancy period in such an altitude. These potatoes migrated through hybridization with other Andean wild species to coastal Chile over time, which allowed the adaptation to temperate climates (reviewed by Ramsay and Bryan 2011). However, cultivars of the *S. tuberosum* L. Phureja Group occurred at the Eastern slope of the Andes from western Venezuela to central Bolivia in an altitude of 2000 to 3400 m (Ochoa 1990), and some cultivars lack tuber dormancy in this group (Ghislain et al. 2006). Thus, the dormancy and subsequently suberization of the tuber could be due to the environmental origins of the Chilean and European potato or due to a selection and breeding process as an adaptation to the Chilean coastal regions and subsequently to temperate climates. Such an adaptation or adaptation/breeding of cassava never occurred as the crop is only grown in the tropical region around the globe (reviewed by McKey and Delêtre 2017). Therefore, the discrepancy in periderm function between *M. esculenta* and *S. tuberosum* remains an interesting scientific as well as important applied research question to be analyzed in the future.

The last sample of suberized tissues analyzed here was the periderm isolated from *M. domestica* shoots. Although this periderm was characterized by the highest amount of wax molecules (Fig. 3) and suberin monomers (Fig. 5) of all samples investigated here, rates of water loss were surprisingly high (Fig. 7h). The permeance was about 1.1×10^{-08} ($\pm 3.4 \times 10^{-08}$) m s^{-1} and there was no significant increase in permeance after the extraction of wax 3×10^{-08} ($\pm 1.8 \times 10^{-08}$) m s^{-1} (Fig. 8). At the moment, we do not have a straightforward explanation as, to why the periderm isolated from *M. domestica* shoots did not represent a reasonable transpiration barrier. Maybe different possibilities must be considered. First, periderms were isolated from still growing and therefore continuously radially expanding shoots, which could be a reason for the failure to establish an efficient transpiration barrier. In addition, compared to the other suberized samples, handling of the periderms isolated from *M. domestica* shoots and mounting to the transpiration chambers was fairly difficult, since shoot periderms were very brittle. It cannot be excluded that this caused some defects or cracks in the investigated periderms, which were not detectable

and visible. Therefore, it is worth investigating the water permeability of periderms isolated from shoots in more detail in the future.

Conclusion

From the data presented and discussed here, it can be concluded that there is no straightforward explanation why certain suberized tissue can form efficient transpiration barriers and others fail. For the soil-grown roots and considering the weak gradients for potential water loss it is understandable that an effective water barrier is not needed, whereas for aerial roots facing the atmosphere an efficient transpiration barrier was established. From a physiological point of view, it can be hypothesized that periderms of tubers, which are acting as storage organs of plants, should have a good transpiration barrier. This was the case for *S. tuberosum* but not at all for *M. esculenta*. This discrepancy remains unclear. Our data also clearly shows that the pronounced variations in wax and suberin amounts and composition do not lead to an easy explanation of why certain suberized tissue represents efficient transpiration barriers and others not. However, for those suberized tissues forming good transpiration barriers, it is evident that wax is essential for barrier formation, since upon wax extraction barrier properties are largely lost. Thus, biotechnological approaches trying to improve the transpiration barriers of suberized tissues should focus on the enhancement of wax biosynthesis.

Author contribution statement LS and TW obtained the grants to support this study. KS, VZ, TW, and LS designed the study and planned the experiments. KS and VZ conducted the experiments. All the authors analyzed the data. KS, VZ, TW, and LS wrote the manuscript. All the authors read and approved the manuscript.

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Availability of data and materials All data generated or analyzed during this study are included in this published article.

Declarations

Conflict of interest The authors declare no competing interests.

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Appendix 3

Chapter 3

Effects of water stress on apoplastic barrier formation in soil grown roots differ from hydroponically grown roots: histochemical, biochemical and molecular evidence

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Effects of water stress on apoplastic barrier formation in soil grown roots differ from hydroponically grown roots: Histochemical, biochemical and molecular evidence

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Abstract

In root research, hydroponic plant cultivation is commonly used and soil experiments are rare. We investigated the response of 12-day-old barley roots, cultivated in soil-filled rhizotrons, to different soil water potentials (SWP) comparing a modern cultivar (cv. Scarlett) with a wild accession ICB181243 from Pakistan. Water potentials were quantified in soils with different relative water contents. Root anatomy was studied using histochemistry and microscopy. Suberin and lignin amounts were quantified by analytical chemistry. Transcriptomic changes were observed by RNA-sequencing. Compared with control with decreasing SWP, total root length decreased, the onset of endodermal suberization occurred much closer towards the root tips, amounts of suberin and lignin increased, and corresponding biosynthesis genes were upregulated in response to decreasing SWP. We conclude that decreasing water potentials enhanced root suberization and lignification, like osmotic stress experiments in hydroponic cultivation. However, in soil endodermal cell suberization was initiated very close towards the root tip, and root length as well as suberin amounts were about twofold higher compared with hydroponic cultivation.

KEYWORDS
apoplastic root barrier, lignin, soil-grown barley root, soil water potential, soil water stress, suberin

1 | INTRODUCTION

Drought severity and frequency have increased as a result of climate change (AghaKouchak et al., 2014). For agriculture, drought is a recurring catastrophic climate occurrence and one of the costliest natural disasters worldwide, according to historical drought trends (Asong et al., 2018). Scientists have examined how plants respond to drought and discovered that, as drought intensifies, there is a stronger nonlinear relationship between vegetative growth and drought (Zhou et al., 2022). To ensure agricultural efficiency and

output in the future, genetic diversity is crucial (Dawson et al., 2015). Given the effects of climate change, this genetic diversity is very valuable since it facilitates the production of more robust and flexible cultivars (Dawson et al., 2015; Newton et al., 2011). Over the years, genetic and genomic tools have been employed to improve drought tolerance. In the current study, we chose barley due to its importance in food production (ranking fourth in the world's food production), and its genetic diversity. The wild progenitor (*Hordeum vulgare* ssp. *spontaneum*) originating from the *Fertile Crescent* region is adapted to various arid and semiarid habitats (Badr et al., 2000). In contrast to the wild progenitor, the modern cultivars of barley, *Hordeum vulgare* ssp. *vulgare*, which are derived from wild barley, have lost important traits related to drought tolerance due to breeding over the years for preferred traits reducing the genetic background (Cai et al., 2020; Zhao et al., 2010). The lost traits during selection can be reintroduced into cultivated barley for crop improvement purposes (Ellis et al., 2000) allowing for the breeding of more drought tolerant barley varieties in the future. Therefore, it was the aim, comparing a wild progenitor of barley (*H. vulgare* ssp. *spontaneum)* rich in genetic diversity, with a modern barley cultivar (*H. vulgare* ssp. *vulgare*), reduced in genetic diversity. This could offer new aims what to consider for breeding more drought tolerant modern barley cultivars.

Roots play an important role in facilitating drought tolerance since they are the organs being directly exposed to water deficiency in soil and they have to respond securing water supply of the plant as good as possible. Thus, water and coupled nutrient uptake efficiency play an important role to mitigate water deficits. Water and nutrient uptake in roots can occur through apoplastic and cell-to-cell pathways (symplastic and transcellular pathways). The apoplastic movement of water and solutes can also be affected by the formation of Casparian strips and suberin in the endodermis (Franke & Schreiber, 2007; Ranathunge et al., 2017). Abiotic and biotic stressors have been demonstrated to enhance suberization in roots (Grünhofer et al., 2022, 2023; Holbein et al., 2019; Kreszies et al., 2020; Lanoue et al., 2010). The suberin polymer is composed of polyaliphatic and polyaromatic domains. Primary alcohols, fatty acids, α,ω-dicarboxylic acids (diacids), and ω-hydroxy acids (ω-OH acids) are the aliphatic monomers, whereas ferulic and coumaric acids are the aromatic components (Graça, 2015; Schreiber et al., 1999). Casparian strips are mainly made up of lignin (Naseer et al., 2012; Schreiber, 1996; Zeier & Schreiber, 1997). Syringyl, guaiacyl, and p-hydroxyphenol are the monomers of lignin, a complex aromatic biopolymer (Rolando et al., 1992). Apoplastic barrier formation leads to water uptake via the cell-to-cell pathway, which is regulated by aquaporins (Steudle, 2000a, 2000b). Previous hydroponic studies showed an earlier onset of root suberization in modern cultivar Scarlett (*H. vulgare* L. ssp. *vulgare*) when compared with wild accession Pakistan (ICB181243; *H. vulgare* L. ssp. *spontaneum*) during osmotic stress induced by polyethylene glycol (PEG) 8000 (Kreszies et al., 2020).

Water stress for plants starts in soil and roots are the first plant organs to sense water limitation in dehydrating soil. Thus, studying roots is of great importance to understand the altered growth and development of plants in response to low soil water content (SWC). In this study, the main intention was to investigate the responses of apoplastic barriers in barley roots to water limitation in soil. Anatomy of root cross sections, chemical composition of apoplastic barriers, and gene expression patterns were analysed over the length of the root. It was the intention of identifying differences in drought stress responses comparing a modern barley cultivar with a wild barley cultivar. In addition, drought effects on root growth in soil investigated here should be compared with recently published data describing root responses to osmotic stress in hydroponic cultivation, mimicking water stress (Kreszies et al., 2020). We hypothesized that (i) the root response to drought stress of wild barley will differ from the modern cultivar (Scarlett and the wild accession), and that (ii) the drought stress response of soil grown roots will differ from that of plants cultivated in hydroponics.

2 | MATERIALS AND METHODS

2.1 | Plant material and growth conditions

The rhizotron experiments were performed in the greenhouse of the Institute of Bio- and Geosciences (Plant Sciences) (IBG-2, Forschungszentrum Jülich GmbH) during the months of June 2020 and June 2021. For detailed growth conditions, refer to Nagel et al. (2012). The rhizotrons were filled with sieved, black peat soil (Graberde; Plantaflor Humus). Seeds of the cultivar Scarlett (*Hordeum vulgare* ssp. *vulgare*) and the wild barley accession Pakistan (ICB181243; *Hordeum vulgare* ssp. *spontaneum*) were soaked in water (Day −1) and transferred to rhizotrons (Day 0) to germinate. A thin layer of perlite or vermiculite was added on top, and only these layers were watered with 20 mL thrice per week for stress treatments to prevent the excess loss of moisture from the soil. For the control condition, the whole rhizotron (vermiculite layer and soil) was watered with 400 mL of tap water three times per week. The plants were grown for 12 days under 16 h/8 h of day/night, temperatures of 25.8/19.7°C, relative air humidity of 48.9/64.5% and average light intensity during the day of 181.5 µmol/m^2 s between 06:00 and 22:00 h local time. The rhizotrons were imaged for physiological root measurements on Days 1, 4, 6, 11 and 12 after transferring the seeds to the rhizotrons with the automated phenotyping platform GrowScreen-Rhizo 1 (Nagel et al., 2012) to quantify noninvasively root-system architecture. Root traits such as visible main root length, visible lateral root length, maximum depth and width of the root system, and convex hull area which represents the area which is covered by the root system were estimated as described in Nagel et al. (2012). After the 12th day, the soil-filled rhizotrons were washed with water to obtain the whole root system with the shoot intact. For each plant, shoot height, leaf length and individual seminal root length were measured using a ruler. The shoot and roots were separated and dried in the oven for 10–12 days at 60°C until the constant weight was measured for dry weight determination.

2.2 | Water stress stimulation: Measurements of SWC and soil water potential (SWP)

A calibration curve (pF curve) was established correlating the SWP in MPa with the corresponding relative SWC in % (Figure 1). Relative SWC in % is calculated by first subtracting the weight of the totally dried soil from the weight of the wet soil and then dividing the difference by the weight of the dry soil. SWC was measured by gravimetry and SWP was measured using a WP4C soil water potentiometer (Decagon Devices). For generating the calibration curve, a larger batch of soil (about 5 L) was homogeneously watered overnight, and a large number of smaller samples (about 10 mL) were prepared in preweighed containers. SWPs and weights of the well-watered soil samples were measured at time 0. Subsequently, the soil samples were allowed to continuously dry out over 2 weeks. Every day from Day 0 to Day 14, a subset of at least three soil samples were taken for determination of the SWP and the corresponding weight. Subsequently, soil samples were dried at 60°C for 1 week, and the final dry weight was measured. Finally, SWC in % of the individual soil samples was calculated. Data pairs (SWP vs. SWC) were plotted and a polynomial curve was fitted (Figure 1).

To obtain larger volumes of soil with a reduced SWC for the planned stress experiments, the sieved black peat soil was spread in the greenhouse and turned around two to three times a day for a couple of days to loose moisture before it was evenly filled into the rhizotrons. This resulted in relative SWCs of about 50% (mild water stress: mild WS) in 2021 and of about 40% (strong water stress: strong WS) in 2022 (Supporting Information S1: Figure S1).

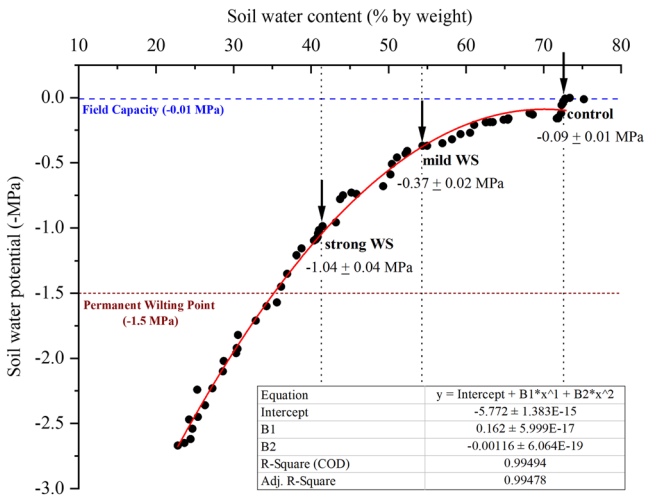


FIGURE 1 Nonlinear correlation of soil water potential (MPa) as a function of relative soil water content (%) established for the soil used in the experiments. The black arrows indicate the soil water content and the corresponding soil water potential for control, mild and strong water stress experiments. The red line gives the polynomial curve fit for the individual data points. The second order polynomial equation for the curve with an adjusted R^2 value is given in the table.

Well-watered soil (SWC of about 70%), close to field capacity (Figure 1), was used for the control experiment.

2.3 | Histochemical detection of suberin lamellae and lignified tissues in roots

Harvested roots were stored in fixation solution and segmented into 1 cm sections, after which they were cut into 50 µm thick cross-sections using a cryostat microtome (Microm HM 500 M, Microm International GmbH). Suberin lamellae were stained for 1 h using 0.01% (w/v) lipophilic Fluorol Yellow 088 (Brundrett et al., 1991). Safranin red 1% (w/v) for 10 min and Astra blue 1% (w/v) for 10 min were used for differential staining to differentiate between lignified and unlignified tissues (Vazquez-Cooz & Meyer, 2002). Before applying the counterstain (Astra blue), the section was washed thrice with 70% ethanol to remove excess Safranin. Safranin red will stain lignified tissues red, while Astra blue will stain unlignified tissues blue. Photographs for both suberin lamellae and lignified tissues were made using a Canon EOS 600D camera and an ultraviolet (UV) filter set (Zeiss) (excitation filter BP 365, dichroic mirror FT 395, barrier filter LP 397). As described in Kreszies et al. (2019), the analysed root segments were expressed as relative lengths of the entire root, with 0% representing the root tip and 100% representing the root base.

2.4 | Chemical analysis of barley root suberin

Following harvesting from the rhizotrons, root samples were kept in 70% ethanol. Seminal root was divided from tip to base into three different zones (zone A: 0%–25%, zone B: 25%–50% and zone C: 50%–100%) as described in Kreszies et al. (2019, 2020), and were enzymatically digested with 0.5% (w/v) cellulase and 0.5% (w/v) pectinase at room temperature under gentle shaking (Zeier & Schreiber, 1997). The enzyme solution was changed every 3–5 days for 3 weeks. The roots were washed in borax buffer and then in deionized water. Subsequently, samples were transferred to chloroform:methanol (1:1) to remove all soluble lipids. Before chemical analysis, root samples were dried, weighed and cut into very fine sections (Ranathunge et al., 2016). For both suberin and lignin analyses, 10 seminal roots sections from three to four plants were pooled per replicate for each zone.

For suberin depolymerization, 2 mg extracted materials were transesterified for 16–18 h at 70°C in 30% (v/v) boron trifluoride-methanol (Zeier & Schreiber, 1998). To stop the depolymerization reaction, 2 mL of saturated $NaHCO_3$ was added. As an internal standard, 10 µg of C_{32} (dotriacontane) were spiked into each sample. Suberin monomers were extracted three times by adding 2 mL chloroform. Free hydroxy groups of released suberin monomers were derivatized using 20 µL of N, O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) and 20 µL of pyridine for 40 min at 70°C before gas chromatographic analysis. Suberin monomers were quantified by injecting 1 µL of sample on a splitter system on a gas chromatography

connected to flame ionization detection (GC-FID; HP 6890N; Hewlett-Packard) and identified by gas chromatography connected to mass spectrometry (GC-MSD; 5977B; Agilent). The obtained mass spectra of suberin monomers were compared with literature mass spectra and an in-house created mass spectral library (Schreiber et al., 2005).

2.5 | Chemical analysis of barley root lignin

The protocol from Foster et al. (2010) was used with slight modifications. For lignin analysis in the three root zones (A, B and C). Only 1–2 mg of extracted dried samples were weighed, incubated in 500 µL of the thioacidolysis reagent on a heat block (105°C) for 4 h in autosampler vials. These vials were sealed with an extra Teflon disc inside the crimp seal with PTFE/silicone septa. Samples were vortexed once per hour and it was made sure that the analyzing materials remained in the liquid reagent throughout the digestion. Upon completion of the reaction, autosampler vials containing the samples were cooled to room temperature, and spiked with 10 µg of C_{32} (Dotriacontane) as internal standard. To stop the reaction 500 µL of saturated $NaHCO_3$ solution was added. Samples were extracted three times with 1 mL ethyl acetate. The combined organic extracts were dried in a heating block, and 500 µL of acetone was added twice to remove all the excess water at 60°C using a gentle nitrogen stream. For lignin monomer analysis by gas chromatography, samples were derivatized with 20 µL pyridine and 100 µL BSA reagent (N,O-bis (trimethylsilyl)acetamide; Sigma Aldrich) for 45 min at 70°C. Suberin monomers were quantified by injecting 1 µL of sample on a splitter system on a gas chromatograph connected to flame ionization detection (GC-FID; HP 6890N; Hewlett-Packard). Lignin monomers were identified by GC-MS (GC-MSD; 5977B; Agilent) according to Rolando et al. (1992) thioacidolysis products prominent fragments.

2.6 | RNA extraction, sequencing and RNA-Seq analyses

RNA was extracted from the 12-day-old barley roots grown under control (well-watered, −0.09 MPa) and strong water stress (−1.04 MPa) of both cultivars using the Zymo Research Plant Easy RNA kit. Based on the histochemical analysis a section from zone A (0%–12.5% of the root length) was harvested for the analysis. The quality was assessed using a nanodrop spectrophotometer (NanoDrop 2000c Spectrophotometer; ThermoFisher Scientific), a gel run and determination of RIN values. Roots were washed with DEPC treated water, and flash frozen using liquid nitrogen. RNA-sequencing was carried out with a total of three biological replicates growing either under control conditions and under water stress. Each replicate consisted of pooled roots from five to six different plants. cDNA libraries were prepared using the QuantSeq. 3′mRNA kit, and the sequencing was done on an Illumina HiSeq. 6000 platform, kindly enabled by the NGS Service, University of Bonn, Germany. Approximately 10–15 million reads were obtained, with a base length ranging up to 1 × 50 bp. The raw RNA-Seq reads were first subjected to a quality check using fastQC, which was then subsequently processed through cutAdapt (Martin, 2011) to remove traces of any sequencing adapters. The processed reads were then aligned against the barley reference genome (EnsemblPlants, v2) using Tophat2 (Trapnell et al., 2012) with the help of a bowtie index designed with the individual chromosome files and default parameters. For mapping statistics, a percentage of at least 90% alignment was considered to be the minimum for further downstream processing in our study. With the alignment files in BAM format, a gene count matrix was obtained using the featureCounts (Liao et al., 2014) function from the Rsubread package (Liao et al., 2019).

With these files, an MDS (Multi-Dimensional Scaling) scaling plot was also generated using the limma (Ritchie et al., 2015) and edgeR (Robinson et al., 2010) packages respectively, which highlighted the nature of the replicates and homogeneity of the samples used in this study. Differential expression analysis was then carried out using the normalized counts using DESeq. 2 (Love et al., 2014) with cutoffs: $|\log_2 FC| > 1$ and FDR < 0.05, where the raw p values obtained using Wald test (Wald, 1943) were subsequently corrected by False Discovery Rate analysis (Benjamini & Hochberg, 1995). For this study, a contrast matrix was designed as stress (−1 MPa) versus control (well-watered). Gene ontology analyses with the obtained differentially expressed genes (DEGs) were carried out with agriGO software (Tian et al., 2017) or shinyGO software (Ge et al., 2020), available online. The raw sequencing data has been deposited at the National Centre for Biotechnology Information (NCBI) database (ID: PRJNA1063280).

2.7 | Statistical analysis

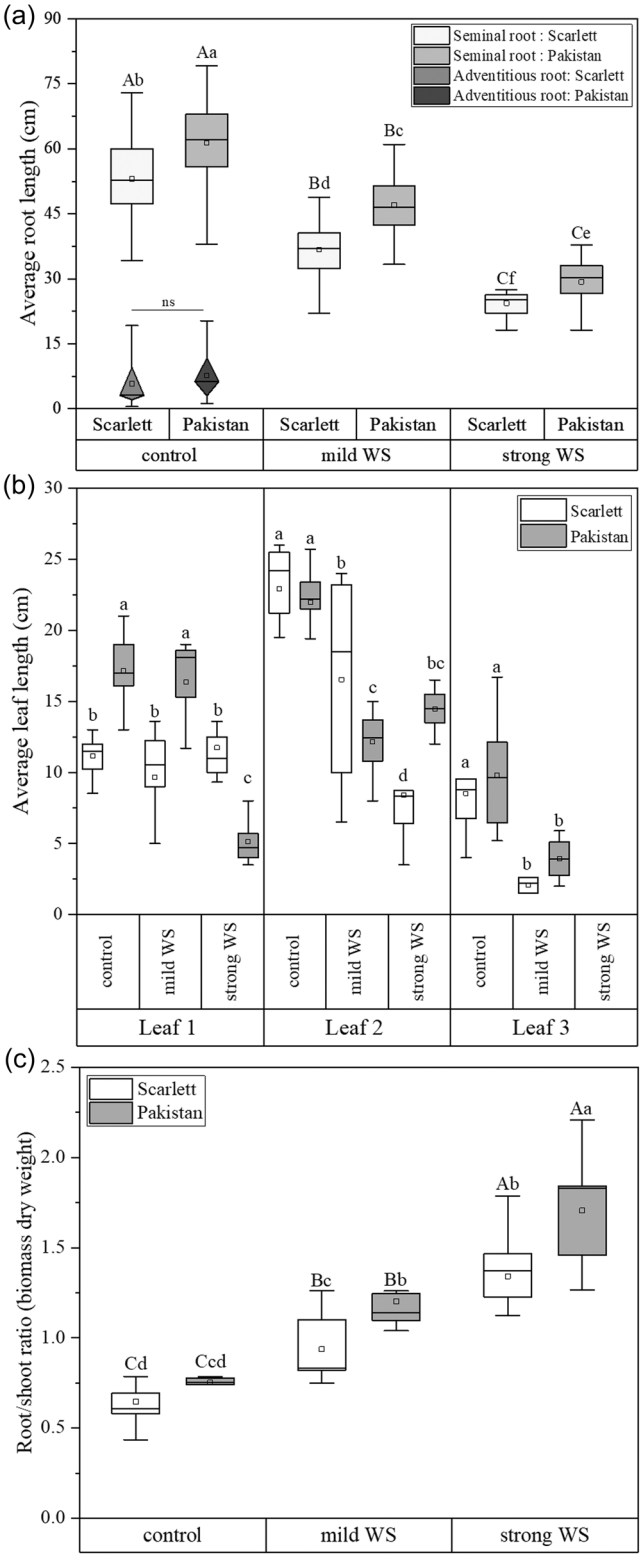
Depending on the type of experiment, the number of replicates varied: more than 10–30 for phenotypic measurements, 10–15 for microscopic examinations, 6–3 for chemical analysis and 3 for RNA-Seq were examined. For the statistical evaluation of the data and figure preparation, OriginPro 2021b (OriginLab Corporation) was used. Using the Shapiro–Wilk test, the normal distribution of the data was tested before checking for statistical significance. All the physiological measurements and chemical analysis were assessed using the analysis of variance (Fisher's least significant difference, LSD) of plants grown under varied water potentials and a significance threshold of 0.05 was used. Means with standard deviations or box plots with medians and means are shown in the graphs.

3 | RESULTS

3.1 | SWC and SWP

After 12 days of plant growth, rhizotrons were opened and soil samples were taken at three different depths in the rhizotrons

(bottom: 15%–25%; middle: 40%–50%; top: 75%–85%) in relation to the maximum root system depth (100%) in length (Supporting Information S1: Figure S1). SWC of the well-watered soil (control experiment) was 72.6% ± 0.4. SWC of the stress treatments was 54.3% ± 0.5 (mild WS) and 41.3% ± 0.8 (strong WS), respectively.



Within each of the three treatments, SWC did not vary significantly between the three different positions (Supporting Information S1: Figure S1). The SWPs of the control and two different treatments could be predicted from the calibration curve using the equation of the curve fit (Figure 1). SWPs for well-watered soil (control) were −0.09 ± 0.01 MPa, and for the stress treatments, they were −0.37 ± 0.02 MPa (mild WS), and −1.04 ± 0.04 MPa (strong WS), respectively (Figure 1). Thus, control conditions were close to field capacity (−0.01 MPa) and stress treatments were between field capacity and the permanent wilting point (−1.5 MPa) defined for crops (Kirkham, 2005).

3.2 | Root and shoot morphology

The average root length of 12-day-old barley seminal roots of both, Scarlett and Pakistan cultivars, negatively correlated with decreasing SWP (Figure 2a). The average seminal root lengths under control, mild WS and strong WS treatment was always shorter in Scarlett (53.1 ± 9.2 cm; 36.8 ± 5.6 cm; 24.4 ± 2.7 cm) compared with Pakistan (61.3 ± 9.7 cm; 47.1 ± 7.4 cm; 29.3 ± 5.2 cm). Only 12-day-old control plants developed very short adventitious roots (Figure 2a). The maximum root system depth (Supporting Information S1: Figure S2a) and the total visible root length (Supporting Information S1: Figure S2b) after 12 days cultivation was higher in Pakistan compared with Scarlett. Maximum root system width was almost similar for control and mild WS plants compared with strong WS (Supporting Information S1: Figure S2c). The total visible lateral root length was not significantly different for cultivars or treatments (Supporting Information S1: Figure S2d). Compared with Scarlett the first leaf of Pakistan was longer in control and mild WS but shorter in strong WS (Figure 2b). Lengths of leaves 2 and 3 were reduced in response to stress in both cultivars and strong WS delayed the formation of the third leaf in both cultivars (Figure 2b). Root/shoot ratios increased significantly upon stress treatments (Figure 2c).

FIGURE 2 Phenotypic characterization of barley roots and shoots. (a) Average length of seminal and adventitious roots of 12-day-old plants grown under control or mild and strong water stress conditions. (b) Average leaf length of 12-day-old plants grown under control or mild and strong water stress conditions. (c) Root/shoot ratios (biomass dry weight) of 12-day-old plants grown under control or water stress. Box plots give the 25th–75th percentiles. Squares inside the boxes represent arithmetic means. The whiskers range to the outliers, and each box plot represents >30 (a) or >10 (b, c) replicates. The different letters indicate significant differences at a significance level of 0.05 in two-way (a, c) and one-way (b) ANOVA (Fischer's least significant difference, LSD). Uppercase letters indicated significant differences between treatments, lowercase letters indicated significant differences between all the means.

3.3 | Root anatomy: Suberin and lignin staining

At 25% and 50% of the root length, almost all endodermal cells were fully suberized except for some passage cells in control and stress treatments (Figure 3a). In control and both WS treatments, first suberized endodermis cells were already detectable at 5%–10% of the total root length (Figure 3a). At 10% of the root length, the endodermal cell wall suberization was higher for roots grown in stress compared with control in both cultivars (Figure 3a). Intensity of the lignin staining of late metaxylem, early metaxylem and endodermal cell walls was increasing from 25 over 50%–90% of the root length and it was increasing with stress intensity (Figure 3b). In response to WS the inner tangential endodermal cell walls were asymmetrically stained at 50% and 90% of the root length (Figure 3b). This is complementary to the suberin staining, where the outer tangential endodermal cell walls showed a much stronger asymmetric staining

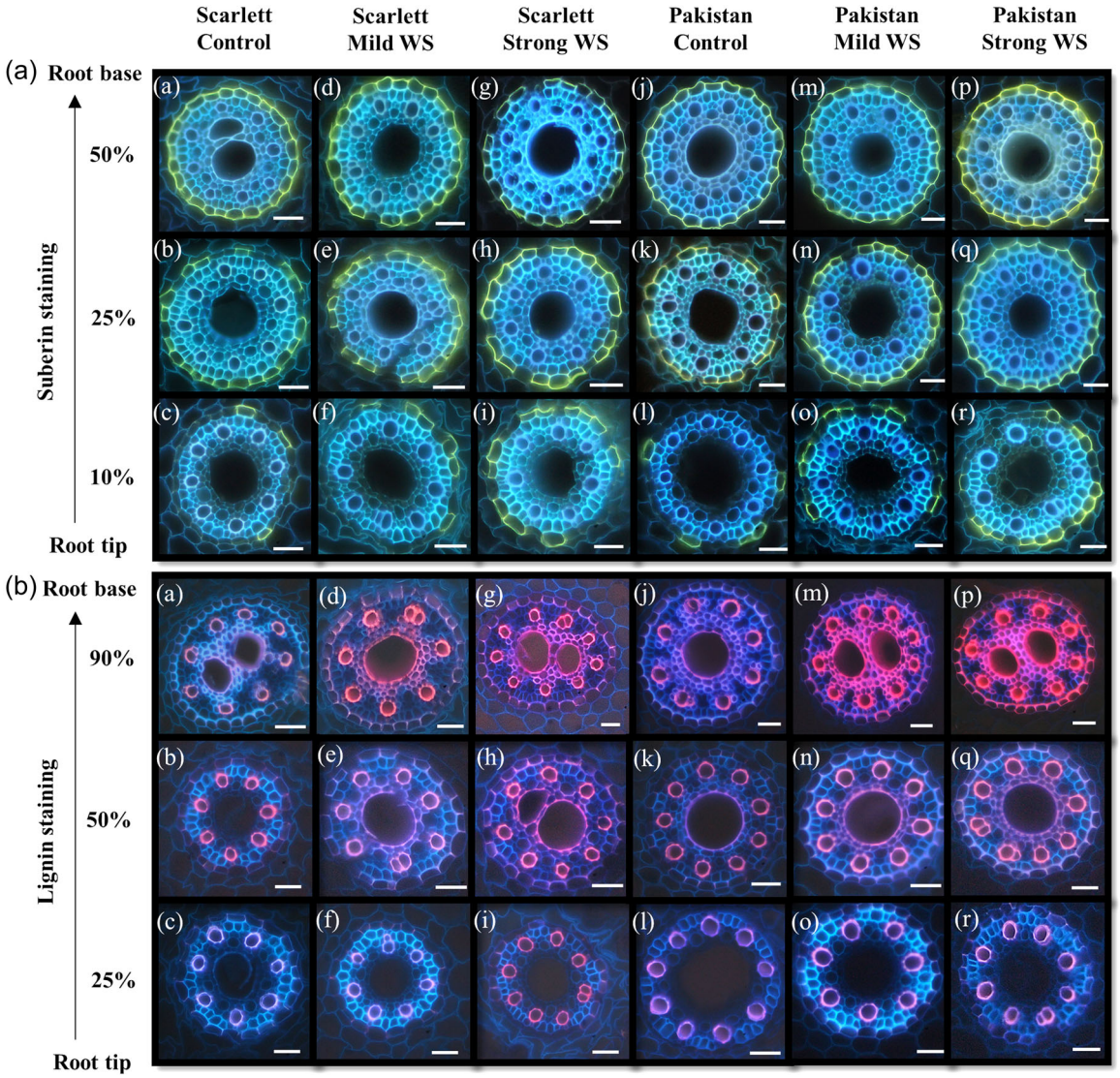


FIGURE 3 Histochemical detection of root suberization and lignification. (a) Suberin-lamellae development in the endodermis of 12-day-old barley roots grown under control or mild and strong water stress. By staining with fluorol Yellow 088, suberin-lamellae presence is indicated by a bright yellow fluorescence. At a distance of 50% from root tip, all the cells are suberized (a, d, g, j, m, p). At a distance of 25% from root tip, endodermal cells of roots grown in control are partially suberized (b, k), whereas the cells grown under stress (mild or strong) are almost completely suberized except for some passage cells (e, h, n, q). At a distance of 10% from root tip, 3–4 cells (c, l) are suberized in the control condition, and partial suberization of the endodermis can be seen. Suberization is stronger in strong WS (i, r) compared with mild WS (f, o). (b) Lignification of 12-day-old barley roots grown under control or mild and strong water stress. Lignified tissues are stained in red and cellulose in blue. At a distance of 90% from root tip, the inner side of the endodermis and Casparian strips are lignified (a, d, g, j, m, p), and metaxylem and protoxylem are lignified. Stress treatments show that the cortical cells inside the endodermis are lignified (d, g, m, p). At a distance of 50% from root tip, the Casparian strips in roots grown in the control condition are hardly stained (b, k), and in stress, stronger lignification can be seen (e, h, n, q). At a distance of 25% from root tip, Casparian strips can be seen mostly under strong WS (i, r) roots compared with mild WS (f, o) and control (c, l). Bars, 50 µm. [Color figure can be viewed at wileyonlinelibrary.com]

compared with a fainter staining of the inner tangential cell walls (Figure 3a).

3.4 | Chemical analysis of barley root suberin

In both cultivars, the total amount of linear long-chain aliphatic suberin monomers released after transesterification continuously increased over root length (Figure 4a), which was not the case for the aromatic suberin amount (Supporting Information S1: Figure S3a). In Scarlett, strong WS significantly enhanced suberization mainly in zone C, whereas in Pakistan both, mild WS and strong WS, significantly enhanced suberization in zones B and C (Figure 4a). In response to strong WS total amounts of aliphatic suberin were higher in Scarlett compared with Pakistan. Aliphatic suberin was composed of four main substance classes (Figure 4a): fatty acids, alcohols, ω-hydroxy acids, and α,ω-dicarboxylic acids (diacids). Most of the total amount of aliphatic suberin was composed of ω-hydroxy acids, followed by diacids, fatty acids and alcohols (Figure 4b). Chain lengths of the aliphatic suberin monomers varied between C16 and C26 (Supporting Information S1: Figure S4a–d) with C18:1 ω-OH, C18:1 diacid and C24 ω-OH hydroxyl fatty acid representing the most abundant aliphatic suberin monomers. Aromatic suberin was mainly composed of coumaric and ferulic acid monomers; trans isomeric monomers of these monomers were present in higher amounts compared with cis isomers (Supporting Information S1: Figure S3b).

3.5 | Chemical analysis of barley root lignin

That amount of lignin which can efficiently be depolymerized in the solid state with minimal condensation is called uncondensed lignin

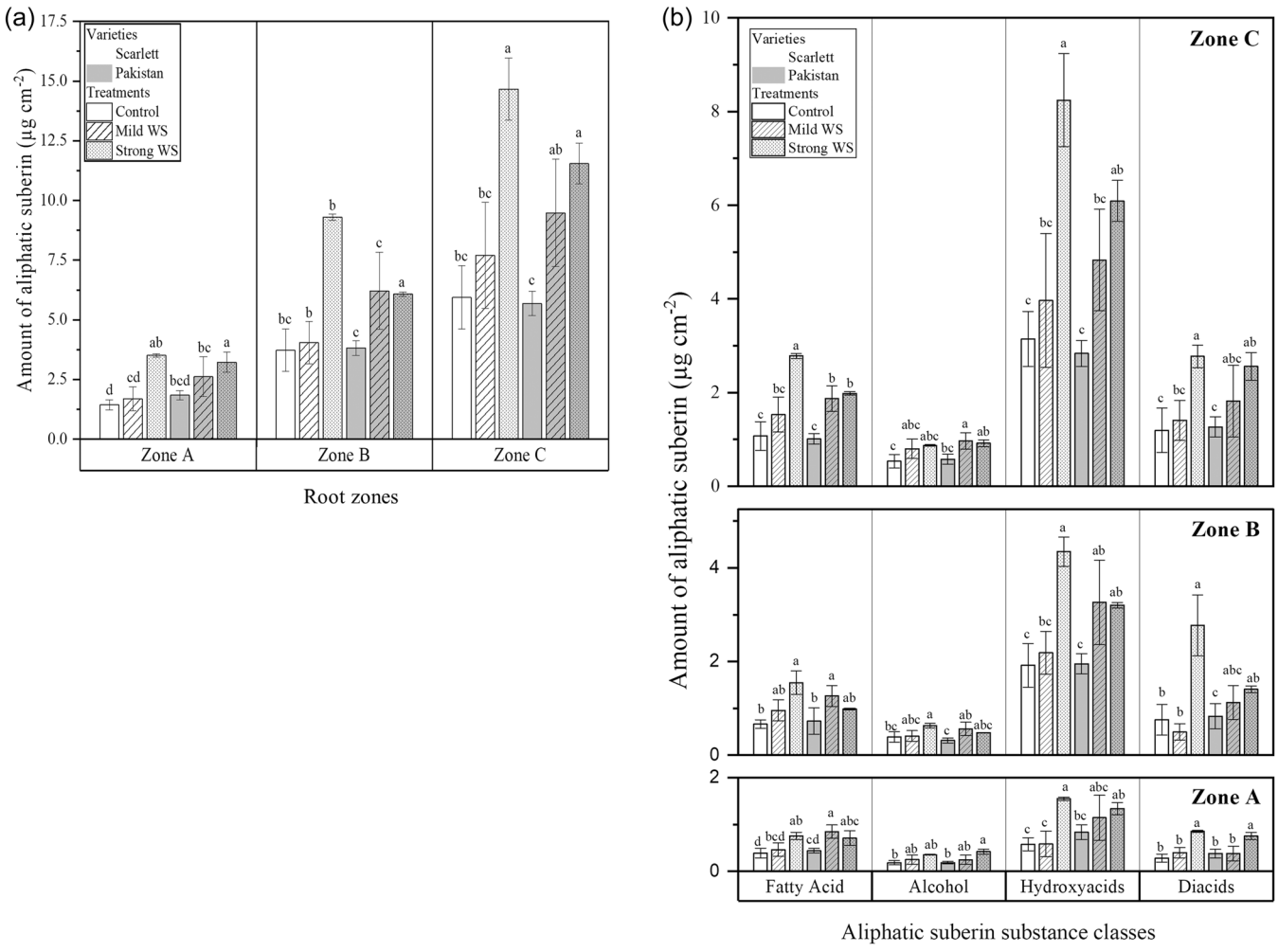


FIGURE 4 Amounts of aliphatic suberin detected in barley seminal roots grown under control or mild and strong water stress. The roots were divided into three root zones (zones A, B and C) from root tip to base. (a) Total amounts of endodermal suberin increase from Zone A to C and they increase with increasing water stress. (b) ω-Hydroxyacids and diacids are the predominant substance classes of the aliphatic suberin, followed by fatty acids and alcohols. Bars represent means with standard deviations of at least three biological replicates (control $n = 6$; stress $n = 3$). Different letters indicate significant differences within each root zone between the means at a significance level of 0.05 in one-way ANOVA (Fischer's least significant difference, LSD).

(Li et al., 2018). Uncondensed lignin increased from zone A to zone C in both cultivars (Figure 5a). Within the same root zone for each of the three stress treatments total amounts of lignin were similar between Scarlett and Pakistan (Figure 5a). The total uncondensed lignin was composed of three main groups of monomers (C_6C_3 aryl glycerol-β-aryl ethers): p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) monomers (Figure 5b). G lignin monomers were the dominant group of monomers in both, Scarlett and Pakistan, followed by S and H lignin (Figure 5b). Further compounds released by thiacidolysis were C_6C_2, C_6C_1 monomeric compounds, and some derived from p-coumaric and ferulic acid aromatic compounds (Supporting Information S1: Figure S5). The H, G, and S core fragments, including all monomers composed only of the H, G, and S aromatic ring, were similar to uncondensed lignin results. Aromatic derived products are not significantly different in between treatments and each root zones, but the amount increases over the root length (Supporting Information S1: Figure S5).

RNA-Seq analyses: comparison of strong water stress versus control of the two barley cultivars.

For RNA-Seq analysis root zone A (0%–12.5%), showing the largest differences in the degree of apoplastic barrier formation (Figures 3–5), was investigated comparing strong WS with control conditions. The MDS plot showed a clear separation of all the control (well-watered) samples from the strong WS samples and clear separation of both cultivars (Figure 6a). About 43 050 expressed genes were identified in the different samples, which corresponds to roughly 50% of the barley reference genome consisting of about 83 381 expressed genes (Sato, 2020). In Scarlett, comparing strong WS versus control, a total of 948 genes were differentially upregulated and 842 genes were downregulated, from which 360 up- and 306 downregulated genes were unique to Scarlett (Figure 6b, Supporting Information S2: Table S1). With Pakistan, 1260 genes were up- and 927 genes were downregulated with 672 up- and 391

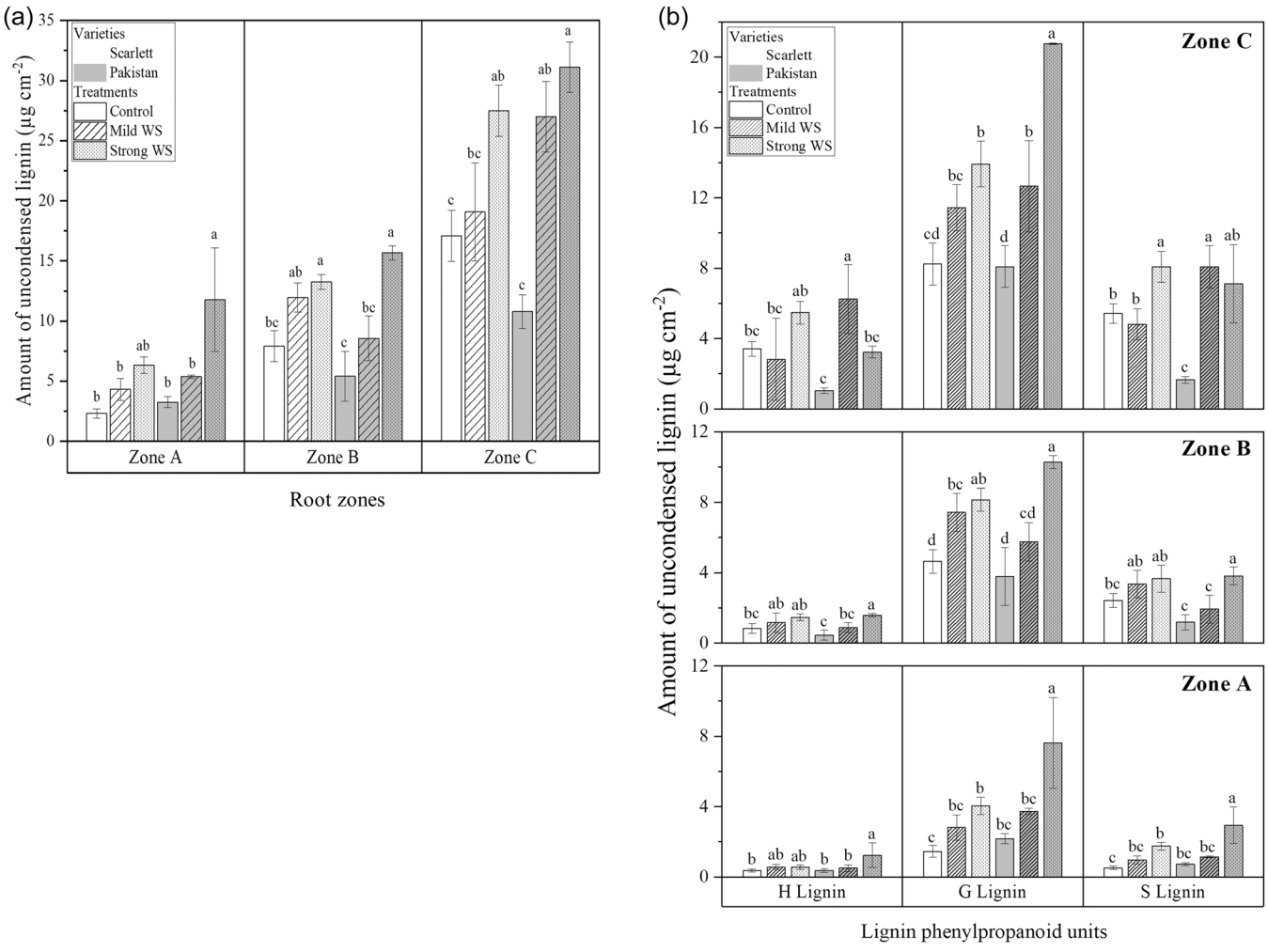


FIGURE 5 Amounts of uncondensed lignin detected in barley seminal roots grown under control or mild and strong water stress. The roots were divided into three root zones (zones A, B and C) from root tip to base. (a) Total amounts of uncondensed lignin increase from Zone A to C and they increase with increasing water stress. (b) The three dominant lignin monomers (H = p-hydroxyphenyl, G = guaiacyl and S = syringyl unit) increase from Zone A to C and they increase with increasing water stress. The bars represent means with a standard deviation of three biological replicates. Different letters indicate significant differences within each root zone between the means at a significance level of 0.05 in one-way ANOVA (Fischer's least significant difference, LSD).

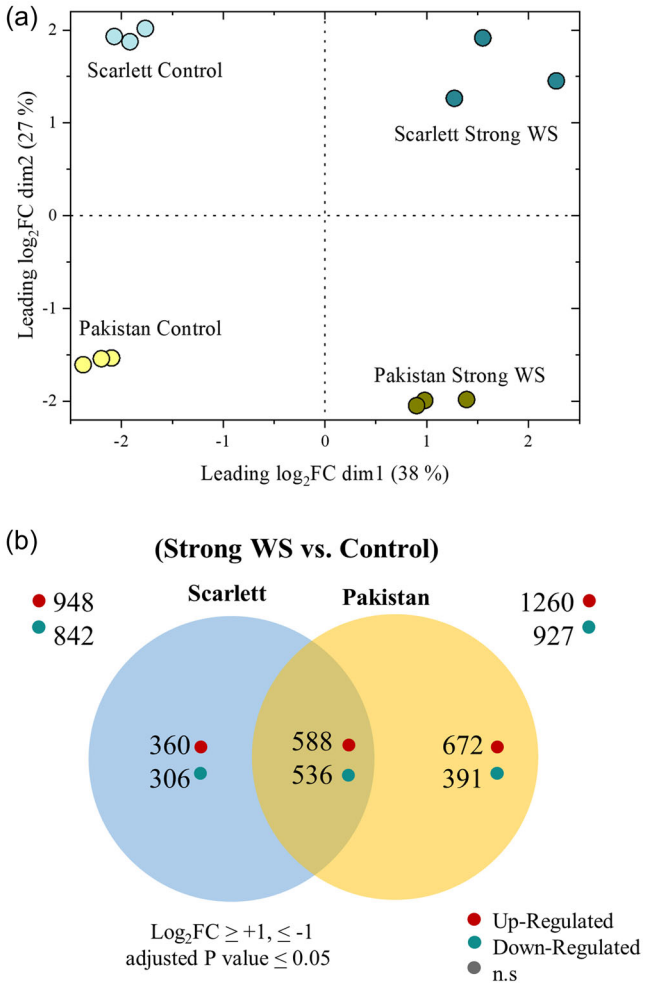


FIGURE 6 Results of the RNAseq analysis showing the differentially expressed genes (DEGs) in the barley roots of Scarlett and Pakistan grown under control or strong water stress conditions. (a) Multidimensional scaling plot of replicated RNA sequencing samples grown under control and strong WS conditions shows that cultivars and growth conditions are clearly separated. (b) Venn diagram representing DEGs (DESeq, Log2FC ≥ 1, ≤−1, and FDR ≤ 0.05) between strong WS and control conditions of Scarlett and Pakistan. Among all DEGs, 588 and 536 genes were commonly up- and downregulated in both Scarlett and Pakistan. Red and cyan dots indicate upregulation, and downregulation of genes, respectively. [Color figure can be viewed at wileyonlinelibrary.com]

downregulated genes being specific for Pakistan (Figure 6b, Supporting Information S3: Table S2). Both cultivars had 588 up- and 536 downregulated genes in common.

A GO (Gene Ontology) analysis was performed with all the DEGs of both cultivars in response to strong WS (Supporting Information S1: Figure S6, Supporting Information S4: Table S3). Most GO terms associated with the upregulated genes in the two cultivars were related to general aspects of abiotic stress responses of plants and associated signalling and transduction aspects. In Scarlett, the top upregulated GO terms were mostly associated with ROS, H_2O_2 metabolism, heat shock proteins (Supporting Information S1: Figure S6a). In Pakistan, the top upregulated GO terms were related to certain aspects of ROS detoxification, glutathione metabolic process, glutathione transferase activity and nicotinamine metabolic activity (Supporting Information S1: Figure S6b). The common upregulated GO-terms in both cultivars were associated with several aspects of stress signalling, phytohormone signal, cold acclimation, actin filament depolymerisation and response to abscisic acid (Supporting Information S1: Figure S6c). In Scarlett GO terms with most downregulated genes were related to aldehyde dehydrogenase activity, nutrient reservoir activity and manganese ion activity (Supporting Information S1: Figure S6d). Further categories included GO terms associated with cell walls, plasmodesmata and the apoplast. The top GO terms contained the most downregulated genes specific to Pakistan included different aspects of detoxification like hydrogen peroxide catabolic processes, ROS metabolic processes and peroxidase activity (Supporting Information S1: Figure S6e). GO terms with most downregulated genes common for both cultivars were related to various aspects of nitrate metabolism and transport including response to nitrate, nitrate transmembrane transporter activity and nitrate transport (Supporting Information S1: Figure S6f).

A number of DEGs (Figure 7, Supporting Information S5: Table S4) specifically upregulated in both cultivars in response to water stress were genes related to suberization (e.g., LACS: fatty acid activation, KCS: fatty acid elongation, FAR: alcohol synthesis, CYP: fatty acid hydroxylation, GPAT: esterification of fatty acids to glycerol, etc.), lignification (C4H: p-coumaric acid synthesis, PAL: cinnamic acid synthesis, HCT: shikimate hydroxycinnamoyl transferase, CCR: p-coumaryl- and coniferyl aldehyde synthesis, OMT: methyltransferase, CAD: p-coumaryl- and coniferyl alcohol synthesis, PER: lignin polymerisation, etc.) and transcription factors known to be involved regulating gene expression in response to abiotic environmental stress (e.g., WRKYs and MYBs). A smaller number of DEGs annotated as genes related to suberization and lignification were downregulated in roots exposed to water stress (Supporting Information S1: Figure S7, Supporting Information S5: Table S4). In addition, a series of DEGs were involved in nutrient (e.g., NRT: nitrate transport, PHT: phosphate transport, KT: potassium transport, MGT: magnesium transport, VIT: iron transport, SULTR: sulfate transport) and water transport (aquaporins) via cell membranes, with many of them being upregulated but some of them also downregulated (Supporting Information S1: Figure S8, Supporting Information S6: Table S5). For further information, the list of all the DEGs related to suberin, lignin, aquaporins, nutrient transporters, ROS signalling and other transcription factors are given in Supporting Information S7: Table S6.

4 | DISCUSSION

In root research, hydroponic cultivation is frequently used since it allows easy, noninvasive access of the root system during growth. But this approach is artificial since roots are continuously immersed in an aqueous solution with homogeneously distributed nutrients.

FIGURE 7 Upregulated genes relevant for suberin and phenylpropanoid biosynthesis, transcription factors, and other associated genes which are commonly upregulated in both Scarlett and Pakistan barley roots upon strong water stress treatment. Empty white cells in the table are nonsignificant (ns). (A detailed description is given in Supporting Information S5: Table S4 with references.) [Color figure can be viewed at wileyonlinelibrary.com]

Cultivating plants in soil-filled rhizotrons are closer to the situation roots face in the field conditions such as mechanical impedances, soil structure, and so forth, when compared with hydroponic cultivation. But rhizotrons still allow the observation of root growth and development noninvasively (Nagel et al., 2012).

4.1 | Nonlinear relationship between SWC and SWP

Water limitation in a substrate leading to drought needs to be quantified in MPa, giving the exact water potential. Only on this basis can different experimental approaches, for example, hydroponics versus soil cultivation, can directly be compared. Whereas, it is easy and straight forward to measure water potential in hydroponics, it is complex in soil. This is due to the fact that relative SWC (in %) versus SWP (in MPa) is not a linear function and this curve is strongly influenced by various factors such as soil texture, organic matter content, and soil moisture patterns (Groenevelt & Grant, 2004; Hewelke et al., 2015). Due to this nonlinearity (Chen et al., 1998), shown here for soil used in our rhizotron experiments, already minor changes in relative SWCs can lead to major changes in SWPs (Figure 1). Decreasing the relative SWC by 1.3-fold (from 54% to 41%) resulted in a decrease of the SWP by nearly threefold (from −0.37 to −1.04 MPa). This shows how important it is to first establish an exact calibration curve correlating SWP with SWC (Nimah & Hanks, 1973; Or et al., 2002). This is also confirmed by our study, with the soil initially drying out to have a relative SWC of approximately 50%, it finally turned out after the experiment 12 days later that relative SWC was only 54.3% in 2021 and 41.3% in 2022, resulting in SWPs of −0.37 MPa (mild WS) and −1.04 MPa (strong WS). SWCs and SWPs were homogeneous in the rhizotron from top to bottom (Supporting Information S1: Figure S1). Thus, in our experiments roots were exposed over their whole length, from tip to base, to nearly the same water deficit. This is still somewhat artificial compared with natural environmental conditions, with the soil drying out from top to bottom leading to an increasing water potential gradient.

4.2 | Comparing root development between wild barley and modern barley

Breeding programmes mostly focus on aboveground plant traits and not on root morphology or architecture (Koevoets et al., 2016). However, root length is an important trait of plants when dealing with water stress (Bengough et al., 2011; Boudiar et al., 2020; Sahnoune et al., 2004). With Pakistan, as the wild barley cultivar, compared with the highly cultivated modern cultivar Scarlett, there was a tendency that in either control or stress conditions, average root lengths (Figure 2a), maximum root system depth (Supporting Information S1: Figure S2a) and total seminal and nodal root lengths (Supporting Information S1: Figure S2b) were higher. Root length is directly correlated to the total root surface area that is accessible to soil water volume and thus dissolved nutrients (Dara et al., 2015). In hydroponic cultivation, similar results were previously observed for different wild cultivars when compared with modern cultivars (Kreszies et al., 2020; Paschen et al., 2022). The formation of longer roots in wild cultivars is obviously genetically fixed and will offer an advantage under drought conditions as the availability of water in deeper soil layers will be higher (Ahmed et al., 2018; Lynch & Wojciechowski, 2015; Naz et al., 2014). Roots of 12-days-old barley plants grown hydroponically under control and osmotic stress were approximately about 1.5–3-fold shorter for both cultivars (Kreszies et al., 2019, 2020) compared with soil grown roots (Supporting Information S1: Figure S9a,b). This clearly indicates that soil cultivation significantly affects root development. A possible explanation might be the fact that in hydroponic cultivation, roots are growing in a homogeneously mixed nutrient solution, which does not require an extensive root system for soil exploration and nutrient and water uptake. The root/shoot ratios increased (Figure 2c) in response to water stress, which was also observed recently with barley cultivated in hydroponics (Kreszies et al., 2019, 2020).

4.3 | Comparing suberization between wild barley and modern barley

In both cultivars, endodermal root suberization detected by histochemistry was already clearly visible in control conditions between 5% and 10% distance from the root tip (Figure 3a). Only in the zone between 0% and 5% from the root tip, no suberized cells were histochemically detected (data not shown). At 25% from the root tip, nearly all endodermal cells in both cultivars were fully suberized in control as well as stress conditions (Figure 3a). These histochemical results are very different from hydroponically grown roots (Kreszies et al., 2019, 2020). In hydroponic cultivation, under control conditions as well as in response to osmotic stress (−0.4, −0.8, and −1.2 MPa) first suberized cells could only be detected earliest at 25% from the root tip in hydroponic experiments. Thus, in soil-grown roots endodermal suberization is extending significantly closer to the root tip, it starts already at 5% from the root tip and 75%–80% of the root length are nearly fully suberized. This observation was confirmed by the detailed chemical analysis (Figure 4; Supporting Information S1: Figure S4). Whereas the qualitative suberin composition (substance classes and chain lengths of the detected monomers) was similar between the two cultivars and the cultivation conditions (soil vs. hydroponic cultivation; Supporting Information S1: Figure S9c), total amounts of suberin were significantly (about twofold) higher in each of the three root zones in soil-cultivated roots. Thus, soil-environment under control conditions stimulates the root to a much higher and much faster root suberization compared with hydroponic cultivation.

4.4 | Comparing lignification between wild barley and modern barley

Besides suberization, lignification represents another well-known nonspecific response of plant cell walls to abiotic stress, which includes oxidative stress besides water limitation (Cabané et al., 2004). Histochemistry (Figure 3b) as well as chemical analysis (Figure 5; Supporting Information S1: Figure S5) showed that the lignification of endodermal cell walls and xylem vessels was significantly increased in response to increasing water deficits. An enhanced lignification of root cell walls in response to water deficit was described earlier in different crop species (Fan et al., 2006; Kováč et al., 2018; Ouyang et al., 2020; Steudle, 2000a; Yang et al., 2006). The increased levels of cell wall lignification can help to prevent embolism of xylem vessels (Lens et al., 2016), stabilize dehydrated tissue (Sharma et al., 2020; Yamaguchi et al., 2010), and provide support and protection against mechanical stress in dehydrating soil (Schneider et al., 2021). The induction of the tertiary developmental state of the endodermis was observed at 90% of the root length close to the base (Supporting Information S1: Figure S10). Histochemically, the inner U-shaped endodermal cell walls (Enstone et al., 2002; Esau, 1953; North & Nobel, 1995; Ouyang et al., 2020) were strongly responding to lignin staining (Supporting Information S1: Figure S10) whereas the suberin signal was limited to the outer periclinal cell walls (Supporting Information S1: Figure S10). Probably due to a strong lignification of the added U-shaped cell wall the suberin signal in the inner periclinal cell wall was masked. This was also observed in roots of other plant species (Zeier & Schreiber, 1998; Zeier, Goll, et al., 1999; Zeier, Ruel, et al., 1999).

4.5 | Comparing differential gene expression between wild barley and modern barley

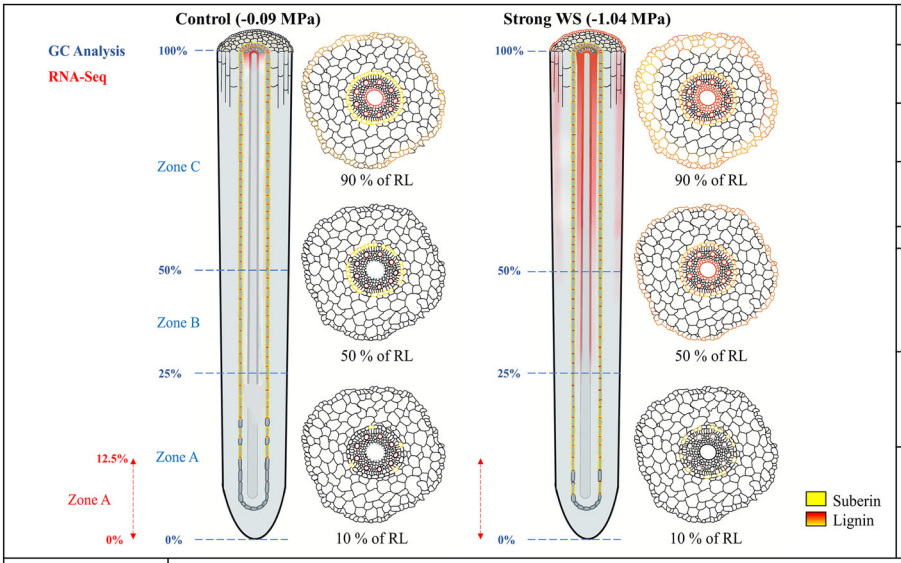
The enhanced lignification and suberization was also supported by the transcriptomic analyses. Several genes related to the biosynthesis of aliphatic monomers of suberin and to the phenylpropanoid pathway were upregulated in both Scarlett and Pakistan (Figure 7; Supporting Information S5: Table S4). This supports the important function of suberization and lignification in dealing with water stress in barley. Apoplastic suberization is also described to prevent the passive

backflow of water from the xylem vessels in the central cylinder into the soil, which is of major importance under water stress conditions (Steudle, 2000a; Steudle & Peterson, 1998). The upregulation of quite a number of genes related to suberization and lignification in soil-grown roots confirmed recent RNAseq studies conducted with hydroponically grown roots (Kreszies et al., 2019, 2020).

Besides the upregulation of suberin- and lignin-related genes, the RNAseq analysis revealed a series of other additional genes that were differentially expressed in both cultivars in response to water stress (Supporting Information S7: Table S6). Additionally, ubiquitous transcription factors (WRKY, MYB, NAC, ERF, etc.) were upregulated, having multiple roles in a variety of plant processes such as development, stress response, and acclimatization to abiotic stress (Dasauni & Nailwal, 2020; Gangola & Ramadoss, 2020; Ramadoss et al., 2020; Wang et al., 2023). Besides osmotic adjustment, tolerance to water deficit also requires that the mechanical stability of the cells and tissues is ensured (Dutta et al., 2016; Izanloo et al., 2008). This explains why besides lignification, many GO terms related to cell wall, plant-type cell wall, plant-type cell wall organization or biogenesis, cell wall organization or biogenesis are differentially enriched in water-stressed roots (Supporting Information S4: Table S3).

Recent studies with hydroponically grown roots showed that the apoplastic water transport in roots decreased with increasing suberization (Kreszies et al., 2019; Ranathunge et al., 2017) and that the nonsuberized or partially suberized root zone close to the root tip is mainly be responsible for water and nutrient uptake. In addition, water transport in roots can be significantly facilitated by aquaporins (Gambetta et al., 2017), which were in fact upregulated in the stressed plants in the root tip (NIP5;1, NIP1;2 and TIP4;1; Supporting Information S1: Figure S8). In parallel genes related to nutrient uptake and transport, including phosphate, ammonium, iron, and magnesium transport, were upregulated. Macro and micronutrients are particularly crucial for plant growth and development, and they are also essential for contributing to several aspects of stress tolerance as osmotic adaptation (Kumari et al., 2022).

Overall, upon decreasing SWP, root lengths decreased, amounts of aliphatic suberin and lignin increased, and genes related to suberin and lignin biosynthesis were upregulated (Figure 8). Compared with hydroponic cultivation, soil-grown roots were longer, suberization started much closer to the root tip and amounts of suberin were higher indicating clear differences between both ways of cultivation (Supporting Information S1: Figure S9). Comparing the drought stress response of the cultivar Scarlett (*H. vulgare* ssp. *vulgare*) with the wild



	Control		Mild WS (-0.4 MPa)		Strong WS (-1.0 MPa)
Root length	S < P (Adventitious roots formed)	>	S < P	>	S < P
Shoot height	S < P	>	S = P	>	S = P (No 3rd leaf development)
Root/shoot ratio	S = P	<	S < P	<	S < P
Zones	GC- Chemical analysis				
Zone C	S = P S = P	<	S = P S = P	<	S = P S = P
Zone B	S = P S = P	<	S = P S = P	<	S > P S = P
Zone A	S = P S = P	<	S = P S = P	<	S = P S = P

RNA seq. analysis	Strong WS (-1.0 MPa) vs Control (Genes that are **up-regulated** during strong water stress when compared to control)
Suberin	FATB, LACS1, LACS17, KCS10, KCS11, FAR5, FAR3, FAR1, GPAT4, GPAT3, CYP75B1, CYP86A1, CYP794D2
Lignin	4CL3, C4H, PAL2, HCT, CCR1, OMT1, CAD1, PER39, PRX52, HXXXD, ABCG40, ABCG32, LOX4, LOX5
Others (suberin/lignin)	WRKY6, WRKY40, WRKY71, WRKY24, MYB's, UGT's, Dirigent-like protein
Aquaporin	NIP5;1, NIP1;2, TIP4;1
Nutrients	NRT, PHT, KT, MGT, SULTR, VIT, ZAT, ZIP

WS - Water stress; S - Scarlett; P - Pakistan; < less than; > more than; = equals to; Font color: Yellow - Suberin, Red – Lignin

FIGURE 8 Summary of the results comparing 12-day-old Scarlett and Pakistan roots grown in soil under control (−0.09 MPa), mild (−0.4 MPa), and strong water stress (−1.04 MPa). In all the treatments, the average root length of Pakistan was greater than Scarlett. The shoot height of Pakistan was only in control higher than Scarlett. Root/shoot ratios were higher in Pakistan compared with Scarlett in stress treatments. Scarlett and Pakistan had similar amounts of suberin and lignin within the same root zones. Aliphatic suberin or uncondensed lignin increased over the root zones and with decreasing water potentials. Suberization is increased during strong WS in zone A compared with the control roots. Relevant upregulated genes related to suberin and lignin biosynthesis, aquaporins and nutrient transporters are listed. Font colour, yellow—suberin, red—lignin; P, Pakistan; S, Scarlett; WS, water stress. [Color figure can be viewed at wileyonlinelibrary.com]

barley accession Pakistan (ICB181243; *H. vulgare* ssp. *spontaneum*) it is obvious that Pakistan developed longer roots. This will be of major advantage under water limited conditions, since it should offer better access to deeper, less dehydrated soil horizons. This aspect of root lengths should be considered in future breeding approaches improving drought resistance in crops.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in NCBI at https://www.ncbi.nlm.nih.gov/, reference number PRJNA1063280.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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Supplementary Tables

Table S1: Summary of different suberized materials from different plant species with their respective substance classes, individual suberin monomers and references. These are arranged based on the material type: Skin (fruit), Fruit periderm, Tuber periderm, Fibres, Bark, Seeds, Aerial roots and Roots.

Source	Material	Aromatics	Fatty acids	Fatty alcohols	ω-hydroxyacids	Diacids	Reference
Adiantum capillus-veneris	Entire root tissues	Coumaric acid, Ferulic acid	C16, C18	C18	-	-	(Su *et al.*, 2023)
Arabidopsis thaliana L.	Root	Hydroxycinnamic acid, Coumaric acid, Ferulic acid	C16-C24, C18:1, C18:2	C18-C22	C16-C24, C18:1, C18:2	C16-C24, C18:1, C18:2	(Baxter *et al.*, 2009; de Silva *et al.*, 2021b; Domergue *et al.*, 2010; Franke *et al.*, 2005; Gully *et al.*, 2024; Höfer *et al.*, 2008; Lu *et al.*, 2011; Ranathunge & Schreiber, 2011; Shanmugarajah *et al.*, 2019)
Arabidopsis thaliana L.	Seed	Coumarate, Ferulate, Sinapate	C16-C24, C18:1	C22	C16, C18, C22, C24, C16:1, C18:1, C18:2	C16, C22, C24, C18:1, C18:2	(Gou *et al.*, 2009)

Ceratopteris richardii	Entire root tissues	Coumaric acid, Ferulic acid	C16, C18	C18	-	-	(Su *et al.*, 2023)
Clivia miniata (Lindl.) Regel	Root periderm	Cis/Trans-Coumaric acid Trans-ferulic acid	C18	C18-C28	C16, C18:1	C18:1	(Suresh *et al.*, 2022)
Crateva benthami Eichl.	RHCWs root tip (0–30 mm)	Cis/Trans-Ferulic acid, S-Lignin	C16, C18, C22, C24, C28, C18:1, C18:2	C18, C22, C28	C16, C20, C22, C18:1	C16, C18, C18:1	(De Simone *et al.*, 2003)
Cucumis melo	Skin discs of 1 cm dia.	Cis/Trans-Ferulic acid	C22-C24	C22-C28	C20-C24	C16-C18	(Cohen *et al.*, 2019; Manasherova & Cohen, 2022)
Cucumis sativus and *Cucumis sativus* var. *sikkimensis*	Skin discs of 1 cm dia.	Coumaric acid, Ferulic acid, Vanillic acid, p-HBA	C16-C28 C18:1, C18:2 C18 ester, C20:1, C22:1, C24:1	C15, C18-C22	C16-C26, C18:1, C18(9,10)-epoxy	C15, C16-C22, C16(1,16), C16(10,16), C18:2(9,10)	(Arya *et al.*, 2022; Nomberg *et al.*, 2022b)
Cycas revoluta	Entire root tissues	Coumaric acid, Ferulic acid	C16-C24	C22	C16, C18, C22, C18:1	C16, C20, C22, C18:1	(Su *et al.*, 2023)
Ginkgo biloba	Entire root tissues	Coumaric acid, Ferulic acid	C16-C24	C20, C22	C16, C18, C20, C22, C18:1	C16, C20, C22, C18:1	(Su *et al.*, 2023)
Glyceria maxima	Root segments (85–155 mm) behind the tip	Cis/Trans-Ferulic acid, Coumaric acid	C18, C24, C28, C18:1, C18:2	C16, C18, C22-C26	C24-C28	C16, C18:1	(Soukup *et al.*, 2007)
Glycine max L. Merr.	Root	-	C16-C22, C16:1, C18:1	-	C16-C24, C18:1	C16, C20, C18:1	(Thomas *et al.*, 2007)

Gossypium hirsutum L.	Green cotton fibers	Ferulate, Caffeate	-	-	C16-C24	C16-24	(Schmutz *et al.*, 1996)
Hordeum vulgare L. cv. 'Golf', L. spp. *vulgare* cv Scarlett,	Root	Cis/Trans-Coumaric and Ferulic acid	C16-C26, C30	C16-C30	C16-C30, C18:1	C16-C26, C18:1	(Kreszies et al., 2019; Ranathunge et al., 2017; Suresh et al., 2024)
Kielmeyera coriacea	Ground and sieved bark (0.250-0.425 mm)	Glycerol, Ferulic acid	9-Epoxyoctadecanoic acid	C26-C30	C18:1, 9-Epoxy-18-hydroxyoctadecanoic acid, 9,10,18-Trihydroxyoctadecanoic acid	C18:1, 9,10-Dihydroxyoctadecane-1,18-dioic acid	(Rios *et al.*, 2014)
Laetia corymbulosa Spruce ex Bent.	RHCWs root tip (0–30 mm)	Cis/Trans-Ferulic acid, *p*-OH-Benzoic acid, S-Lignin	C18-C24, C18:1, C18:2	C18-28	C16-C24, C18:1	C16, C18, C22, C18:1	(De Simone *et al.*, 2003)
Malus × *domestica* Borkh	Bark	Benzoic acid, Ferulic acid, Cinnamic acid, Linoleic acid	C16-C28	C16-C28	C16-C24, C18:1	C16-C22, C18:1	(Straube *et al.*, 2021; Suresh *et al.*, 2022)
Malus × *domestica* Borkh	Fruit periderm	Hydrocinnamic acid, Cis/Trans-Coumaric acid	C16-C26	C26-C28	C16-C24, C18:1, C16: di (10,16), C18: tri (9, 10, 18)	C16, C16 (9,10), C18 (9,10)	(Chen *et al.*, 2022; Straube *et al.*, 2021)
Manihot esculenta Crantz	Tuber periderm	Trans-Coumaric acid, S-Syringyl-	C22-C32	C26-C30, C29	C16, C18:1, C24-C30	C16-C18, C18:1	(Suresh *et al.*, 2022)

		Lignin-Keton, cis-Ferulic acid					
Metasequoia glyptostroboides	Entire root tissues	Coumaric acid, Ferulic acid	C16-C24	C20	C16, C18, C22, C18:1	C16, C20, C22, C18:1, C18:2	(Su *et al.*, 2023)
Monstera deliciosa Liebm.	Aerial root periderm	Cis-Coumaric acid Trans-Ferulic acid	C18-C30	C20-C28	C16-C30, C18:1	C16-C18, C18:1	(Suresh *et al.*, 2022)
Nephrolepis auriculata	Entire root tissues	Coumaric acid, Ferulic acid	C16, C18, C24	C18	-	-	(Su *et al.*, 2023)
Ophioglossum vulgatum	Entire root tissues	Coumaric acid, Ferulic acid	C16, C18	C18	-	-	(Su *et al.*, 2023)
Oryza sativa L. cv. Kinmaze	Root exodermis	Cis/Trans-coumaric and ferulic acid	C18-26, C30, C18:1, C18:2	C16-C28	C16-C30, C18:1	C16-C20, C18:1	(Jiménez *et al.*, 2024)
Phaseolus vulgaris	Root	Cis/Trans-Ferulic and Trans Coumaric acid	C18-C26	C18-C26	C16-C22, C18:1	C16-C22, C18:1	(Carvajal et al., unpublished)
Phragmites australis	Root segments (85–155 mm) behind the tip	Cis/Trans-Ferulic, Coumaric acid	C18-C24, C28, C30, C18:1, C18:2	C16, C18, C22, C24	C16-C28, C18:1	C16, C18:1	(Soukup *et al.*, 2007)
Polystichum tsus-simense	Entire root tissues	Coumaric acid, Ferulic acid	C16, C18	C18	-	-	(Su *et al.*, 2023)
Populus tremula x *P. alba*	Bark tissues	Ferulic acid	C22-C24	-	C16, C18:1, 10,16-OH 16:0	C18-C20, C18:1	(Rains *et al.*, 2018)

P. × *canescens* (Aiton) Sm. clone "84K" (*P. alba* × *P. tremula* var. *glandulosa*)	Root	Cis/Trans-Ferulic acid	C16, C24, C18:1, C18:2	C18-C22	C16-C24, C18:1, C18:2	C16-C22, C18:1	(Grünhofer *et al.*, 2021a)
Quercus suber L.	Ground and sieved bark, (<0.425 mm)	Ferulic acid	C16-C24, C18:1, C18:2	C16-C26, C18:1	C16-C24, C18:1, C20:1, C21, C23	C16-C24, C18:1, C20:1	(Lopes *et al.*, 2000)
Salix martiana Leyb.	RHCWs root tip (0–30 mm)	Trans-Ferulic acid, *p*-OH-Benzoic acid, S-Lignin	C22-C26, C18:1, C18:2	C22-C26	C16-C26, C18:1	C16, C18, C18:1	(De Simone *et al.*, 2003)
Salvinia cucullata	Entire root tissues	Coumaric acid, Ferulic acid	C18, C20, C24	C18	C16	-	(Su *et al.*, 2023)
Selaginella kraussiana	Entire root tissues	Coumaric acid, Ferulic acid	C16, C18, C24	C18	-	-	(Su *et al.*, 2023)
Solanum lycopersicum and *S. pennellii*	Root	Trans coumaric, Cis/Trans-Ferulic acid	C18-C28	C18-C28	C16-C26, C18:1	C16-C18, C18:1	(Cantó-Pastor et al., 2024; Carvajal et al., unpublished)
Solanum tuberosum	Hairy root cultures	-	C18-C24	C18-22	C16-C24, C18:1	C16-C22, C18:1	(Bjelica *et al.*, 2016)
Solanum tuberosum L.	Tuber periderm disc	Cis/Trans-Ferulic acid, Glycerol, Coniferyl alcohol	C16-C30, C29	C16-C30, C19, C21, C23, C27	C16-C28, C18:1, C18:2 (9,10), C23	C16-C28, C18:1, C18:2 (9,10)	(Company-Arumi *et al.*, 2023; Graça & Pereira, 2000b; Schreiber *et al.*, 2005a; Suresh *et al.*, 2022)

Tabernaemontana juruana Schumann ex J.F. Macbride	Rhizodermal cell walls (RHCWs) root tip (0–30 mm)	Cis/Trans-Ferulic acid, S-Lignin	C18-C26, C18:1, C18:2	C18-C28	C16, C22-C28, C18:1	C16, C18, C22, C24, C18:1	(De Simone *et al.*, 2003)
Triticum aestivum	Root	Cis/Trans-Coumaric and Ferulic acid	C18-C26, C30	C16-C30	C16-C30, C18:1	C16-C26, C18:1	(Carvajal et al., unpublished)
Vigna radiata	Root	Cis/Trans-Ferulic acid and Trans Coumaric acid	C18-C26	C18-C26	C16-C24, C18:1	C16-C24, C18:1	(Carvajal et al., unpublished)
Vitis riparia root	Root section 5–20 cm from the root tip	-	C16-C22, C18:1, C18:2, C18:3	C18-C22	C16-C22, C18:1	C16-C22, C18:1, C18:2	(Zhang *et al.*, 2020)
Zea mays	Suberized bundle sheath strands	Coumarate, Ferulate, Caffeate	C16-C32	C18-C22	C16-C30, C18:1	C16-C18, C18:1	(Mertz *et al.*, 2020)
Zea mays	Root	Cis/Trans-Coumaric and Ferulic acid	C18-C26, C30	C16-C30	C16-C30, C18:1	C16-C26, C18:1	(Carvajal et al., unpublished)
Zea mays L. *cv. Mutin*	Root endodermis, hypodermis	p-Coumaric and Ferulic acid	C16-C26, C18:1, C18:2	C16-C24	C16-C30, C18:1	C16, C24, C18:1	(Zeier *et al.*, 1999)

Table S2: Summary of studies reporting about effects of different biotic stress on suberization of different crop species.

Species	**Biotic factor**	**Findings related to suberin**	**Reference**
Arabidopsis thaliana (Col-0)	*Verticillium longisporum*	Lignification of the secondary cell wall structures	(Floerl *et al.*, 2012; Reusche *et al.*, 2012)
Arabidopsis thaliana (Col-0)	*Heterodera schachtii* and *Meloidogyne incognita*	Suberin biosynthesis genes activation and periderm formation or ectopic suberization at nematode infection sites	(Holbein *et al.*, 2019)
Arabidopsis thaliana (Col-0)	*Pseudomonas protegen*s and *Ralstonia solanacearum*	Lignin and suberin deposition in the endodermis as immune responses	(Zhou *et al.*, 2020)
Arabidopsis thaliana	Bacterial isolates	Modify endodermal suberization and enhance performance under abiotic stress	(Salas-González *et al.*, 2021)
Arabidopsis thaliana (Col-0)	*Verticillium longisporum*	*V. longisporum* downregulate genes involved in Casparian strip formation and suberin biosynthesis	(Fröschel *et al.*, 2021)

Glycine max L. Merr. Conrad × OX760-6	*Phytophthora sojae*	Suberin deposition in root tissues as a response to *P. sojae infection*	(Thomas *et al.*, 2007)
Glycine max cv. Conrad and L., var OX760-6	*Phytophthora sojae*	Suberin was formed subsequent to the initial infection process. Conrad had more resistance and aliphatic suberin than the line OX760-6	(Ranathunge *et al.*, 2008)
Hordeum vulgare cv. Jana	*Chaetomium globosum*	Hypodermal suberization stopped fungal invasion in barley root compared to unsuberized root tip	(Reissinger *et al.*, 2003)
Solanum tuberosum L.	*Erwinia carotoäora subsp. Carotoäora* and *Fusarium sambucinum*	Both aliphatic and aromatic domain of suberin play different role in development of resistance against bacterial and fungal infection during suberization	(Lulai & Corsini, 1998)
Solanum lycopersicum var. Marmande, Hawaii 7996 (H7996) and Moneymaker	*Ralstonia solanacearum*	Vascular structural barrier formed by a ligno-suberin coating and tyramine-derived hydroxycinnamic acid amides restricted bacterial movement	(Kashyap *et al.*, 2022)
Oryza sativa germplasm Phule Radha	*Meloidogyne graminicola*	Enhanced suberin deposition and biosynthesis of genes were greatly expressed in the exodermis	(Singh *et al.*, 2021)