Stress responses of black locust (*Robinia pseudoacacia* L.) to drought and/or pathogen attack

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Table of contents

List of acronyms and definitions

ADP	Adenosine diposphate
ATP	Adenosine triposphate
ANOVA	Analysis of Variance
bp	Base pair
CBL	Column boundary layer
CBS	Centraalbureau voor Schimmelcultures
CDIAC	Carbon Dioxide Information Analysis Center
Ci	Intercellular carbon dioxide
CO_2	Carbon dioxide
CODIT	Compartmentalization of Decay in Trees
CRIDA	Central Research Institute for Dryland Agriculture
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DNA	Deoxyribonucleic acid
dw	Dry weight
E	Transpiration
EtBr	Ethidiumbromide
EMBL	European Molecular Biology Laboratory
FAO	Food and Agriculture Organization
g_s	Stomatal conductance
g_m	Mesophyll conductance
IPCC	Intergovernmental Panel on Climate Change
IRGA	Infra-Red Gas Analyzer

IRI	International Research Institute for Climate and Society
ITS	Internal transcribed spacers
LSD	Least significant difference
NADP	Nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
Nm	Nanometer
PCR	Polymerase Chain Reaction
P_N	Net photosynthesis
Ppm	Parts per million
PVPP	Polyvinyl-polypyrrolidone
rDNA	Ribosomal deoxy ribose nucleic acid
RFLP	Restriction fragment length polymorphism
rpm	Rounds per minute
tax for	Taxon forward (Species specific primers)
tax rev	Taxon reverse (Species specific primers)
UNCCD	United Nations Convention to Combat Desertification
USDA	United States Department of Agriculture
UV	Ultraviolet
µl	Microliter
WMO	World Meteorological Organization
WWF	World Wide Fund For Nature

1. Summary

The impact of drought on trees and its consequences in the event of an additional pathogen attack were repeatedly studied. Based upon published work, the hypothesis was put forward by Desprez-Loustau et al. (2006) that trees are predisposed to pathogen attacks weakened by drought stress. The underlying details, however, are mainly based on field observations but not yet substantiated by experimental evidence. Therefore, the present project was designed with saplings of black locust (*Robinia pseudoacacia* L.) to study their responses to drought with and without wounding and fungal infestation.

In detail, seven-year old black locust saplings cultivated in pots on an experimental plot of the Thünen-Institute and Centre of Wood Sciences, University of Hamburg, were kept well-watered or put under drought stress. Additionally, wooden dowels either sterile or infected by the pathogen *Armillaria mellea* were introduced into the stems of saplings via bore holes. Also the influence of the season of wounding on the process of compartmentalization was studied by setting the bore holes and infecting the saplings in July (season of activity) or in February (season of dormancy).

Morphology, phenology, physiology and biomass of the saplings were severely affected by drought, but not by the pathogenic fungus. Drought has been reducing the overall growth, leaf area and total biomass as well as gas exchange and stomatal conductance resulting in a down-regulation of photosynthesis. However, the saplings stressed by drought and by a pathogen at the same time were found to be drastically more affected, showing the lowest values for nearly all variables measured.

With regards to host responses to the wounding and to the fungal infestation at different seasons, the shape and extent of discoloration and the intensity of callus formation were observed or measured. Both in well-watered and drought-stressed saplings, the area of dysfunctional and discoloured wood was larger in axial than in radial or tangential direction. The discoloration was slightly larger when the infection occurred in February as compared to July. The axial discoloration was much longer in drought-stressed than in well-watered saplings. Callus formation being the visible sign of compartmentalization

was measured in all saplings. Well-watered saplings infected either in February or in July formed more callus tissue than drought-stressed saplings, whereby drought-stressed saplings inoculated in February were even not at all able to form a callus.

There are only small amounts of non-structural carbohydrates detectable after wounding and infection in the decay and reaction zones of wounds (non-infected or infected) in well-watered saplings inoculated in July. This is interpreted as an active defence reaction against wounding and infection. The disappearance of the previously existing non-structural carbohydrates is based on their conversion into phenols/flavonoids which are strong fungicides. Well-watered and drought-stressed saplings inoculated in February and drought-stressed ones in July were unable to convert their reserves into defence substances. In drought-stressed saplings inoculated in February, the presence of considerable amounts of starch in the reaction zone emphasizes the inability of the saplings to convert them into phenols/flavonoids for an adequate biochemical defence.

By applying molecular techniques, the pathogen was neither detected in the decay and reaction zone nor in the sound wood of the saplings that were inoculated in July, no matter if they were well-watered or drought-stressed. However, the pathogen was detected in well-watered saplings inoculated in February, but only in the decay zone, inoculated dowels and in the adjacent callus. However, most frequently the pathogen was detected in almost all zones in wood samples of drought-stressed saplings, inoculated in February.

In conclusion, black locust saplings were severely affected by drought; however, saplings stressed by drought and a pathogen simultaneously were drastically more affected. In addition, well-watered saplings can be considered as stronger compartmentalizers than drought-stressed saplings, and drought-stressed saplings inoculated in February were proven to be the weakest compartmentalizers.

Zusammenfassung

Stressreaktionen von Robinie auf Trockenheit und/oder Pathogen-Befall

Die Wirkung von Trockenheit auf Bäume und ihre Folgen im Falle eines zusätzlichen Pathogen-Befalles wurden wiederholt untersucht. Auf der Grundlage von publizierten Studien haben Desprez-Loustau et al. (2006) die Hypothese formuliert, dass durch Trockenstress geschwächte Bäume für einen Pathogen-Befall prädisponiert sind. Die dieser Annahme zugrunde liegenden Details beruhen jedoch zumeist auf Feld-Beobachtungen und sind noch nicht experimentell bestätigt worden. Daher wurden im vorliegenden Projekt junge Robinien auf ihre Reaktion auf Trockenstress mit und ohne Verwundung und mit und ohne nachfolgenden Pilzbefall untersucht.

Sieben Jahre alte getopfte Robinien wurden auf ein Freilandversuchsfeld des Thünen-Institutes und Zentrums Holzwirtschaft der Universität Hamburg gestellt, wo sie ausreichend bewässert oder unter Trockenstress gesetzt worden sind. Zusätzlich wurden sterile oder mit dem pathogenen Pilz *Armillaria mellea* infizierte Holzdübel in Bohrlöcher in die Sprossachse der Versuchspflanzen eingeführt. Auch ein jahreszeitlicher Einfluss der Verwundung auf die Abwehrprozesse der Versuchspflanzen wurde untersucht, indem die Pflanzen während des aktiven Wachstums im Juli bzw. während der Ruheperiode im Februar verletzt und infiziert worden sind.

Morphologie, Phänologie, Physiologie und Biomasse der Testpflanzen wurden durch Trockenstress beeinflusst, aber nicht durch den Pilz. Trockenstress hat das gesamte Wachstumsgeschehen, aber auch die Blattentwicklung und Biomassebildung ebenso behindert wie den Gaswechsel und die stomatäre Leitfähigkeit, was zu einer Herunterregulation der Photosynthese geführt hat. Jedoch sind die Versuchspflanzen, die gleichzeitig von Trockenheit und einem Pathogen-Befall gestresst waren, deutlich stärker beeinträchtigt und zeigten für fast alle erhobenen Parameter die niedrigsten Werte.

Im Hinblick auf die Reaktionen der Versuchspflanzen auf die Bohrlöcher und Pilzinfektionen zu verschiedenen Jahreszeiten wurden die Form und Ausdehnung von

Verfärbungen im Holz und die Intensität der Kallus-Bildung beobachtet und gemessen. Sowohl in ausreichend bewässerten als auch in Trocknis-gestressten Versuchspflanzen war die funktionslos gewordene und verfärbte Holzsäule größer in axialer als in radialer und tangentialer Richtung. Sie war geringfügig größer, wenn die Infektion im Februar geschah als wenn sie im Juli erfolgte. Die axiale Verfärbung war deutlich länger in Trocknis-gestressten als in ausreichend bewässerten Versuchspflanzen. In ähnlicher Weise haben ausreichend bewässerte, im Februar oder im Juli infizierte Versuchspflanzen mehr Kallus-Gewebe gebildet als Trocknis-gestresste Versuchspflanzen, wobei diese sogar gänzlich unfähig waren, einen Kallus zu bilden, wenn sie im Februar verwundet bzw. infiziert worden sind.

In den ausreichend bewässerten, im Juli verwundeten Versuchspflanzen waren nur kleine Mengen von nicht-strukturellen Kohlenhydraten in den Abbau- und Reaktionszonen um die sterilen bzw. infizierten Wunden herum nachweisbar. Dies wurde als aktive Abwehrreaktion gegen die Verwundung interpretiert. Das Verschwinden der zuvor vorhandenen Kohlenhydrate beruht auf deren Umwandlung in Phenole/Flavonoide, die als starke Fungizide gelten. Ausreichend bewässerte und Trocknis-gestresste Versuchspflanzen, die im Februar verwundet worden sind, sowie Trocknis-gestresste Versuchspflanzen, die im Juli verwundet worden sind, waren unfähig, ihre Reservestoffe in Abwehrsubstanzen umzuwandeln. In Trocknis-gestressten, im Februar verwundeten Versuchspflanzen wurde der Nachweis von beträchtlichen Stärke-Mengen in der Reaktionszone als Unfähigkeit interpretiert, Stärke in Phenole/Flavonoide als angemessene biochemische Abwehr zu verwandeln.

Der pathogene Pilz war weder in der Befalls- und Reaktionszone noch im gesunden Holz der Versuchspflanzen molekularbiologisch nachweisbar, die im Juli verwundet worden sind, unabhängig davon ob sie ausreichend bewässert oder Trocknis-gestresst waren. Der Pilz wurde dagegen in ausreichend bewässerten und im Februar verwundeten Versuchspflanzen gefunden, aber nur in den Befallszonen, in infizierten Dübeln und im Kallus. Jedoch am häufigsten wurde das Pathogen in fast allen Zonen des Holzes von Trocknis-gestressten und im Februar verwundeten Versuchspflanzen nachgewiesen.

Zusammenfassend kann gesagt werden, dass die Robinien-Versuchspflanzen stark durch Trockenheit beeinträchtigt wurden, wobei Pflanzen unter Trockenstress bei gleichzeitigem Pathogen-Befall noch weit stärker geschädigt worden sind. Die ausreichend bewässerten Versuchspflanzen können als stärkere “Kompartimentierer” von Wunden betrachtet werden als die durch Trockenheit gestressten bzw. die durch Trockenheit und eine Infektion zur Zeit der Kambiumruhe belasteten Versuchspflanzen.

2. Introduction

Trees are exposed to abiotic and biotic stress lifelong. Drought is one of the most relevant abiotic factors, impairing many physiological and biochemical processes in trees (Larcher 2003; Ohashi et al. 2006; Reddy et al. 2004) and in consequence causing a substantial reduction in their overall vigor, growth, and productivity (Boyer 1982; Kramer and Boyer 1995). During the past 30 years, Central Europe has been affected by a number of major drought events, among them the summer heat wave in 2003 having caused severe tree mortality. Such drought effects are expected to increase with climate change and increasing water shortage (IPCC 2007). Among biotic stress events, pathogen attacks are playing a major role. *Armillaria mellea* is such a pathogen. It is ubiquitous and affects trees, shrubs and herbaceous plants causing root rot, root-collar rot and butt rot (Fox 2000). To cope with, trees have evolved a variety of defence strategies.

In nature, trees are subjected to abiotic and biotic stress successively or simultaneously. The fungus *Armillaria mellea* grows on and derives its nourishment from trees weakened by some prior stress factors such as drought, temperature extremes, other pathogens, or reduction in site quality (Wargo and Harrington 1991; Popoola and Fox 1996; Wargo 1980). Drought stress makes trees more susceptible to infections (Ayres 1991; Hepting 1963; Schoeneweiss 1975). Studies on drought/disease interactions in forest trees have been reviewed by Desprez-Loustau et al. (2006) who concluded that drought-stressed trees are predisposed to diseases because of their weakened defence potential. Nevertheless, such interactions between various stressors need more attention to enrich our understanding of tree pathology.

Forest trees are suffering from a wide range of injuries caused by wind, snow, ice, fire, animals, and insects; in cities and alongside roads, trees additionally face damages caused by human activities (Dujesiefken and Stobbe 2002; Lonsdale 2004; Smith and Lewis 2005). If a tree is unable to seal-off a wound from the surrounding sound tissue by a self-generated compartmentalization, damages spread quickly and the tree vitality is declining. The processes involved in this encapsulation of any kind of damage were

firstly summarized by Shigo and Marx (1977) under the concept called CODIT (Compartmentalization of Decay in Trees) which later was modified/advanced by Dujesiefken and Liese (2008) to Compartmentalization of Damage/Decay in Trees. The main characteristic is the formation of a discolored reaction zone as an active host response at the dynamic interface between the living sapwood and the damaged wood (Shain 1979). This comprises the closure of vessels by plugs or tyloses in the case of broad-leaved trees (Schmitt and Liese 1995) or the closure of bordered pits in conifers, as well as cell-wall alterations by suberization (Schmitt and Liese 1995). Additionally, antimicrobial polyphenolic compounds are deposited in the reaction zone (Pearce 1991; 1996; Frankenstein and Schmitt 2006). Finally, a wound is closed by the formation of a callus induced by cambial cells which develop from the parenchymatic callus tissue. The extent of discoloration and damage/decay in the wood considerably reduces its economic value even if the wounded tree continues to grow (Shortle et al. 2003). The efficiency of compartmentalization depends primarily on the tree species (Eckstein and Dujesiefken 1998/99) but also on the type, severity and season of wounding (Dujesiefken et al. 2005) as well as on tree vigour, environmental conditions and aggressiveness of the pathogens (Shigo and Hillis 1973). But up to now, no information is available whether drought impacts the efficiency of compartmentalization of damage/decay in trees.

Stress by droughts and pathogen has been extensively studied in the field but details have still to be supported by experimental evidence. To our knowledge, this is the first study on the influence of a long-term drought and of an aggressive fungal pathogen on trees, both separately and in combination. The objectives of this project were to monitor various growth parameters as well as leaf traits, phenology, gas exchange and biomass and to study how black locust saplings, well-watered or under drought conditions, respond to wounding in combination with the attack by a pathogen whereby the pathogen was introduced either in the dormant or in the active season of growth. Moreover, a biochemical approach to the compartmentalization of saplings and to the spread of *A. mellea* were studied to compare the effectiveness of compartmentalization of well-watered and of drought-stressed saplings.

For this purpose, black locust (*Robinia pseudoacacia* L.) was selected as a ‘’relatively drought tolerant’’ species (Veste and Kriebitzsch 2010), and a wide spectrum of various techniques was applied to measure and compare morphological, physiological and biochemical variables of the control saplings and of differently treated saplings.

2.1. Objectives of this PhD project

The overall purposes of this study were to explore the impact of two important stressors (drought and pathogen) on black locust, separately and in combination, and in return the responses of the tree.

- Assessing the influence of long-term drought and of the pathogen *Armillaria mellea,* both separately and simultaneously, on the overall vigor, growth, morphology, phenology, physiology and biomass of black locust (*Robinia pseudoacacia* L.).

- Observing and discussing the defense responses, visible as discoloration and callus formation, of well-watered and drought-stressed black locust, to injuries and infections, applied in different seasons.

- Analyzing the occurrence and distribution of non-structural carbohydrates (glucose, fructose, sucrose and starch), both in non-infected and in infected wounds in all saplings of black locust to determine an active defence reaction against wounding and infection.

- Studying the effectiveness of compartmentalization, both in well-watered and drought-stressed saplings, against the spread of *Armillaria mellea* by designing specific primers using molecular techniques.

3. Literature review

3.1. Background scenario and exposure of the problem

It is assumed that the increasing concentration of gases in the atmosphere has caused a warming of the ambient air worldwide ("greenhouse effect"). This global warming coincides with the increasing emission of carbon dioxide and other greenhouse gases from about 275 in 1800 to 370 ppm of today (CDIAC 2002). Greenhouse gas emissions are substantially changing the global climate and resulting in an increasing rate of warming as particularly reported for the last three decades (Fig. 3.1). Climate models for the 20th century suggest that there was little change prior to around 1915, and that a considerable fraction in the early 20th century was contributed by natural influences including solar radiation changes and volcanism. The increasing industrialization from about 1940-1970 following World War II increased the air pollution in the Northern Hemisphere, and carbon dioxide and other greenhouse gases dominated the observed warming after the mid-1970s (IPCC 2007).

Global warming progresses and produces both higher temperatures and increased drought. Observations over the past one and a half century manifested that temperatures at the surface have risen globally. An increase in global mean temperature (about 0.58°C since 1970) and changes in the world's hydrological cycle are on the record (IPCC 2007).

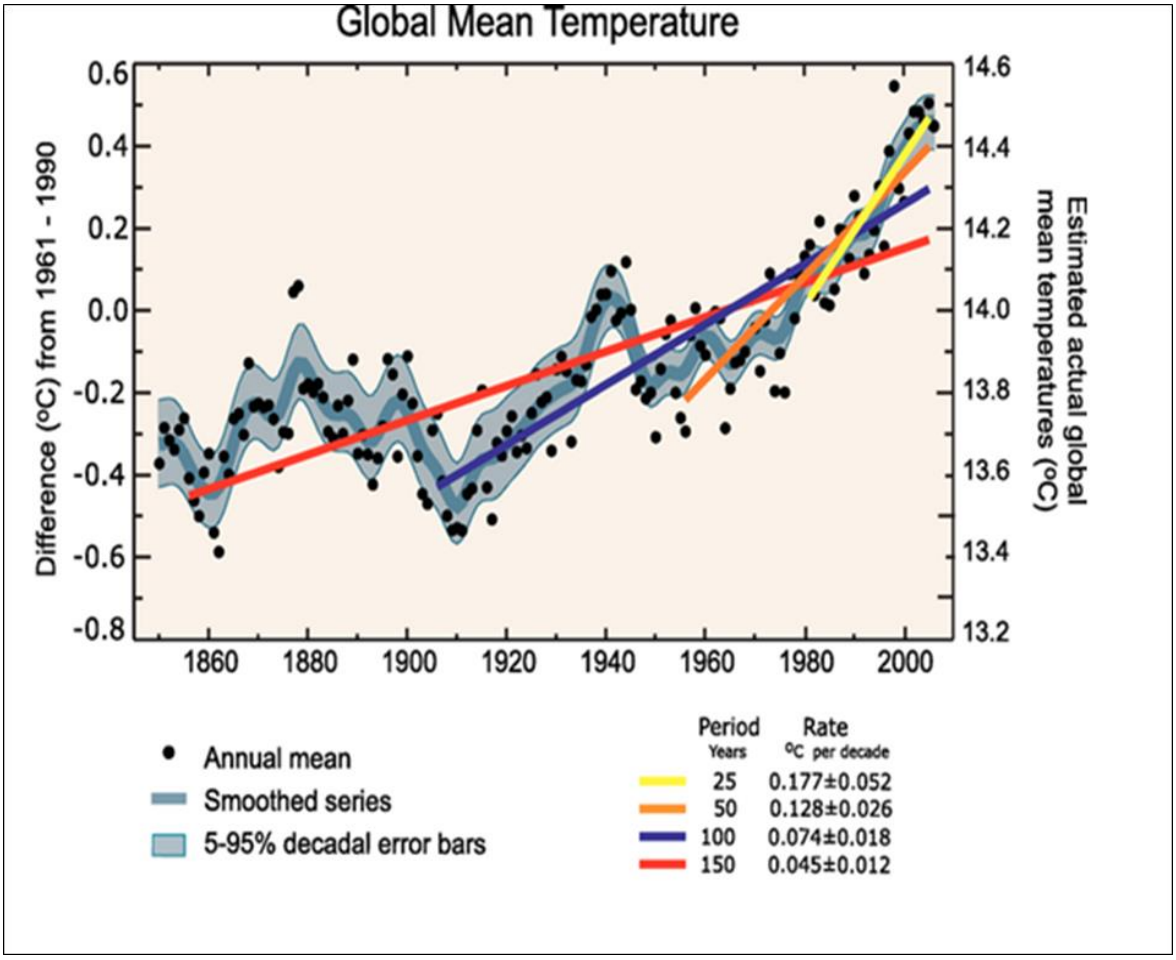


Fig. 3.1: Observed annual global mean temperatures (black dots). The left hand y-axis shows differences relative to the 1961-1990 average, and the right hand y-axis shows the estimated temperature (°C). Linear trends are calculated for the last 25 years (1981-2005) (yellow), 50 (1956-2005) (orange), 100 (1906-2005) (purple) and 150 years (1856-2005) (red). Note that for the shorter recent periods (yellow and orange) the slope is steeper, indicating an accelerated warming. The smoothed curve in blue captures the decadal variations. To make clear whether the fluctuations are meaningful, decadal 5 to 95% (light grey) error ranges around that line are given (accordingly, annual values may exceed those limits) (IPCC 2007).

http://www.ipcc.ch/publications_and_data/ar4/wg1/en/faq-3-1-figure-1.htm

However, conclusions made in the 5th assessment report by the IPCC (accepted but not published yet) have downgraded the degree of threat, however, it is emphasized that climate change has not stopped and human activities are the main cause. The rate of warming over the past 15 years (1998–2012) is only 0.05°C per decade; this is smaller than the trend from 1951 to 2012 that is 0.12°C per decade. Similarly, the temperature range given for a doubling of CO_2 in the atmosphere, provided in 2007, was 2.0 to 4.5°C, and the range has changed from 1.5 to 4.5°C in the latest report. Von Storch and Krauß (2013) stated that the rate of warming from 1998-2012 is smaller than anticipated by the IPCC (2007). Anyhow, the report agrees that warming is projected to continue in future under all scenarios and the global surface temperature changes by the end of the 21st century by at least 1.5°C, relative to 1850-1990. Moreover, climate changes are expected to include a further increase in mean temperature (about 2-4°C globally) with a significant drought in some regions as emphasized by Christensen et al. (2007).

3.2. Drought and its impacts on trees

According to the World Meteorological Organization (WMO 1986), drought means an extended deficiency in precipitation. The United Nations Convention to Combat Drought and Desertification (UNCCD; UN Secretariat General 1994) defines drought as a naturally occurring phenomenon that exists when precipitation has been significantly below normal levels, causing hydrological imbalances that adversely affect land and resource production systems. Passioura (2002) has summarized how various geologists, meteorologists, historians, farmers, plant physiologists and biochemists have defined a drought (Table 3.1). Accordingly, plant physiologists, plant biochemists and molecular biologists are interested in very short time scales and more concerned with the survival than with the productivity of trees; in terms of days and hours, drought events could be triggered by rapid desiccation and sudden exposure to strong osmotica.

Table 3.1: Drought: definitions and significance (adapted from Passioura (2002).

Practitioner	Time scale of interest	Common meaning of drought	Significance
Geologist, paleontologist	Millennia	Aridity	Major climatic change
Historian, geographer, relief agency	A decade to one century or two	Sequence of many years of low rainfall	Migration Desertion Famine
Meteorologist, farmer, insurer	Years	Rare event (the lowest seasonal rainfall)	Risk management
Farmer, agronomist, crop physiologist, breeder	Weeks to months, growing season	Yield strongly limited by water	Water productivity
Plant physiologist	Days	Pots not watered	Mild shock, survival
Biochemist, molecular biologist	Hours	Rapid desiccation, sudden exposure to strong osmotica	Severe shock, survival

Droughts are becoming a severe problem in many regions of the world (Passioura 1996, 2007) as they can reduce the crop yield by up to 50% (Boyer 1982; Chaves and Oliveira 2004) and are associated with tree mortality (Allen et al. 2010). According to Isendahl and Schmidt (2006), the percentage of drought-affected areas became double from the 1970s to 2000 in the world and will still increase in future (Hennessy et al. 2008; Allen et al. 2010). A conceptual assessment of tree mortality due to global climate change is provided by Allen et al. (2010) (Fig. 3.2); it shows increases in extreme drought and temperature events, and indicates the high risk of drought-induced die-off in the future. Longer drought duration and higher drought intensity are conceptualized as causal agents of tree mortality.

Based on historical evidences, it is easy to conclude that droughts are a frequent phenomenon globally. During the past three decades, Europe has experienced a

number of major drought events, especially in Northern and Western Europe in 1976, in most of Europe in the years 1989 and 1991, and more recently over large parts of Europe associated with a heat wave in the summer of 2003 (Feyen and Dankers 2009), that caused a high mortality of fir, spruce, oak, beech, and pine in France, Switzerland and Italy (Breda et al. 2006; Bigler et al. 2006; Landmann and Dreyer 2006).

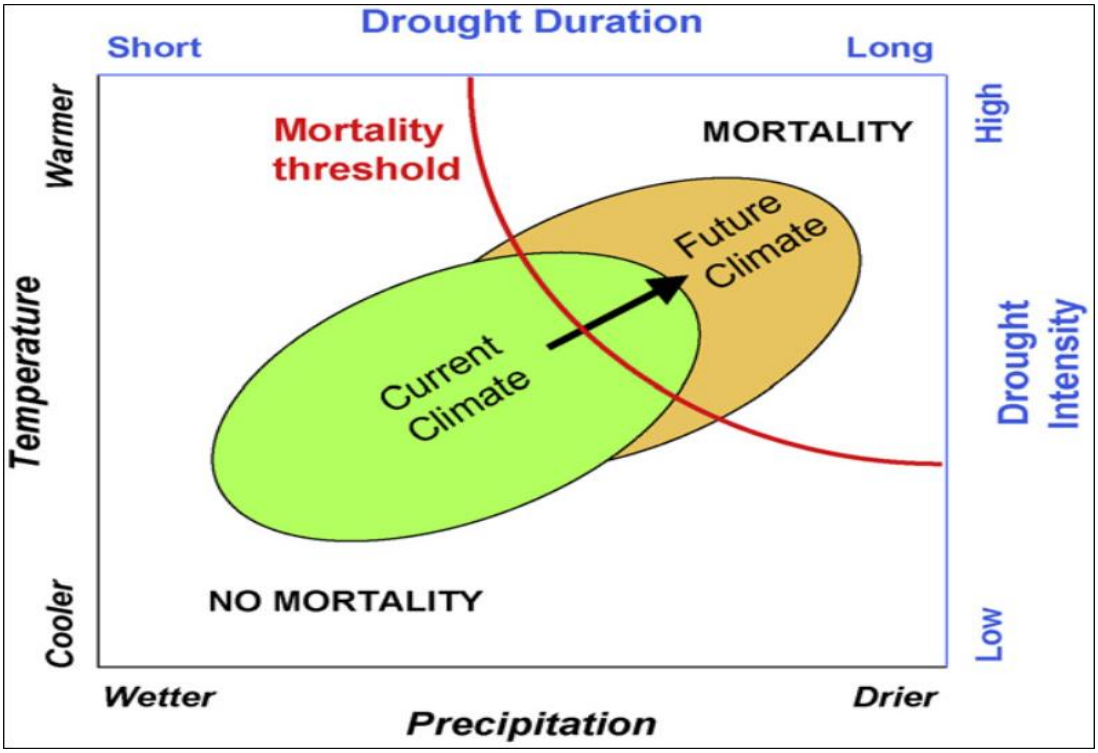


Fig. 3.2: Conceptual diagram, showing the range of variability of ‘‘Current Climate’’ parameters (precipitation and temperature) and alternatively of drought duration and intensity. ‘‘Future Climate’’ shows increases in extreme drought and temperature events associated with the projected global climate change, indicating higher risk of drought-induced die-off for current tree populations (Allen et al. 2010).

Since 1991, the economy has been affecting by drought in Europe, with an economic damage by the 2003-drought amounting to €8.7 billion (European Community 2007). Moreover, Anenkhonov in 2008 reported about a decline of birch stands in southeast Siberia. Similarly, vegetation die-off in response to global-change-type droughts was

presented for over a million hectares by Breshears et al. (2005) in the United States. Likewise, the mortality of *Populus tremuloides* was caused by a regional drought in Canadian forests (Hogg et al. 2008), and a well-known Millennium Drought (2001–2009) is described as one of the worst droughts for southeast Australia as mentioned by Van Dijik et al. (2013). According to the International Research Institute of Climate and Society (IRI 2001), from 1999–2000 a persistent drought and its severe impacts were experienced in Western Pakistan, Iran, Afghanistan, Tajikistan, Uzbekistan, and Turkmenistan. In addition, the Food and Agricultural Organization of the United Nations (FAO 2002) and the World Bank (2003) reported that the frequency of droughts has risen in India. Droughts in 1997, 1999 to 2002 in large areas of northern China were responsible of large economic losses (Zhang 2003).

Drought-induced forest decline and die-off during the last decades is illustrated in a global overview by Allen et al. (2010) (Fig. 3.3); for some regions, this review is obviously incomplete, particularly for mainland Asia and Russia.

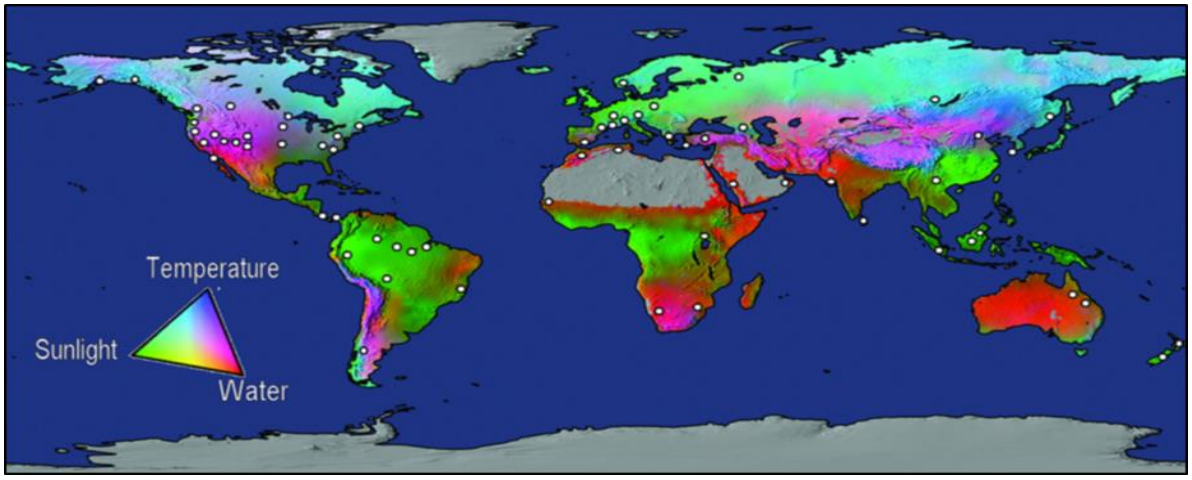


Fig. 3.3: Background map showing potential environmental limits to vegetation net primary production (Boisvenue and Running 2006). Drought and heat-driven forest mortality is documented in dry regions (red/orange/pink), but also occurs outside these regions. White dots indicate localities with documented forest mortality due to drought and high temperatures (Allen et al. 2010).

Drought is the notable environmental factor limiting plant growth and yield through the combination of photosynthetic and biochemical limitations. Inadequate availability of water damages plant tissues and metabolic processes. Drought escape, avoidance, tolerance, and resistance are different strategies that plants have evolved under short-term (hours to days) and long-term (days to weeks and months) drought conditions (Fig. 3.4). In short-term droughts plants minimize water loss or exhibit metabolic protection. In long-term droughts plants escape dehydration by shortening their life cycle or through acclimation responses. Severe droughts lead to catastrophic biological/metabolic failures and even to plant death. A lot of literature is available on plant responses to drought (Mittler 2006; McDowell et al. 2008).

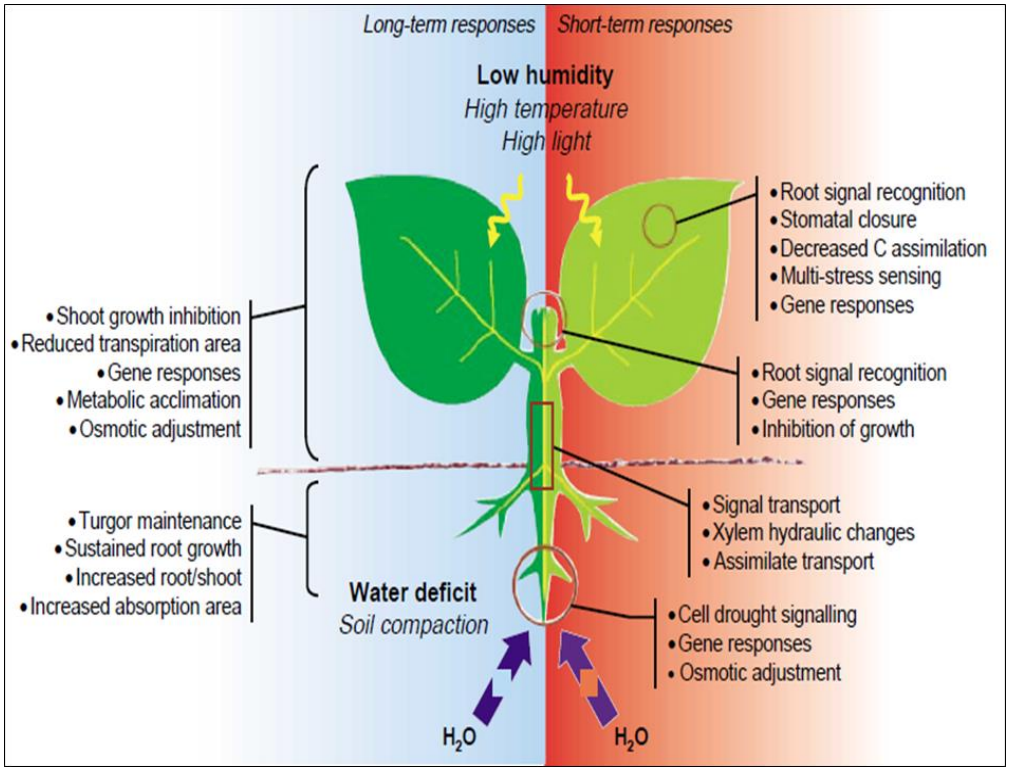


Fig. 3.4: Whole plant responses to drought stress. Left, long-term or acclimation responses; right, short-term responses (Chaves et al. 2003).

Droughts are considered as one of the major abiotic factors, negatively affecting many plant processes, such as photosynthesis, transpiration, stomatal conductance, and metabolite accumulation (Larcher 2003; Ohashi et al. 2006). In addition, it limits plant growth and performance and causes substantial reductions in yield (Boyer 1982; Bray et al. 2000; Yordanov et al. 2000; Wang et al. 2003; Reddy et al. 2004). Stomatal closure resulting in a decreased flow of CO_2 into the mesophyll or in an impairment of metabolic activities (Chaves et al. 2003; Flexas et al. 2004) is because of drought. Similarly, stomatal closure and loss of leaf turgor to prevent desiccation reduces carbon uptake and in consequence the assimilation (Chaves et al. 2009; McDowell et al. 2008; Galmes et al. 2007). Stomatal closure is the main limiting factor for photosynthesis under moderate water availability, but under severe condition, metabolic impairment occurs (Medrano et al. 2002; Chaves et al. 2003). Adverse effects of drought on photosynthesis are mediated by the response of the respiration system (electron transport and ATP synthesis) in the mitochondria, the accumulation of metabolites and through gene expression and protein synthesis (Atkin and Macherel 2009; Lawlor and Tezara 2009).
Plants respond to water stress by acclimation in non-severe cases and by damage and loss of plant parts in severe cases (Chaves et al. 2002), and even mortality in extreme situations (Allen et al. 2010).

3.3. Impacts of pathogens on trees

Pathogens can reduce the yield of trees, extensive timber losses and even tree mortality. This can happen by the direct loss of tissue, damaging xylem, restricting water and nutrient uptake or reducing phloem transport or both, inducing defences (Kozlowski 1969; Froelich et al. 1977; Franceschi et al. 2005) that divert resources, thus affecting growth and physiology of their host trees. Severe infections can eventually kill the host. However, the time required for the pathogen to kill a tree varies considerably and depends on many factors, including vigor of the host, host and parasite combination, severity of disease, and climatic situation under which the host tree is growing. Drought can increase the frequency of tree pathogens through effecting the host physiology (Ayres and Lombardero 2000; Lloyd and Bunn 2007; Scholze et al. 2006).

Armillaria root disease, of both trees and crops, is known to occur everywhere except Antarctica (DeLong 1995). Hundreds of species of trees and shrubs are hosts for this aggressive pathogen. The disease is caused by the fungus, living parasitic on the host tissue. Saprophytic fungi on dead woody material are another source of disease for healthy trees. Most frequently, the identified fungus causing a disease is *Armillaria mellea*. But several different and closely related species can also be involved. Therefore, the generic term *Armillaria* is used for this group. As parasitic fungus, it causes growth reduction, wood decay and mortality of the tree. *Armillaria* living as saprophyte on dead wood, spreads through rhizomorphs by contacting non-infected roots of host or when non-infected roots get into contact with infected ones (Fig. 3.5). Rhizomorphs can grow over distances of up to 10 feet (3 m) through the top soil layers, and penetrate the roots by mechanical pressure and enzymatic actions. According to Williams et al. (1989), the ability of rhizomorphs to penetrate into roots depends upon the specific fungus, the soil environment and the host species.

Fig. 3.5: Spread of *Armillaria* from a dead to a living tree via root contacts; infected roots are painted white; blue ribbons mark the infection points (Wargo and Shaw 1985).

There are more than 30 *Armillaria* species worldwide (Watling et al. 1991) causing root rot, root-collar rot and butt rot. In general, losses (mainly mortality) attributed to

Armillaria root disease are most severe in forests in dry Mediterranean or continental climates (Kile et al. 1991). *Armillaria* is considered as an important contributor to tree mortality and has resulted in significant economic losses (Bendel and Rigling 2008). However, early studies revealed that the fungus usually acts as a pathogen on trees weakened by some stress factor (Day 1929; Raabe 1966).

Similarly, the virulence of *Armillaria* is assumed to depend on environmental conditions stressing the host (Popoola and Fox 1996; Wargo 1980). Some *Armillaria* species are primary pathogens and infect healthy trees, whereas other species act as secondary pathogens invading trees after their resistance has been impaired by drought, temperature extremes, other pathogens, or reduction in site quality (Wargo and Harrington 1991). Moreover, the physiological resistance of healthy tissues against *A. mellea* is also the part of the literature, according to that, penetration by the fungus is not preventable but the subsequent development and spread can be limited (Thomas 1934).

3.4. Abiotic and biotic stresses and their interactions

In their natural environment, trees are exposed to various stresses (abiotic, biotic). Droughts, wind, frost, nutrient deficiency, overwatering or planting too deep may act as abiotic stressors. Biotic stressors are living organisms, such as viruses, bacteria, fungi, insects, and animals to which a tree may be exposed during its lifetime. Abiotic stress often occurs on many species but does not spread from tree to tree like biotic stressors that can spread throughout a tree and even to neighboring trees of the same species.

Plants may be injured by a stress and exhibit metabolic impairment. The injury may be temporary in case of a moderate and short-term stress and the plant may recover after the stress is over. Intense stress may prevent flowering, seed formation, and induce senescence and may lead to plant death. Such plants are known as susceptible.

Some plants like ephemeral, short-lived, desert plants escape drought stress by germinating, growing, and flowering quickly following rains. Thereby, they complete their

life cycle during a period of appropriate moisture and form dormant seeds before the beginning of dry period. Similarly, many arctic annuals rapidly complete their life cycle during the short arctic summer and survive over winter in form of seeds, thus these plants survive by avoiding stress. Deep, extensive roots, thick cuticles, small stomatal openings and physiological adjustments are the salient features that enable the plants to escape unfavorable conditions.

Plants that can tolerate a particular stress are considered to be stress-resistant as these organisms adjust (avoid or survive) or acclimate to stress. In this case, plants apply an avoidance strategy through biochemical and physiological processes, but plants that cannot survive such an extreme situation starve to death (Fig. 3.6).

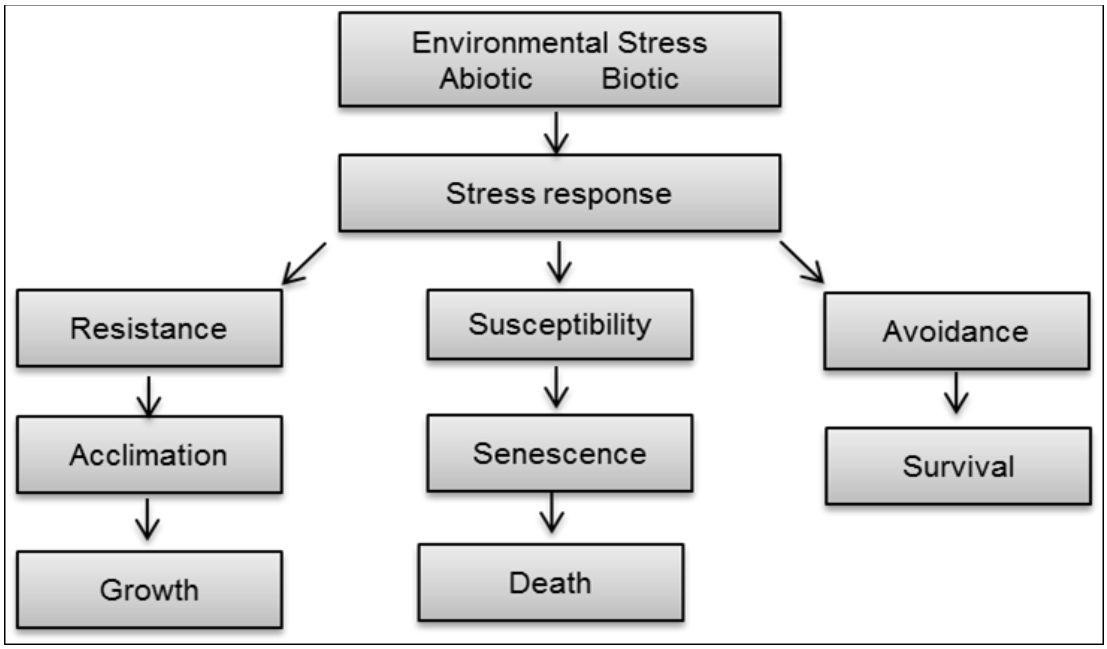


Fig. 3.6: Effects of environmental stress on plants (Hopkins and Hüner 2009).

In nature, plants are often subjected to multiple or simultaneous stresses whose influences are not easily understood neither if studied under controlled conditions nor in the field. Stresses that occur in the field can be additive or can interact positively or negatively (Niinemets and Valladares 2004; Mittler 2006; Rennenberg et al. 2006). The

influence of heat and drought can act additive, and stresses that cause stomatal closure or the formation of a thicker cuticle may prevent invasion by pathogens, especially by obligate parasites (Gäumann 1950), thus interacting positively.

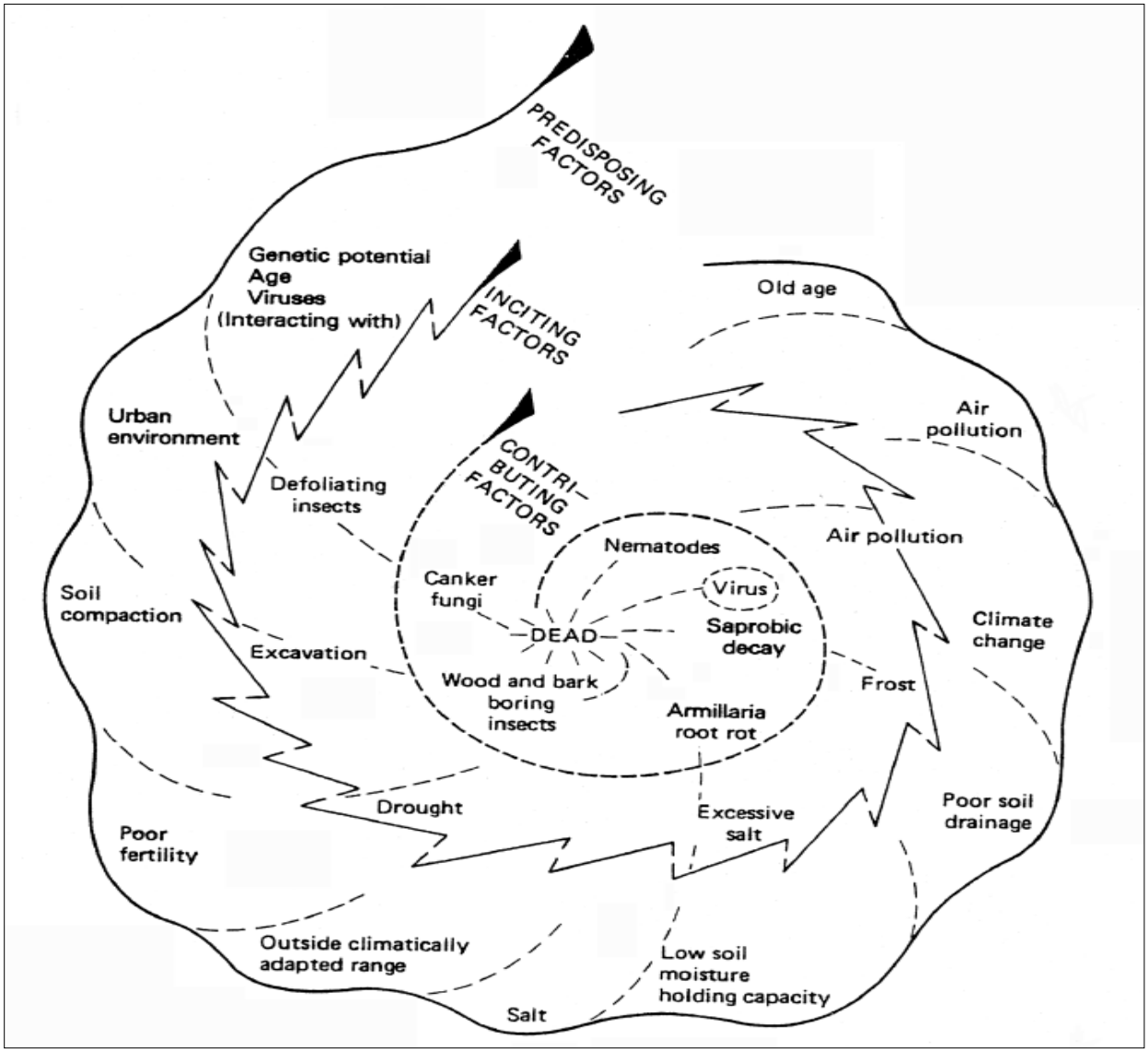


Fig. 3.7: The Manion Decline Spiral, showing three sets of stressors that may contribute in the complex process of decline (Manion 1981).

For the first time, Yarwood (1959) used the term predisposition by illustrating the environmental influence on the genetically controlled response of a host plant to the

presence of a pathogen or of its metabolites. The concept of predisposition was then introduced into the field of plant pathology by Sorauer (1974), who emphasized the importance of environmental factors in relation to plant diseases. Later on, Manion (1981; 1991) categorized plant diseases into biotic, abiotic and decline. Biotic and abiotic diseases are related to symptoms, host specificity and spatial distribution, whereas decline diseases are caused not from a single agent but from an interacting set of factors (Fig. 3.7). In this process of decline, climate or site factors are almost always major predisposing or inciting factors that make the host vulnerable to contributing factors like pathogens. More recently, such drought/disease interactions have been reviewed by Desprez-Loustau et al. (2006), who hypothesized that trees impacted by drought are predisposed to biotic diseases because of their weakened defence potential.

3.5. Drought-pathogen interactions

Climate warming is thought to increase disease and mortality of plants by pathogens, particularly fungi (Schoeneweiss 1981, 1983, 1986; Ayres and Lombardero 2000; Desprez-Loustau et al. 2006; Garrett et al. 2006; McDowell et al. 2008). For example, water stress was proven to increase the development of canker in sycamore (*Platanus occidentalis*). Similarly, significant drought effects on the formation of diseases in red pine (*Pinus resinosa*) have been reported (Blodgett et al. 1997). Recently, Linares et al. (2010) observed *Heterobasidion abietinum*-related mortality of *Abies pinsapo* following a drought. Similarly, Lindberg and Johansson (1992) highlighted that drought may predispose conifers to *Heterobasidion* attacks through the reduction of the endogenous defence mechanisms of the trees. *Phytophthora* species as predisposing or triggering agents are considered to play a role in oak declines (Delatour 1983; Wargo 1996).

Global environmental changes are likely to have a deep impact on the host-pathogen interactions at several levels. Based on a review of 270 scientific publications, La Porta et al. (2008) concluded that climatic conditions giving advantages to a pathogen may at the same time giving disadvantages to a host tree; such situations are often intensifying

tree damage. According to Desprez-Loustau et al. (2006), three main types of drought-disease interactions are expected, (1) direct effects of drought on the pathogens, (2) indirect effects through community interactions, and (3) interactions by predisposing the host to pathogen attacks. *Armillaria* is an aggressive killer of healthy trees and shrubs throughout the world but on the other hand, it is known as a secondary pathogen of trees that are stressed and as its saprophytic mode of action on dead trees. *Armillaria* following drought was found to be associated with declines (Wargo et al. 1991). In another study, species such as *A. gallica* and *A. cepistipes* were recognized as secondary pathogens, i.e. pathogens can invade trees when they are stressed by another factor (Gregory at al. 1991). Deciduous and coniferous trees weakened by abiotic factors like drought, waterlogging, soil compaction, air pollution or by biotic factors like insects, foliage diseases, stem cankers and bark-sucking are colonized and eventually killed by *Armillaria*. Pathogen as saprophyte can also be a cause to spread infection through rhizomorphs to weak trees, and this process is intensified after a severe stress such as drought. A spread by basidiospores also occurs but it is limited. Mostly, the rhizomorphs can spread from a diseased tree to a neighboring tree (see Fig. 3.5), if it has already been under some stress. However, colonization does not occur and tree mortality ceases, if the stress is abated and tree health is restored. The fungus thus depends a lot on stressed hosts to play its pathogenic role. Moreover, differences in site, soil factors, and tree vigor are mitigating influences, and different species of *Armillaria* can behave differently (Wargo and Shaw 1985).

Similarly, the virulence of some *Armillaria* species depends on environmental changes stressing the host (Popoola and Fox 1996; Wargo 1980). Fox (2000) reported that symptoms of infections by many *Armillaria* species appear after physiological injury from environmental stress. Host plants treated like drought stress or their roots kept constantly flooded were more susceptible. Moreover, amounts of carbohydrates, fatty acids and amino-acids were also changed in water-stressed Lawson cypress as compared to control plants, favoring increased growth of *Armillaria mellea* and *A. gallica* on root extracts (Popoola and Fox 2003). Drought as an inciting factor and pathogens, such as *Armillaria*, was recognized as contributing factor (see Fig. 3.7).

3.6. Defence strategies of trees against drought and pathogens

Trees are the tallest, massive and longest living organisms on Earth. Their longevity is due to their unique defence responses against destructive forces. Trees suffer from injuries caused by wind, snow, ice, fire, animals, insects, and by man all over the planet. These injuries provide a pathway to surrounding microorganisms to invade. Effective defence responses of the trees are in action at this stage to restrict the development of decay. Various models are proposed for better understanding of these processes. A protective barrier and chemical changes at the margin between decay lesions and the living sapwood were differently named by different authors. Such mechanisms were found to be very dynamic (Shain 1967; 1979). Shortle and Smith (1990) proposed to term them as column boundary layers (CBL), and Pearce (1996) used the term reaction zone. Later on, this model was refined, indicating that lesions formed by reaction zones can retain their function for an extended time.

These lesions can expand under even little host response, before a new reaction zone boundary is formed (Pearce 1987; 1991; 2000; Boddy 1992). However, among these models, Compartmentalization of Decay/Damage in Trees (CODIT), proposed by Shigo and Marx (1977), Shigo (1979; 1984) and modified/advanced by Dujesiefken and Liese (2008), is widely applied. According to this model, trees attempt to wall-off the injured or infected portion, thus trees respond by compartmentalization (Fig. 3.8).

Fig. 3.8: Compartmentalization of a wound in *Robinia pseudoacacia* L.

Trees respond to injuries by strengthening existing walls or forming new walls to encapsulate the subsequent spread of air or of an infection. Wall 1 resists the vertical spread by anatomical and chemical means thus plugging the axially running cells; it is rather weak. Wall 2 exists continuously around each growth ring and from top to bottom of a tree and resists the radial spread of an infection. Wall 3 is built up of the radially oriented ray cells and hence resists the tangential spread of an infection. Wall 4 does not exist at the time of wounding but is built to separate the infected wood from the newly formed healthy wood; it is the strongest of all these walls and acts as a barrier against microorganisms. Walls 1 to 3 are equivalent to reaction zones, but wall 4 is clearly noticeable comprising a tissue laid down by the cambium in the vicinity of wounds. The strengthening of all these walls is achieved by biochemical conversion of carbon compounds into phenolic. Phenols act antimicrobial and discolor the wood. Trees grow continuously after injury and infection, if they have enough time, energy and genetic capacity to recognize and compartmentalize the injured and infected tissue (Shigo and Marx 1977).

The strong host response in form of compartmentalization depends primarily on the tree species (Eckstein and Dujesiefken 1998/99) but also on the type, severity and season of wounding (Dujesiefken et al. 2005). Compartmentalization of autumn or winter wounds is considered as weak compared to summer and spring wounds (Leben 1985; Shain and Miller 1988; Mireku and Wilkes 1989). Moreover, tree vigour, environmental conditions and aggressiveness of the pathogens are important in this regard (Shigo and Hillis 1973).

Compartmentalization is not always successful and does not function perfectly all the time. When it fails, some tree part or even the whole tree will die. When a tree stays alive for years after injury and infection, compartmentalization is functioning well. As long as the tree generates new rings over the older infected ones, and keeps strong durable boundaries between the infected areas. Successful respond of a tree depends greatly on its genetic program and its ability to generate and allocate energy. Compartmentalization is the framework for a tree defence system that consumes much

energy. The system is unique because the interaction between trees and pathogens usually takes place within the tissues present at the time of injury and infection, and the tree sets a boundary between these tissues and newly forming tissues. The newly forming tissues act as new "tree" growing over the older "trees". This system has long-term survival benefits (Shigo 1984).

Survival of a tree after injury or infection depends on its ability to compartmentalize pathogens. The virulence of pathogens depends on their ability to occupy as much tissue as possible before they are compartmentalized. There is always an interaction between host and pathogen in an ever-changing environment. Trees cannot 'move away' and many types of wounds accumulate on or in them during their long life. It is compartmentalization that makes long-term survival possible, after hundreds or even thousands of infections. Compartmentalization is a defence process that has the potential to be effective for millennia. Otherwise, trees would not be thousands of years old, full of rot, and still growing (Shigo 1984). Gäumann (1950) summarized the situation succinctly: "Man is able to destroy the pathogen in many infectious diseases, whereas the plant can only localize it."

Moreover, drought stress reduces tree vigour, and thus reduces the ability to compartmentalize. The water status of a tree is a fundamental factor in plant pathology. Decay processes are mostly initiated by an injury, leading to infection, whereby the host water status plays a pivotal role. Climatic changes over a period of years have been addressed along with severity of many diseases. Ash dieback, maple decline, birch dieback, oak decline, dry face of slash pine, and pitch streak in slash pine were found to be associated with an extended period of below normal precipitation in the 1930s in the United States (Hepting 1963; Ross 1966). Similarly, Leaphart and Stage (1971) concluded that extended drought from 1916 to 1940 in the United States, played crucial role in the severity of pole blight of western white pine. The importance of water in relation to canker development was reported and canker caused by various microorganisms depended on the relative turgidity of the bark (Bier 1959).

Drought-stressed trees were described as more susceptible to disease than well-watered ones (Bier 1959; Hepting 1963; Schoeneweiss 1975). Moreover, cankers were

significantly larger on drought-stressed trees than on unstressed trees (McIntyre 1996). Patterns of callus formation in various hosts in response to infection and water stress were reported by Bevercombe and Rayner (1980); accordingly, callus often failed to form effectively around diamond-cankers during the dry summer of 1976 in Devon, Britain and this was associated with death of the whole or upper part of the tree. Eventually, this resulted in a huge destroy of large trees in this area.

Drought/disease interactions in trees have again been reviewed in response to the Europe-wide drought in 2003 (Desprez-Loustau et al. 2006). Disease-related variables, severity of infection and timing of water stress were recognized as significant factors influencing the drought-infection interaction, and it was hypothesized that drought-stressed trees are predisposed to diseases because of their weakened defence potential. This problem may increase if drought episodes will occur more often along with global warming, as anticipated by the IPCC (2007).

Unfortunately, most reports having indicated water stress as a predisposing effect on the host's susceptibility to a disease are based on field observations and are not supported by experimental evidences.

3.7. Biochemical aspects of compartmentalization

Compartmentalization involves both stable and dynamic, wounding-induced, anatomical, and physiological and biochemical changes. On the whole, compartmentalization 'walls-off' infections and tends to resist the spread of the decay process into the wood formed after wounding (Shigo 1984).

Generally, the wood (xylem) in a living tree is protected from pathogen by the periderm and rhytidome and by defence mechanisms in the bark. Only a few pathogens may penetrate through these tissues directly, whereas most xylem pathogens gain entry through open wounds. Biochemical aspects of wounding in tree species are not much available. However, a series of predictable and coordinated events are concluded from

histochemical investigations in the tree bark, with the formation of a ligno-suberized boundary layer, and/or a wound periderm through cell division, of callus tissue, and/or a new vascular cambium, and possibly a closure of the wound, which may function both as inhibitory and as barrier to a further pathogen spread (Biggs 1992).

Injured trees are at a great risk of infection. Wounded wood is exposed for infection by many microorganisms from the surroundings. Only a small number of fungi and bacteria act as ‘pioneers’ consuming the nutrients in the wood cells but are unable to degrade and digest the wood itself. During time, these pioneers are replaced by other microorganisms including further non-decay fungi but also decay fungi. Some of the decay fungi can degrade only certain components of the woody cell wall and some others can degrade most or all of the wood substance. Such patterns of succession vary from place-to-place and with the prevailing circumstances. Few decay fungi effectively infect and spread from wounds even without the preparatory impact of pioneers. However, the first few days after wounding mean a maximum risk of infection to sapwood. Therefore, at this time, protective treatments that accelerate or stimulate tree defence responses have the potential to reduce the establishment of infection in sapwood.

Changes in the anatomy and chemistry of xylem cells, undergoing differentiation at the time of wounding (Frankenstein et al. 2006), are part of the process of barrier-zone formation. The barrier zone tends to resist the outward spread of wound-initiated discoloration and decay into the wood formed after an injury. As a result, the wood-decay process takes place in wood present at the time of injury (Smith 2006).

Histochemical analyses of heartwood and discolored wood were presented by Shigo and Hillis (1973). From this comparison, main similarities and differences are mentioned here. Tyloses in vessels and parenchymal necrosis, formation of phenolic substances, separation with a transition zone/reaction zone to the sapwood are common in both kinds of tissues. Discolored wood differs from heartwood by a lower content of phenols and a higher content of ash minerals and of water, an increase in the pH value, and by the occurrence of fungal hyphae. Moreover, heartwood contains compounds of low-

molecular mass; in contrast, compounds with a high-molecular weight are accumulated in discolored tissue due to the reaction of oxidative polymerization (Smith 1997). In a wounded tree, discoloration is compartmentalized from the sound sapwood by the tissue known as reaction zone (Shain 1967; Shortle and Smith 1990). Biochemical changes lead to the production of phenol-based chemical defences. Brown-colored polyphenolic materials are commonly deposited in reaction zones (Pearce 1990). These processes make the attacked tissues resistant against microbial decay and fungal invasion, and may lead to acquire resistance against future attacks (Krokene et al. 1999). Some of the key fungicide phenolic compounds in reaction zones are produced as a result of an infection, not just injury (Barry et al. 2002). According to Vance et al. (1980), the production of phenolic derivatives is a universal response after injury or infection of the plants. Substances synthesized as a reaction to wounding neutralize, inhibit or confine the effect of pathogens (Klepzig et al. 1996). Carbohydrates stored in parenchymatous tissues of wood and bark, mainly as starch (Kozlowski 1992; Hoch et al. 2003), are converted to phenols and flavonoids.

There is a discrepancy between the on-site limited existence of non-structural carbohydrates and an increasing content of phenolic constituents in *Robinia*, in the heartwood/sapwood transition zone during heartwood formation. The increased content of phenols and flavonoids in this zone require an import of carbon skeletons (Magel et al.1991; Magel and Hübner 1997; Hauch and Magel 1998). The source used in the heartwood/sapwood transition zone exists on-site reserve materials and the imported sucrose. After intercalation of heartwood substances, the sapwood transition zone turned into dead heartwood (Magel et al. 1994). Magel et al. (1997) stated that starch and sucrose, accumulated in young living woody cells, deplete abruptly in the oldest ones. Therefore, the formation of heartwood phenolics coincides with the transformation of sapwood into heartwood, and sugars are metabolized for the synthesis of phenolics (Niamke et al. 2010). Similarly, it has also been stated that discolored wood of *Fagus sylvatica* is not physiologically different from heartwood (Magel and Höll 1993). According to the literature, both in conifers and in deciduous trees, the outermost sapwood contains a high amount of sugars and starch, whereas the heartwood is almost free of storage material (Magel and Höll 1993; Islam et al. 2012) and that starch is

consumed or withdrawn during sapwood-heartwood transformation (Datta and Kumar 1987; Magel et al. 1994). Thus, it is obvious that phenolic substances are derived from carbohydrates.

Seasonal variations in the susceptibility of trees to fungal attacks were exhibited, in the effectiveness of endogenous defence (Spiers et al. 1998). Seasonal alterations in the availability of starch reserves or changes in a tree's internal microenvironment may influence the ability of a tree to establish durable defensive barriers. Similarly, abiotic stress such as drought stress might impair the ability of the compromised xylem to re-wet - apparently an important stage in the formation of a structurally continuous reaction zone barrier (Pearce 2000).

3.8. Molecular investigation of the pathogen

The DNA-based PCR, particularly, taxon-specific primers technique (Garbelotto et al. 1996; Schmidt and Moreth 2000; Gardes and Bruns 1996) is valuable appliance to identify fungi in their natural substrates as only a small amount of wood is used. For this purpose the internal transcribed spacer (ITS) region (ITSI, the 5.8S ribosomal DNA and ITSII) is analyzed. This region is frequently used because of multicopy arrangement and highly conserved priming sites in the genome of fungi. In addition, the high variability of ITSI and ITSII facilitates to generate restriction fragment length polymorphism (RFLP) patterns to identify wood decay fungi or to design taxon-specific primers. Fungus-specific primers were initially designed to identify fungal symbionts directly from ectomycorrhizae and to identify rusts that are obligate parasites, in the host tissue by Gardes and Bruns (1993).

However, in this case, taxon-specific primers were used to investigate the spread of *Amillaria mellea* in inoculated stems to study the effectiveness of compartmentalization.

4. Materials and methods

4.1. Site of experiment

The research was conducted at an experimental plot of the Thünen Institute and the Centre of Wood Sciences in Hamburg, North Germany (about 53.3° northern latitude and 10.0° eastern longitude). The average annual temperature is 8.9°C; July and August are the warmest (23°C) and January and February the coldest months (-3°C). The annual sum of rainfall is 716 mm (Fig. 4.1).

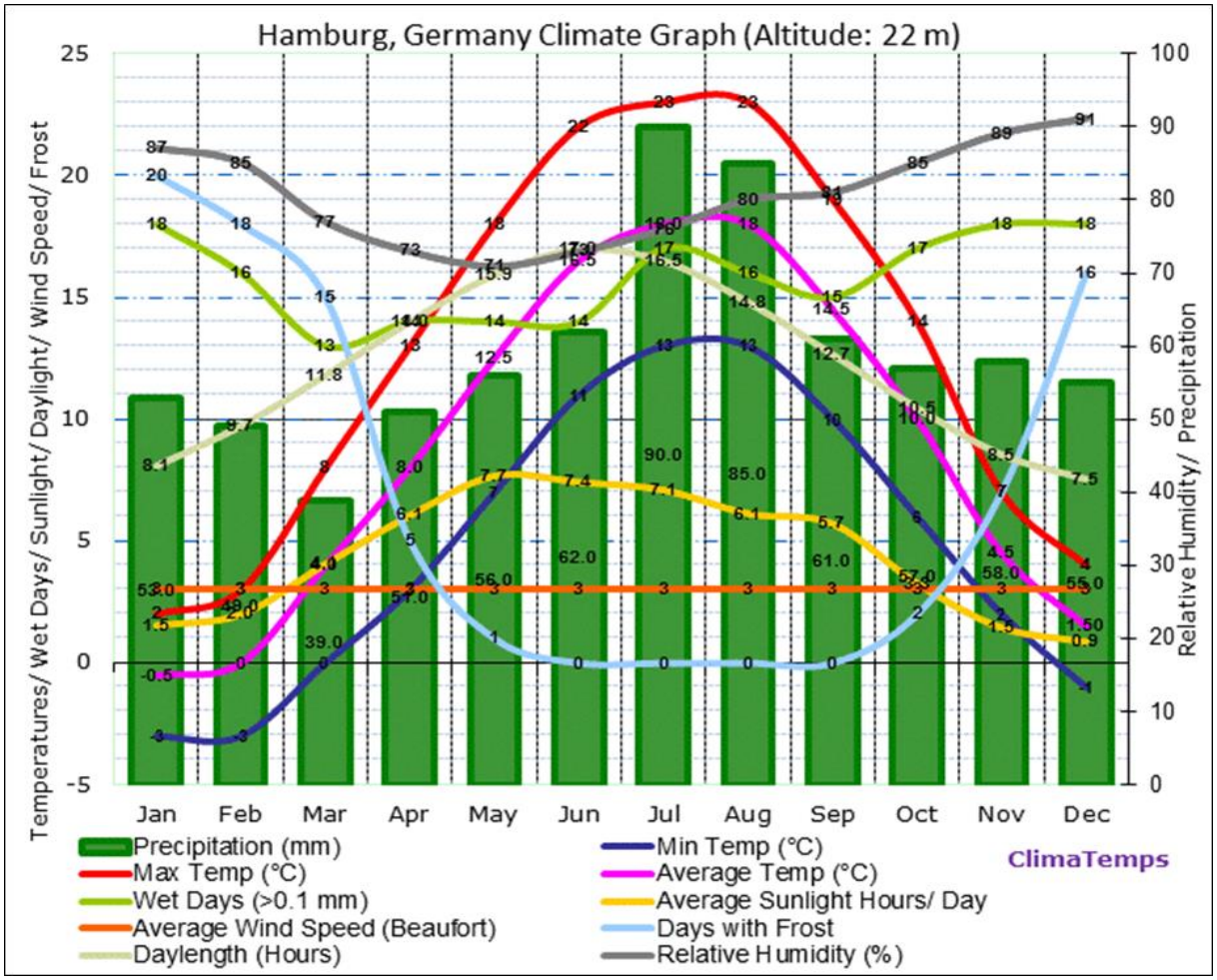


Fig. 4.1: Climate graph of Hamburg, Germany;

http://www.climatetemp.info/germany/hamburg.html.

4.2. Experimental design and treatments

The experiment, based on 22 seven-year old saplings of black locust (*Robinia pseudoacacia* L.) growing in 65 l pots, lasted for two years from June 2010 until July 2012. One half of the plants were kept well-watered and one half of them were submitted to drought stress. From the well-watered and drought-stressed saplings, three, were infected by *A. mellea* in July and in February. Five saplings of well-watered and drought-stressed each, remained unwounded and uninfected (Fig. 4.2). The experimental design consisted of four treatments, T1 (well-watered), T2 (well-watered + infected with *A. mellea*), T3 (drought-stressed), and T4 (drought-stressed + infected with *A. mellea*), with five (T1, T3) and three (T2 July, T2 February; T4 July, T4 February) replicates, respectively.

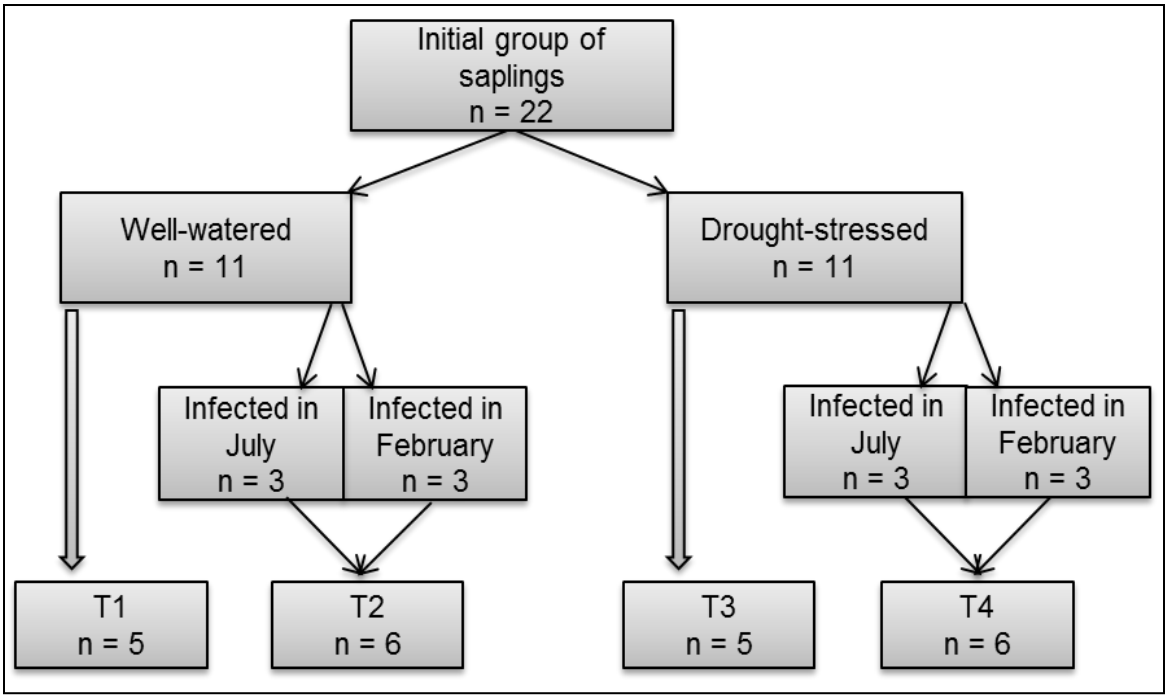


Fig. 4.2: Experimental design, treatments along with number of saplings (n).

The well-watered plants were irrigated daily to field capacity, with a maximum of ten liters of water; irrigation was withheld on rainy days. The drought-stressed saplings were

irrigated according to the prevailing daily temperature, i.e. 825 ml at up to 20°C, 1100 ml from 21 to 25°C, 1650 ml from 26 to 30°C, and 2200 ml at and above 31°C (Table 4.1, adopted from Veste and Kriebitzsch (2013). To avoid any influence of rainfall, the pots of the drought-stressed saplings were covered with plastic sheets from June to October (Fig. 4.3).

Table 4.1: Water regime for well-watered and drought-stressed saplings of black locust.

Temperature (°C)	Drought-stressed saplings (ml)	Well-watered saplings (l)
20	825	10
21 to 25	1100	10
26 to 30	1650	10
≥ 31°C	2200	10

Fig. 4.3: Well-watered saplings (control) (left) and drought-stressed saplings (right), whose pots were covered with plastic sheets to avoid the effect of rainfall.

4.3. Inoculum

Armillaria mellea (120.59) obtained from CBS (Centraalbureau voor Schimmelcultures, Holland) was cultivated on 2% malt extract agar (consisting of 15 g of malt extract and 11.25 g of agar dissolved in 750 ml of water and autoclaved at 121°C for 30 minutes).

Beech wood dowels (8 mm diameter, 15 mm long) were autoclaved. Six were placed on the growing mycelium. After six weeks, the dowels were sufficiently covered and penetrated by the fungus (Fig. 4.4) and were used as inoculum as shown in 4.4.

Fig. 4.4: Dowels on malt extract agar covered by *Armillaria mellea* mycelium.

4.4. Wounding and artificial inoculation

In July 2010 and February 2011, three well-watered and three drought-stressed saplings were wounded and inoculated. For this purpose, in each sapling, two holes, 8 mm wide and 15 mm long, were drilled at 30 and 60 cm above ground (Fig. 4.5). Then, an autoclaved dowel was introduced into the upper drill hole as non-infected; similarly,

dowel containing the inoculum was introduced into the lower hole (Fig. 4.6). To avoid any interaction the holes were slightly displaced at each level. After inoculation, each wound was sealed with wound dressing.

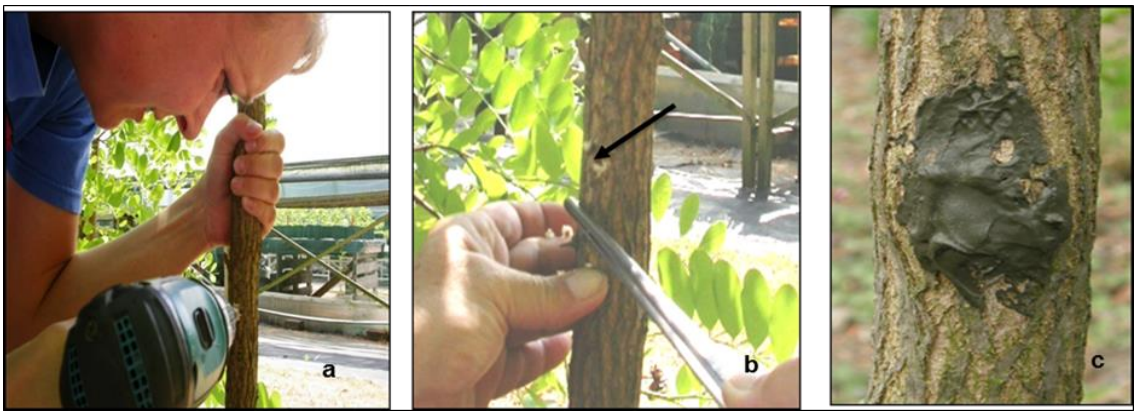


Fig. 4.5: Artificial inoculation: (a) bore holes were drilled into the stem, (b) a dowel was introduced into the bore holes; (c) wounds were sealed with a wound dressing.

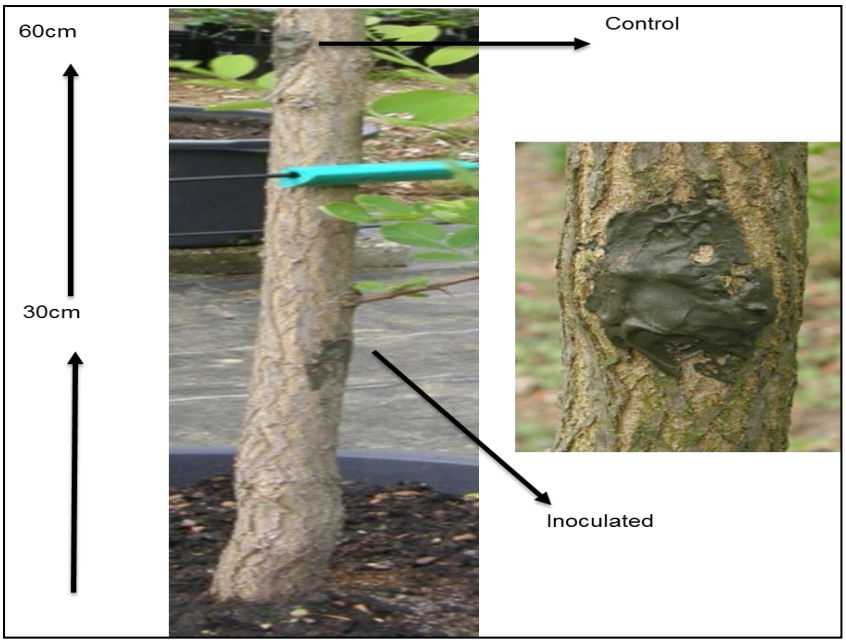


Fig. 4.6: Wounding and infection design: Two bore holes were drilled in the stems at 30 cm above ground for *Armillaria mellea* containing dowels and at 60 cm above ground for the control dowels without an inoculum.

Thus to study compartmentalization, the experimental design consisted of four treatments, T2, T2c, T4 and T4c. The statistical sample size in each experimental variant was n = 3 (Fig. 4.7).

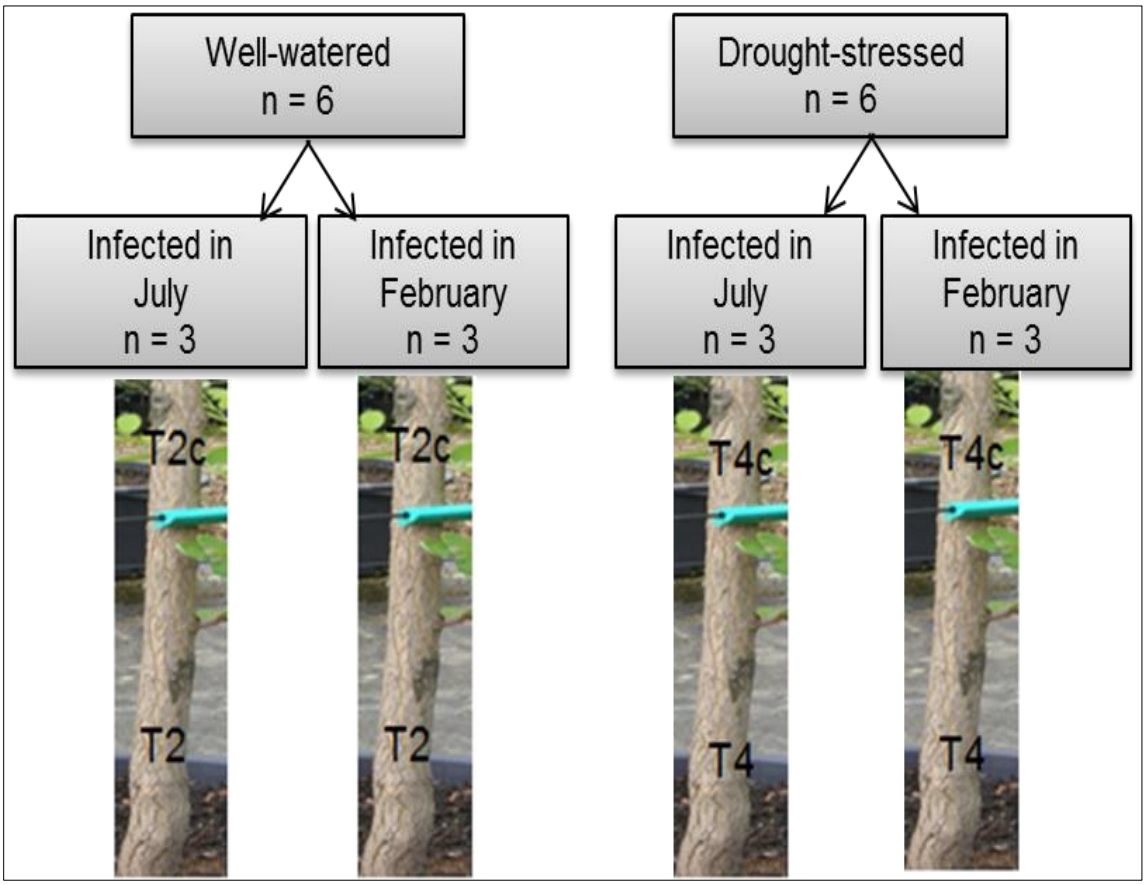


Fig. 4.7: Wounding and artificial inoculation scheme; treatments along with the number of saplings T2 (well-watered with infected dowel), T2c (well-watered with non-infected dowel), T4 (drought-stressed with infected dowel), and T4c (drought-stressed with non-infected dowel). The statistical sample size in each experimental variant was n = 3.

4.5. Morphological measurements

4.5.1. Leaf parameters

Morphological measurements were made on all 22 saplings. One year after the inoculation, the area, length and width of seven leaves per sapling were measured non-destructively using a portable leaf-area meter (Fig. 4.8).

Fig. 4.8: Leaf measurements by a Leaf Area Meter (Li-Cor model 3000, Lincoln, NE USA).

4.5.2. Stem diameter

The stem diameter were measured with a Vernier caliper at 40 cm above the soil in August 2010 and 2011 for all saplings (Fig. 4.9) to know their average diameter per treatment and to calculate the increase in diameter per treatment in one year.

Fig. 4.9: Measuring diameter of black locust saplings by a Vernier caliper.

4.5.3. Tree height

The height of all saplings of each treatment was measured and noted after leaf fall with a meter ruler by determining the distance from the soil level to the top of the saplings in November 2011.

4.5.4. Number of seed pods

The number of seed pods (Fig. 4.10) of all the saplings per treatment was counted at maturity to study any difference in the yield of seed pods.

Fig. 4.10: Seed pod of well-watered black locust.

4.5.5. Morphological and phenological aspects

Along with the measurements, observations about the initiation of the leaves and of the inflorescence, as well as of senescence and leaf abscission were noted and photographed with a digital camera (Olympus SP-55OUZ) at various intervals.

4.6. Gas exchange measurements

Gas exchange measurements were made on all saplings using a portable Infra-Red Gas Analyzer (IRGA; Model LI 6400; LI-Cor Inc., Lincoln, NE, USA) fitted with a standard leaf chamber of 2 cm^2 (Fig. 4.11). The light intensity was 800 $\mu molm^{-2}s^{-1}$ provided by a red-blue light source, and the CO_2 was 380 ppm. Net photosynthesis (P_N), transpiration (E), and stomatal conductance (g_s) were measured in June, July, August and September 2011 of at least 2 to 3, fully expanded healthy leaves per sapling. All measurements were made between 1 and 4 o'clock pm. The mesophyll conductance (g_m) was calculated by dividing the photosynthesis (P_N) data by the intercellular CO_2 (Ci) data (Fischer et al. 1998).

Fig. 4.11: Gas exchange measurements by IRGA; Model LI 6400; LICor Inc., Lincoln, NE, USA.

4.7. Harvesting

All well-watered and drought-stressed saplings of black locust were harvested in July 2012 and separated into leaves, branches, stems and roots.

The soil was carefully shaken off the roots and the remaining soil was washed off in a separate container by careful rinsing. Soil was removed from roots by gently washing over a 2 mm sieve placed on top of a container under running tap water (Fig. 4.12). Care was taken to ensure that all roots were collected from the sieve and container.

Fig. 4.12: Washed roots of black locust saplings after harvesting.

4.8. Biomass determination and root-to-shoot ratio

The biomass of the saplings was determined for all saplings per treatment after the harvest. The fresh weight of all fractions (leaves, stems, branches and roots) was determined to calculate the water content and the dry weight biomass. For this purpose, the biomass fractions of each sapling were oven-dried at 70°C until a constant weight was reached. In addition, the root-to-shoot ratio was calculated for all treatments by dividing the average dry weight of the roots by the average dry weight of the above ground biomass of the saplings.

4.9. Statistical analysis

The data collected were subjected to Analysis of Variance (ANOVA) using Microsoft Excel 2007 (Data analysis). After the rejection of the null hypothesis, the treatment means were compared using Fisher's LSD (Least Significance Difference) at p = 0.05 for morphological traits. Multiple ANOVA (pair-wise comparisons) was performed for physiological parameters to investigate any difference between treatments.

4.10. Freeze drying of inoculated stem sections

After harvesting, stem sections containing the wounds, both aseptic and infected, were frozen at -20°C and processed for freeze drying prior to other experiments (Fig. 4.13).

Fig. 4.13: Stem sections containing the wound and inoculation sites are freeze dried.

4.11. Measurement of discoloration and callus formation

After freeze drying, longitudinal and cross sections of the stems were prepared and the extent of discoloration and decay was measured in axial, radial, and tangential direction, as mentioned by Deflorio et al. (2008). Discoloration in axial, radial and tangential

direction was also observed under UV light. In addition, the thickness of the callus in the vicinity of each wound was also measured in all trees of each treatment (Fig. 4.14).

Fig. 4.14. Measuring the callus formation in the vicinity of wounds in all samples of black locust; measurements were taken from a to b.

As the statistical sample size was too small for a rigorous statistical analysis, results were graphically presented to show the ranges of the data for each experimental variant.

4.12. Homogenizing material for biochemical and molecular analysis

For biochemical and molecular analysis, wood samples from the decayed zone, reaction zone, sound wood and callus (Fig. 4.15) were collected from wounded and infected stems. Freeze dried samples were stored in airtight plastic bags (see Fig. 4.19) and were reopened after thawing, just before proceeding with the analysis of the samples.

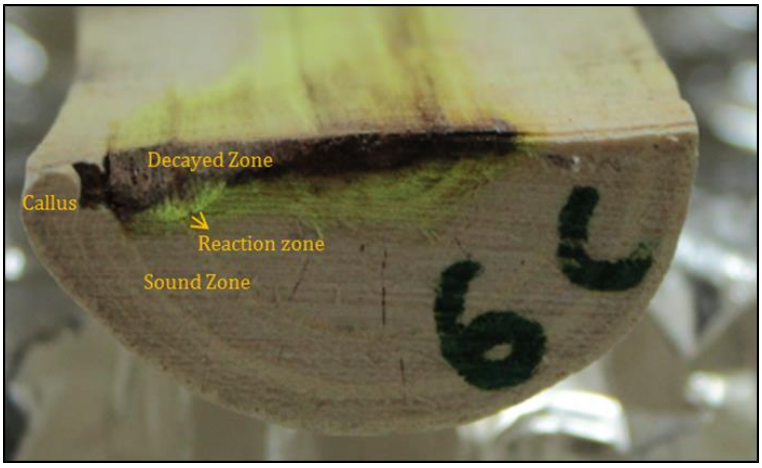


Fig. 4.15: Decayed zone, reaction zone, sound wood and callus in an inoculated wood sample under UV light.

4.13. Determination of soluble carbohydrates and starch

The soluble carbohydrates (glucose, fructose, and sucrose) and starch were quantitatively analyzed by an enzyme-based method as presented by Magel et al. (2001). For that purpose, 10 mg homogenized fine powder from the respective tissues were taken in a 2 ml Eppendorf tube and a pinch of activated charcoal and of PVPP (polyvinyl-polypyrolidone) was added to bind polyphenols and to eliminate pigments; then, 1 ml of 65% ethanol was added. This mixture was incubated for 60 min at 60°C. Then the extract was centrifuged for 10 min at 4°C and 12500 rpm. The supernatant was separated from the pellets for sugar analysis and the pellets were prepared for starch analysis. Chemicals and solutions along with respective concentrations are given (Table 4.2).

Table 4.2. Chemicals and solutions with concentrations.

Chemicals and Solutions	Concentrations
PVPP	20 mg (SERVA)
Ethanol	65%
Charcoal	Pinch
Acetate buffer	0.1 M, pH 4.6 10 ml (1N) acetic acid, 5ml (1N) NaOH fill up to 100 ml with bidest; adjust pH to 4.6
Glucose	1mM
Fructose	1mM
Sucrose	1mM

Table 4.3. Test mixture for sugar and starch analysis.

For Sugar analysis; TRIS-Buffer (500mM pH 7.5) For Starch analysis; TRIS-Buffer (500mM pH 8.8)	10 ml
1 M $MgSO_4$	60 µl
200 mM ATP	200 µl
200 mM NADP	100µl
Glucose-6-phosphate Dehydrogenase (G6P-DH; 140 U/mg, 5mg/ml)	10µl

To the pellets, 200 µl Acetate buffer (0.1 M, pH 4.6) was added and homogeneously mixed for 15 min in a boiling water bath. This mixture was cooled down to room temperature, 10 µl of the original Amyloglucosidase solution (Roche Diagnostic GmbH 102857) was added, mixed carefully and incubated overnight at 37°C. Then, the extract was centrifuged for 10 min at 12500 rpm at room temperature. Test mixture for sugar and starch analysis is provided (Table 4.3). Background was adjusted by mixing 75 µl test mixture and 30 µl H_2O, and standard was maintained with 75 µl test mixture and 10 µl of each, Glucose (1mM), Fructose (1mM), and sucrose (1mM). 75 µl test mixture

along with 20 µl test sample was used for sugar analysis, whereas 75 µl test mixture along with 10 µl test sample was used for starch analysis.

By successive addition of the enzymes hexokinase, phosphoglucoisomerase and ß-fructosidase, the carbohydrates glucose, fructose and sucrose were determined, respectively. The amount of the carbohydrates was calculated based on the respective formation of NADPH at 340 nm wavelength. Enzymes along with their respective concentrations and manufacturer are provided (Table 4.4). All experiments were performed in triplicate and quantified using Lambert-Beer's law. The extinction coefficient of NADPH at 340 nm is 6.27 $mmol^{-1}cm^{-1}$.

Table 4.4. Enzymes with respective concentrations and manufacturer.

Enzymes	Concentrations	Amount	Manufacturer
Amyloglucosidase	1.4 U	10 µl	Roche
Hexokinase	0.15 U	10 µl	Roche
Phosphoglucoisomerase	0.7 U	10 µl	Roche
ß-Fructosidase	6 mg in 1.2 ml Acetate buffer (pH 4.6; 0.1 M)	30 µl	Roche
Glucose-6-phosphateDehydrogenase	5 mg in 1 ml	10 µl	Roche

4.14. Investigation of *Armillaria mellea* by molecular techniques

To study the effectiveness of compartmentalization, the spread of *Armillaria mellea* was followed in inoculated stems through molecular technique. This was done in collaboration with Corinna Gebarowski during her B.Sc. thesis work.

Selective amplification of the genomic DNA by the polymerase chain reaction (PCR) is a sensitive and specific tool appropriate to investigate fungal pathogens in wood with the help of specific primers.

Taxon-specific primers are to identify the pathogen. For this purpose, the internal transcribed spacer (ITS region; ITS1, the 5.8S ribosomal DNA and ITS2) was analyzed (Fig. 4.16), and suitable taxon primers were designed to investigate the pathogen in question.

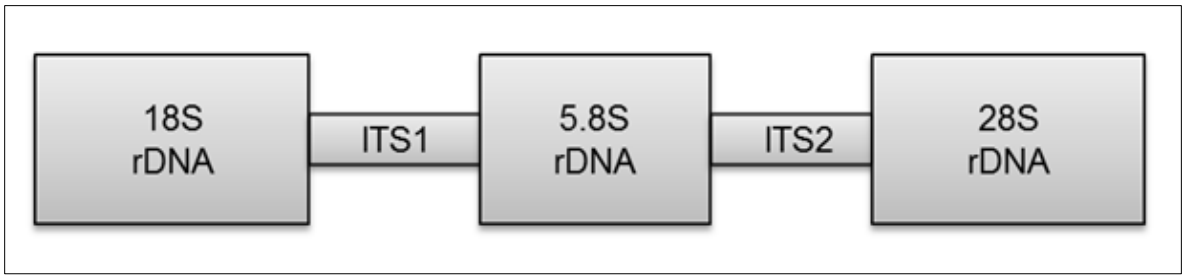


Fig. 4.16: Map of the ribosomal DNA region containing ITS1 and ITS2, and the 5.8S rDNA.

4.14.1. DNA extraction from pure culture

DNA of *Armillaria mellea* grown on 2% malt agar at room temperature was extracted by using 30 mg fresh mycelium and DNeasy® Plant Mini Kit from Qiagen. The experimental procedure was applied according to the manufacturer's instruction. Kits and solutions used are provided here (Tables 4.5, 4.6).

Table 4.5. Kits used with solutions and manufacturers.

Kit	Solutions	Manufacturer
DNeasy® Plant Mini Kit	Buffer AP1 Buffer AP2 Buffer AP3 Buffer AW1 (+Ethanol) Buffer AE	Qiagen
PCR Core Kit	5 x Q Solution 10 x PCR Buffer dNTP Mix Taq-Polymerase	Qiagen
Qiaquick® PCR Purification Kit	Buffer PB Buffer PE (+Ethanol) Buffer EB	Qiagen

Table 4.6. Chemicals and solutions used during molecular work.

Chemicals and Solutions	Manufacturer
Agarose	SERVA
DNA AWAY ™	Carl Roth
Ethanol (≥ 99.8 % p.a.)	Carl Roth
Ethidiumbromide	Carl Roth
GelPilot 100bp Plus Ladder (Marker)	Qiagen
Loading buffer (0.25 % Bromphenolblau, 30% Glycerin)	Lab setting
TAE-Buffer (50 x Stock-Solution: 2M TRIS, 1M Acetate, 50mM EDTA	Lab setting

4.14.2. Amplification of desired DNA regions

Amplification of desired regions was performed using PCR Taq Core Kit from Qiagen (Table 4.5); solutions and concentrations for master mix for a test with the Taq Core Kit from Qiagen are given (Table 4.7). Master mixture was adjusted up to 12 µl and 0.5 µl template was used.

For this purpose, ITS forward primer (ITS1.1; GAACCTGCGGAAGGATCAT) and ITS reverse primer (ITS4; TCCTCCGCTTATTGATATGC), fungal-specific forward primer (pilzfor 1; AACTTTCAACAACGGATCTCTT) and fungal-specific reverse primer (pilzrev 1; AAGAGATCCGTTGTTGAAAGTT) were used (White et al. 1990). These universal primers along with respective melting temperatures are provided (Table 4.8). Amplification was carried out with the following conditions: an initial denaturing for 4 min at 94°C, 40 cycles of 30 sec at 94°C, 30 sec at 55°C for annealing of primers, respectively, 45 sec at 72°C for elongation. The final extension was for 7 min at 72°C.

Table. 4.7. Master mixture for a test with the Taq Core Kit from Qiagen.

Solutions and concentrations	Volume (µl)
Bidest	7.775
Q-Solution	2.5
10 x Reactions buffer	1.25
dNTP Mix (10mM/dNTP)	0.25
Primer for (100 pmol/µl)	0.075
Primer rev (100 pmol/µl)	0.075
Taq-polymerase (5 units/µl)	0.075
Total volume	12

Table 4.8. Primers along with sequences and melting temperatures.

Primer name	Primer sequence	Melting temperature
Forward primer (ITS1.1)	GAACCTGCGGAAGGATCAT	56.7°C
Reverse primer (ITS4)	TCCTCCGCTTATTGATATGC	55.3°C
Fungal specific for	AACTTTCAACAACGGATCTCTT	54.7°C
Fungal specific rev	AAGAGATCCGTTGTTGAAAGTT	54.7°C

4.14.3. Purification of PCR products

PCR products were cleaned by means of the Qia-quick purification kit (Qiagen, Valencia, CA) and sequenced by MWG-Biotech (Ebersberg, Germany).

4.14.4. Taxon-specific primers design and testing

The sequence was used to compare the query sequence with database sequences. The sequence of the test strain and sequence information of objective and related species from the European Molecular Biology Laboratory (EMBL) and the Gene Bank (NCBI) database (www.ncbi.nlm.nih.gov/nucleotide) were aligned by Mega 5.1 and ClustalX (1.81) to search *A. mellea* taxon-specific sequence. Along with *A. mellea*, sequences of *A. hinnulea*, *A. ectypa*, *Pleurotus pulmonarius*, *Ganoderma pseudoferrum*, *Laetiporus sulphureus* and *Phellinus robiniae* were used to find out specificity. Thus, taxon-specific PCR primers based on a suitable informative area of the sequenced ITS region was designed for *A*. *mellea* (Garbelotto et al. 1996; Moreth and Schmidt 2000; Nicolotti et al. 2009).

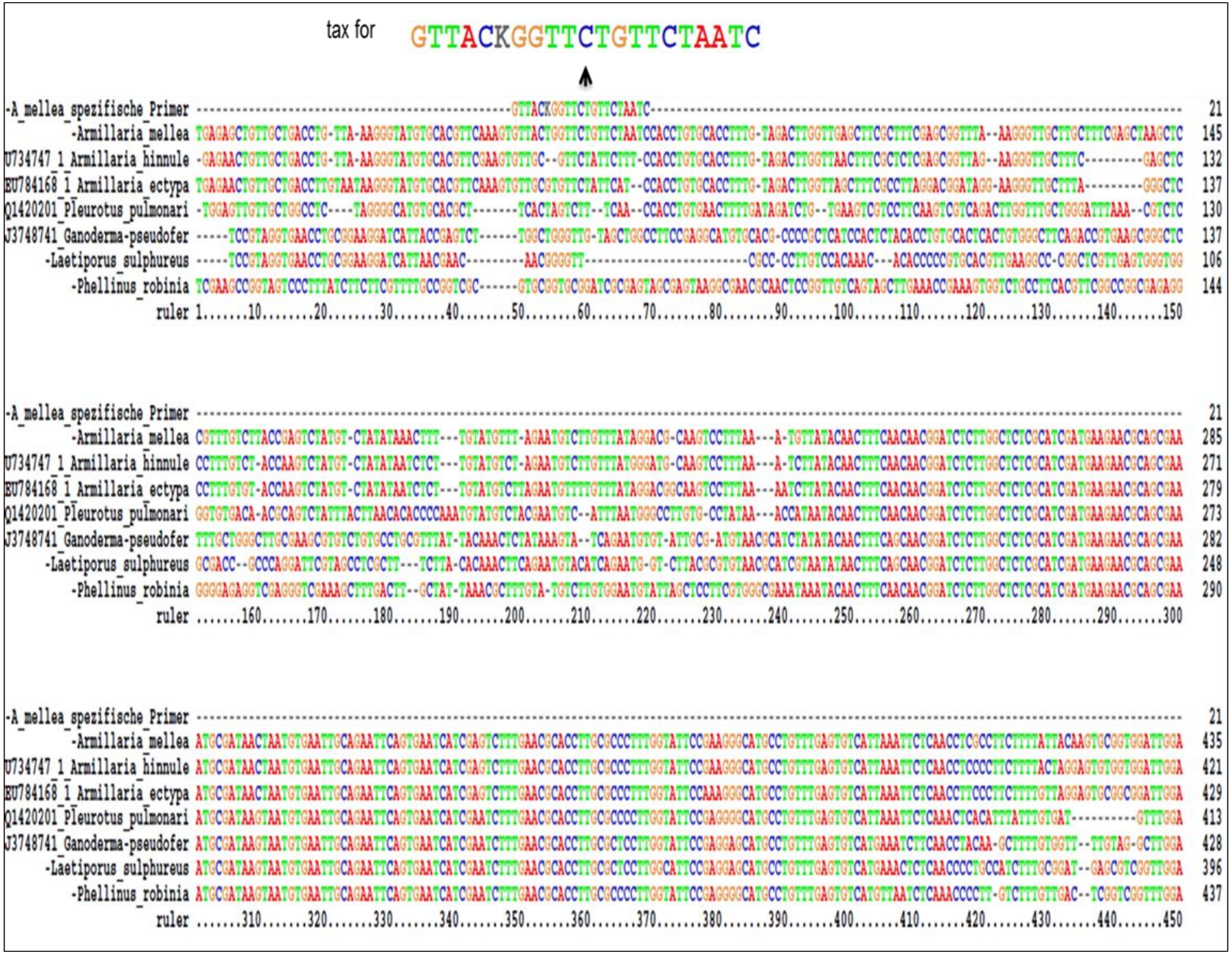
The primers were designed as forward (tax for) and reverse primers (tax rev) using the software PRIMER3 (www.genome.wi.mit.edu/cgibin/primer/primer3) to be specific for *A. mellea* (Fig. 4.17; Table 4.9). The length of specific primers was between 20 and 22 basepairs.

PCR amplification with taxon-specific primers forward and reverse (Amtaxforb; GTTACKGGTTCTGTTCTAATC) and (Amtaxrevb; CCAAGAGTTTCTTGTTACSG) was carried out in a thermal cycler by Biometra by using 0.5 µl template and test mixture given in table (4.7) and with the following conditions: an initial denaturing for 4 min at 94°C, 40 cycles of 30 sec at 94°C, 30 sec at 55°C for annealing of *A. mellea* specific primers, respectively, 40 sec at 72°C for elongation. The final extension was for 7 min at 72°C.

Table 4.9. Taxon-specific primers of *A. mellea* along with sequences and melting temperatures.

Taxon-specific primer of *A. mellea*	**Primer sequence**	**Melting temperature**
Amtax-for b	GTTACKGGTTCTGTTCTAATC	54.9°C
Amtax-rev b	CCAAGAGTTTCTTGTTACSG	55.3°C

To analyze PCR products, aliquots of 2.5 µl were loaded to gel electrophoresis on 2.5 % (w/v) agarose gel and visualized with ethidium bromide (0.00015%). A DNA marker (100 bp) was used for size estimation.


tax for GTTACKGGTTCTGTTCTAATC
-A_mellea_spezifische_Primer
-Armillaria_mellea
U734747_1_Armillaria_hinnule
EU784168_1_Armillaria_ectypa
Q1420201_Pleurotus_pulmonari
J3748741_Ganoderma-pseudofer
-Laetiporus_sulphureus
-Phellinus_robinia
ruler 1.....10.....20.....30.....40.....50.....60.....70.....80.....90.....100.....110.....120.....130.....140.....150
ruler .....160.....170.....180.....190.....200.....210.....220.....230.....240.....250.....260.....270.....280.....290.....300
ruler .....310.....320.....330.....340.....350.....360.....370.....380.....390.....400.....410.....420.....430.....440.....450

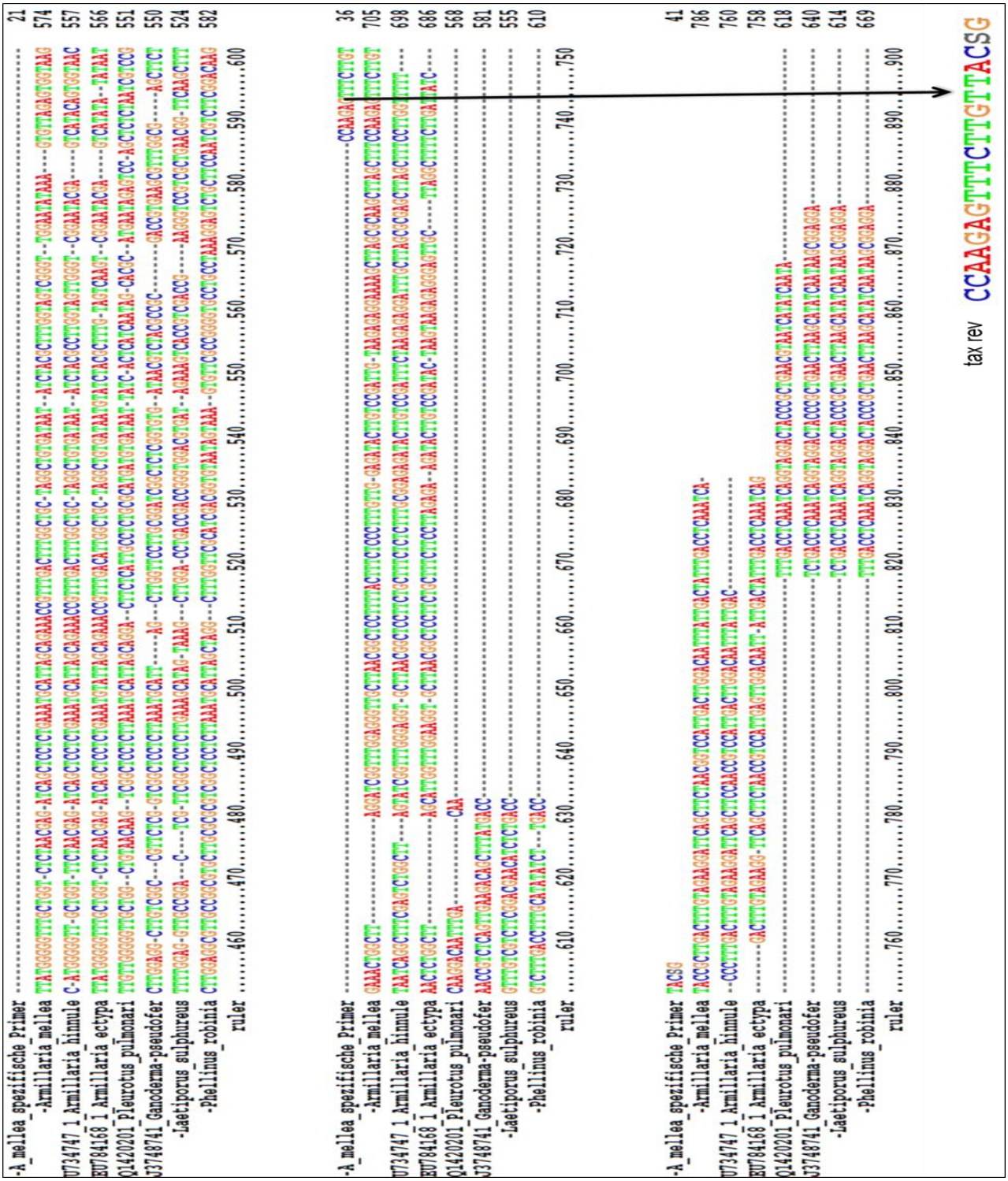


Fig. 4.17: Multiple sequence alignment of ITS regions of *Armillaria* species of our own and from the Genebank (NCBI) using ClustalX (1.81) to design and analyse *A. mellea* species-specific primers. Boxes highlight primers (tax for) and (tax rev) that contribute to the specificity of pathogen.

4.14.5. DNA extraction from different wood zones

DNA was extracted from 60 mg of homogenized material from all zones (decayed zone, reaction zone, sound wood and callus) as mentioned in 4.12 (see Fig. 4.18) with the DNeasy® Plant Mini Kit from Qiagen. The experimental procedure developed by the manufacturer was applied. It should be added, that the elution of DNA from the column with AE buffer was repeated and elution two is mentioned in results section as 2nd elution.

Fig. 4.18: Homogenized material from decayed and reaction zones as well as sound wood and callus.

4.14.6. PCR amplification of *A. mellea* from wounded and inoculated stems

To detect the fungus in question by PCR amplification, the total DNA was isolated from infected plant material and NanoDrop was performed to quantify nucleic acid. Furthermore, the presence or absence of *A. mellea* was investigated with specific primers (tax for and tax rev) designed for *A. mellea* (Fig. 4.17) in the callus, decay zone, reaction zone and sound wood. For this purpose, 0.5 µl to 2µl template was used.

PCR conditions were set as mentioned in 4.14.4 and products were visualized with ethidium bromide (0.00015%).

5. Results

5.1. Morphology and phenology

There were no significant differences in the morphological and phenological parameters of well-watered and drought-stressed saplings infected in summer or in winter. Therefore, saplings infected in summer and winter of the individual treatment were considered together.

5.1.1. Leaf parameters

All morphological leaf parameters (area, length and width) were significantly smaller in drought-stressed saplings (non-infected or infected; T3, T4) than in well-watered ones (non-infected or infected; T1, T2). Moreover, the leaves of drought-stressed saplings emerged on April 12, 2011 (emergence of leaf was just started in drought-stressed ones; Fig. 5.1) compared to well-watered ones whose leaves unfolded already on March 25, 2011.

Fig. 5.1: Effect of drought stress in the form of delayed bud break of black locust saplings in April 2011; drought-stressed saplings (right) vs. well-watered ones (left).

In addition, a change in morphology and a significantly smaller number of leaflets of the drought-stressed saplings was found. The average number of leaflets per leaf ranged from 17 in the case of well-watered leaf and 10 in the case of drought-stressed saplings (Table 5.1). Moreover, the individual leaflets were shorter and broader and their surfaces did not overlap each other as compared to the leaves of well-watered saplings (Fig. 5.2).

Fig. 5.2: Morphology of leaves under well-watered (a) and drought-stressed (b) growth conditions.

The average leaf area varied from 160 to158 cm^2 in T1 and T2 and from 72 to 64 cm^2 in T3 and T4, respectively. The average leaf area and the average number of leaflets were not statistically different between the infected and non-infected well-watered saplings (T1, T2). In the drought-stressed saplings, the leaf area was significantly smaller by more than 50%, and the leaves were shorter by more than 30%.

The average leaf length varied from 32 to 30 cm in T1 and T2 and from 22 to 20 cm in T3 and T4, respectively. The average leaf width varied from 7.3 to 7.2 cm in T1 and T2 and from 6.1 to 5.2 cm in T3 and T4, respectively. The average leaf length was not statistically different between the infected and non-infected well-watered saplings (T1, T2) or between the infected and non-infected drought-stressed saplings (T3, T4). However, the average leaf length and the average leaf width of well-watered saplings were significantly different than the drought-stressed ones (Table 5.1).

The smallest area, length, and width of leaves were recorded for saplings under the combined effects of drought and infection (T4; Table 5.1).

Table 5.1: Mean value ± standard deviation of various leaf parameters of black locust saplings, well-watered (T1), well-watered + infected (T2), drought-stressed (T3), and drought-stressed + infected (T4). All measurements were taken on seven leaves from five saplings in each treatment; LSD = least significant difference at 5% probability.

Treatments	Leaf area (cm^2)	Leaf length (cm)	Leaf width (cm)	Number of leaflets
T1	160 ± 15	32 ± 1.2	7.3 ± 0.7	17 ± 1.3
T2	158 ± 14	30 ± 4.4	7.2 ± 0.7	17 ± 1.2
T3	72 ± 16	22 ± 3.1	6.1 ± 0.7	10 ± 1.8
T4	64 ± 8	20 ± 1.1	5.2 ± 0.7	10 ± 0.7
LSD	13	2.7	0.67	1

Additionally, the flowering of the saplings was impaired by water shortage. In drought-exposed saplings, inflorescence started on June 6, 2011, and thus was delayed by more than two weeks compared to the well-watered saplings that started to flower already on May 19, 2011.

In addition, drought stress resulted in a stunted growth of inflorescences which are looking somehow ball-shaped, whereas well-watered saplings got long and fresh inflorescences (Fig. 5.3).

Fig. 5.3: Morphology of the inflorescence of drought-stressed (right) vs. well-watered saplings (left) in June 2011.

Moreover, early senescence was observed in drought-stressed saplings. Whose leaves started to become yellow in July, compared to well-watered saplings (Fig. 5.4).

Similarly, leaf abscission was more rapid in drought-stressed saplings compared to well-watered ones (Fig. 5.5).

Fig. 5.4: Early senescence in drought-stressed (right) vs. well-watered saplings (left) in July.

Fig. 5.5: Accelerated leaf abscission in drought-stressed (right) vs. well-watered saplings (left) in November.

5.1.2. Stem diameter

Well-watered saplings, both infected and non-infected (T1, T2; Fig. 5.6), showed a diameter increase of about 10 mm within one year. Drought-stressed saplings, however, had only half the diameter increase compared to well-watered ones in one year. This is a significantly smaller average diameter for the drought-exposed saplings (T3) by 48%. In the case of the saplings stressed by both drought plus a pathogenic fungus, diameter growth was even reduced by 54% (Fig. 5.6).

After one year of inoculation, the average diameter varied from 45.7 to 45.9 mm in T1 and T2 respectively. In T3 and T4, the diameter was 33.9 to 34.1 mm, respectively (Table 5.2).

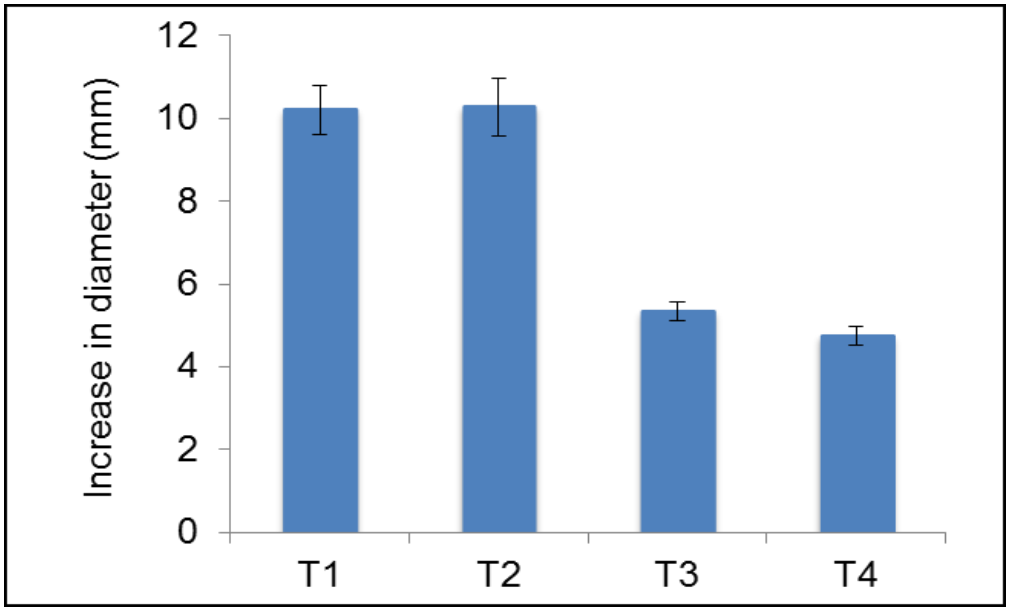


Fig. 5.6: Average growth in stem diameter ± standard deviation of the saplings T1 to T4 within one year (n=5 for T1 and T3; n=6 for T2 and T4); for the definition of the treatments T1 – T4 see Table 5.1.

Table 5.2: Mean value ± standard deviation of stem diameters of saplings T1 to T4; the mean values are calculated from five saplings per treatment; LSD = least significant difference at 5% probability; for the definition of the treatments T1 – T4 see Table 5.1.

Treatments	Stem diameter (mm)
T1	45.7 ± 0.5
T2	45.9 ± 0.2
T3	33.9 ± 0.2
T4	34.1 ± 0.1
LSD	3.0

5.1.3. Tree height

The height was measured on five saplings from each treatment after leaf fall in November. The well-watered saplings, both infected and non-infected (T1, T2), were significantly taller than the drought-stressed ones (T3, T4) (Table 5.3; Fig. 5.7). The average height varied from 423 to 434 cm in T1 and T2 and from 317 to 312 cm in T3 and T4, respectively.

Table 5.3: Mean value ± standard deviation of the height of the saplings T1 to T4. Values are the average from five saplings per treatment; LSD = least significant difference at 5% probability; for the definition of the treatments T1 – T2 see Table 5.1.

Treatments	Sapling height (cm)
T1	423 ± 13
T2	434 ± 13
T3	317 ± 27
T4	312 ± 28
LSD	21.6

Fig. 5.7: Drought-stressed saplings (right) were by 30% shorter than the well-watered ones (left).

5.1.4. Number of seed pods

The drought-exposed saplings (T3, T4) produced significantly less seed pods than the well-watered ones (T1, T2). An impact by the pathogen was not recorded, neither in well-watered nor in drought-stressed saplings. The average number of seed pods varied from 28 to 24 in T1 and T2, and from 11 to 13 in T3 and T4 (Table 5.4).

Table 5.4: Mean value ± standard deviation of the number of seed pods of the black locust saplings T1 to T4. Values are the average of five saplings per treatment; LSD = least significant difference at 5% probability; for the definition of the treatments T1 – T4 see Table 5.1.

Treatments	Number of seed pods
T1	28 ± 11
T2	24 ± 11
T3	11 ± 10
T4	13 ± 9
LSD	9.6

Moreover, the impact of drought became apparent in the color of the seed pods. Well-watered saplings formed dark-brown and bright seed pods, whereas drought-stressed ones formed pale-brown pods (Fig. 5.8).

Fig. 5.8: Seed pods of well-watered black locust saplings were dark-brown and bright (a) and of drought-stressed saplings were pale-brown in color.

5.2. Gas exchange

All gas exchange processes such as net photosynthesis (P_N), stomatal conductance (g_s), mesophyll conductance (g_m), and transpiration (E) were reduced in T3 and T4 saplings as compared to T1 and T2 saplings, measured from June to September. Both in well-watered and in drought-stressed saplings the season of infection, July or February, did not significantly change the gas exchange parameters.

5.2.1. Net photosynthesis

The net photosynthesis (P_N) of all saplings was measured once in June, July, August and September (Fig. 5.9a, b). In June, its average was 8.89 and 8.23 µmol CO_2 $m^{-2}s^{-1}$ in T1 and T2, and 6.76 and 5.26 µmol CO_2 $m^{-2}s^{-1}$ in T3 and T4, respectively. In July, it was 8.31 and 8.22 µmol CO_2 $m^{-2}s^{-1}$ in T1 and T2, and 6.62 and 4.5 µmol CO_2 $m^{-2}s^{-1}$ in T3 and T4, respectively. In August, it was 10.5 and 10.2 µmol CO_2 $m^{-2}s^{-1}$ in T1 and T2, and 4.2 and 3.19 µmol CO_2 $m^{-2}s^{-1}$ in T3 and T4, respectively. In September, it was 10.37 and 10.27 µmol CO_2 $m^{-2}s^{-1}$ in T1 and T2, and 6.94 and 5.9 µmol CO_2 $m^{-2}s^{-1}$ in T3 and T4, respectively.

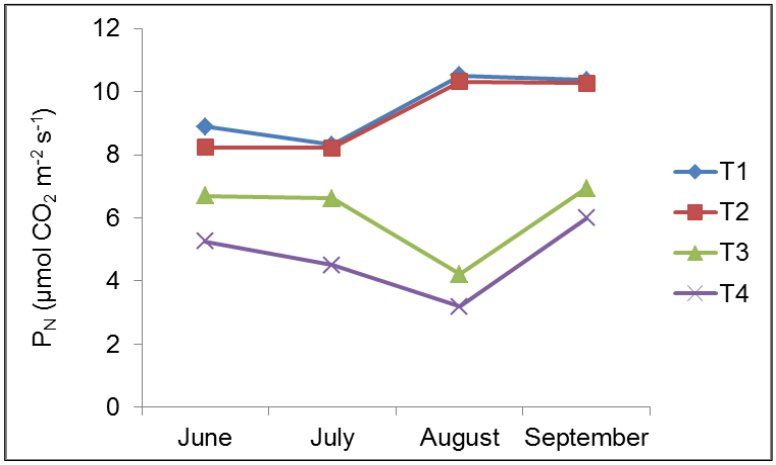


Fig 5.9a: Net photosynthesis (P_N, µmol CO_2 $m^{-2}s^{-1}$) of the saplings of the treatments T1 to T4 (n=10-12) in June, July, August, and September.

The net photosynthesis declined significantly under drought by 36% and under simultaneous drought and pathogen stress, even more, by 48% (Fig. 5.9b). In detail, a significant limitation of the net photosynthesis was noticed in the T4 than that of T3 saplings in June, July and August, but no longer in September (Table 5.5; T3xT4).

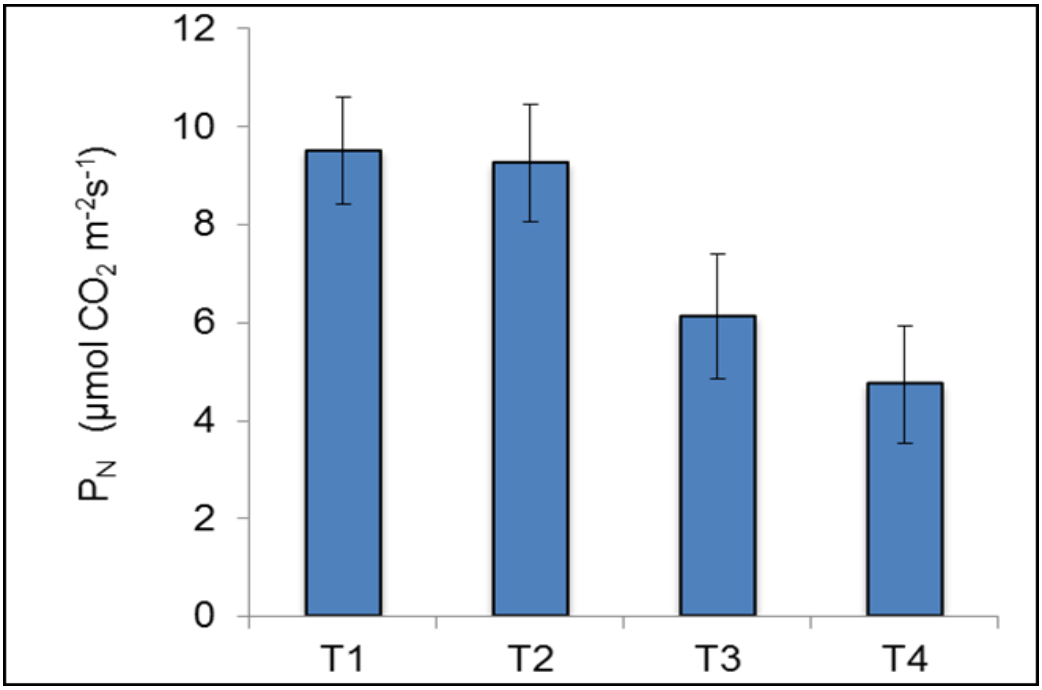


Fig. 5.9b: Net photosynthesis (P_N, µmol CO_2 $m^{-2}s^{-1}$) of treatments T1 to T4. Mean value ± standard deviation of June + July + August + September; n=42; for the definition of the treatments T1 – T4 see Table 5.1.

The highest rate of net photosynthesis occurred in T1 and T2 saplings in August and September (Fig. 5.9a). Nevertheless, the net photosynthesis in T1 and T2 was not significantly different throughout the season (Table 5.5; T1xT2). This means that the pathogen did not affect the net photosynthesis of these saplings. Conversely, declined net photosynthesis was noticed in drought-stressed saplings (T3, T4) in June, July, August and September. The lowest values were measured in T4 saplings throughout the season (Fig 5.9a).

It should be added, that there was no significant difference in net photosynthesis of well-watered saplings, no matter if they were infected in July or in February. Similarly, the photosynthesis of drought-stressed saplings was not affected by the season of infection (Table 5.5; T2FxT2J, T4FxT4J). Moreover, according to ANOVA results T1xT3, T1xT4, T2xT3, T2xT4, and T1/T2xT3/T4 were significantly different throughout the season and collectively (JJAS) as well due to drought stress Table 5.5).

Table 5.5: ANOVA results of the net photosynthesis (P_N, µmol CO_2 $m^{-2}s^{-1}$) of all saplings of the treatments T1 to T4 (n=10-12) of June, July, August, and September measurements, both separately and seasonalized (JJAS = June + July + August + September; n=42). F = February inoculation; J = July inoculation; n=6); n.s. = not significant, * = 0.05, ** = 0.01, *** = 0.001; for the definition of the treatments T1 – T4 see Table 5.1.

P_N	T1xT2	T1xT3	T1xT4	T2xT3	T2xT4	T3xT4	T1/T2x T3/T4	T2FxT2J	T4FxT4J
June	n.s	***	***	***	***	**	***	n.s	n.s
July	n.s	***	***	***	***	***	***	n.s	n.s
August	n.s	***	***	***	***	*	***	n.s	*
September	n.s	***	***	***	***	n.s	***	n.s	n.s
JJAS	n.s	***	***	***	***	***	***	n.s	n.s

5.2.2. Stomatal conductance

An average stomatal conductance (g_s) in June was 0.2 and 0.18 mol H_2O $m^{-2}s^{-1}$ in T1 and T2, and 0.16 and 0.14 mol H_2O $m^{-2}s^{-1}$ in T3 and T4, respectively. In July, it was 0.17 and 0.13 mol H_2O $m^{-2}s^{-1}$ in T1 and T2, and 0.1 and 0.09 mol H_2O $m^{-2}s^{-1}$ in T3 and T4, respectively. In August, it was 0.2 and 0.19 mol H_2O $m^{-2}s^{-1}$ in T1 and T2, and 0.09 and 0.07 mol H_2O $m^{-2}s^{-1}$ in T3 and T4, respectively. In September, it was 0.1 and 0.15 mol H_2O $m^{-2}s^{-1}$ in T1 and T2, and 0.14 and 0.12 mol H_2O $m^{-2}s^{-1}$ in T3 and T4, respectively (Fig. 5.10a).

Stomatal conductance (g_s) remained higher in well-watered saplings (T1) than in drought-stressed ones, except in September. However, it was reduced in drought-stressed saplings and lowest values were measured in T4 saplings in June, July and August (Fig. 5.10a).

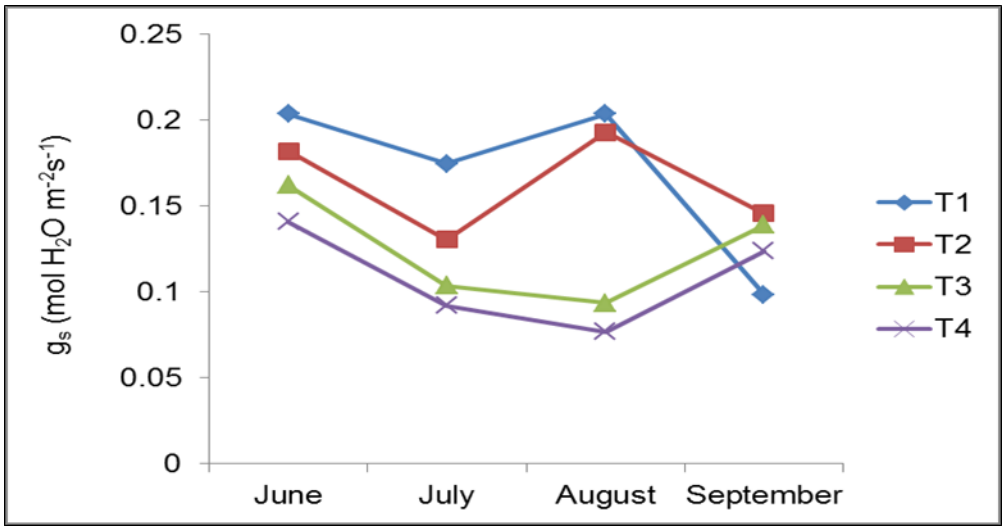


Fig 5.10a: Stomatal conductance (g_s, mol H_2O $m^{-2}s^{-1}$) of the saplings of the treatments T1 to T4 (n=10-12) in June, July, August, and September.

The average seasonal stomatal conductance (g_s) was significantly reduced by 26 and 34% in the drought-exposed saplings T3 and T4, respectively (Fig. 5.10b). This difference remained throughout the season as well (Fig 5.10a).

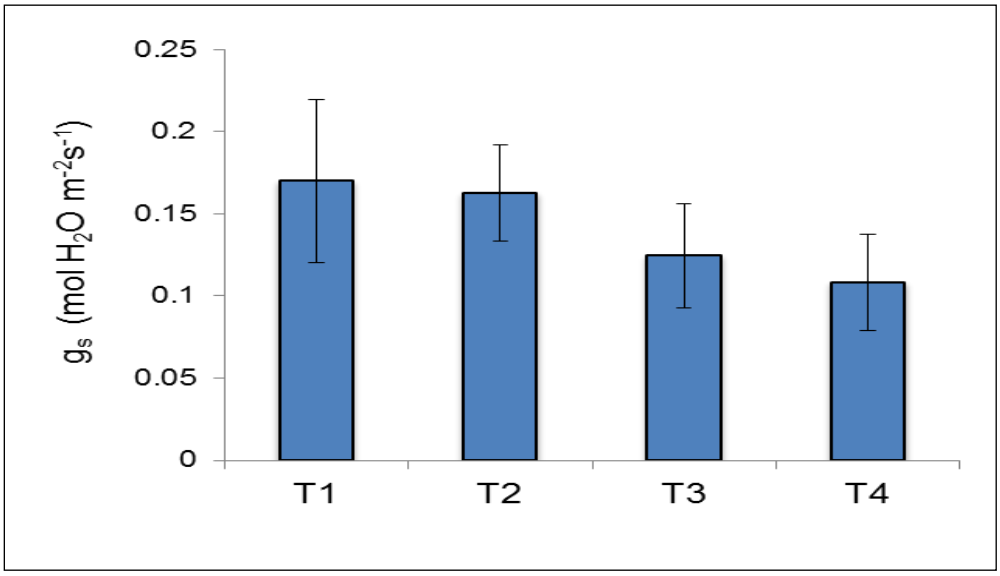


Fig. 5.10b: Stomatal conductance (g_s, mol H_2O $m^{-2}s^{-1}$) of treatments T1 to T4. Mean value ± standard deviation of June + July + August + September; n=42; for the definition of the treatments T1 – T4 see Table 5.1.

Overall, the average stomatal conductance in well-watered saplings, infected or non-infected (T1, T2) was not significantly different in JJAS (June + July + August + September), but variations can be seen on the monthly basis (Table 5.6; T1xT2). Similarly, stomatal conductance in drought-stressed saplings, infected or non-infected (T3, T4) was significantly not different between each other (Table 5.6; T3xT4).

There were no significant alterations in stomatal conductance of well-watered saplings, no matter if they were infected in July or in February. Similarly, stomatal conductance of drought-stressed saplings was not either affected by the season of infection (Table 5.6; T2FxT2J, T4FxT4J). Moreover, according to ANOVA results T1xT3, T1xT4, T2xT3, T2xT4, and T1/T2xT3/T4 were significantly different due to drought stress (Table 5.6).

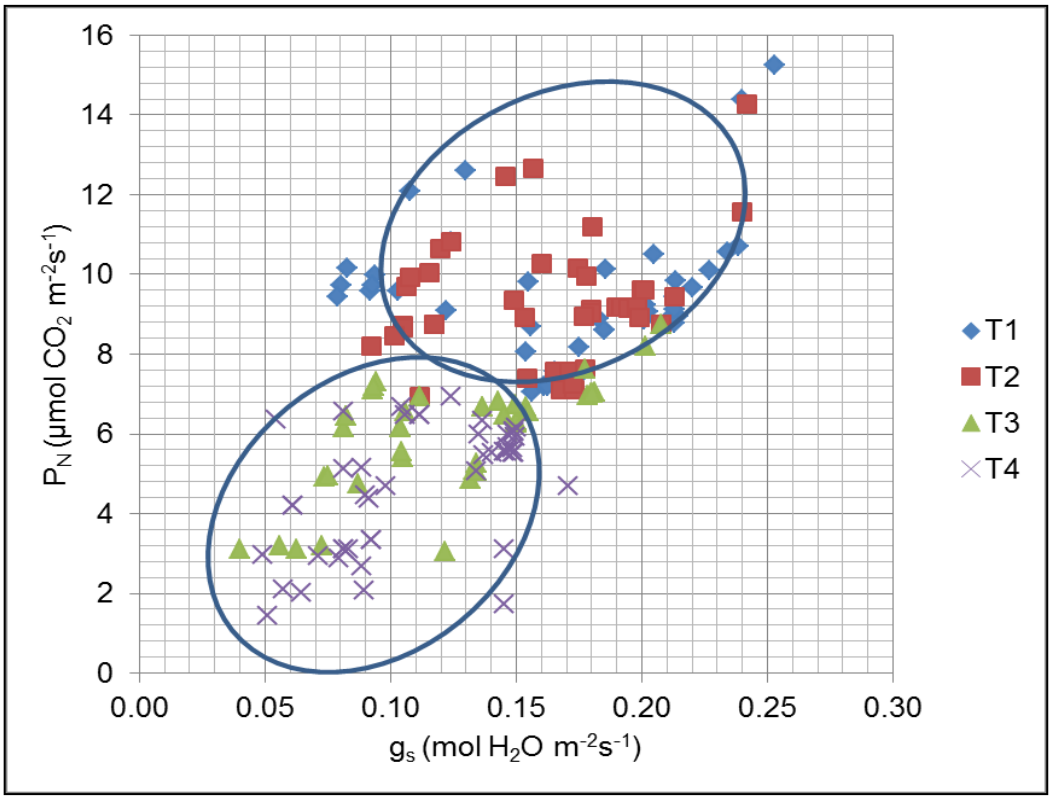


Fig. 5.11: Net photosynthesis (P_N) vs. stomatal conductance (g_s); for the definition of the treatments T1 – T4 see Table 5.1.

Drought stress reduced the stomatal conductance (g_s) resulting in a reduction of net photosynthesis (P_N); this cause/effect relationship is visualized in Fig. 5.11, where P_N was plotted versus g_s. In this graph, the cause/effect relationship between stomatal conductance (g_s) and photosynthesis (P_N) of well-watered saplings (T1, T2) and drought-stressed saplings (T3, T4) clearly differed from each other. However, this relationship is almost same for well-watered saplings infected or non-infected (T1, T2) and drought-stressed saplings infected or non-infected (T3, T4); again confirming, that the black locust mainly have been affected by drought stress.

Table 5.6: ANOVA of the stomatal conductance (g_s, mol H_2O $m^{-2}s^{-1}$) of saplings of the treatments T1 to T4 (n=10-12) of June, July, August, and September measurements, both separately and seasonalized (JJAS = June + July + August + September; n=42), F = February inoculation; J = July inoculation; n=6); n.s. = not significant, * = 0.05, ** = 0.01, *** = 0.001; for the definition of the treatments T1 – T4 see Table 5.1.

g_s	T1xT2	T1xT3	T1xT4	T2xT3	T2xT4	T3xT4	T1/T2x T3/T4	T2FxT2J	T4FxT4J
June	**	***	***	**	**	***	***	n.s	n.s
July	**	***	***	n.s	*	n.s	***	n.s	n.s
August	n.s	***	***	***	***	n.s	***	n.s	n.s
September	**	**	**	n.s	n.s	n.s	n.s	n.s	n.s
JJAS	n.s	***	***	***	***	n.s	***	n.s	n.s

5.2.3. Transpiration

An average transpiration in June was 3.39 and 3.06 mmol H_2O $m^{-2}s^{-1}$ in T1 and T2, and 2.99 and 2.46 mmol H_2O $m^{-2}s^{-1}$ in T3 and T4, respectively. In July, it was 2.54 and 1.38 mmol H_2O $m^{-2}s^{-1}$ in T1 and T2, and 1.1 and 0.85 mmol H_2O $m^{-2}s^{-1}$ in T3 and T4, respectively. In August, it was 2.4 and 2.39 mmol H_2O $m^{-2}s^{-1}$ in T1 and T2, and 1.22 and 1.07 mmol H_2O $m^{-2}s^{-1}$ in T3 and T4, respectively. In September, it was 0.64 and 1.62 mmol H_2O $m^{-2}s^{-1}$ in T1 and T2, and 1.88 and 1.76 mmol H_2O $m^{-2}s^{-1}$ in T3 and T4, respectively (Fig. 5.12a).

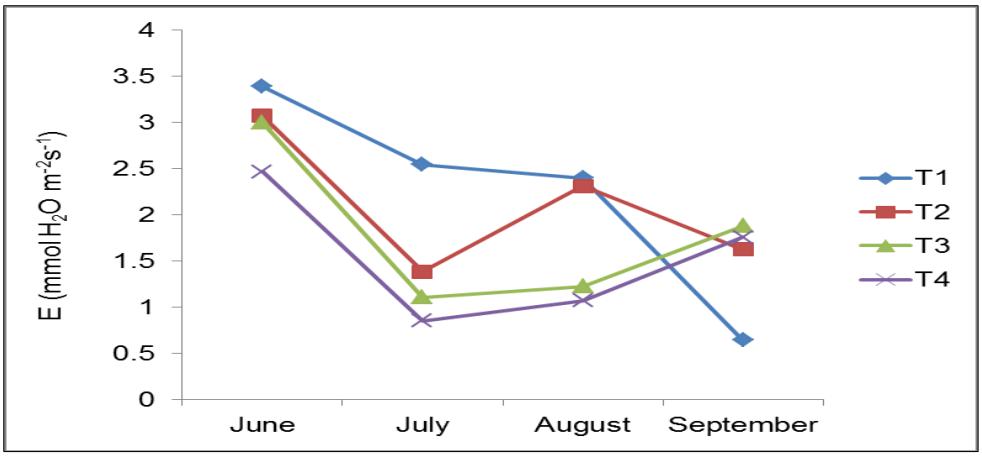


Fig. 5.12a: Leaf transpiration (E, mmol H_2O $m^{-2}s^{-1}$) of the saplings of the treatments T1 to T4 (n=10-12) in June, July, August, and September.

The average seasonal transpiration (E) was significant reduced by 13 and 22% in the drought-exposed saplings T3 and T4, respectively (Fig. 5.12b). It remained higher in well-watered saplings (T1) except in September, whereas it was variable in T2 saplings. However, it was reduced in drought-stressed saplings and the lowest transpiration was measured in T4 saplings in June, July and August (Fig. 5.12a, b).

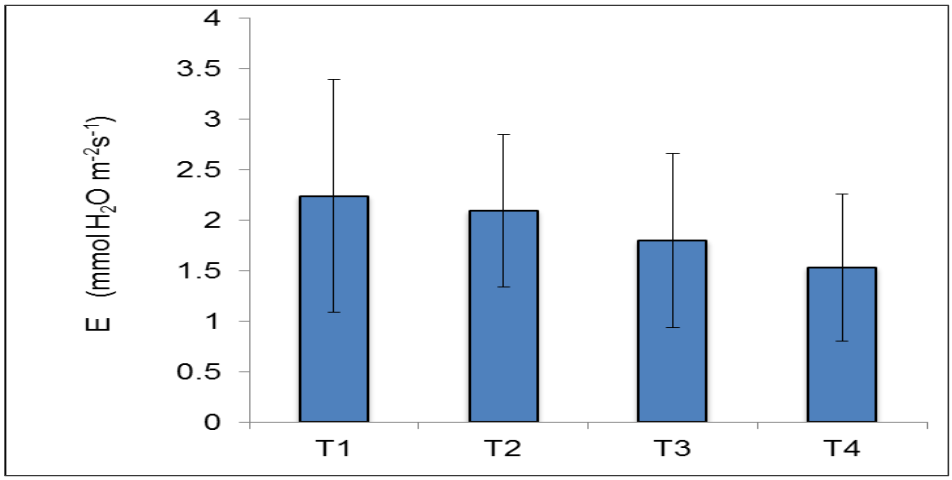


Fig. 5.12b: Leaf transpiration (E, mmol H_2O $m^{-2}s^{-1}$) of treatments T1 to T4. Mean value ± standard deviation of June + July + August + September; n=42; for the definition of the treatments T1 – T4 see Table 5.1.

According to ANOVA, the average seasonal transpiration in well-watered saplings, infected or non-infected (T1, T2) was not significantly different in JJAS (June + July + August + September) (Table 5.7; T1xT2). Similarly, transpiration in drought-stressed saplings, infected or non-infected (T3, T4), was also significantly not different either (Table 5.7; T3xT4).

There were no significant alterations in stomatal conductance of well-watered saplings, no matter if they were infected in July or in February. Similarly, stomatal conductance of drought-stressed saplings was not either affected by the season of infection (Table 5.7; T2FxT2J, T4FxT4J). Moreover, ANOVA results of T1xT3, T1xT4, T2xT3, T2xT4, and T1/T2xT3/T4 were significantly different due to drought stress (Table 5.7).

Table 5.7: ANOVA of leaf transpiration (E, mmol H_2O $m^{-2}s^{-1}$) of saplings of the treatments (T1 to T4; n=10-12) of June, July, August, and September measurements, both separately and seasonalized (JJAS = June + July + August + September; n=42), F = February inoculation; J = July inoculation; n=6); n.s. = not significant, * = 0.05, ** = 0.01, *** = 0.001; for the definition of the treatments T1 – T4 see Table 5.1.

E	T1xT2	T1xT3	T1xT4	T2xT3	T2xT4	T3xT4	T1/T2x T3/T4	T2FxT2J	T4FxT4J
June	**	**	***	n.s	**	*	***	n.s	n.s
July	**	***	***	n.s	n.s	n.s	***	n.s	n.s
August	n.s	***	***	***	***	n.s	***	*	n.s
September	*	***	***	n.s	n.s	n.s	***	n.s	n.s
JJAS	n.s	**	***	n.s	**	n.s	***	n.s	n.s

5.2.4. Mesophyll conductance

An average mesophyll conductance in June was 0.03 and 0.028 mol $m^{-2}s^{-1}$ in T1 and T2, and 0.022 and 0.017 mol $m^{-2}s^{-1}$ in T3 and T4, respectively. In July, it was 0.029 and 0.032 mol $m^{-2}s^{-1}$ in T1 and T2, and 0.025 and 0.016 mol $m^{-2}s^{-1}$ in T3 and T4, respectively. In August, it was 0.037 and 0.036 mol $m^{-2}s^{-1}$ in T1 and T2, as well as 0.014 and 0.01 mol $m^{-2}s^{-1}$ in T3 and T4, respectively. In September, it was 0.052 and 0.041 mol $m^{-2}s^{-1}$ in T1 and T2, as well as 0.023 and 0.019 mol $m^{-2}s^{-1}$ in T3 and T4, respectively (Fig. 5.13a).

The mesophyll conductance remained higher in well-watered saplings (T1, T2), in September it was highest in T1. It was, however, reduced in drought-stressed saplings and the lowest stomatal conductance was measured in T4 saplings in June, July, August and September (Fig. 5.13a, b).

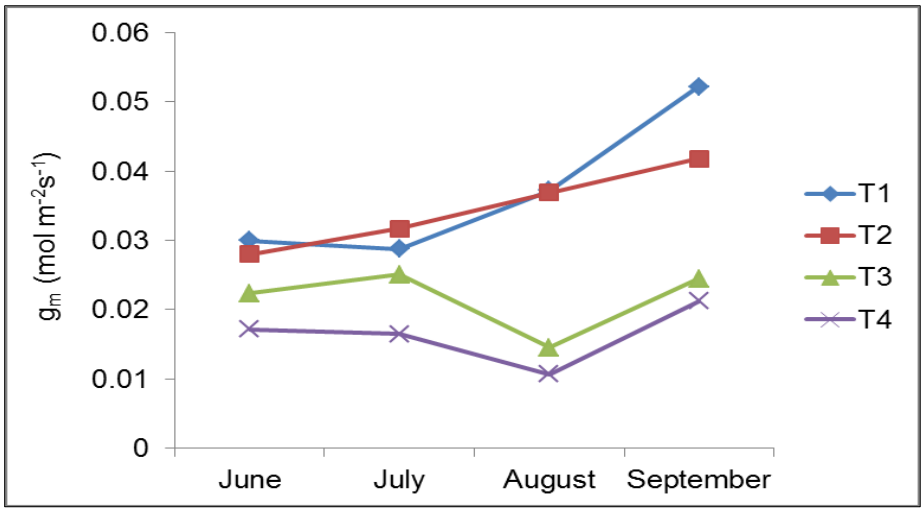


Fig. 5.13a: Mesophyll conductance (g_m, mol $m^{-2}s^{-1}$) of the saplings of the treatments T1 to T4 (n=10-12) in June, July, August, and September.

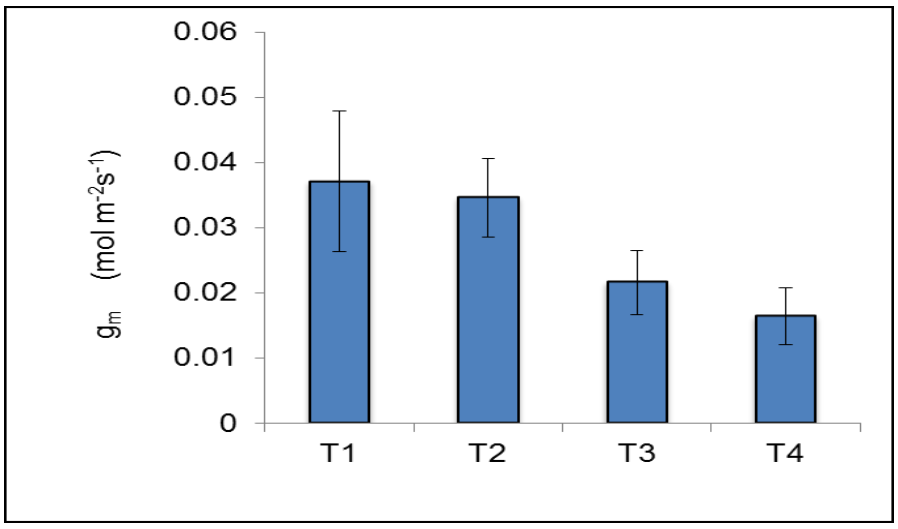


Fig. 5.13b: Mesophyll conductance (g_m, mol $m^{-2}s^{-1}$) of treatments T1 to T4. Mean value ± standard deviation of June + July + August + September; n=42; for the definition of the treatments T1 – T4 see Table 5.1.

According to ANOVA (Table 5.8), the average mesophyll conductance in well-watered saplings, infected or non-infected (T1, T2) was not significantly different throughout the season, except in September (T1xT2). However, mesophyll conductance in drought-stressed saplings, infected or non-infected (T3, T4), was significantly different throughout except in September (Table 5.8; T3xT4).

There were no significant alterations in mesophyll conductance of well-watered saplings, no matter if they were infected in July or in February. Similarly, mesophyll conductance of drought-stressed saplings was not either affected by the season of infection (Table 5.8; T2FxT2J, T4FxT4J). Moreover, according to ANOVA results T1xT3, T1xT4, T2xT3, T2xT4, and T1/T2xT3/T4 were significantly different throughout the season and collectively (JJAS) as well due to drought stress (Table 5.8).

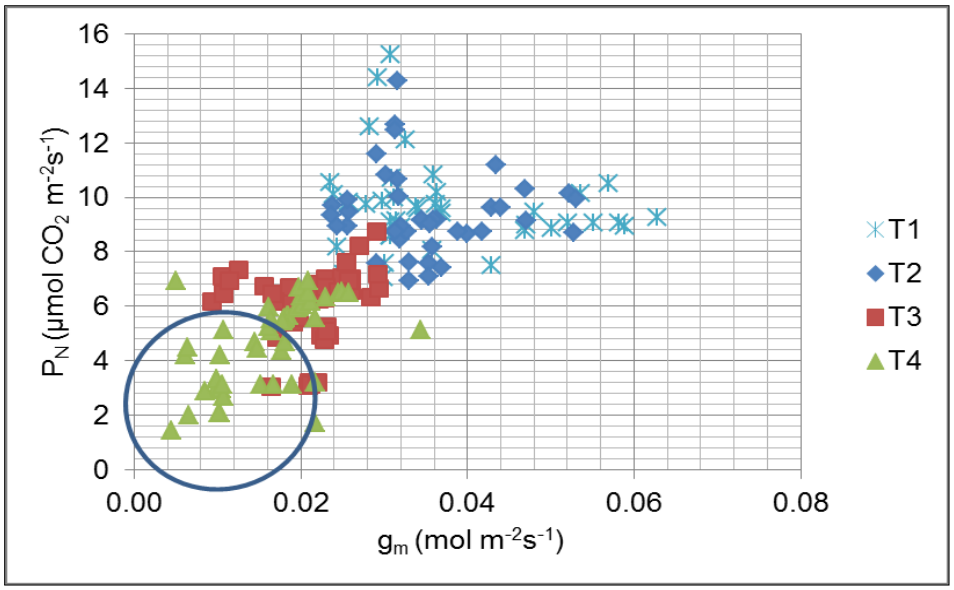


Fig. 5.14: Net photosynthesis (P_N) vs. mesophyll conductance (g_m); T4 saplings exhibited lowest photosynthesis associated with the lowest mesophyll conductance (as shown in the circle); for the definition of the treatments T1 – T4 see Table 5.1.

The pathogen did not affect the mesophyll conductance of the well-watered saplings from June to August but in September. In the drought-stressed saplings, however, a significant limitation of mesophyll conductance was noticed in the T4 saplings as compared to T3 saplings in June, July and August, but not in September. Similarly, the mesophyll conductance averaged over JJAS (June, July, August, and September) significantly differed between T3 and T4 (highlighted in Table 5.8). Moreover, a reduced net photosynthesis was associated with a lowered mesophyll conductance (Fig. 5.14); it clearly depicts the role of pathogen in the physiology of drought-stressed saplings. It should be added, that net-photosynthesis and mesophyll conductance were significantly different between drought-stressed saplings infected and non-infected.

Table 5.8: ANOVA of mesophyll conductance (g_m, mol $m^{-2}s^{-1}$) of saplings of the treatments (T1 to T4; n=10-12) of June, July, August, and September measurements, both separately and seasonalized (JJAS = June + July + August + September; n=42), F = February inoculation; J = July inoculation; n=6); n.s. = not significant, * = 0.05, ** = 0.01, *** = 0.001; for the definition of the treatments T1 – T4 see Table 5.1.

g_m	T1xT2	T1xT3	T1xT4	T2xT3	T2xT4	T3xT4	T1/T2x T3/T4	T2FxT2J	T4FxT4J
June	n.s	***	***	***	***	***	***	n.s	n.s
July	n.s	*	***	***	***	***	***	n.s	n.s
August	n.s	***	***	***	***	**	***	n.s	n.s
September	**	***	***	***	***	n.s	***	*	n.s
JJAS	n.s	***	***	***	***	***	***	n.s	n.s

5.3. Biomass

The total biomass (leaves, branches, stems and roots) of drought-stressed saplings was significantly smaller than of non-stressed ones.

5.3.1. Average dry weight of leaves

The average dry weight of leaves was 0.46 and 0.48 kg in T1 and T2, respectively, whereas in T3 and T4 it was 0.12 and 0.13 kg, respectively (Fig. 5.15). The average dry weight of leaves of well-watered and drought-stressed saplings was highly significantly different, but there was no difference due to the pathogen, neither in well-watered nor in drought-stressed saplings.

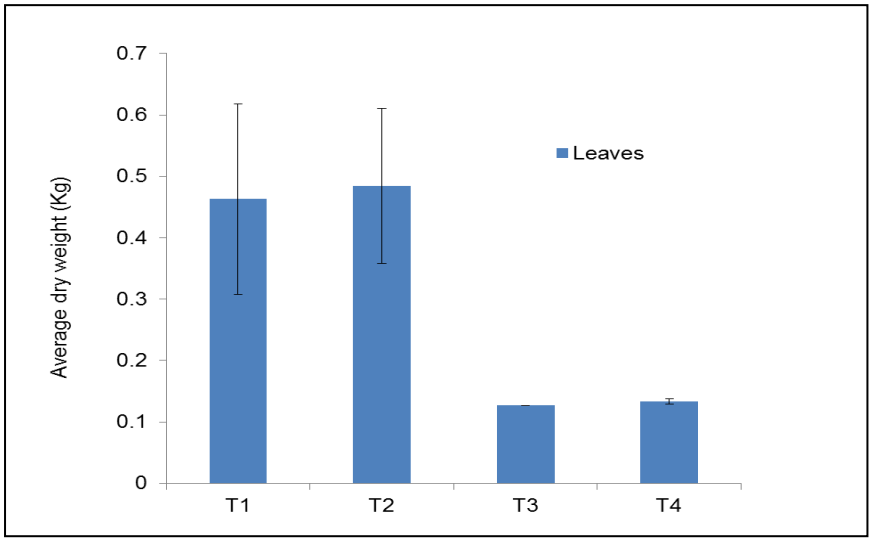


Fig. 5.15: Average dry weight ± standard deviation of leaves of T1 to T4 saplings after harvest and oven drying (n=3 for T1 and T3; n=6 for T2 and T4); for the definition of the treatments T1 – T4 see Table 5.1.

5.3.2. Average dry weight of stems

The average dry weight of stems was not different for T1 and T2, as it was 1.9 kg for both treatments, whereas in T3 and T4 saplings the average dry weight was only 0.85 and 0.78 kg, respectively (Fig. 5.16). These differences between well-watered and drought-stressed saplings were highly significant, but there was no difference resulting from the infection, neither in the well-watered nor in the drought-exposed saplings.

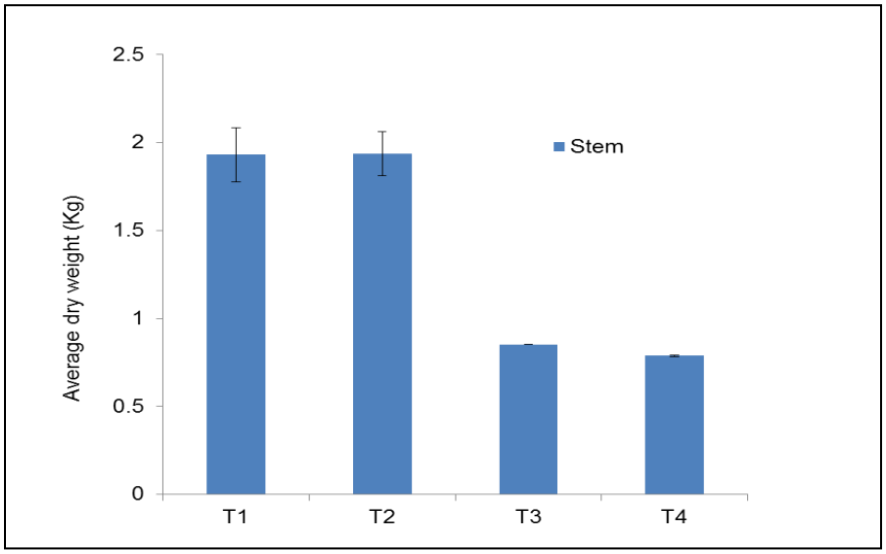


Fig. 5.16: Average dry weight ± standard deviation of stems of T1 to T4 saplings after harvest, and oven drying (n=3 for T1 and T3; n=6 for T2 and T4; for the definition of the treatments T1 – T4 see Table 5.1.

5.3.3. Average dry weight of branches

The average dry weight of branches was significantly different between well-watered (T1, T2) and drought-stressed saplings (T3, T4) but not between T1 and T2, as it was 1.4 kg for both treatments; also between T3 and T4 saplings, the difference was insignificant (0.37 and 0.36 kg, respectively) (Fig. 5.17).

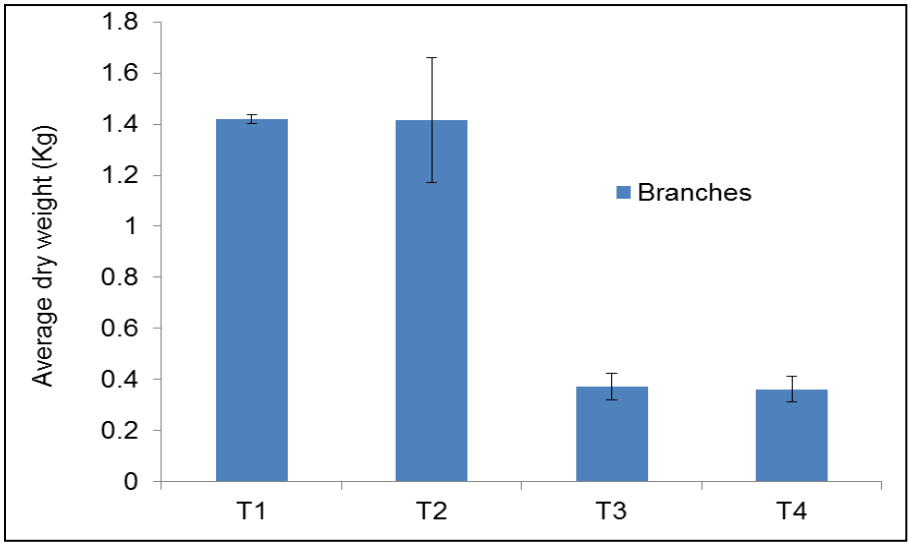


Fig. 5.17: Average dry weight ± standard deviation of branches of T1 to T4 saplings after harvest, and oven drying (n=3 for T1 and T3; n=6 for T2 and T4; for the definition of the treatments T1 – T4 see Table 5.1.

5.3.4. Average dry weight of roots

The average dry weight of roots was 1.7 and 1.8 kg in T1 and T2, respectively, whereas in T3 and T4 it was 0.59 and 0.53 kg, respectively (Fig. 5.18). The difference between well-watered and drought-stressed saplings was highly significant, but there was no significant difference resulting from the pathogen infection, neither in well-watered nor in drought-stressed saplings. However, the average dry weight of roots was slightly lowered when the saplings grew up under drought stress plus a pathogen infection.

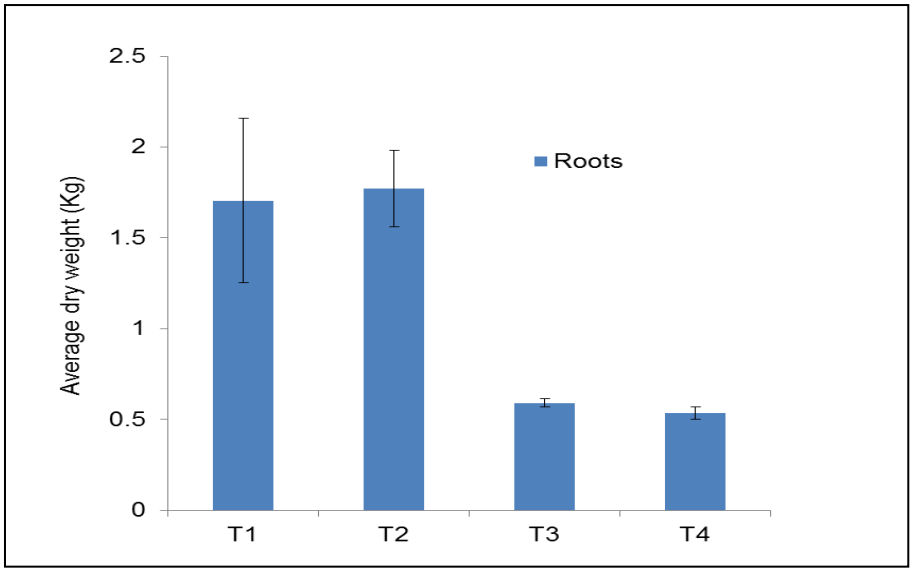


Fig. 5.18: Average dry weight ± standard deviation of roots of T1 to T4 saplings after harvest and oven drying (n=3 for T1 and T3; n=6 for T2 and T4); for the definition of the treatments T1 – T4 see Table 5.1.

5.3.5. Total biomass dry weight and root-to-shoot ratio

The total biomass dry weight (leaves, branches, stems and roots) of drought-stressed saplings was significantly reduced by 64% than of non-stressed ones (Fig. 5.19). The presence of the pathogen did not significantly affect the biomass neither of the well-watered nor of the drought-stressed saplings. However, the lowest biomass was recorded for the saplings that were simultaneously stressed by drought and infection. A shift of the root-to-shoot ratio was not found in any of the treatments. Calculated root-to-shoot ratio varied from 0.45 to 0.46 for T1 and T2 as well as 0.43 to 0.42 for T3 and T4, respectively.

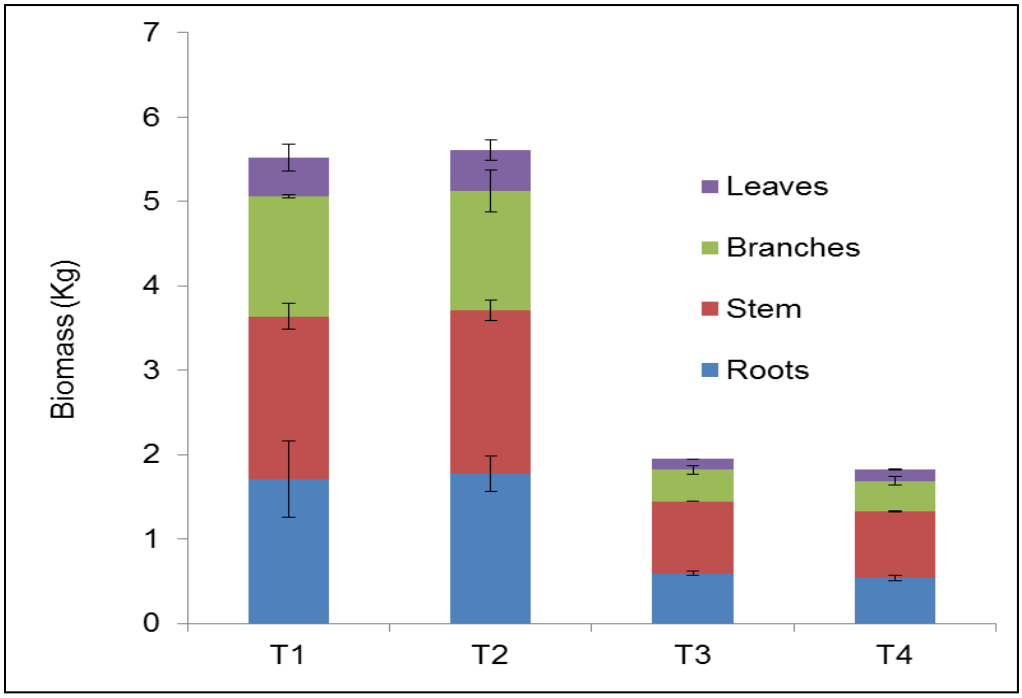


Fig. 5.19: Mean values of total biomass dry weight ± standard deviations of leaves, branches, stems, and roots of T1 to T4 saplings after harvest and oven drying (n=3 for T1 and T3; n=6 for T2 and T4); for the definition of the treatments T1 – T4 see Table 5.1.

5.4. Host reaction

Trees are able to encapsulate wounded and decayed tissues by specific defence reactions. This part of the present study was conducted to reveal the impacts of drought stress on the efficiency of compartmentalization of damage or decay in black locust. For this purpose, host reactions to non-infected and infected wounds, visible as discoloration and callus formation both of well-watered and drought-stressed saplings were measured after harvesting of the stems.

5.4.1. Discoloration

Both non-infected and infected wounds induced dark-brown discolorations in well-watered and drought-stressed saplings (Figs. 5.23 and 5.24). In general, the

discoloration was longer in axial direction, ranging from 39 to 75 mm, than in tangential (12-16 mm) and radial (14-16 mm) direction (Figs. 5.20, 5.21, and 5.22).

5.4.1.1. Discoloration in axial direction

There was no difference in the average extent of axial discoloration in drought-stressed saplings between infected and non-infected wounds (T4, T4c) no matter if they were inoculated in July or in February. In well-watered saplings a slight difference was visible between infected and non-infected wounds (T2, T2c) inoculated in February (Fig. 5.20).

In well-watered saplings, inoculated in July, the total average extent of axial discoloration (above and below the hole) was 39 and 40 mm, in infected and non-infected wounds (T2 and T2c), respectively. In drought-stressed saplings, in infected and non-infected wounds (T4 and T4c), the total average extent of axial discoloration was 75 and 72 mm, respectively (Fig. 5.20).

In well-watered saplings, inoculated in February, the average extent of axial discoloration (above and below the hole) was 55 and 45 mm, in infected and non-infected wounds (T2 and T2c), respectively. In drought-stressed saplings, due to infected and non-infected wounds (T4 and T4c); the average extent of axial discoloration was similar to the saplings that were inoculated in July (Fig. 5.20).

The discoloration columns in the well-watered saplings were longer when the infection occurred in February as compared to July. In drought-stressed saplings the axial discoloration was distinctly longer, 75 mm, than in the well-watered saplings without any difference as to the time of wounding (Fig. 5.20).

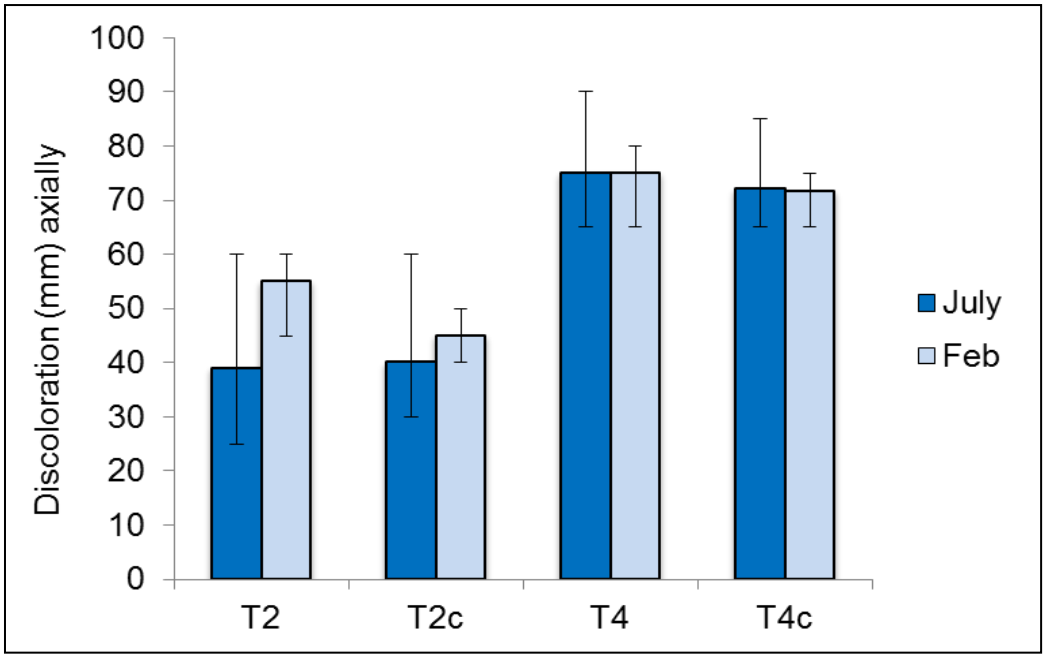


Fig. 5.20: Average extent of axial discoloration in saplings inoculated in July 2010 and February 2011. T2 = well-watered with infected wound, T2c = well-watered with non-infected wound, T4 = drought-stressed with infected wound, T4c = drought-stressed with non-infected wound; error bars indicate maximum and minimum values around the mean (n=3).

5.4.1.2. Discoloration in radial (inward) direction

All in all, there were no distinct differences in the radial extension of the discoloration between infected and aseptic wounds both in well-watered and drought-stressed saplings, no matter if they were inoculated in July or in February (Fig. 5.21).

In well-watered saplings inoculated in July, around infected and non-infected wounds (T2 and T2c), the average extent of radial discoloration was 15 and 14 mm, respectively. In drought-stressed saplings, around infected and non-infected wounds (T4 and T4c), the average extent of radial discoloration was 15 mm for both infected and aseptic wounds (Fig. 5.21).

In well-watered saplings inoculated in February, the average radial discoloration both in infected and non-infected wounds (T2 and T2c) was 16.3 mm, whereas in drought-

stressed saplings the average radial discoloration, both in infected and non-infected wounds (T4 and T4c), was 15.6 mm (Fig. 5.21).

The average radial discoloration columns were slightly larger in all saplings infected in February than in July. There was no distinct difference in the average radial discoloration between infected and aseptic wounds, both in well-watered and drought-stressed saplings (Fig. 5.21).

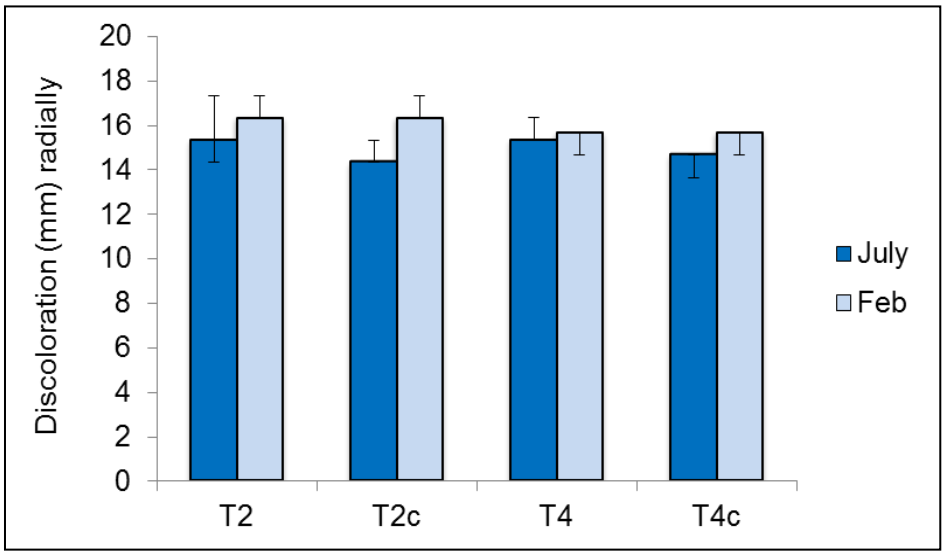


Fig. 5.21: Average extent of radial discoloration in saplings inoculated in July 2010 and February 2011. T2 = well-watered with infected wound, T2c = well-watered with non-infected wound, T4 = drought-stressed with infected wound, T4c = drought-stressed with non-infected wound; error bars indicate maximum and minimum values around the mean (n=3).

5.4.1.3. Discoloration in tangential direction

There was no difference in average tangential discoloration between infected and non-infected wounds, both in well-watered and drought-stressed saplings.

In well-watered saplings inoculated in July, the average tangential discoloration was 13.3 mm for both infected and non-infected wounds (T2, T2c), whereas in drought-

stressed saplings the average tangential discoloration was 12 mm in infected (T4) and 12.3 mm in non-infected wounds (T4c) (Fig. 5.22).

There was a slight difference in average tangential discoloration between infected and non-infected wounds both in well-watered and drought-stressed saplings.

In well-watered saplings inoculated in February, the average tangential discoloration was 15.6 mm in infected (T2) and 15 mm in non-infected wounds (T2c), whereas in drought-stressed saplings the average tangential discoloration was 14 mm in infected (T4) and 13 mm in non-infected wounds (T4c) (Fig. 5.22).

The average tangential discoloration columns were slightly wider in all saplings infected in February as compared to July. There was no distinct difference in the average tangential discoloration between infected and non-infected wounds both in well-watered and drought-stressed saplings (Fig. 5.22).

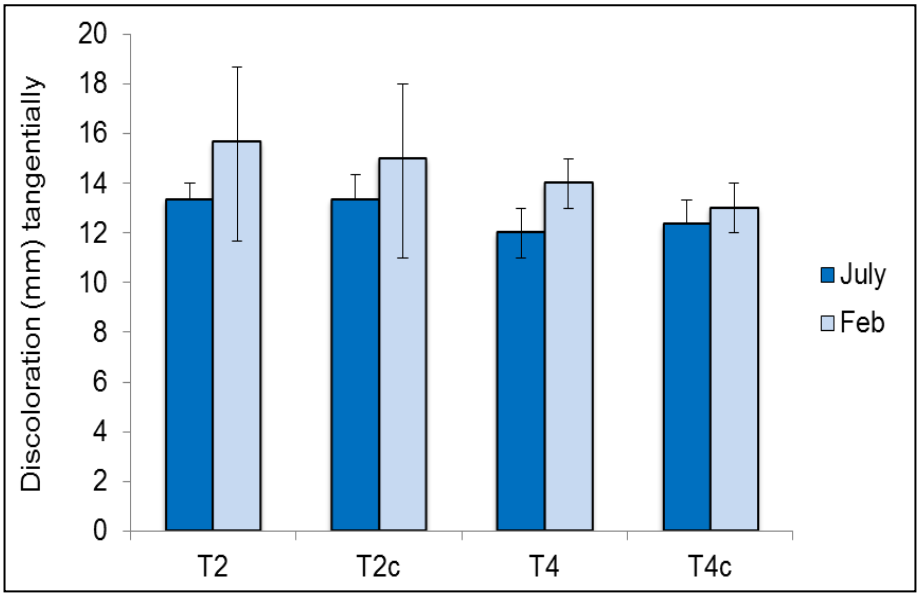


Fig. 5.22: Average extent of tangential discoloration in saplings inoculated in July 2010 and in February 2011. T2 = well-watered with infected wound, T2c = well-watered with non-infected wound, T4 = drought-stressed with infected wound, T4c = drought-stressed with non-infected wound; error bars indicate maximum and minimum values around the mean (n=3).

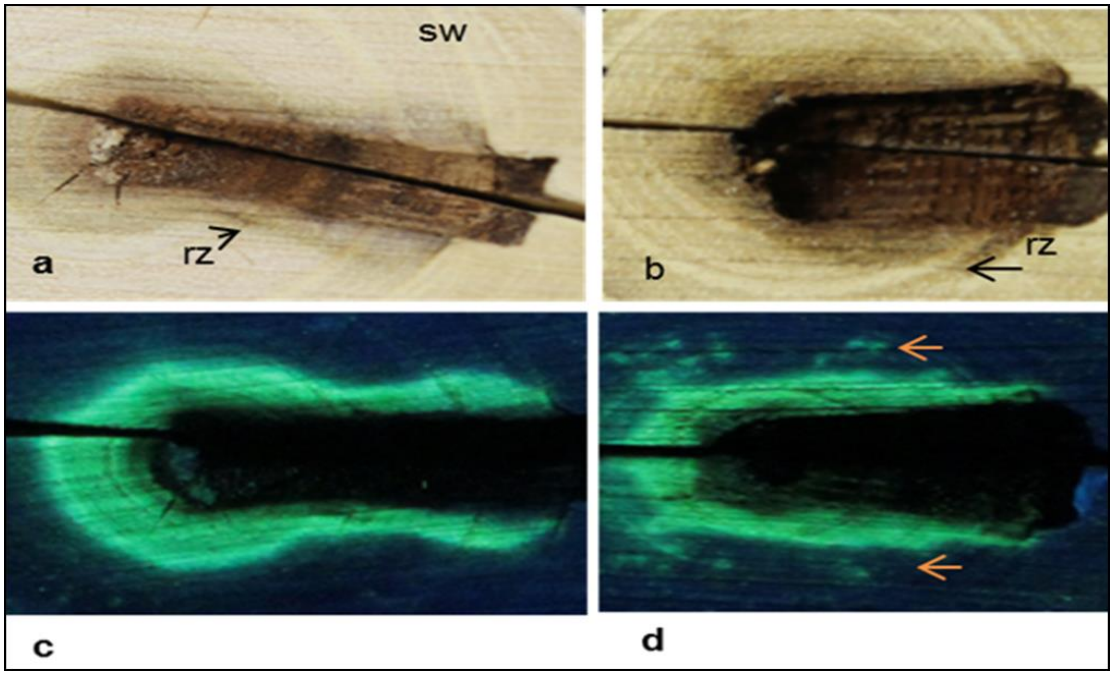


Fig. 5.23: Cross-sections through black locust saplings inoculated in February with *Armillaria mellea*; well-watered (a, c), drought-stressed (b, d); c and d under UV light; rz, reaction zone; sw, sound wood.

5.4.1.4. Discoloration in radial and tangential direction under UV light

There was no distinct difference in radial and tangential discoloration between infected and non-infected wounds both in well-watered and drought-stressed saplings (Fig. 5.21, 5.22). However, under UV light (Fig. 5.23 a, c) a stronger host response in form of a distinct reaction zone is visible in the well-watered saplings, whereas in drought-stressed saplings that were infected in February (Fig. 5.23 d) the reaction zone apparently might not be effective enough to protect the functional sapwood around the bore holes by means of flavonoids components, scattered outside the reaction zone. Without an effective barrier, there is a high possibility that the inoculated pathogen could have breached the defence line.

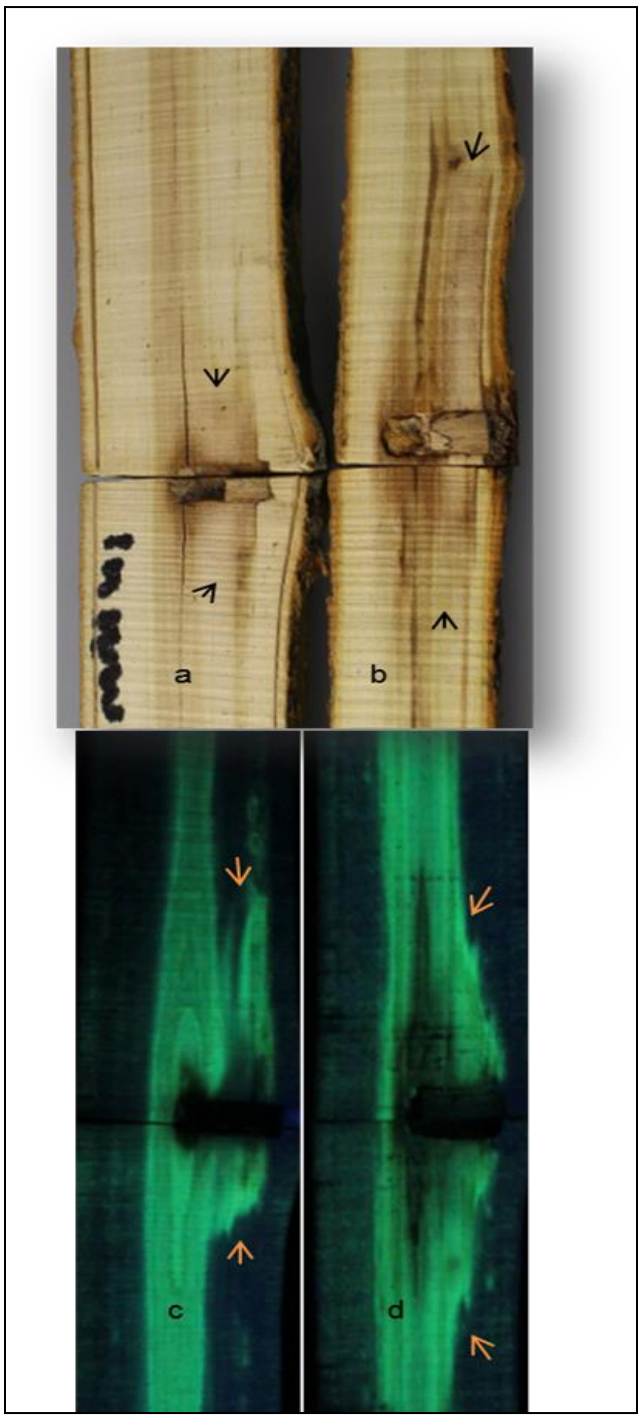


Fig. 5.24: Longitudinal section through black locust inoculated with *Armillaria mellea*: well-watered (a, c), drought-stressed (b, d); a and b depict the axial extent of discoloration (arrows) in well-watered and drought-stressed saplings, respectively; c and d are photographed under UV light indicating a larger axial discoloration and decay in drought-stressed saplings (d, arrows).

5.5. Callus formation

The callus formed in direct vicinity around the wound was remarkably more intense in well-watered than in drought-stressed saplings. In well-watered saplings with non-infected dowels (T2c) and with infected dowels (T2), in the July bore holes, the callus was 10.3 mm and 9.3 mm thick, respectively. However, in drought-stressed saplings with non-infected dowels (T4c) and with infected dowels (T4), the callus was only 5.7 and 3.3 mm thick, respectively, in July bore holes (Fig. 5.25).

The callus formation was distinctly less intense in saplings inoculated in February than in July, both in well-watered and drought-stressed saplings. The callus was 4.7 and 5.3 mm in T2 and T2c, respectively, whereas there was no callus formation at all in T4 but of 3.3 mm thickness in T4c (Fig. 5.25).

All in all, the callus formation was distinctly more intense when the saplings were inoculated in July as compared to February (Fig. 5.25), no matter if they were well-watered or drought-stressed.

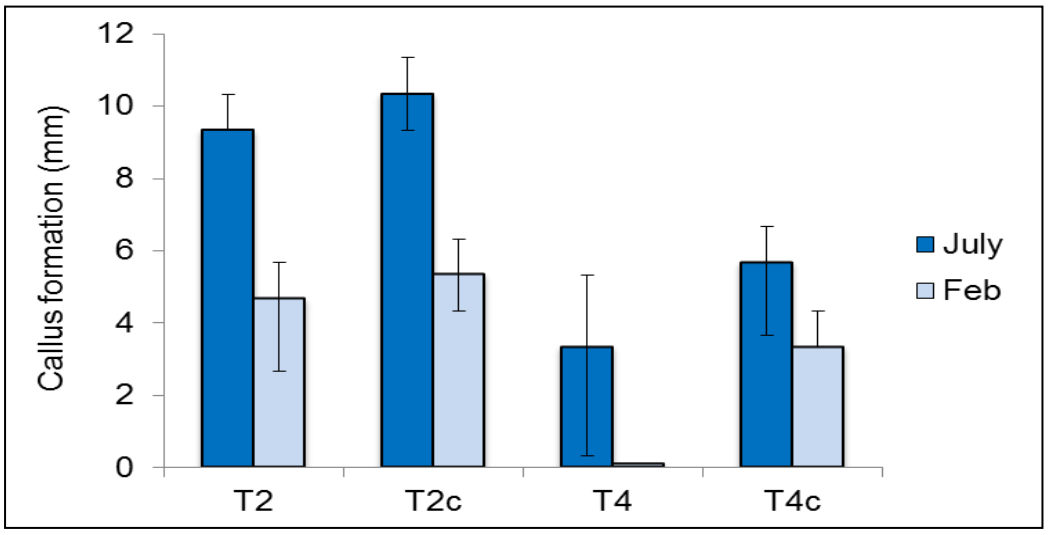


Fig. 5.25: Callus formation in black locust inoculated in July 2010 and February 2011 and harvested in July 2012. T2 = well-watered with infected wound, T2c = well-watered with non-infected wound, T4 = drought-stressed with infected wound, T4c = drought-stressed with non-infected wound; error bars indicate the maximum and minimum values around the mean (n=3).

The situation in the saplings inoculated in February (T4 and T4c) highlights the difference between an aseptic wound vs. an infected wound in view of the intensity of callus formation; directly around the infected wound no callus tissue was built, instead the callus formation ended further apart from the wound (Fig. 5.26).

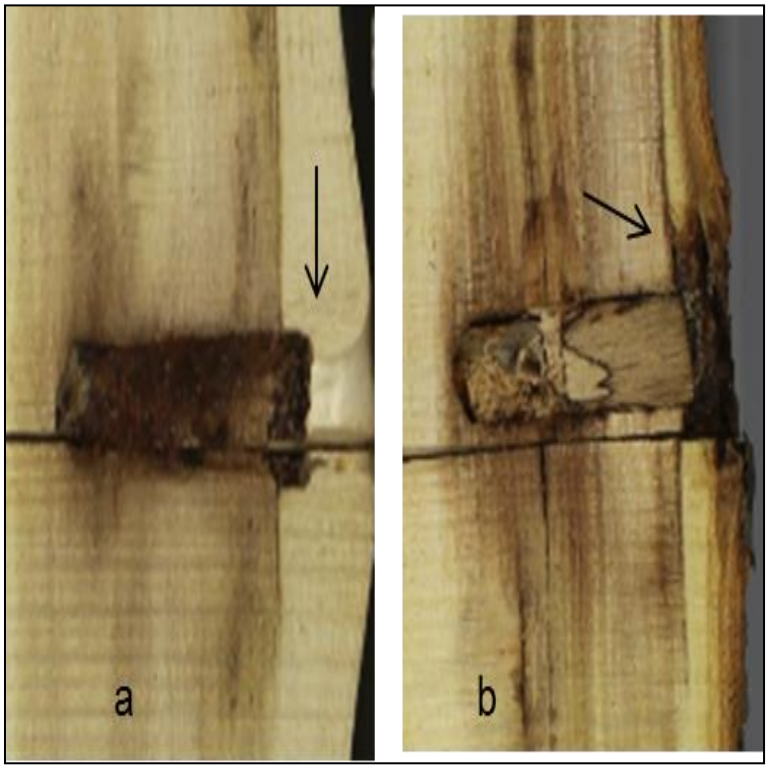


Fig. 5.26: Callus formation around a non-infected wound of a drought-stressed sapling (T4c) (arrow) (a); no callus formation around an infected wound of a drought-stressed sapling (T4) inoculated in February (b, arrow).

5.6. Determination of non-structural carbohydrates

The occurrence and distribution of non-structural carbohydrates (glucose, fructose, sucrose and starch) were studied both in non-infected wounds and in infected wounds in all saplings of black locust.

The amounts of non-structural carbohydrates were 0-20 nmol/mg in the decay and reaction zones of non-infected as well as in infected wounds of well-watered saplings (P7, P10 and P11), inoculated in July. Their amounts were similarly high in bark and sapwood, except in sapling P11 where the amount of starch was negligible in sapwood of infected wound. In adjacent bark of non-infected wounds, amount of sucrose varied from 150 to 204 nmol/mg, and in infected wound, it varied from 142 to 165 nmol/mg. Similarly, in adjacent bark of non-infected wounds, amount of starch varied from 126 to 231 nmol/mg, and in infected wound, it varied from 169 to 237 nmol/mg. However, in sapwood, amount of starch varied from 46 to 306 nmol/mg, and 16 to 257 nmol/mg in non-infected wound and infected wound, respectively (Fig. 5.27).

In well-watered saplings inoculated in February (P4, P5 and P6), the amounts of non-structural carbohydrates were also lower in decay and reaction zones of non-infected wounds and in infected wounds, except in sapling P4, where the amount of starch was slightly higher in decay and reaction zones than in the other saplings. In P4, in decay and reaction zones of non-infected wound the amount of starch was 61 and 77 nmol/mg; and of infected wound it was 59 and 91 nmol/mg, respectively. Similarly, their amounts coincide in adjacent bark and sapwood, both in non-infected wounds and in infected wounds with minor differences. In all saplings of this treatment, both in adjacent bark of infected and non-infected wounds, the amount of sucrose varied from 80 to 195 nmol/mg and amount of starch varied from 104 to 269 nmol/mg. Moreover, both in non-infected and infected wounds, the amount of starch varied from 94 to 377 nmol/mg in sapwood (Fig. 5.28).

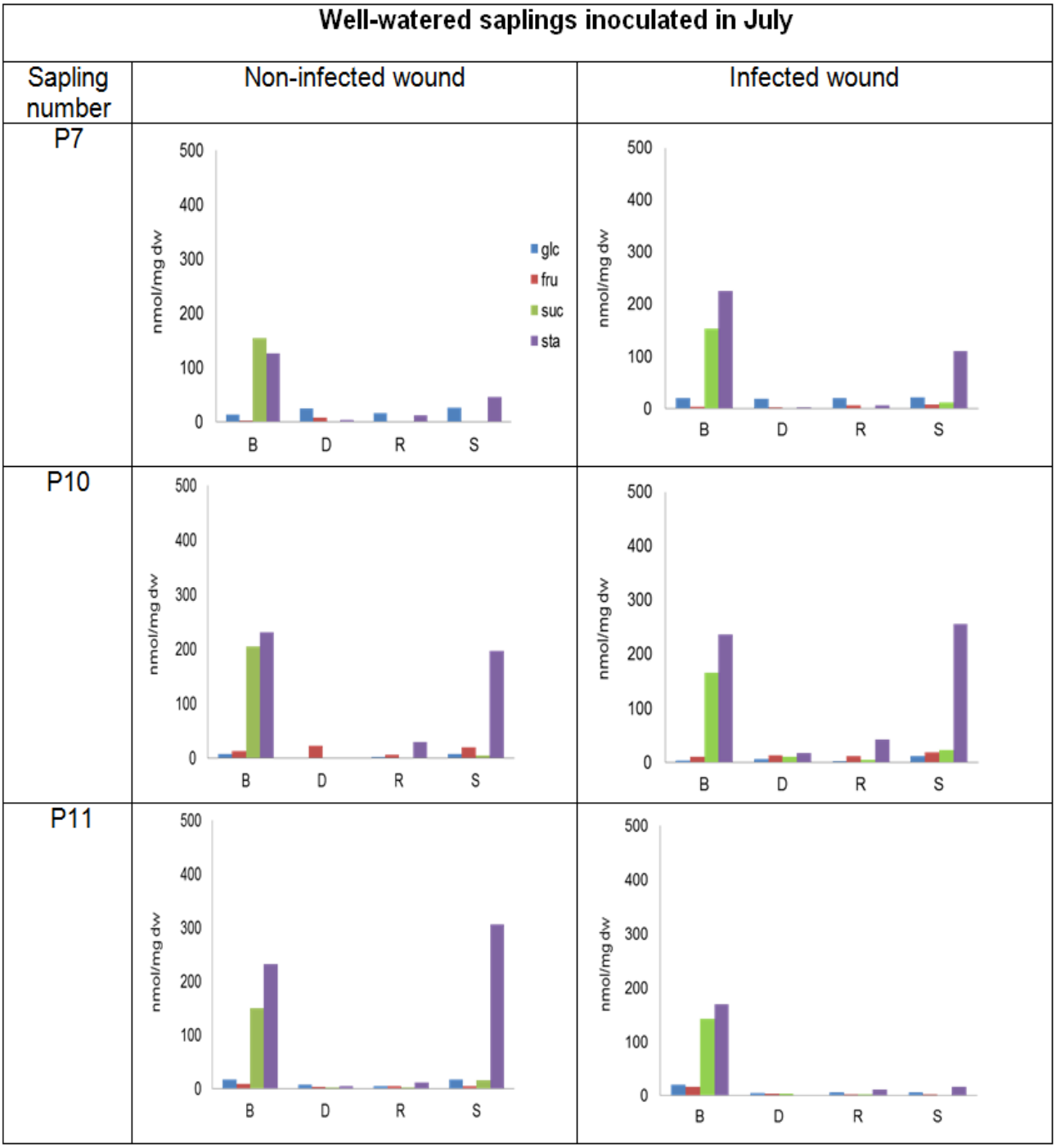


Fig. 5.27: Non-structural carbohydrates (glucose, fructose, sucrose, and starch) in various tissues of well-watered black locust inoculated in July 2010 and harvested in July 2012. B = bark, D = decay zone, R = reaction zone, S = sapwood.

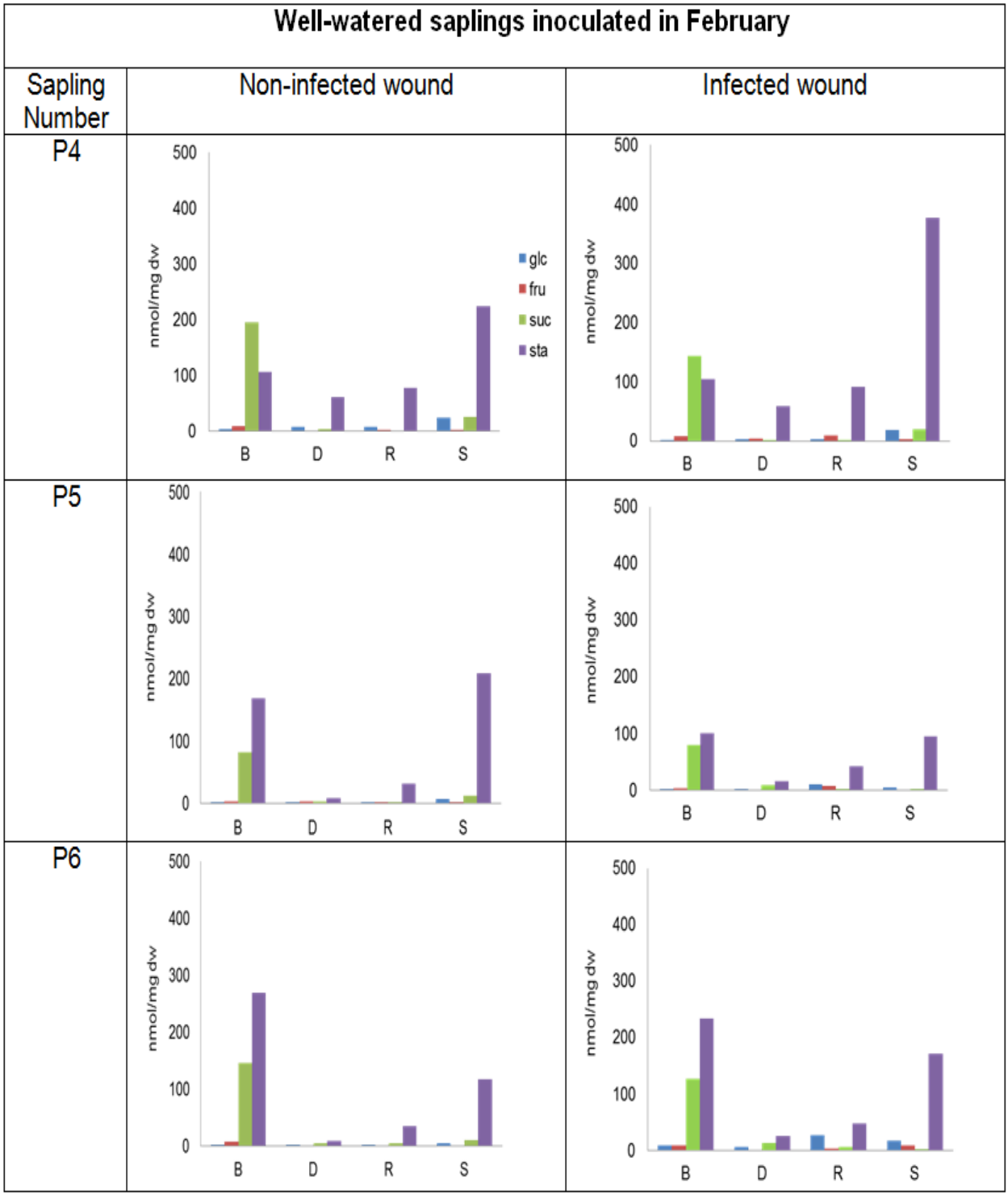


Fig. 5.28: Non-structural carbohydrates in well-watered black locust inoculated in February 2011 and harvested in July 2012; for abbreviations, see Fig. 5.27.

In drought-stressed saplings (P7, P8 and P9) inoculated in July, variable amounts of non-structural carbohydrates were measured in decay and reaction zones. In P9, the amount of starch was higher (128 nmol/mg) in the reaction zone of non-infected and 78 nmol/mg in infected wound. In P8 and P9, in adjacent bark of non-infected wound, amounts of starch varied from 99 to 253 nmol/mg, respectively; while in adjacent bark of infected wound it ranged from 294 to 328 nmol/mg. Additionally, in these saplings, both in non-infected and infected wounds, the amount of starch varied from 203 to 300 nmol/mg in sapwood, except in P7, where the lowest amount of non-structural carbohydrates was noted in adjacent bark, decay zone, reaction zone and sapwood (Fig. 5.29).

In drought-stressed saplings (P1, P3 and P4) inoculated in February, the trend in the amounts of non-structural carbohydrates was coinciding in the different zones of non-infected and infected wounds in almost all saplings. The amounts of sucrose and starch were not conspicuous in adjacent bark. However, in P4, the amounts of sucrose and starch were 203 and 88 nmol/mg, respectively. Moreover, the amount of starch was higher in decay and reaction zones, both in non-infected wounds and in infected wounds. In decay and reaction zones, it varied from 18 to 63 nmol/mg and 17 to 193 nmol/mg, respectively. In these saplings, in sapwood surrounding non-infected and infected wounds, amount of starch remained higher; varied from 145 to 393 nmol/mg (Fig. 5.30).

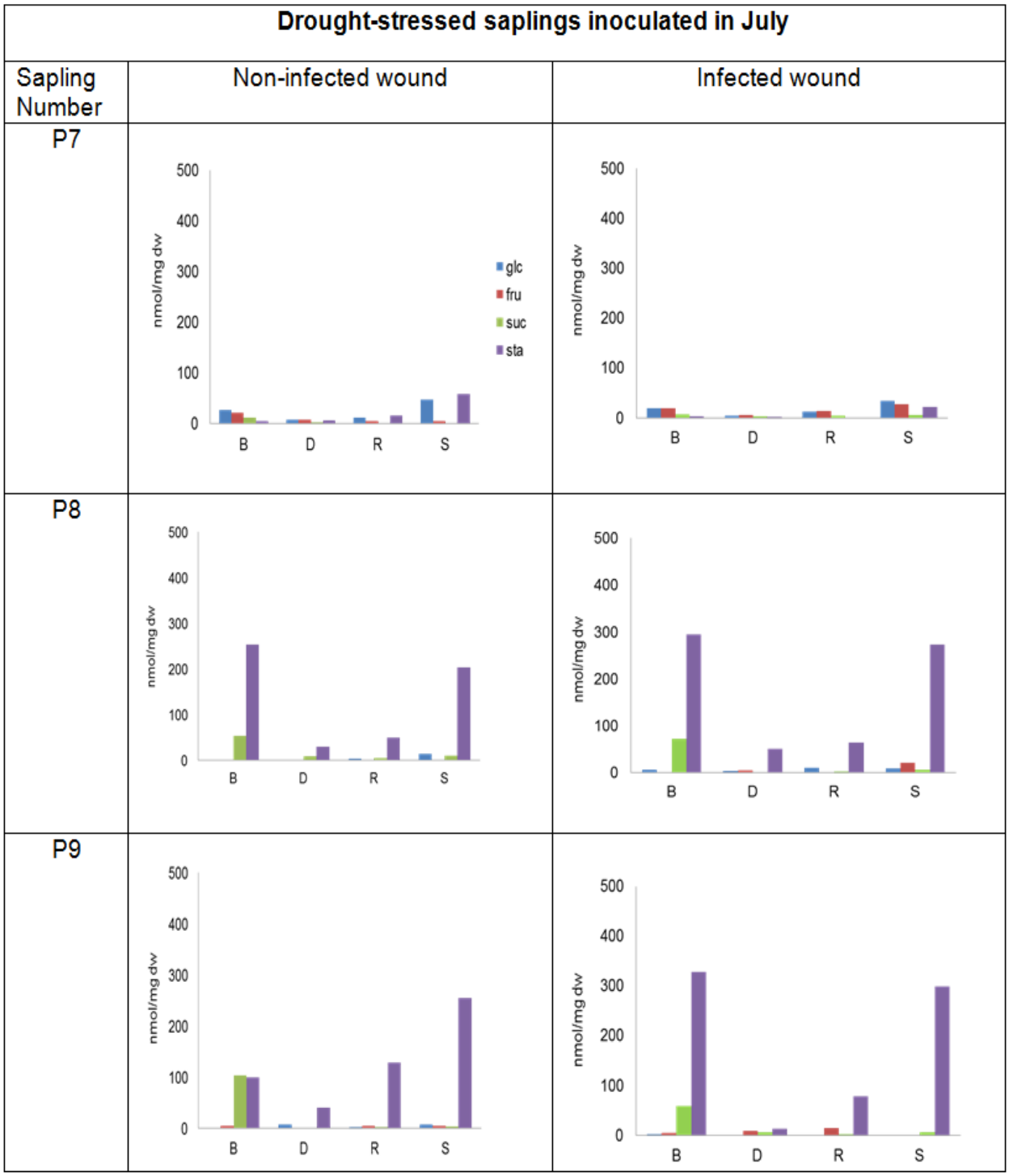


Fig. 5.29: Non-structural carbohydrates in drought-stressed black locust inoculated in July 2010 and harvested in July 2012; for abbreviations, see Fig. 5.27.

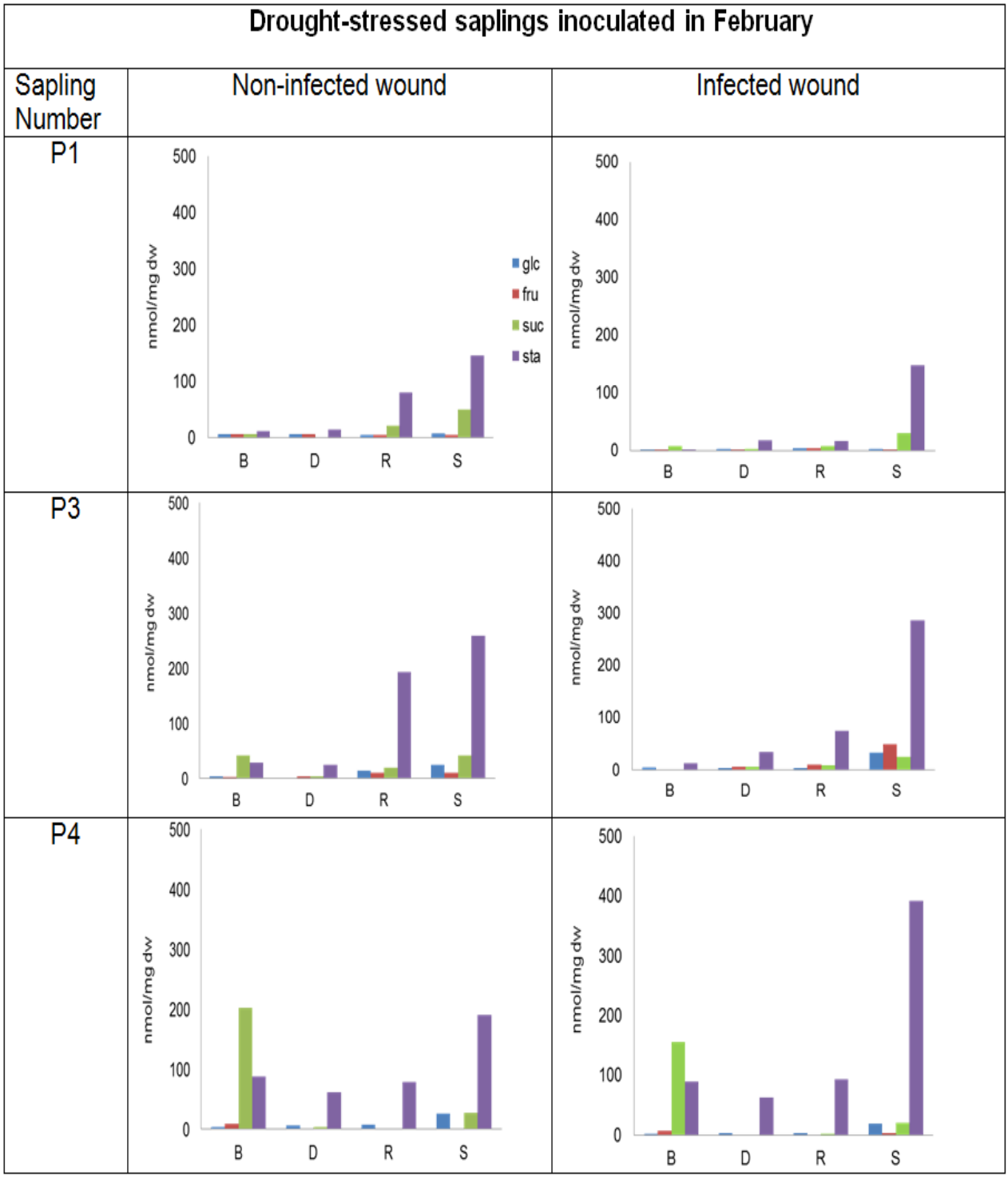


Fig. 5.30: Non-structural carbohydrates in drought-stressed black locust inoculated in February 2011 and harvested in July 2012; for abbreviations, see Fig. 5.27.

Overall, the starch and sucrose content in the bark of well-watered saplings was higher than of drought-stressed saplings, and a large amount of starch was found in sapwood, both in well-watered and drought-stressed saplings. In contrast, non-structural carbohydrates in decay and reaction zones of well-watered saplings inoculated in July were lower compared to drought-stressed saplings (Figs. 5.31). However, in well-watered saplings inoculated in February, overall a reasonable amount of starch, 48 to 60 nmol/mg can be seen in the reaction zones of non-infected and infected wounds, respectively (Fig. 5.32). Similarly, in drought-stressed saplings inoculated in July, the amount of starch in reaction zones ranged from 28 to 47 nmol/mg (Fig. 5.33). Additionally, in drought-stressed saplings inoculated in February, the amount of starch was considerably higher; 118 nmol/mg and 62 nmol/mg in non-infected and infected wounds, respectively (Figs. 5.34).

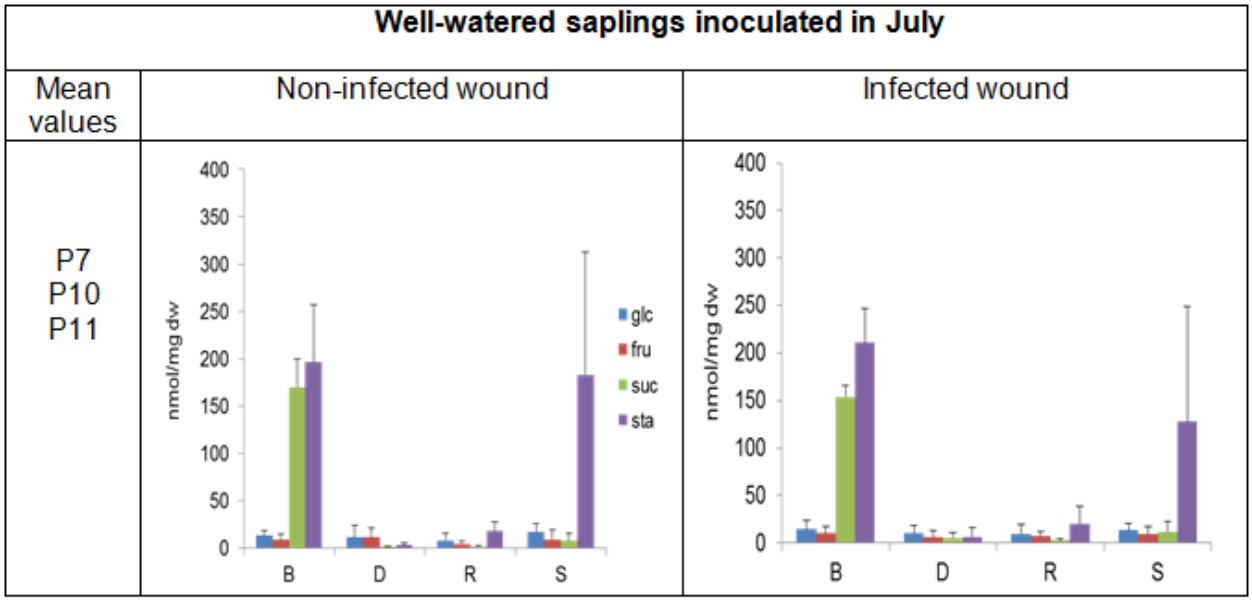


Fig. 5.31: Mean values + standard deviation (n=3) of non-structural carbohydrates in well-watered black locust saplings (P7, P10 and P11) inoculated in July 2010 and harvested in July 2012; for abbreviations, see Fig. 5.27.

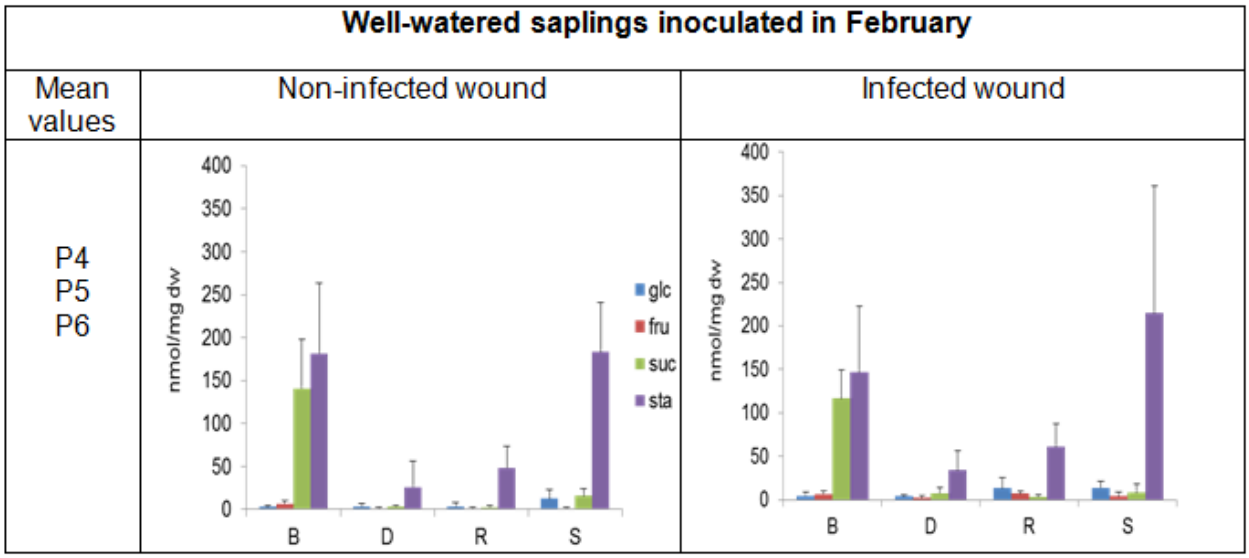


Fig. 5.32: Mean values + standard deviation (n=3) of non-structural carbohydrates) in well-watered black locust saplings (P4, P5 and P6), inoculated in February 2011 and harvested in July 2012; for abbreviations, see Fig. 5.27.

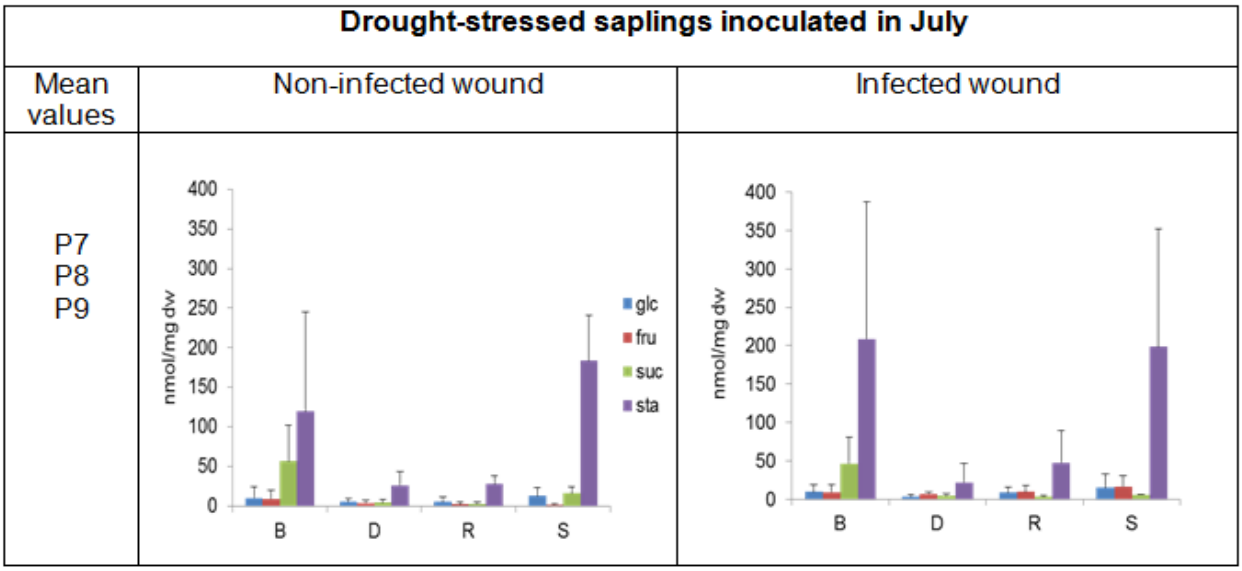


Fig. 5.33: Mean values + standard deviation (n=3) of non-structural carbohydrates in drought-stressed black locust saplings (P7, P8 and P9) inoculated in July 2010 and harvested in July 2012: for abbreviations, see Fig. 5.27.

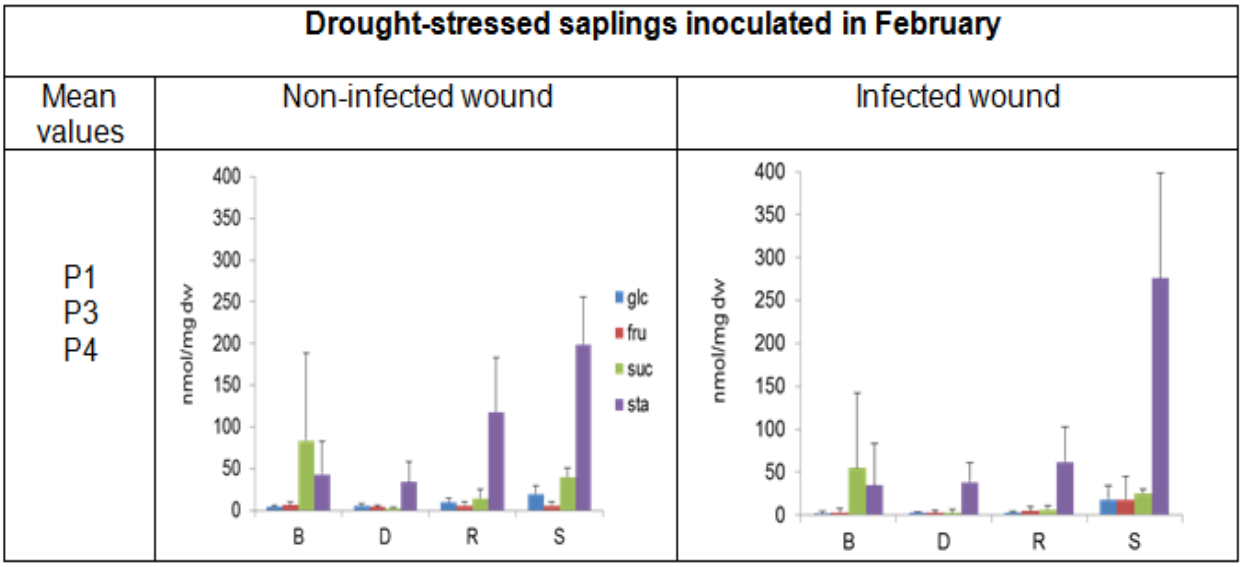


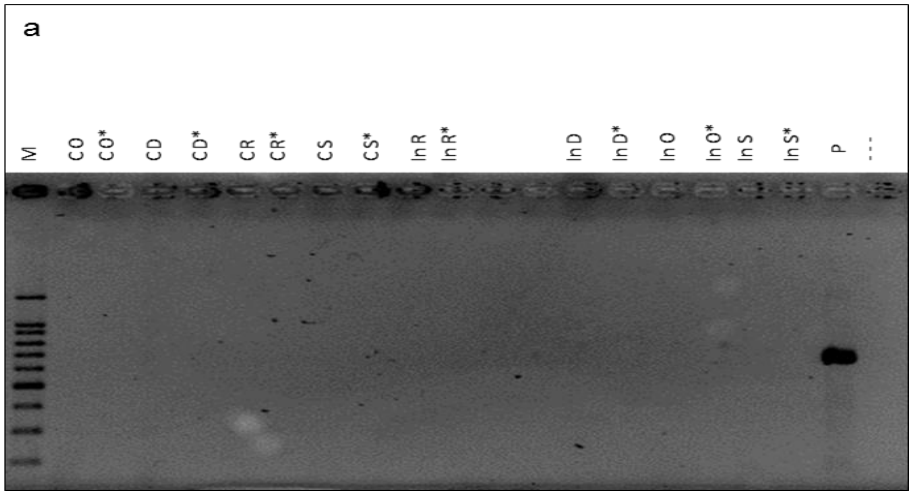
Fig. 5.34: Mean values + standard deviation (n=3) of non-structural carbohydrates in drought-stressed black locust saplings (P1, P3 and P4) inoculated in February 2011 and harvested in July 2012; for abbreviations, see Fig. 5.27.

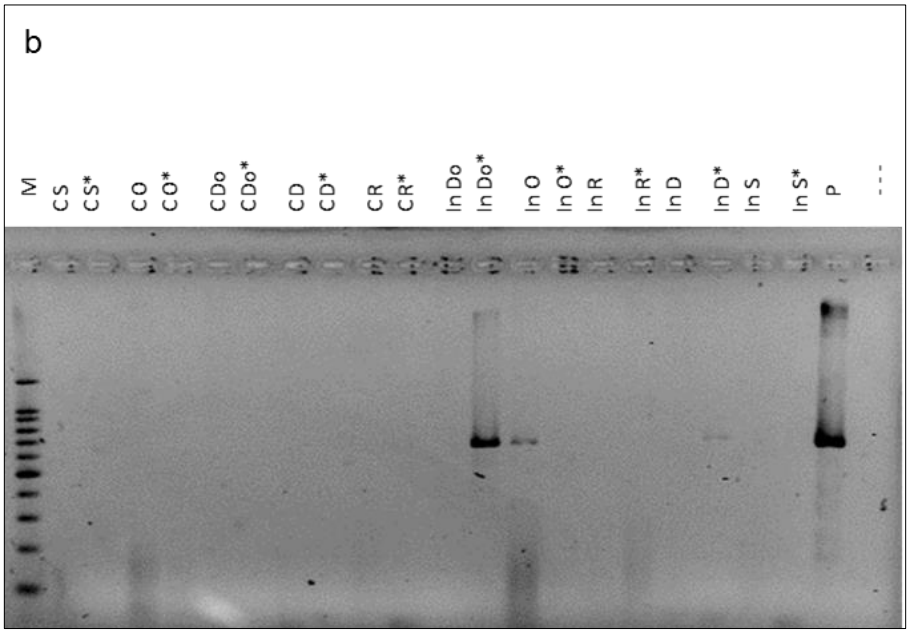
5.7. Investigating the spread of *Armillaria mellea* by taxon-specific primer

To study the effectiveness of compartmentalization spread of *Armillaria mellea* was studied in inoculated stems by using molecular technique in collaboration with Corinna Gebarowski during her B.Sc. thesis work. Taxon-specific primers were used to investigate *A. mellea* in the callus, sapwood, and decay and reaction zones as well as in infected dowels.

In the well-watered saplings P10 and P11, inoculated in July (Fig. 5.35a; Table 5.9), the pathogen was not found in any of the tissues tested. In the well-watered sapling P4, inoculated in February, the pathogen was found in the infected dowel but also in the adjacent callus and in the decay zone, however not in sapwood and in the reaction zone of inoculated tissue (Fig. 5.35b; Table 5.9). In the well-watered sapling P5, inoculated in February, the pathogen was also confirmed in the callus formed after wounding and inoculation (Table 5.9). In the drought-stressed saplings P8 and P9, inoculated in July,

the pathogen was not found in any zones of inoculated and control wounds (Fig. 5.35c; Table 5.9). In the drought-stressed sapling P3, inoculated in February, the pathogen was confirmed in decay, reaction and sapwood zones of inoculated tissue. In P4, the pathogen was confirmed in the infected dowel and in the decay zone. Interestingly, the viability of the pathogen was identified in the callus of the control tissue in both saplings P3 and P4 (Fig. 5.35d; Table 5.9).





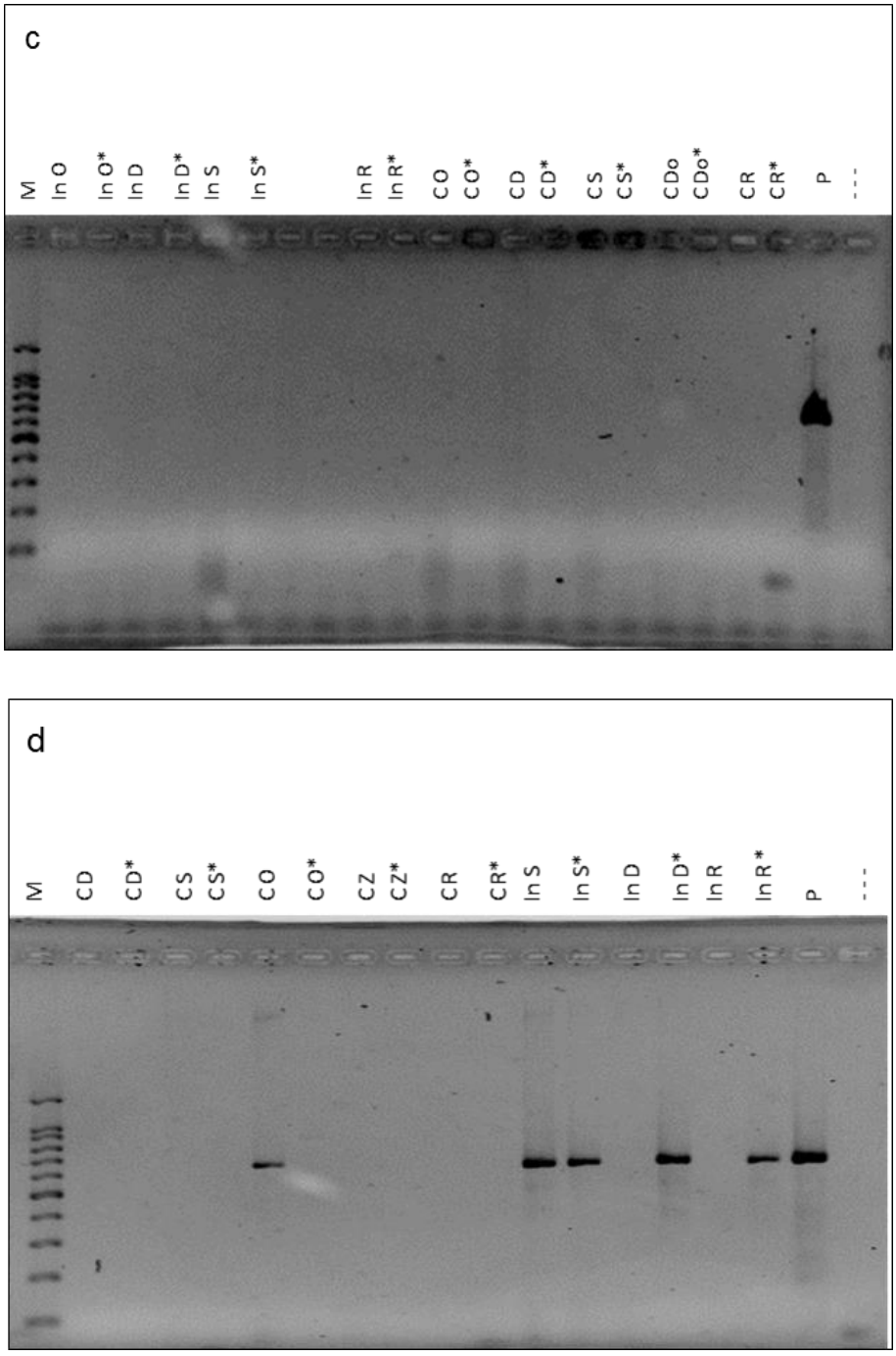


Fig. 5.35 a-d: A taxon-specific primer applied to determine the presence or absence of the pathogen, *Armillaria mellea*, in various tissues of the differently treated saplings. P: Positive control, C: Control, In: Inoculated, O: Callus, D: Decay zone, S: Sapwood, Do: Dowel, R: Reaction zone, and * = 2nd elution. a. well-watered sapling P10, inoculated in July, b. well-watered sapling P4, inoculated in February, c. drought-stressed sapling P8, inoculated in July, d. drought-stressed sapling P3, inoculated in February; bands around 700bp are visible.

Table 5.9: Presence or absence of the pathogen, *Armillara mellea,* in the specifically chosen zones of the saplings. C: Control, In: Inoculated, O: Callus, O*: Callus (2nd elution), D: Decay zone, D*: Decay zone (2nd elution), S: Sapwood, S*: Sapwood (2nd elution), R: Reaction zone, R*: Reaction zone (2nd elution), Do: Dowel, Do*: Dowel (2nd elution). Presence of the pathogen = +, absence of the pathogen = -, x = samples were not available.

Zones	Well-watered				Drought-stressed			
	July		February		July		February	
	Sapling number		Sapling number		Sapling number		Sapling number	
	10	11	4	5	8	9	3	4
CO	-	-	-	-	-	-	+	+
CO*	-	-	-	-	-	-	-	-
CD	-	-	-	-	-	-	-	-
CD*	-	-	-	-	-	-	-	-
CR	-	-	-	-	-	-	-	-
CR*	-	-	-	-	-	-	-	-
CS	-	-	-	-	-	-	-	-
CS*	-	-	-	-	-	-	-	-
CDo	-	-	x	x	-	-	x	x
CDo*	-	-	x	x	-	-	x	x
InO	-	-	+	+	-	-	x	x
InO*	-	-	-	-	-	-	x	x
InD	-	-	-	-	-	-	-	+
InD*	-	-	+	-	-	-	+	+
InR	-	-	-	-	-	-	-	-
InR*	-	-	-	-	-	-	+	-
InS	-	-	-	-	-	-	+	-
InS*	-	-	-	-	-	-	+	-
InDo	x	x	-	x	x	x	x	+
InDo*	x	x	+	x	x	x	x	+

6. Discussion

Trees are exposed to all kinds of abiotic stressors and biotic aggressors whose intensity and viability, respectively, is changing on short-term to long-term time scales. In their endeavors to survive, trees have to cope continuously with their ever changing environmental growth conditions by various defence strategies of which several have been experimentally studied in the preceding chapters and discussed in the following.

6.1. Morphological and phenological adaptations

Morphology and phenology of the saplings of black locust were severely affected by drought, but not by the pathogenic fungus. Drought has been reducing the overall growth and leaf area of the saplings. However, the saplings stressed by drought and by a pathogen were found to be drastically more affected, showing the lowest values for nearly all morphological and phenological variables. For the drought-exposed saplings (T3), a 48% smaller average diameter was recorded. In the case of the saplings stressed by both drought and a pathogenic fungus, the diameter growth was even reduced by 54% (Fig. 5.6). Moreover, drought-stressed saplings were by 30% shorter than the well-watered ones (Table 5.3, Fig 5.7).

It is a general adaptation strategy of plants to avoid drought by reducing their size and minimizing drought-induced injuries. Plants respond to water stress by acclimation in non-severe cases but in severe cases a damage and loss of plant parts can happen (Chaves et al. 2002). This fits well with a number of studies, as for example by Meenakshi et al. (2005) who described that drought stress reduced the growth of *Albizzia* seedlings by affecting various physiological and biochemical processes. Similar observations are reported for water-stressed *Eucalyptus microtheca* (Li et al. 2000), several *Populus* species (Yin et al. 2005) and *Citrus* (Wu et al. 2008). Similarly, diameter and height of *Pinus radiata* was also reduced because of drought stress (Nanayakkara et al. 2013).

Moreover, the drought-stressed black locust saplings adapted themselves by delaying leaf initiation, reducing the leaf number, and increasing leaf senescence and abscission (Figs. 5.1, 5.4, 5.5); this is in line with earlier observations by Boyer (1976). A delay in leaf initiation, reduction in leaf number, and an increase in leaf senescence (Fernandez-Conde 1998) as well as a higher number of leaf abscissions (Kozlowski 1976; Grice 1998; Arndt et al. 2001) are adaptation strategies to minimize the transpiration surface and restricting water loss.

Drought avoidance by drastically reducing the leaf area by more than 50% (Table 5.1) is consistent with recent findings by Veste and Kriebitzsch (2013). Similarly, the total leaf area was reduced, when black locust was subjected to a reduced availability of soil water (Mantovani et al. 2014). Black locust plants subjected to 35% and 70 % availability of water developed smaller leaves by 60 % and 42 %, respectively compared with the plants subjected to the 100% availability of water.

Experiments with herbaceous plants and trees have manifested that reducing the leaf area is a common response to soil-water limitation (Fischer and Turner 1978; Lof and Welander 2000; Otieno et al. 2005) and thus avoiding a severe decrease in cell-water potential and turgor pressure (Hinckley et al. 1981; Kozlowski and Pallardy 1997). Significant differences between two sympatric *Populus* species were found in the number of leaves, leaf area and leaf biomass under drought stress (Yin et al. 2005). The lowest average leaf area in drought-stressed black locust saplings, infected with a fungus, is noteworthy and indicates a trend of decline under dual stresses. It has also been reported that drought stress increased the susceptibility of plants for pathogens by reducing plant growth (Boyer 1995). A significant reduction in the number of leaflets (Table 5.1, Fig. 5.2) as confirmed for drought-stressed black locust saplings was also observed for *Leucaena* (Hegde 1983). Moreover, drought stress resulted in a stunted growth of inflorescences, morphologically somehow ball-shaped, whereas well-watered saplings got long and fresh inflorescences (Fig. 5.3). A delayed flowering was described for container-grown drought-stressed mango trees by Chaikiattiyos et al. (1994). Moreover, drought stress caused a reduction in the number of seed pods. It is mentioned in the literature that drought avoidance by reducing the leaf area may also

reduce the yield (Blum 2005). As similar decrease in the number of pods due to drought stress was seen with cowpea (Turk et al. 1980; Turk and Hall 1980) and soybean (Specht et al. 2001).

6.2. Physiological responses

The black locust plants responded to drought stress also by physiological parameters. Water stress decreased the net photosynthesis (P_N) by decreasing the stomatal conductance (g_s) for CO_2. The net photosynthesis (P_N) ranged from 7 to 15 µmol CO_2 $m^{-2}s^{-1}$ in well-watered saplings and from 3 to 7 µmolCO_2 $m^{-2}s^{-1}$ in drought-stressed ones (Fig. 5.11). This is consistent with findings of another study on black locust by Veste and Kriebitzsch (2013); who reported that the net photosynthesis (P_N) ranged from 6 to 14 µmol CO_2 m^{-2} s^{-1} in well-watered and from 2 to 10 µmol CO_2 m^{-2} s^{-1} in drought-stressed saplings. Similarly, the transpiration (E) varied from 0.62 to 3.39 mmol H_2O m^{-2} s^{-1} in well-watered saplings and from 0.84 to 2.99 mmol H_2O m^{-2} s^{-1} in drought-stressed ones (Fig. 5.12a). Veste and Kriebitzsch (2013) measured 0.5 to 4.5 mmol H_2O m^{-2} s^{-1} in well-watered and 0.4 to 0.9 mmol H_2O m^{-2} s^{-1} in drought-stressed black locust trees.

Stomatal closure is one of the crucial events taking place during drought (Chaves et al. 2002). Under moderate water stress, the photosynthetic apparatus is considered to be very resistant (Chaves et al. 2002, 2009; Warren et al. 2004). However, as water deficit progresses, like in the drought-stressed black locust saplings, the biochemistry of the CO_2 fixation is affected. Flexas et al. (2004; 2007) reported that drought stress primarily down-regulates the photosynthesis by increasing the diffusive resistances to CO_2 entry into the chloroplasts, and thus causes a lowered mesophyll conductance for CO_2. Throughout the experiments, the mesophyll conductance was significantly reduced in drought-stressed black locust trees, like in many other plant species (Flexas et al. 2002; Galmés et al. 2007a; Warren et al. 2004). As water deficit progresses, the biochemical limitations to photosynthesis might have also been involved.

The presence of a pathogen did not affect the gas exchange of the well-watered saplings, but of the drought-stressed ones. It might be because of little or no physiological damage in well-watered saplings infected in July and February, so that significant alterations were not exhibited in gas exchange parameters. Conversely, the significant limitation of gas exchange in drought-stressed saplings inoculated with a pathogen (T4) as compared to the drought-stressed saplings T3 indicated that the pathogen has affected the respective saplings physiologically (Table 5.8). A reduced net photosynthesis and a significantly lowered mesophyll conductance for CO_2 in drought-stressed saplings inoculated with pathogen (T4; Fig. 5.14) might be due to an increased susceptibility of drought-stressed saplings to pathogen attack. Boyer (1995) described that drought stress increased the susceptibility of plants to attacks by pathogens by reducing the assimilate production or by reducing plant growth. This is also supported by Popoola and Fox (2003) who showed an increased susceptibility of host plants from which water had been withheld. Therefore, these results strengthen the hypothesis presented by Desprez-Loustau et al. (2006) that drought-stressed trees are predisposed to diseases. Moreover, the physiological resistances of healthy tissues restrict the development and spread of the pathogen (Thomas 1934).

6.3. Alterations in biomass

Alterations in growth, leaf morphology and physiology resulted in a reduction of the total biomass of drought-stressed saplings by 64% (Fig. 5.19). This is in line with the findings of Mantovani et al. (2014); the total above-ground biomass of black locust was reduced under less availability of soil water. The biomass reached only 46% and 48% of the black locust saplings with 35% and 70% availability of water, respectively than that of the biomass yield obtained in 100% availability of water.

This is also consistent with findings for *Tribulus terretris*, where water stress hindered dry matter accumulation and decreased the biomass (Yang et al. 2010). A biomass reduction due to drought is also reported for *Jatropha* (Niu et al. 2012; Achten et al. 2010). A reduction in total biomass is recently recorded for *Pinus radiata* due to drought

stress (Nanayakkara et al. 2013). However, in this study the total biomass was even more reduced when the black locust saplings were loaded by two stressors, drought and pathogenic infection.

None of our experimental treatments led to an effect on the root-to-shoot ratio, as reported for other plant species under drought stress (Jackson et al. 2000). Kozlowski (1982) recorded a larger reduction of shoot growth compared to root growth under water shortage for a number of woody species, but the root-to-shoot ratio was not altered in perennial grasses and *Leymus chinensis* (Xu and Zhou 2005). Joslin et al. (2000) could not confirm an increase in the root-to-shoot ratio due to a long-term drought exposure. Recently, the root-to-shoot ratio was found to be unaffected in drought-stressed *Pinus radiata* (Nanayakkara et al. 2013).

6.4. Host reaction in form of discoloration

The longer discoloration in the stems of black locust in axial direction (the amount of dysfunctional wood) than in tangential and radial direction (Figs. 5.20, 5.21, 5,22) is similar to a number of previous studies (e.g., Gibbs 1968; Kile and Wade 1974; Armstrong et al. 1981; Boddy and Rayner 1983; Smith and Shortle 1993; White and Kile 1993; Shortle et al. 1995; Deflorio et al. 2008). The vertical extension of discoloured wood was equal or longer upward than downward from the bore hole, both around the control holes and the infected holes (Fig. 5.24). This was also observed earlier by Mireku and Wilkes (1989). Similarly, the vertical spread of discoloration induced by fall and spring wounds was longer upward than downward from the injury (Armstrong et al. 1981).

Furthermore, the discoloration columns were slightly larger in all saplings inoculated in winter than in summer. Several studies supported this finding such as by Leben (1985), Shain and Miller (1988) and Mireku and Wilkes (1989) who reported that discoloration around autumn or winter wounds was largest, around summer wounds intermediate, and around spring wounds minimal. Roots of oak and linden compartmentalized weaker

when wounded in winter than at the beginning of the growing season (Balder et al. 1995). However, according to Santamour (1985), no difference was observed for sweet gum and plane trees injured in the dormant and in the growing season.

Distinctly shorter axial discoloration in well-watered as compared to drought-stressed saplings indicated that drought-stress increased the vulnerability of the saplings for wound and pathogens. The well-watered saplings have shown a high potential for developing a strong reaction zone on the basis of a high energy reservoir, and responded successfully against the fungal attack, proving to be strong compartmentalizers. The poorer compartmentalization of drought-stressed saplings might be the result of colonization by the pathogen on one hand and a weak performance of the host on the other hand. According to the literature, the rate of pathogenic colonization can be increased by drought stress (Towers and Stambaugh 1968; Lindberg and Johansson 1992). The stimulation or inhibition of fungal decay may depend on the level of stress as well as on the host and pathogen species (Wahlstrom and Barklund 1994; Desprez-Loustau et al. 2006). Drought stress enhanced the severity and progression of the symptoms when *Parthenocissus quinquefolia* was infected by *Xylella fastidiosa* (McElrone et al. 2001). Larch subjected to drought stress appeared to show an increased susceptibility to infection by the bark beetle *Ips cembrae* which acts as a vector for the fungus *Ceratocystis laricicola* (Redfern et al. 1987).

6.5. Callus formation

The cambium around a wound continues to produce cells more than elsewhere on the same level of the stem. This new tissue is essential for defence. The wound callus, produced around the wound expands faster tangentially than radially that allows the wound to close whereas the tree expands in girth. Due to an effective compartmentalization, infected and decaying trees can live and contribute to the human landscape for many years (Lonsdale 2004).

The larger callus formation in well-watered than in drought-stressed black locust saplings (see Fig. 5.25) makes it easy to understand vitality of trees. When trees are growing up without any abiotic stress like drought, the cambium effectively produces cells around a wound and vice versa.

Less callus formation in trees inoculated in February might be due to weaker compartmentalization ability in winter when trees are dormant. A similar finding has been reported for injuries of oak and linden roots in winter as compared to the beginning of the season (Balder et al. 1995). Callus formation of drought-stressed saplings with non-infected dowels (T4c), saplings, inoculated in February, is also not similar to saplings inoculated in July. Thus, a poorer compartmentalization ability of drought-stressed saplings is obvious even in different seasons. In T4c (inoculated in February), the significantly more intense callus formation as compared to T4, is interesting to understand the role of pathogens in drought-stressed saplings. Moreover, the failing of callus formation in T4, inoculated in February, might be because of a high susceptibility of drought-stressed saplings to the pathogen, or *A. mellea* can be considered as 'cambium killer'. In this situation, chances of a fungus to establish are higher when the host is already under stress and passing through dormancy. In contrast, saplings inoculated in July have a chance to seal the wound in the highly active time of the year. The wound surfaces of drought-stressed saplings (T4c) are admittedly also colonized by spores from the ambient air, but the infection potential is by many times lower compared to drought-stressed saplings inoculated with a pathogen (T4).

6.6. Distribution of non-structural carbohydrates

From the biochemical approach in the context of compartmentalization, the sapling-related results per treatment were highly similar (see Figs. 5.27 to 5.30), that is why they were averaged to variant-specific mean values (see Figs. 5.31 to 5.34). Only these will be discussed, unless it is necessary to refer to an outlier value.

In the well-watered saplings, no matter whether the wounds were kept sterile or were infected in July, the amounts of non-structural carbohydrates (glucose, fructose, sucrose and starch) in the decay and reaction zones were between 0 and 20 nmol/mg (Figs. 5.27, 5.31). These results are consistent with findings by Busch H (1999); in black locust after 360 days of inoculation by a pathogen the amount of non-structural carbohydrates (glucose, fructose, sucrose and starch) present in decay and reaction zones was also only 0 to 20 nmol/mg. In contrast, in the bark starch and sucrose and in the sapwood only starch were found in appreciable amounts. From these observations, it can be concluded that all three saplings have converted the non-structural carbohydrates in the decay and reaction zones into phenolic substances for defence activities against the wounding. In the sapwood and in the bark, however, high amounts of carbohydrates are present, except in P7 and P11. The starch in the sapwood of P7 and P11 (see Fig. 5.27) may have been consumed during an untimely sprouting or may have been transformed into phenolic substances for some defence activity against any other causes. The appreciable amount of starch in the reaction zone of all three saplings inoculated in February (Fig. 5.28) indicates that they were unable to convert all reserves available into defence substances. Presumably, their metabolism was still in winter dormancy and not yet sufficiently active.

In contrast to the well-watered saplings, the drought-stressed saplings show a less consistent appearance as to the amount of carbohydrates between individuals and between the season of inoculation (see Figs. 5.29 and 5.30). First of all, it is striking that in P7 nearly no carbohydrates were detectable. According to protocol notes, P7 has been declining before the harvest. The most obvious difference, particularly regarding the high amount of starch in the reaction zone, exists between drought-stressed saplings inoculated in February and the saplings of the other three experimental variants. This observation can be taken as evidence for a weakened potential of the drought-stressed saplings infected in February to react against wounding. Drought-stress in combination with wounding in February, that is to say during dormancy, might be the reason that starch was insufficiently converted into phenolic substances in the reaction zone especially in sterile wounds compared to infected ones.

The interaction between carbohydrates and phenols, including flavonoids, is widely discussed in the literature. Carbohydrates are not only potential carbon and energy sources for processes taking place during defence actions but they also affect the expression and activity of enzymes in the sucrose metabolism and in the phenol synthesis (Koch et al. 1992, Ehneß et al. 1997). In our saplings, from the tissues selected, the decay and the reaction zone contain only traces of non-structural carbohydrates. A maximum of non-structural carbohydrates might have been converted into phenols and flavonoids during the active defence reactions against wounding and pathogen attacks, as the production of phenolic derivatives is a ubiquitous response of plants when injured or infected (Vance et al. 1980). According to Klepzig et al. (1996), substances synthesized as a reaction to wounding neutralize, inhibit or confine the effect of pathogens.

In this way, discolored wood of well-watered saplings inoculated in July (Fig. 5.27) is physiologically similar to heartwood, as decay and reaction zones of all three saplings are almost free of storage material. Both in softwood and hardwood species, the outermost sapwood contain high amounts of sugars and starch, and starch is consumed or withdrawn during the sapwood-heartwood transformation process (Datta and Kumar 1987; Magel and Höll 1993; Magel et al. 1994). Similarly, Magel and Höll (1993) described that discolored wood, in the case of *Fagus sylvatica,* is physiologically not different from true heartwood. Moreover, the low amount of starch in the bark near wounds of drought-stressed saplings inoculated in February reveals about shortage of resources around the wound (fig. 5.30).

6.7. Investigation of *Armillaria mellea* by molecular technique

In order to follow the spread of *Armillaria mellea,* its presence or absence within the different zones of wood was tested by molecular technique. The pathogen was not confirmed in the control and in the inoculated tissues (decay, sap, reaction zones and callus) of well-watered and drought-stressed saplings inoculated in July. In these

saplings, absence of the pathogen in the decay zone of inoculated tissue is reflecting towards an effective encapsulation of the *A. mellea* during the active season.

Well-watered saplings, inoculated in February, were also competitive enough to repel the pathogen. Only in decay zones, in inoculated dowels and in the callus adjacent to the dowel, the pathogen was confirmed. However, most frequently the pathogen was present in drought-stressed saplings inoculated in February. These saplings were unable to close the wounds and stop the growth of the pathogen. According to Shigo (1986), an incompletely closed wound provides an excellent environment for the growth and spread of pathogens. *A. mellea* even occurred in the callus around sterile wounds 30 cm above the inoculum. In addition, the higher amount of starch in the reaction zones of control saplings compared to infected ones reflects that stored resources might be consumed by the pathogen. Most frequently, pathogens occurred in inoculated and surrounding tissue; its spread up to the callus of control wounds and the inability to close inoculated wounds confirms the high susceptibility of drought-stressed saplings inoculated in February. In these saplings, the presence of starch and phenols might be favorable for the fungal growth, as the fungus is able to use oxidized phenols as an additional carbon source and can grow more vigorously than on glucose alone (Wargo 1980b, 1981a, 1981b). In contrast, drought-stressed saplings inoculated in July showed active defence, wounds were closed, and the spread of the pathogen restricted.

7. Conclusions and outlook

The experimental approach of applying two stressors, both separately and in combination, was interesting as evidences were found to be supporting field observations. This study suggests that most strikingly water stress is by far the most severe impairment for young black locust trees.

A long-term drought stress has severely affected growth parameters, leaf traits, phenology, and gas exchange, yield and biomass of the saplings. A limitation in stomatal conductance regulated the response of photosynthesis. However, a declining trend in leaf area, a more pronounced physiological stress and average biomass tended to show, at least, a weak detrimental effect of the pathogen superimposed in drought-stressed saplings.

Furthermore, drought-stressed black locust saplings with a reduced metabolic activity, however, are not efficient enough to properly compartmentalize the wounds or tissues infected by the pathogen. Similarly, the axial spread of discoloration was small and the callus formation was almost double in well-watered saplings as compared to drought-stressed saplings. In contrast, the drought-stressed saplings, inoculated in February, failed to form a callus around inoculated wounds entirely.

The non-structural carbohydrates very likely have been converted into phenols and flavonoids during the active defence reactions against wounding and pathogen attack in well-watered saplings inoculated in July. An obvious amount of starch in the reaction zones of drought-stressed saplings inoculated in February is due to a low rate of biochemical conversion. Similarly, the very low amount of starch in bark in the vicinity of wounds revealed least or no physiological activity to keep the saplings functional. In addition, the presence of the pathogen in drought-stressed saplings inoculated in February confirms their susceptibility. Incompletely closed wounds have provided a suitable environment for the growth and spread of pathogen, as the presence of *A. mellea* was also confirmed in the callus of sterile wounds. Therefore, the well-watered saplings inoculated in July can be taken as strong compartmentalizers whereas the

drought-stressed saplings, inoculated in February can be taken as poor compartmentalizers.

These findings give experimental evidence for the hypothesis that trees impacted by drought are predisposed to biotic diseases because of their weakened defence potential, presented by Desprez-Loustau et al. (2006), and that drought-stressed trees are more susceptible to attacks by pathogens (McDowell et al. 2008). Our results also support Dujesiefken et al. (2005) who mentioned that the efficacy of compartmentalization depends upon the season of wounding.

As drought conditions are expected to increase with climate change and rising water shortage in many areas of the world, quantifying the impacts of drought or water stress on tree species and their interaction with pathogens is of core importance and need to be elucidated in further experiments, so that the aggressiveness or virulence could be offset by a concurrent increase in host resistance. Therefore, detailed information about the tolerance of tree species to environmental stresses is urgently wanted.

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