POPLAR RESPONSES TO BIOTIC AND ABIOTIC STRESS

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María Escalante Pérez

aus Miranda de Ebro (Spanien)

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Eingereicht am:

Mitglieder der Promotionskommission:

Vorsitzender: Prof. Dr. Thomas Dandekar

1. Gutachter: Prof. Dr. Rainer Hedrich

2. Gutachter: Prof. Dr. Ute Hentschel

Tag des Promotionskolloquiums: ……

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Würzburg, im September 2009

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María Escalante Pérez

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List of abbreviations

%	percentage
ºC	**C**elsius grad
µ	micro-
A.th.	***A****rabidopsis* ***th****aliana*
ABA	**Ab**cisic **a**cid
AGI	**A**rabidopsis **G**enome **I**nitiative
AKT	**A**rabidopsis thaliana **K^+**-**t**ransporter
Ca^{2+}	**Ca**lcium ion
cDNA	**c**opy-**DNA**
Cl^-	Chloride ion
CO_2	Carbon dioxide
Col 0	**Col**umbia **0**
COR6.6	**Co**ld **r**egulated **6.6**
DEPC	**Die**thyl**p**yro**c**arbonat
dS/m	Electric conductivity (d-Siemens per meter)
DNA	**D**eoxyribo**n**ucleic **a**cid
DW	**D**ry **w**eight
EFN	**E**xtra**f**loral **n**ectar
EFNs	**E**xtra**f**loral **n**ectarie**s**
ER	**E**ndoplasmic **r**eticulum
et al.	**e**t **a**lii
Fig.	Figure
g	Gram
GABA	**g**-**a**mino**b**utyric **a**cid
ha.	Hectare
HPLC	**H**igh-**p**erformance **l**iquid **c**hromatography
JA	**J**amonic **a**cid
K^+	Potassium ion
L	**L**itre
M	**M**olar
M	**m**ili-
tair	**T**he ***A****rabidopsis* **I**nformation **R**esource
Mg^{2+}	Magnesium ion
mRNA	**m**essenger **RNA**

Na^{2+}	Sodium ion
NaCl	Sodium chloride
PCR	**P**olymerase **c**hain **r**eaction
P.t.	***P**opulus **t**richocarpa*
P.t.t.	***P**opulus **t**remula **t**remuloides*
PR	**P**athogen **r**elated
RNA	**R**ibo**n**ucleic **a**cid
RT	**R**oom **t**emperature, **r**everse **t**ranscriptase
RT-PCR	**R**eal **t**ime **PCR**
SE	**S**tandard **e**rror
sp.	**sp**ecie
TEM	**T**ransmission **e**lectron **m**icroscopy
WT	**W**ild **t**ype

1. INTRODUCTION

Plants are renewable sources of food and fibers essential to the survival and well being of humans. Lacking the mobility of animals, higher plants are products and victims of the environment to which they are physically bound (Tainter & Baker 1996). In nature, plants are under permanent stress. The term stress has no clear definition. It might be described as any factor that decreases plant growth and reproduction below the genotype's potential (Osmond 1987). There are two major groups of stress factors: one is biotic stress, resulting from living organism, such as viruses, fungi, bacteria, parasitic weeds or insects which can harm plants. The other is abiotic stress, caused by environmental factors like drought, extreme temperatures or pollutants. In the plant, biotic- as well as abiotic stresses, might feedback on each other and thus lead to complex responses (Abuqamar *et al.*, 2008). For example, plants under drought stress would be more sensitive to a variation in temperature and plants affected by pollutants contamination are more easily infected by pathogens. One example of how the plant-pathogen-environment relationships can extremely affect human life is the so called "Great Hunger" also known as "Irish Potato Famine". This incident took place all over Europe during the 1840s and was caused by a fungal infection by pathogenic water mould, *Phytophthora infestans*, the "potato blight". Even though *P. infestans* ravaged potato crops in Europe in the 17th century, its human cost in Ireland was exacerbated by climatological factors: extreme cold and rainy weather in successive years. The result of these biotic/abiotic factors interaction was a reduction in the population of Ireland by 20 to 25 percent between 1845 and 1852. Its effects extended well beyond its immediate demographic impact and permanently changed the island's political and cultural landscape (Reader, 2008).

In order to defend themselves from these harmful elements, plants evolved a wide range of adaptations to improve their survival and reproduction by reducing or avoiding the impact of "stress elements". Plant defenses can be classified generally as "induced" or "constitutive". Constitutive defenses are always present, while induced defenses require de novo synthesis or mobilization of different substances to the site where the plant is injured. There are wide variations within these types of defenses. Constitutive or inducible defenses can again be divided into mechanical or chemical responses. Most external mechanical defenses, such as thorns or spines that discourage herbivores or waxes that prevent desiccation, are constitutive, as they require large amounts of resources to be produced and are difficult to mobilize. Chemical defenses include synthesis of secondary metabolites, which are not part of the common growth

processes, development program or reproduction machinery of the plant (Benett and Wallsgrove, 2006). Induced defenses in general include secondary metabolic products, as well as morphological and physiological changes.

In recent years many attempts have been made to understand the mechanism underlying stress tolerance (Kessler and Baldwin, 2002; Munns, 2005; Yamaguchi and Blumwald, 2005). *Arabidopsis* has been used as a model for studying a wide range of abiotic and biotic stress factors. Research on the molecular basis of salt stress resistance in plants has focused on *Arabidopsis* seedlings (Flowers, 2004; Zhang *et al.*, 2004; Liu *et al.*, 2007; Attia *et al.*, 2008). Also drought or cold stress was investigated mainly with *Arabidopsis* (Welin *et al.*, 1994; Seki *et al.*, 2001; Choh *et al.*, 2008; Qin *et al.*, 2008). Using this model early genes encoding potential regulators of pathogen- and herbivore-induced plant responses have been identified (Staskawicz, 2001; Kwon *et al.*, 2008; van Poecke and Dicke, 2004; Mewis *et al.*, 2006; Vogel *et al.*, 2007). Owing to their long lifespan and large size, forest trees present more opportunities for suffering different kinds of stresses during their lifetime than herbaceous annual plants. The genus *Populus*, has become a useful tool for the research on molecular biology, forest biotechnology, and most recently tree genomics. Populus provides an excellent model in order to gain insights into stress management of trees. A member of the genus, *Populus trichocarpa*, with its genome sequenced, has been adopted as the model tree species (Tuskan *et al.*, 2006). Attributes making *Populus spp* that useful for research are their rapid growth, easy vegetative propagation, a relatively small genome, and tractability to *Agrobacterium*-mediated transformation. In addition this genus contains some of the most economically important tree species in the Northern hemisphere. Therefore here poplar has been chosen to study the effect of biotic and abiotic stress on growth and developmental processes of wood producing plants.

1.1 BIOTIC STRESS

Plants respond to heterotrophic attacks with a bewildering variety of responses. Aggressors are divided into two different groups. One denotes the “pathogens”, organisms comprising fungi, oomycetes, bacteria, viruses, viroids, virus-like organisms, phytoplasmas, protozoa, nematodes and parasitic plants that cause infectious diseases. The other group includes insects, mites, vertebrate or other pests that affect plant health by consumption of plant tissues, the so called “herbivores”. Plant-

heterotrophic interactions take place on cellular level (de Wit, 2007; Friedman and Baker, 2007) as well as on that of whole organ, plant or even at community (De Moraes *et al.*, 2001; Kunkel and Brooks, 2002 ; Mithofer *et al.*, 2005; Baldwin *et al.*, 2006; Gershenzon, 2007).

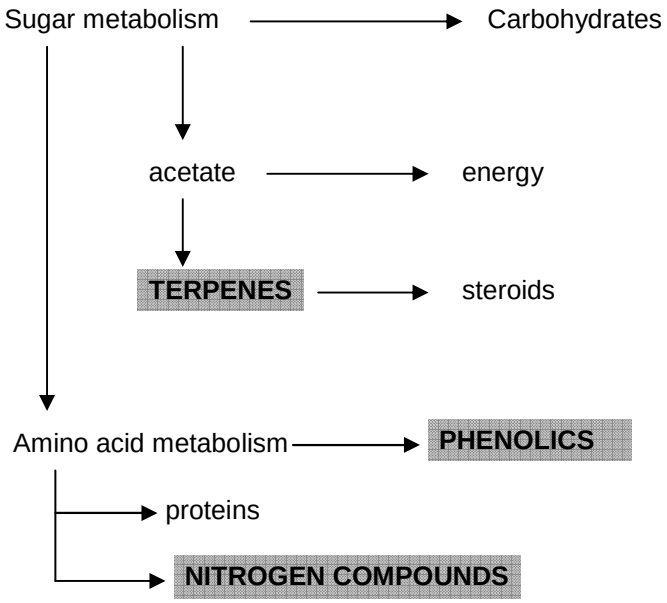


Fig 1_1 Origin of plant secondary metabolites involved in herbivore defence. Nitrogen compounds are alkaloids, amines, non-protein aminoacids, cyanogenic glycosides and glucosinolates. Terpenoids are monoterpenoids, cartenoids. Phenolics are simple phenols, polyphenols. All these defense chemicals are usually synthesized as by-products of the primary metabolites.

Plants developed a wide range of physical barriers to avoid being eaten or invaded. These mechanical structures such as spines, trichomes and very hard, very sticky, or very smooth surfaces discourage heterotrophs from attacking the plant (Lucas *et al*. 2000). The chemical defense involves secondary metabolites which are organic compounds that are not directly involved in the normal growth, development or reproduction of the organism, and often are produced as by-products during synthesis of primary metabolic products (Berenbaum and Zangerl, 2008; Hartmann, 2008). In some plants, structures such as glandular trichomes and secretory canals are combinations of mechanical and chemical defences that interact to avoid plant damage (Pillemer and Tingey, 1976; Gershenzon *et al.*, 1992; Eisner *et al.*, 1998). These secondary metabolites can be classified into three sub-groups as depicted in Figure 1.1.

Of these, terpenoids belong to the most diverse class of plant secondary metabolites (>40.000 known structures), and are used extensively for their aromatic qualities. Terpenoids are formed in large volumes in conifer oleoresins and are the major components of volatiles emitted by all higher plants (Mumm *et al.*, 2008).

The alkaloids, e.g. caffeine, nicotine, morphine and cocaine, are widely distributed and known for their metabolic effects in mammals (Cavin *et al.*, 1987; Chabner and Horwitz, 1990; Cardoso, 1997). Some of the crop plants we use to eat frequently are basically toxic, for example beans or potatoes (Mandimika *et al.*, 2007). To prevent herbivory by the larvae of some Lepidoptera species, Cinchona trees produce a variety of alkaloids, of which the most familiar is quinine.

The production of cyanogenic chemicals in grasses is primarily a defense against herbivores. Glucosinolates occur as secondary metabolites of almost all *Brassicaceae*. They are natural pesticides and also responsible for the bitter or sharp taste of many common foods such as mustard or radish (Hopkins *et al*., 2009).

Amines have strong, characteristic odors and are toxic for insects. Polyamines conjugated to phenolic compounds have been shown to accumulate in incompatible interactions between plants and a variety of pathogens. A role for the free polyamine spermine in the hypersensitive response of barley to powdery mildew and of tobacco to TMV has been suggested (Edreva, 1997; Cowley and Walters, 2002).

1.1.1 Pathogen defence

Pre-formed physical barriers such as the cuticle, suberin lamellae, or callose plugs are the first line of defenses which protect the plant against pathogen penetration. The chemical defense, however, includes the use of a wide array of different antimicrobial structures (Veronese *et al.*, 2003). A common feature of the defense system of such taxonomically diverse organism like mammals, insects or plants is the ability to coordinate the accumulation of antipathogen proteins and peptides when an invasion of a foreign organism occurs (Hoffmann *et al.*, 1999).

The expression of genes encoding most of these antimicrobial proteins and peptides is induced by the invasion of the pathogen. Many studies of plant-pathogen interaction focus on gene-for-gene interactions, thereby pathogen produced elicitors are detected, and result in transcription of pathogen related (PR) proteins which finally confer local or

systemic resistance. The general concept of PR proteins was introduced to designate any plant protein induced in response to a pathological situation (Antoniw *et al.* 1980). PR proteins were basically grouped into 5 different families according to sequence similarities, serological relationships, and/or enzymatic or biological activities. Each of these groups comprised two subclasses. One class is usually localized in the extracellular space and its members are induced by salicylic acid. The other class, found in the cell vacuole, is modulated by ethylene or jasmonic acid (Thomma *et al.*, 1998; Santamaria *et al.*, 2001; Selitrennikoff, 2001). The function of many PR proteins remains still unknown.

Another strategy to prevent pathogens from propagating or even killing of invaders is the production of free radicals such as superoxide and hydrogen peroxide called oxidative burst. In pathogen interactions, the major short term common response is the hypersensitive response (HR), wherein apoptosis-compromised cells commit suicide in order to create a physical barrier for the invader (Mehdy, 1994; Lam *et al.*, 2001).

1.1.2 Herbivore defence

Since the late 17th century it has been known that plants contain chemicals that are avoided by insects.

Plants can also use indirect ways to defend themselves by enhancing the probability of attracting the natural enemies of the herbivores. These “bodyguards” as payment defend the plant from the invasion of damaging insects. One example of indirect defense structure is the extrafloral nectaries (EFNs). These specialized tissues secrete a sweat diet to attract animals, mainly ants, keeping herbivores away (Almeida and Figueiredo, 2003; Mathews *et al.*, 2007).

In response to herbivores attack plants release volatile organic compounds (VOCs) that can attract predatory arthropods and/or repel herbivores (Arimura *et al.*, 2000;De Moraes *et al.*, 2001; Kessler and Baldwin, 2002; Gershenzon, 2007). VOCs have been demonstrated to be part of a “plant-to-plant communication” network, they can act as a message for neighboring plants yet undamaged. Lima Beans for instance release volatile chemical signals from leaves infested with spider mites, which lead to the formation of EFN in uninfected leaves (Heil and Silva Bueno, 2007; Kost and Heil, 2008). This message allows even neighboring plants to prepare themselves by

activating different defense genes, making them less vulnerable to a future attack and also attracting predators of the herbivores.

1.1.2.1 Extrafloral nectaries

Caspary (1848) distinguished between floral and extrafloral nectaries, the former occurring in the flowers and are involved in pollination whereas the latter appear on vegetative organs and are not involved in pollination but in defence. Nevertheless, both have the same basic function, to reward animals that provide the mobility which plants lack. Extrafloral nectar (EFN) is supposed to attract ants, which then would protect the plant from damage by herbivores (Heil *et al.*, 2001; Linsenmair *et al.*, 2001; Mathews *et al.*, 2007; Radhika *et al.*, 2008). Several studies have shown that extrafloral nectar secretion increases in response to herbivory which in turn leads to a decrease of herbivory rates.

In general nectaries consist of three components, an epidermis, with or without stomata or trichomes, where nectar is released to the exterior, a specialized parenchyma that produces or stores nectar. Within this tissue usually the secretory cells are located and a vascular bundle for nutrient supply usually can be found (Stpiczynska *et al.*, 2005; Kaczorowski *et al.*, 2008). Mechanical leaf damage, volatile compounds exposure, and application of external jasmonic acid induce EFN secretion (Heil *et al.*, 2001; Choh *et al.*, 2006; Choh and Takabayashi, 2006; Kost and Heil, 2008; Radhika *et al.*, 2008).

Nectar is basically of phloem origin and is believed to move to the secretory cells through numerous plasmodesmata (Fahn, 1988). This conventional view that nectar originates from phloem sap and may be modified by the nectary parenchyma is possibly oversimplified. There is an enormous variability in nectar features (volume, concentration and composition). Nectar is mainly composed of sugars and amino acids but may also contain inorganic ions, proteins, lipids, organic acids, phenolics or alkaloids (Jones, 1983).

Extrafloral nectar, unlike floral nectar, is always present on the surface of the nectary. There are two types of secretion, the holocrine and the merocrine. The first, involves cell death at the moment of secretion. In this case, the nectar is produced within the cell followed by the rupture of the plasma membrane, thus releasing the cellular contents (Vesprini *et al.*, 2008). The merocrine secretion allows the secreting cells to survive and to continue with their secretory activity (Nepi and Stpiczynska, 2007;

2008). Within the merocrine type the following distinction can be made: The Eccrine secretion, which comprises transport of individual molecules across the cell membrane, possibly by transport via specialized proteins. And the granulocrine secretion, where transport of solutions within vesicles from ER or dictyosomes occurs that subsequently fuse with the plasmalemma and finally release nectar into the wall area (Dauwalder and Whaley, 1982; Sauer *et al.*, 1994; Jurgens and Geldner, 2002) .

All extrafloral nectaries so far ultrastructurally described have chloroplasts in their parenchyma (Pacini *et al*., 2003). Therefore part of the nectar sugar content might be produced by the nectary itself. Another explanation is that these chloroplasts serve as energy sources for the high demand of these extremely active cells. Nectar can be produced for days (annual plants) or months (perennial plants). Due to the long nectar production the nectary might be a portal for pathogens attracted by the sugar. This disadvantage is avoided by the presence of antimicrobial proteins in nectar (Naqvi *et al.*, 2005; Carter *et al.*, 2007).

Poplar is known as host for many herbivorous insects and pathogens (Arimura *et al.*, 2004; Lawrence *et al.*, 2006; Ralph *et al.*, 2006). Nectaries of the genera *Populus* were first described by Trealease in 1880. Since that time not much attention has been paid to this interesting defense mechanism in poplar (Wooley *et al.*, 2007). A deeper knowledge of how poplars defend themselves against insect herbivores will provide a basis for understanding defense mechanisms of woody plants in general. How defence in long-lived plants such as trees compares with that in short-lived annuals and crops is one of the outstanding questions in the field of plant-herbivore/pathogen interaction. To gain new insides into this field poplar nectary ecology, physiology, and molecular biology together with the nectar composition and biochemistry were explored in the present study.

1.2 ABIOTIC STRESS

In the recent years cellular and molecular responses of plants to environmental stresses have been studied in detail (Munns, 2005; Liu *et al.*, 2007; Attia *et al.*, 2008; Dinneny *et al.*, 2008; Fabienne *et al.*, 2008). Abiotic stress is a major constraint in crop and food production. A common factor limiting growth of crops worldwide is low water supply (Boyer, 1982). However, other abiotic stresses, notably salinity, are becoming increasingly significant (data from www.fao.org, Food and Agriculture Organization of the United Nations). Nevertheless, most studies on water stress signaling have focused

on salt stress, primarily because plant responses to salt and drought are closely related (Zhu, 2002).

Water supply is a critical factor to all life forms. Plants subjected to water stress undergo numerous physiological and metabolic changes. In response to decreased soil water potential the roots produce the phytohormone, abscisic acid (ABA), which leads to stomatal closure in leaves. In turn transpirational water loss is reduced. As matter of fact CO_2 intake and thus photosynthectic carbon gain is lowered too (Christmann *et al*., 2006).Besides drought cold or high salt, reflect conditions of low water potential too.

About two thirds of the world's landmass is annually subjected to temperatures below the freezing point, and about half of it suffers from temperatures below -20ºC (data from www.fao.org). Plants living in temperate climates require tolerance to the seasonal appearance of cold. However, tropical or sub-tropical species may also experience sudden cold temperatures. In general low temperatures may impose stress on plants by effects of low temperature and additionally by dehydration of the cells and tissues when cellular water starts to freeze (Beck *et al.*, 2004). Therefore cold stress is often accompanied by osmotic stress. Plants have evolved different strategies to avoid or decrease the impact of cold stress by, for example, altering the lipid composition of the biomembranes to maintain their fluidity (Williams, 1990), synthesis and accumulation of compatible solutes (Shinozaki and Yamaguchi-Shinozaki, 2000), changes in the carbohydrates metabolism (Frankow-Lindberg, 2001), or boosting the radical scavenging potential of cells (Hernandez-Nistal *et al*. 2002; Baek and Skinner, 2003).

1.2.1 Salt stress

One of the most abundant abiotic factors affecting plants is high salt content in soil. According to the FAO (www.fao.org) over 6% of the world's land is affected by either salinity or sodicity (Table 1). A "saline soil" is defined as having concentrations of soluble salts that are high enough to affect plant growth. The ions responsible for salinization are: Na^+, K^+, Ca^{2+}, Mg^{2+} and Cl^-. If Na^+ (sodium) predominates, the soil is often also called *sodic*.

Regions	Total area	Saline soils	%	Sodic soils	%
Africa	1899.1	38.7	2.0	33.5	1.8
Asia, the Pacific and Australia	3107.2	195.1	6.3	248.6	8.0
Europe	2010.8	6.7	0.3	72.7	3.6
Latin America	2038.6	60.5	3.0	50.9	2.5
Near East	1801.9	91.5	5.1	14.1	0.8
North America	1923.7	4.6	0.2	14.5	0.8
Total	12781.3	397.1	3.1%	434.3	3.4%

Table 1. Regional distribution of salt-affected soils in million ha. Over 6% of the World's land is salt affected. Data from the FAO, 2007.

Primary salinity occurs as the result from accumulation of salts through natural processes over long periods of time in soil or groundwater. Secondary salinity results from human activities that change the balance of water applied (irrigation) and water used by crops (transpiration). Clearing and irrigation provide more water than the crops could use. The excess watering raises ground water table and mobilizes salts previously stored in the subsoil bringing them up to the root zone. Subsequently the plants take up the water leaving the salt behind until the soil water becomes too salty for further uptake. Symptoms of soil salinity include slow and spotty seed germination, reduced total number of germinating seeds and postponed initiation of germination processes, sudden wilting, stunted growth, marginal burn on leaves (especially lower, older leaves), leaf yellowing, leaf fall, restricted root development, and sudden or gradual death of plants (Qu *et al*.2008; Kozlowski 1997). Salinity often alters the morphology and anatomy of woody plants by inhibition of the cambial growth rate and formation of proper shaped cambial derivates (Kozlowski, 1997; Escalante *et al.*, 2009).

Salinity imposes two major stresses on the plant: one is due to the presence of salt in the soil solution which leads to a high osmotic pressure and low water potential that reduces the ability of the plant to take up water. When the salt is taken up, salinity also lowers the water potential of the roots, which quickly causes growth reductions along with a set of metabolic changes identical to those caused by water stress. The second stress is more ion-specific and is due to high NaCl concentrations in the soil, which

make it difficult for the plant to discriminate Na^+ and Cl^- from other cations. Uptake of Na^+ is accompanied by a decreased root intake of the essential macro-nutrients K^+ and Ca^+ (Rubio *et al.*, 1995). The role of chloride in salinity stress is less clear. Uptake of Cl^- may alter uptake of other anions such as phosphate and nitrate. These effects are, however, complex and vary between species (Kozlowski 1997 and references therein). If excessive amounts of salt are translocated via the transpiration stream, injuries of leaf cells and photosynthesis are unavoidable which may cause further growth reductions. Many of the observed effects associated with salt stress are probably due to the reduction of the photosynthetic capacity feeding back on the carbon- and energy supply demanding sink cells (Escalante *et al.*, 2009).

Plants can adapt to salinity by taking up salt (tolerance) or by largely excluding it (avoidance), or by both. Absorbed salts are sequestered in vacuoles to reduce the salt concentration within cytoplasm and chloroplasts. In return the cytoplasm often has to build up high concentrations of compatible osmolytes that counterbalance high salt concentrations in the vacuoles (Koslowski 1997). As a further mechanism of salt tolerance the regulation of K^+/Na^+ selectivity by carrier proteins has been discussed (Munns, 2002). A large number of salt-stress-responsive genes have a function in ion homeostasis, including, e.g., plasma membrane Na^+/H^+ antiporters for Na^+ exclusion (Shi *et al.*, 2000; Yokoi *et al.*, 2002), vacuolar Na^+/H^+ antiporters for compartimentation in the vacuole (Apse *et al.*, 1999; Sottosanto *et al.*, 2004; Ottow *et al.*, 2005a) and high affinity K^+ transporters and channels for K^+ acquisition (Becker *et al.*, 2003; Langer *et al.*, 2004).

Salt exclusion is particularly important for perennial species with leaves required to perform photosynthesis during the entire live span. These species need to well control the incoming salt load. Studies on the physiological basis of genetic variation in salinity tolerance of the model tree *Populus* revealed an important role for the phytohormone ABA in this event (Bolu and Polle 2002; Ottow et al. 2005a, b and references therein).

1.2.2 The role of ABA

Although ABA has broad functions in plant growth and development, its main role is to regulate plant water balance and to enhance osmotic stress tolerance (Zhu, 2002). Several ABA-deficient mutants have been reported for *Arabidopsis*, such as *aba1*, *aba2* and *aba3*. Under salt exposure, they readily wilt and die if the stress persists (Leon-Kloosterziel *et al.*, 1996; Schwartz *et al.*, 1997). ABA levels increase in response

to drought, salt and cold stress. (Shinozaki and Yamaguchi-Shinozaki, 2000; Chen *et al*. 2001 and 2002; Chang *et al*. 2006 and references therein). Under drought and salt stress ABA induces stomata closure, and thus reduces water loss by transpiration (Zhang *et al.* 1987; Becker *et al.*, 2003; Roelfsema *et al.*, 2004). Simultaneously this phytohormone triggers cellular dehydration tolerance by induction of genes involved in the synthesis of compatible osmolytes (Xiong *et al.*, 2001). In this context it should be mentioned that ABA induces biosynthesis of a large number of osmolyte regulating genes as for example members of the KIN family (also named COR, cold-regulated). In particular KIN2 has been identified to be drought and cold inducible in *Arabidopsis* (Webb *et al.*, 1996; Seki *et al.*, 2001). The small KIN proteins are extremely hydrophilic, harbor repetitive amino acid sequence motifs and remain soluble upon boiling in dilute aqueous buffer. The characteristics of these polipeptides suggest that they act in the cytoplasm during severe cellular dehydration by binding water molecules to their surface and thus prevent dehydration damage of the protein motifs and structural components the membrane systems (Kurkela and Borg-Franck, 1992; Wang and Cutler, 1995; Thomashow, 1998; Gong *et al.*, 2001; Zhu, 2001).

GOALS

The aim of this work was to study the response of poplar trees to different (abiotic or biotic) stress factors.

To reach the first part goals

1. The effect of salt stress on changes in wood morphology was studied.
2. The questions “how does high salinity affect the mineral transport/profile” and
3. “How does high salinity affect ABA levels of this woody species” were followed?

Concerning biotic stress in the second part of this study was focused on the role of extrafloral nectaries of *Populus* in herbivore control.

To reach the second part goals the following topics were examined

1. The composition of nectars from both species.
2. Mechanisms of nectaries and nectar induction.
3. Microarray analysis with poplar nectaries to gain the expression profile of the first specialist secretory plant structure.

2. MATERIAL AND METHODS

If not otherwise indicated, all chemicals are from Sigma (Sigma-Aldrich, Steinheim, Germany) or AppliChem (AppliChem, Darmstadt, Germany).

2.1 BIOTIC STRESS

2.1.1 Plant material and growing conditions

2.1.1.1 Poplar field-grown culture

Populus tremula × *P. tremuloides* plants (clone T89) and *Populus trichocarpa* were grown in soil under natural conditions (Fig.2.1).

Fig 2.1 *Populus tremula tremuloides* field- culture. Spring 2007.

2.1.2 Light microscopy

Nectaries from *P. trichocarpa* and *P. tremula tremuloides* were harvested and fixed by passing through ascending grades of ethanol. The time of exposure at each stage was 45 min. Following dehydration, nectaries were embedded in 2-hydroxyethyl methacrylate (HEMA)/GMA, using the AGAR GMA (HEMA) KIT (Agar Scientific, Stansted, England) according to the protocol.

For microscopy, 20µm sections of extrafloral nectaries were cut with a profile c-16cm knife (Leica, Nussbloch, Germany) in a Leica RM2165 microtome (Leica, Nussbloch,

Germany) and heat-fixed to microscope slides. Stained with toloudine blue and then mounted in immersion oil under coverslips.

Sections were examined with a Keyence VHX-100k digital microscope (Keyence Corporation, Osaka, Japan).

2.1.3 SudanIII Staining

100µm thick sections of extrafloral nectaries from P. tremula tremuloides were cut in a Hand microtome (Leica) and stained with Sudan III (Sigma-Aldrich) 0.3µg/ml. Sections were examined with a Keyence VHX-100K digital microscope (Keyence Corporation).

2.1.4 FM 4-64 Staining and confocal microscopy

Nectaries from *Populus tremula tremuloides* were immersed for 1 minute in a solution containing 5 µl/ml Fm 4-64 dye (Invitrogen), the staining solution was kept on ice during this time. After 1 minute incubation the nectaries were washed and subsequently cut into 100µm sections with a Hand microtome (Leica).

Sections were examined with confocal microscopy using a laser scanning microscope, Axioskop2 mot Plus (Zeiss), with an excitation λ of 558nm and an emission λ of 734.

2.1.5 TEM analysis

Nectaries from P. trichocarpa and P. tremula tremuloides from field-grown cultures were collected. Fixation, embedding and microscopy pictures were performed in collaboration with Prof. Fromm workgroup in the “Technische Universität München” (Munich, Germany), for details see Arend and Fromm, 2003.

2.1.6 Quantification of nectar sugars

For nectar sugar quantification, different amounts of nectar were diluted in HPLC water until 1ml, boiled at 105ºC for 5 min and subsequent centrifuged 10 min at maximal speed. The supernatant was treated with 10mg per 100 µl sample Serdolit MB1 (Serva, Heidelberg, Germany) and the sugar concentration was measured by Eva Wirth (Julius-v.-Sachs-Institut, Universität Würzburg) using PED (pulse electrochemical detector) Dionex 4500i (Dionex, Idstein, Germany).

2.1.7 Quantification of nectar ions

For nectar anion quantification different amounts of nectar were diluted in HPLC water until 1ml, centrifuged at maximal speed for 10 min at 4ºC. Boiled at 105ºC for 5 min. Insoluble components were removed by centrifugation at maximal speed for 10 min at 4ºC. The concentration of anion in the supernatant was determined by Eva Wirth (Julius-v.-Sachs-Institut, Universität Würzburg) by HPLC anion chromatography, Biotronik IC1000 (Biotronik, Maintal, Germany). Nectar cation quantification was performed by Elfriede Reisberg (Julius-v.-Sachs-Institut, Universität Würzburg) using an ICP-emission spectrometer JY70 plus (Division dínstruments S.A., Jobin Yvon, France).

2.1.8 Quantification of nectar amino acids

Nectar amino acids quantification and probes preparation was performed by Elfriede Reisberg (Julius-v.-Sachs-Institut, Universität Würzburg). Amino acid quantity and quality was measured using an Amino acid analyser Biochrom 20 Plus (Biochrom Ltd., Cambridge Science Park, England).

2.1.9 Nectar proteome analysis

Proteome analyses were performed by Dr. Yvonne Reinders and Dr. Joerg Reinders (Prof. Mueller workgroup, Pharmazeutische Biologie, University of Wuerzburg, Germany).

Samples of *P. trichocarpa* nectar were collected from field-grown cultures between May and June 2007, and stored at -20ºC until further use. Aliquots of 100µl diluted nectar were resuspended in SDS-PAGE buffer (see below), heated to 90℃ for 10min and separated in a 4%-20% 1D-Tris-HEPES-SDS-Gel (Precise Protein Gel, Pierce, IL, USA). Gels were stained as described by Neuhoff *et al*. (1990) and washed to remove detergent and dye previous to tryptic digestion. The obtained peptide mixture was separated by nano-RP-HPLC using an ultimate 3000 nano-HPLC system (Dionex, Idstein, Germany) and further analyzed using a LCQ DecaXP^plus iontrap mass spectrometer (ThermoElectron, Dreiech, Germany). The MS/MS spectra were analyzed using the Mascot algorithm (version 2.1; Matrixscience, London, U.K.) which searched against the JGI Populus trichocarpa v1.1 database. For details see Schönleben et al. (2007).

2.1.10 Anti-microbial test

The assay was carried out in Petri plates (100x15mm) containing 10ml YEP media supplemented with 50 µg/ml ampicillin. Before the mycelial colony developed, sterile blank paper disk had been placed, Sample Discs SS-033 (Wescor, Utah, USA). An aliquot of nectar or nystatin 50mg/ml was added to the disk. The plates were incubated at room temperature about 72 hours until mycelial growth had enveloped the disk containing the water control.

YEP media
For 1 liter
-5g Trypton -5g Yeast extract
-5g Sucrose
-50mM $MgSO_4$
-15g Agar, Dänisch (Carl Roth, Karlsruhe, Germany)

To exclude that the fungal growth inhibition was caused by the high sugar concentration present in nectar, a solution containing sugars in the same amount as in nectar was added to a disk. After 72 hours mycelial growth was observed on those disks.

In a further setup the nectar was boiled for 15 minutes to denaturize the proteins. After 72 hours of room temperature incubation of the plates, mycelia growth inhibition could be detected around the disks containing nystatin (positive control) and untreated nectar while the fungi perfectly grew around the disks containing water (negative control) or pre-boiled nectar (Fig 2.2).

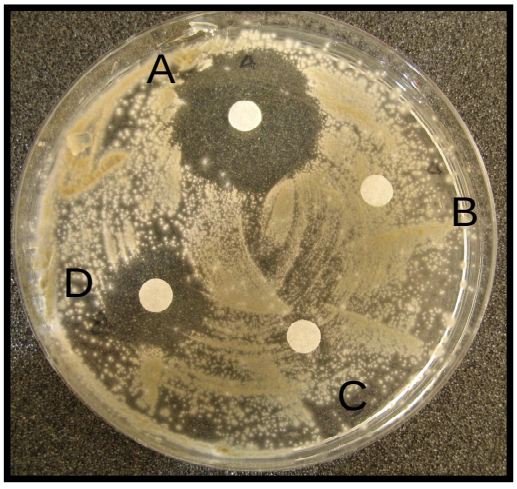


Fig 2.2 Nectar lost its antifungal properties when proteins were denaturized. A= nystatin, B= water, C= denaturized nectar, D= nectar.

2.1.11 Induction of extrafloral nectaries and nectar

The experiment was conducted under natural conditions in about one year old *P. trichocarpa* soil-grown plants. The four youngest fully expanded leaves of each plant were induced, either puncturing 100 times with a needle (1mm diameter) or by cutting the leaf tip (about 10% of the total leaf area). Plants were observed using a Keyence VHX-100k digital microscope (Keyence Corporation, Osaka, Japan). Every 15 min pictures were taken in order to know the exact time of induction.

For “herbivory-induction”, three caterpillar (of undetermined specie) were placed in *P.trichocarpa* soil-grown plants.

2.1.12 RNA isolation and amplification for microarrays

Total RNA of leaves was extracted from ground plant material using the E.Z.N.A. Plant RNA Mini Kit (Omega bio-tek, GA, USA) according to the manufacturer’s protocol with minor modifications. 700µl RB buffer, supplemented with 2% β- Mercaptoethanol, 1% PVP and 70 mM K-Ethylxantogenate, was added to 30mg of homogenized leaf tissue and incubated for 30 min at room temperature.The sample was briefly centrifuged before transfer to homogenation column. The supernatant was centrifuged for 30 minutes at 4ºC at maximal speed, and further treated according to the protocol.

For nectaries total RNA extraction the RNeasy MicroKit (Qiagen, Hilden, Germany) was used according to the manufacturer’s protocol with minor modifications. 500 µl RTL buffer supplemented with 1% β- Mercaptoethanol, 1% PVP and 70 mM K-Ethylxantogenate, was added to 120 homogenized nectaries and incubated at room temperature for 30 min. After in incubation the samples were centrifuged for one hour at 4ºC at maximal speed, and further treated according to the protocol.

1 µg total RNA was used for RNA amplification based on the BD-SMART mRNA amplification kit (BD Bioscience, www.clontech.com). The mRNA was reverse-transcribed using SuperScript II Reverse Transcriptase (Invitrogen, CA, USA) and the kit’s buffer. To pre-amplify full-length cDNAs prior to in vitro transcription, an additional 10 cycle PCR (95ºC for 30 sec, 60ºC for 1 min, and 68 º C for 10 min) was introduced using t7 extension and PCR primer IIA of the kit.

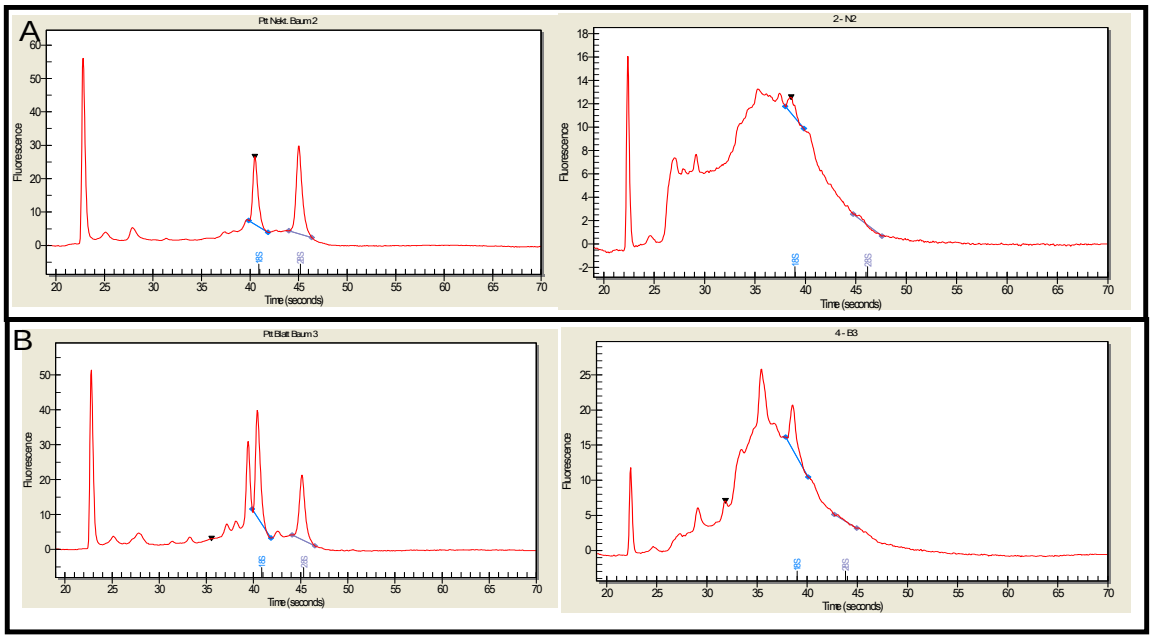


Fig.2.3 Electropherogram of RNA. Quality control for RNA prior to and after RNA amplification (A) left, Total RNA from nectaries. Right, its amplification. (B) left, total RNA from leaves. Right, its amplification. In both tissues the distribution of fragment length of the sample hasn't changed result of the amplification procedure.

RNA integrity and concentration was monitored using a Experion Automated Electrophoresis System (BIO-RAD) with the Experion RNA Highsense Analysis Kit according to the manufacture's protocol (Fig.2.3).

2.1.13 Microarrays

Microarray analyses were conducted at the Microarray Facility, University of Tübingen, Germany. Samples of leaves and nectaries from *P. tremula tremuloides* field-culture were analysed. All samples were amplificated using the "One-Cycle Target Labellig Assay" (Affymetrix) according to the manufacture's protocol and hybridized to the Gene Chip Poplar Genome (Affymetrix), for details see Deeken *et al.* 2008.

Microarrays were scanned using the GCS3000 GeneChip scanner (Affymetrix) and GCOS software, version 1.4. Scanned images were subjected to visual inspection to control for hybridation artefacts and proper grind alignment. Files of quality control were generated using the program "Expression Console" (Affymetrix). The intensity files were analysed using Microarray Suite 5.0 (MAS5; Affymetrix) to calculate the signal value for each probe set. Biostatistic analyses were performed by Dr. Tobias Müller and Marcus Dittrich from the Bioinformatic department of Wuerzburg University.

2.1.14 Volatile compounds measurements

Measurements of volatile compounds of *Populus trichocarpa* under different conditions were obtained in collaboration with Prof. Boland workgroup in the Max Planck Institute for Chemical Ecology in Jena, Germany. All experiments were done with help of Dr. Kunert.

Headspace volatiles emitted by Poplar treated with caterpillar, MecWorm or Jasmonic acid were continuously collected in charcoal traps and analysed by gas chromatography (GC)-mass spectrophotometry (MS) as described in (Mithofer *et al.*, 2005).

2.1.14.1 Animal material and sample preparation

For the experiments larvae of *Spodoptera Littoralis* and *Helicoverpa sp*. (Lepidoptera, Noctuidae) were used. For details of growing and feeding conditions see Bergomaz and Boppré 1986.

P. trichocarpa plantlets were cut with a razor blade and transferred to a vial, the caterpillar were placed on the leaf blade and allowed to feed. For headspace analyses, the vials containing the plants were enclosed in dessicators (750ml).

2.1.14.2 Mechanical caterpillar

The mechanical caterpillar, MecWorm (Fig.2.4), was engineered in Max Plack Institute to user specification for the imitation of wounding that emerges upon caterpillar feeding, for details see Mithofer *et al.*, 2005.

Fig.2.4 Mecworm, the mechanical caterpillar. Left, overview of the MecWorm system. Right, section of the punching unit of the Mecworm, including the aglet treating a *P. trichocarpa* leaf.

For these experiments a damage programme was set up to punche every 10sec during 25h, as result damage of 80% the leaf surface was caused. Plants used for continuous mechanical damage were enclosed in a Plexiglas cabinet (approximately 500ml) and punched for the time indicated (Fig 2.5B).

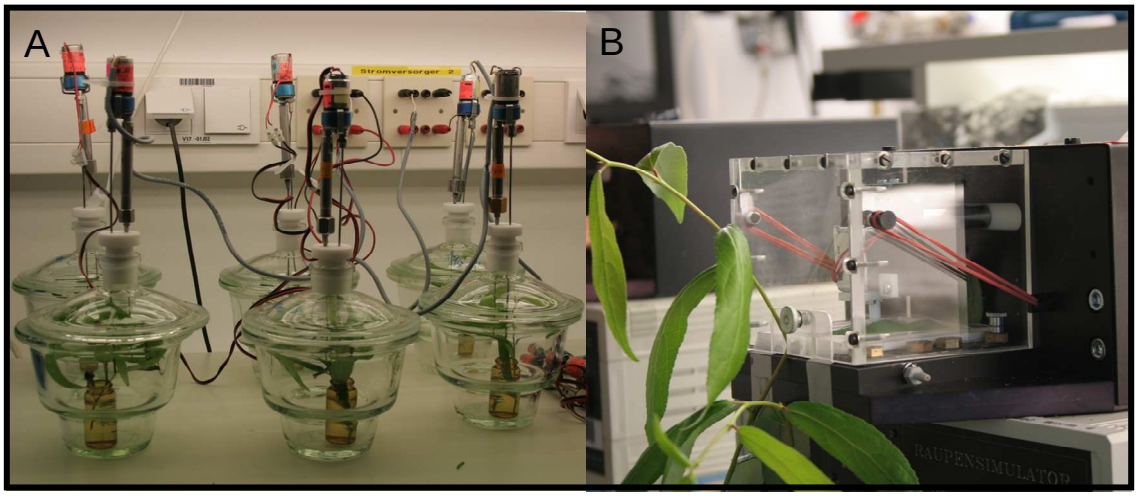


Fig.2.5 Headspace volatile collection. (A) Plantlet treated with Jasmonic acid inside the dessicator connected to the volatile collection unit. (B) Plexiglas cabinet for the MecWorm volatile collection.

2.1.14.3 Jasmonic acid treatments

P.trichocarpa plantlets were cut with a razor blade and transferred to a vial containing either 1mM or 0,5mM jasmonic acid. The vials containing the plants were enclosed in dessicators (Fig 2.5A). The jasmonic acid was kindly supplied by Dr. Kunert.

2.2 ABIOTIC STRESS

Material and methods on poplar salt stress experiment in detail in Escalante *et al*. 2009 (see supplement).

2.2.1 Plant material

2.2.1.1 Poplar agar-culture.

The poplar hybrid *P.tremula L. x P. tremuloides* Michx. T89 and *P.canescens* (*P.alba x P. tremula*), INRA clone no. 717 1B4, were sterile cultivated in media containing hormones. In the first stages of development in Petri plates (Media I, for 3-4 weeks), afterwards the plants were transplanted to Medium II for the development of the

vegetative structures. The plants were transferred again after 3-4 weeks to weckglass containing Media III for the root system development (Fig.2.6). The plants were grown in a culture room at 22ºC/16ºC day/night temperature in a 16 hour photoperiod (25W, 230V OSRAM; TL70, F32T8/TL 741, Philips)

Agar-culture medium I (per 1 liter):

4.4g MS-medium (Murashige and Skoog, macro- and micro-elements)
0.2mg BAP (6-Benzylaminopurine)
0.1 mg IBA (Indolbutiric acid, Duchefa)
0.01 mg TDZ (Thiadiazuron, Duchefa)
20g Sucrose
pH 5.8 (with 1M KOH)
7g Agar Dänish (Roth#4508), autoclave

Agar-culture medium II (pro 1 liter):

4.4g MS+MES with vitamins (Duchefa #M0255)
0.2mg BAP
0.1mg IBA
20g sucrose
pH 5.8 (with 1M KOH)
10g Agar Dänish (Roth#4508), autoclave

Agar-culture medium III (per 1 liter):

4.4g MS+MES with vitamins (Duchefa #M0255)
20g sucrose
pH 5.8 (with 1M KOH)
10g Agar Dänish (Roth#4508), autoclave

Fig 2.6 Sterile culture *Populus tremula* L. x *Populus tremuloides* Michx. In Weckglass containing rooting media (Medium III).

2.2.1.2 Poplar hydro culture.

Plants with roots were transferred from the weckglass containing rooting medium to shade flasks with hydro culture medium. In the first month plants were kept inside a wet bag to prevent desiccation. The hydro culture media were exchanged every week (Fig.2.7).

Hydroculture medium:

2.0mM KNO_3
1.0mM $CaCl_2$
1.0mM $MgSO_4$
18µM FeNaEDTA
8.1µM H_3BO_3
1.5µM $MnCl_2$
pH 6.0

Fig 2.7 *Populus canescens* hydroculture

2.2.1.3 *Arabidopsis thaliana* plant material.

If not indicated differently, wild type plants of *Arabidopsis thaliana* ecotype Columbia 0 or aba 3.1 mutants (for details see León-Kloosterziel *et al.* 1996) were grown in potting soil in the greenhouse under an 8-h-light/16-h-dark regime at 22℃ and 60% relative humidity.

2.2.2 Cold stress treatments and *A.th*. sample preparation

One month old soil-grown plants of *Arabidopsis thaliana* ecotype Columbia (Col-0) and aba 1-3 mutants were used for cold-stress experiments. All plants were grown at 22ºC in a greenhouse under an 8h light/ 16h dark regime. Low-temperature treatments were performed by transferring the plants to a chamber set to 4ºC. Plant material was collected at the indicated times, sampling started with shock freezing (in liquid nitrogen) for additional analysis.

Total RNA of *Arabidopsis thaliana* was extracted from ground plant material using the Plant RNeasy mini kit, according to the manufacturer's instruction (Qiagen, Hilden, Germany). Remaining DNA was digested using RNase-Free DNase (Amersham, Freiburg, Germany) according to the manufacturer's protocol.

First-strand cDNA was prepared using 2.5µg RNA with the M-MLV-RT kit (Promega, Mannheim, Germany)

As standard the product of each primer pair were used, each fragment were in a 3% agarose gel tested and the concentration in the GeneQuant pro (Ge-healthcare) spectrophotometer was measured and diluted by serial dilutions until 20fg/µl. All quantifications were normalised to actin cDNA fragments amplified by ACTfwd and ACTrev. Each transcript was quantified using the individual standards. To enable detection of contaminating genomic DNA, PCR was performed with RNA as template. These DNA-free RNA samples were subsequently used for cDNA synthesis.

Real time PCR was performed either using the LightCycler (ROCHE, Basel, Switzerland) with the LightCycler-FastStart DNA Master SYBR Green I Kit (ROCHE) or the Realplex^{2}Mastercycler (Eppendorf, Hamburg, Germany) with the Absolute SYBR Capillary Mix (Thermo Scientific, UK). All kits were used according to the manufacturers' protocols.

3. RESULTS

3.1 BIOTIC STRESS

Poplar provides an important system for large-scale, short-rotation plantation forestry in the Northern Hemisphere. To sustain productivity and ecosystem health of natural and planted poplar forests, it is of critical importance to better understand the molecular mechanisms of poplar defense and resistance against insect pests or pathogens.

3.1.1. Extrafloral nectaries

Poplars are known to serve as host for many herbivorous insects. Extrafloral nectaries are one effective way of indirect defence against herbivorous. Since the first description of the extrafloral nectaries of the genera *Populus* in 1880 no further attention has been paid to nectary biology with this species. The main focus of this part of the study was, therefore, to gain a deeper insight in nectary-based plant defence.

3.1.1.1 Morphology and structure

From the anatomical point of view nectaries vary widely in morphology and structure, both among and within species. So far, only the structure of floral nectaries has been investigated in detail (Stpiczynska *et al.*, 2005; Wist and Davis, 2006; Wenzler *et al.*, 2008). Therefore, in the first step nectaries´ structure and ultrastructure were studied using two different poplar species: *P. trichocarpa and P. tremula tremuloides.*

Locations of extrafloral nectaries vary from species to species, but most often they occur on the petiole or rachis near the attachment of the leaf (Caspary, 1848). In many cases, the extrafloral nectary is a modified stipule or a trichome (plant hair) on a stipule. Other nectary types are modifications of leaf margins or trichomes on the blade surface (Bentley and Elias, 1983).

In both poplar species studied, *P.trichocarpa* and P*. tremula tremuloides*, nectaries were located on the base of the leaf blade near the petiole, always in pairs of two (Fig 3.1_1), one on each side*. P. tremula tremuloides* nectaries are bigger, while those of *P.trichocarpa* released more nectar (Fig 3.1_1).

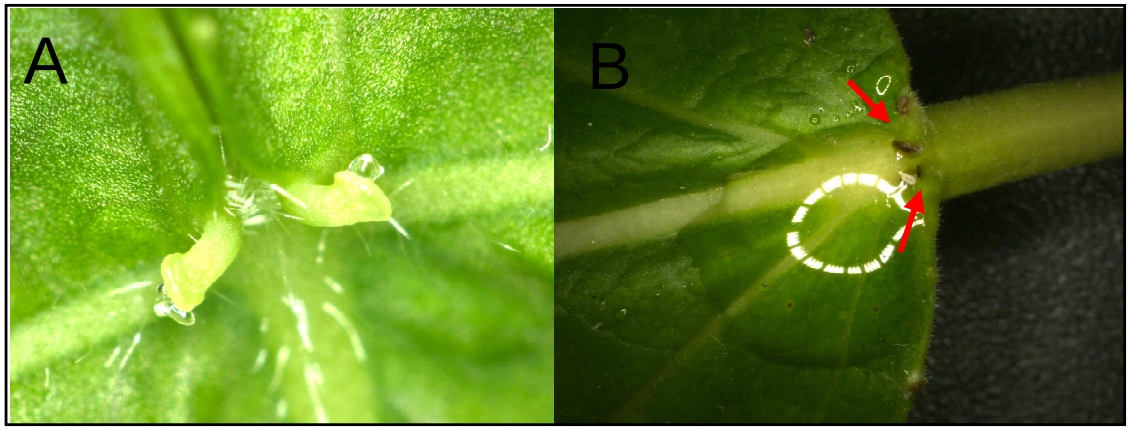


Fig 3.1_1 Disposition of extrafloral nectaries (A) *P.t.t*. nectaries are big but release only drops of nectar. (B) Nectaries of *P.t*. (red arrows) are smaller but release larger volumes of nectar.

The nectaries´ typical structure consists of three different tissues: a nectary epidermis, one layer of small and polyhedric cells which may have an anticlinal orientation (Razem and Davis, 1999). Beneath the nectary epidermis is the nectary parenchyma which consists of layers of small isodiametric cells. Within this layer usually the secreting cells are located (D´Amato, 1984). The layer below is the subnectary parenchyma that generally consists of larger cells, which are more loosely packed than those of the nectary parenchyma.

Light microscopy analyses of the *P. trichocarpa* nectaries revealed a structure that perfectly meets those described above (Fig 3.1_2 A). *P. tremula tremuloides* nectaries also developed a nectar- and subnectary parenchyma. But in contrast to *P. trichocarpa* the outer layer consisted of modified epidermal cells which seemed to be connected to nectar secretion (Fig 3.1_2 B). This nectary morphology has not been described before.

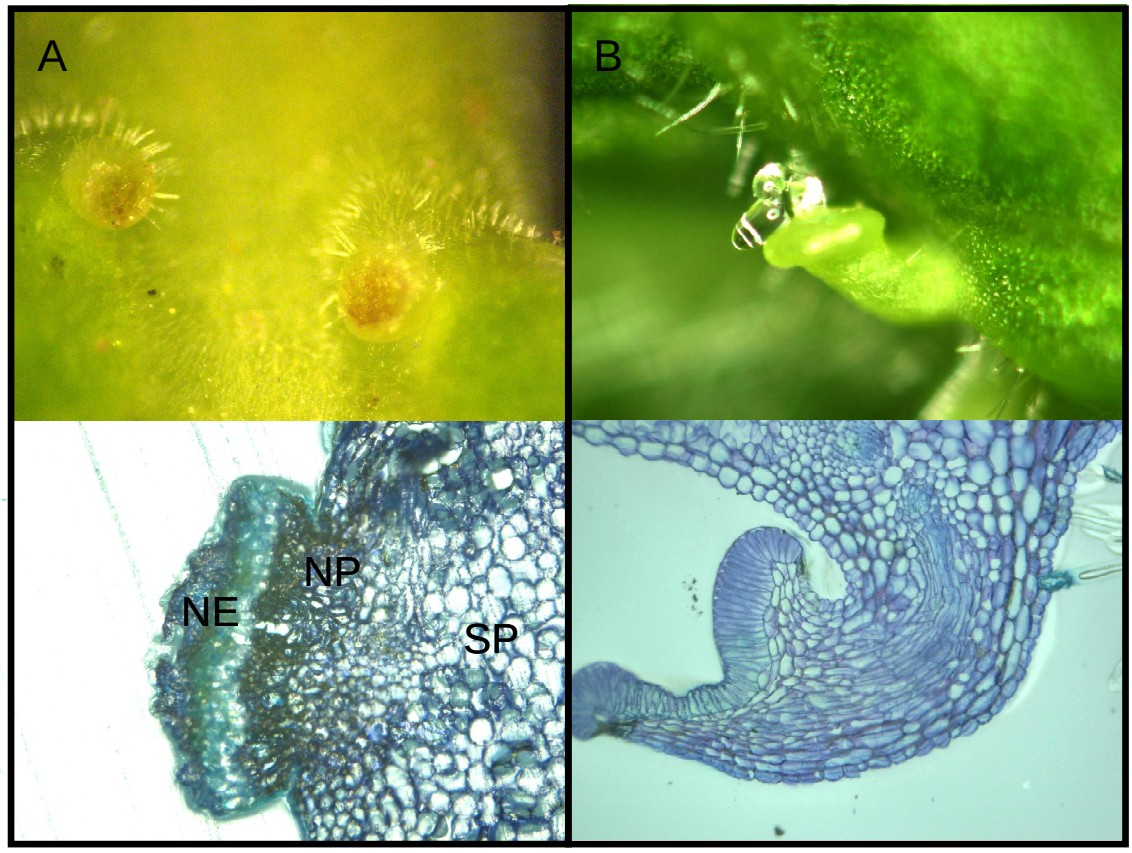


Fig 3.1_2 Light microscopy on extrafloral nectaries. (A) *P.t.* nectaries structure, NE (nectary epidermis) NP (nectary parenchyma), SP (subnectary parenchyma). (B) *P.t.t.* nectaries have secretory cells in the nectary epidermis.

To study the morphology in more detail, nectaries were subjected to TEM analysis. The surface of nectary epidermal cells of *Populus trichocarpa* is entirely covered by a cuticle on their so called micro-channels seems to represent the pathway for nectar release to the outer surface (Fig 3.1_3). These micro-channels appear as fibrillar outgrowths of the outer epidermal cell wall, a structure that has been previously described in floral nectaries of *Platanthera chlorantha* (Orchidaceae), *Abutilon sp*. and *Helleborous foetidus* (Ranunculaceae, Kronestedt *et al.*, 1986; Stipiczynka, 2003; Koteyeva, 2005). Micro-channels are narrow tubular interruptions of the cuticle in continuity with the cell wall.

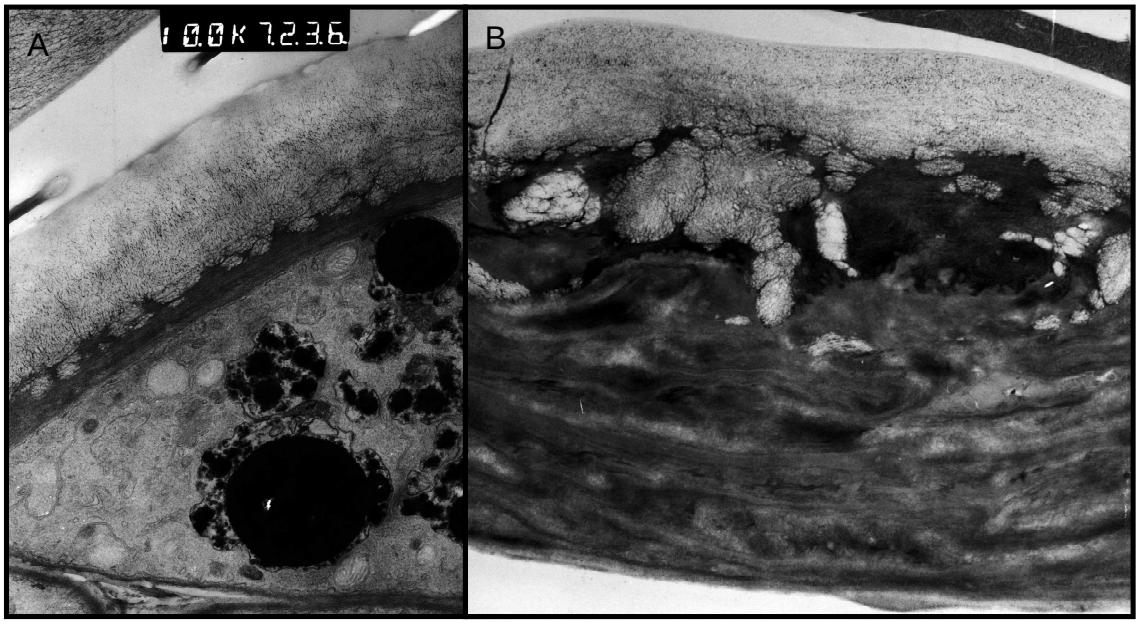


Fig 3.1_3 Ultrastructure of *P. trichocarpa* nectary epidermis. (A) Overview of an epidermal cell. (B) Detail view of the cell wall and cuticle with micro-channels.

Ultrastructural analyses of *P. tremula tremuloides* nectaries showed that the secretory cells (specialized epidermal cells) at their lateral side are interconnected by a large number of plasmodesmata. In contrast, no such connections with the nectary parenchyma at the basolateral side could be found (Fig 3.1_4 B and C). The structure indicates that the pre-nectar is loaded into the nectary parenchyma apoplastically and reaches the secretory cells via a symplastic route. These brush boarder-like cells have very elaborate ER systems, dictyosomes and vesicles, features characteristic for secretory cells. Also excreted vesicles are visible in the outer apoplastic space (Fig 3.1_4 D)

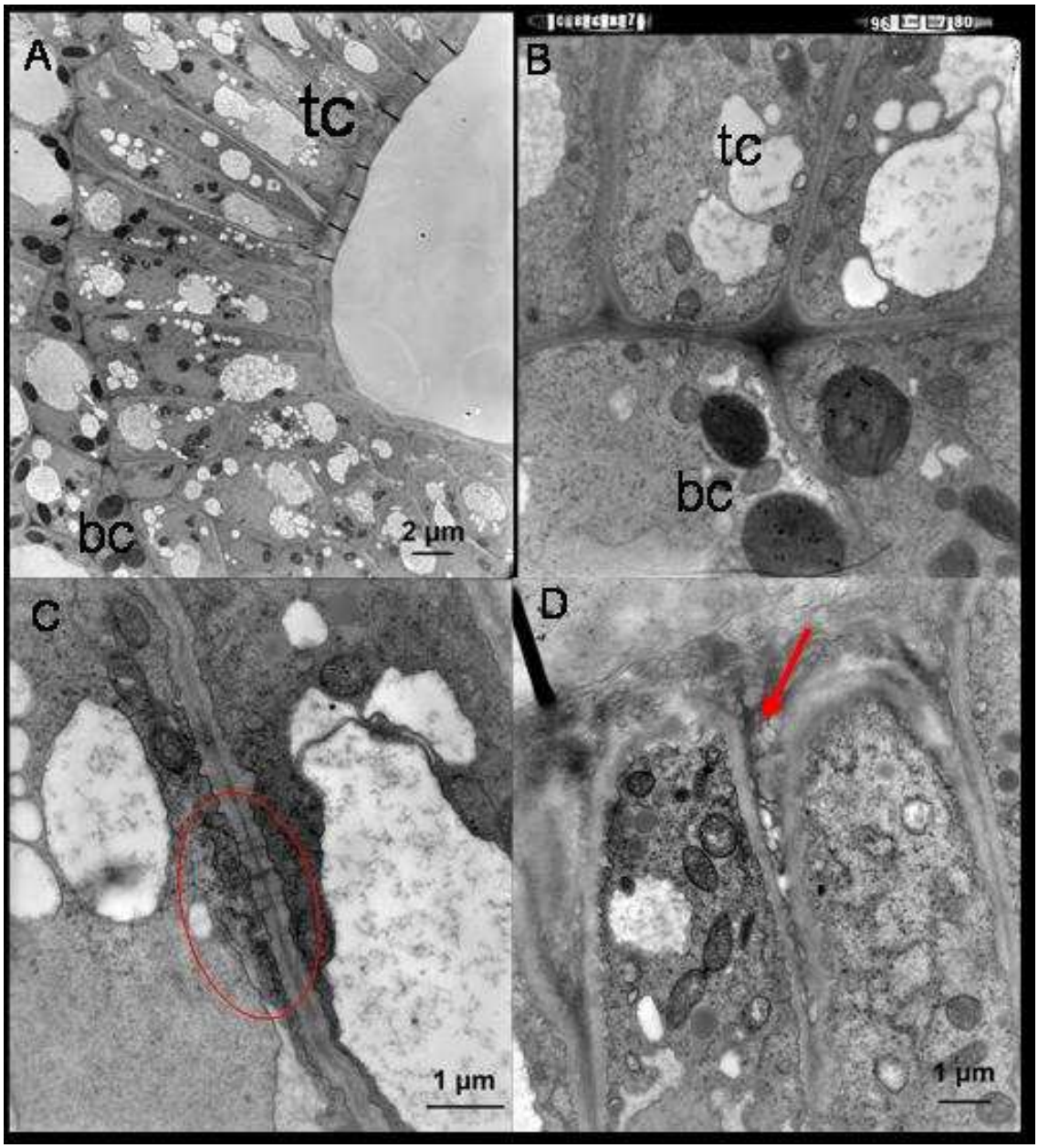


Fig 3.1_4 Ultrastucture of *P.t.t.* nectary epidermis.(A) Overview of secretory cells. (B) Absence of connections between the secretory cell and the basolateral neighbours.(C) Symplastic connections between two secretory cells. Note, the plasmodesmata inside the red circle. (D) Endo/exocytosis vesicles of secretory cells (red arrow), secretory cell (tc), basal cell (bc).

In the TEM analysis and Sudan III staining with *P.t.t.* nectaries, surprisingly, no cuticle covered the epidermal secretory cells. Only a lid protruding from the nectary seemed to protect the secretory cells from the environment (Fig 3.1_5).

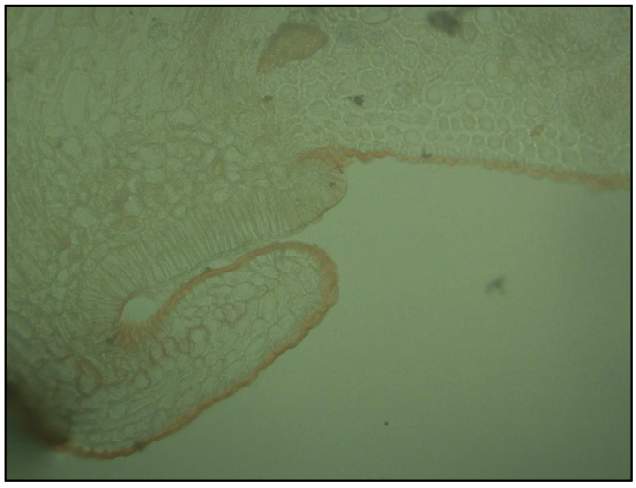


Fig 3.1_5 *P.t.t.* secretory cells are not covered by a cuticle. Nectaries stained with Sudan III. A lid of parenchyma cells covers the secretory cell

Some floral nectaries are able to reabsorb the unconsumed nectar by endocytosis (Nepi and Stpiczynska, 2007 and 2008). To test whether *P. tremula tremuloides* belongs to this group, nectaries were stained with a fluorescents dye. FM4-64 is a styryl chromophore, readily soluble in water but essentially non fluorescent until bound to membranes. Following absorption into the outer leaflet of the membrane fluorescence quantum yield is 50- to 100-fold enhanced (Schote and Seelig, 1998). Ammonium groups prevent the dye from crossing the bilayer, making FM4-64 a suitable endocytosis marker. Longitudinal-sections with stained nectaries revealed typical structural characteristics for endocytotic events in the secretory cells. The dye didn't cross the apoplastic space between the secretory and the neighbouring cells pointing to a pronounced endocytotic capacity of the secretory cells only (Fig 3.1_6).

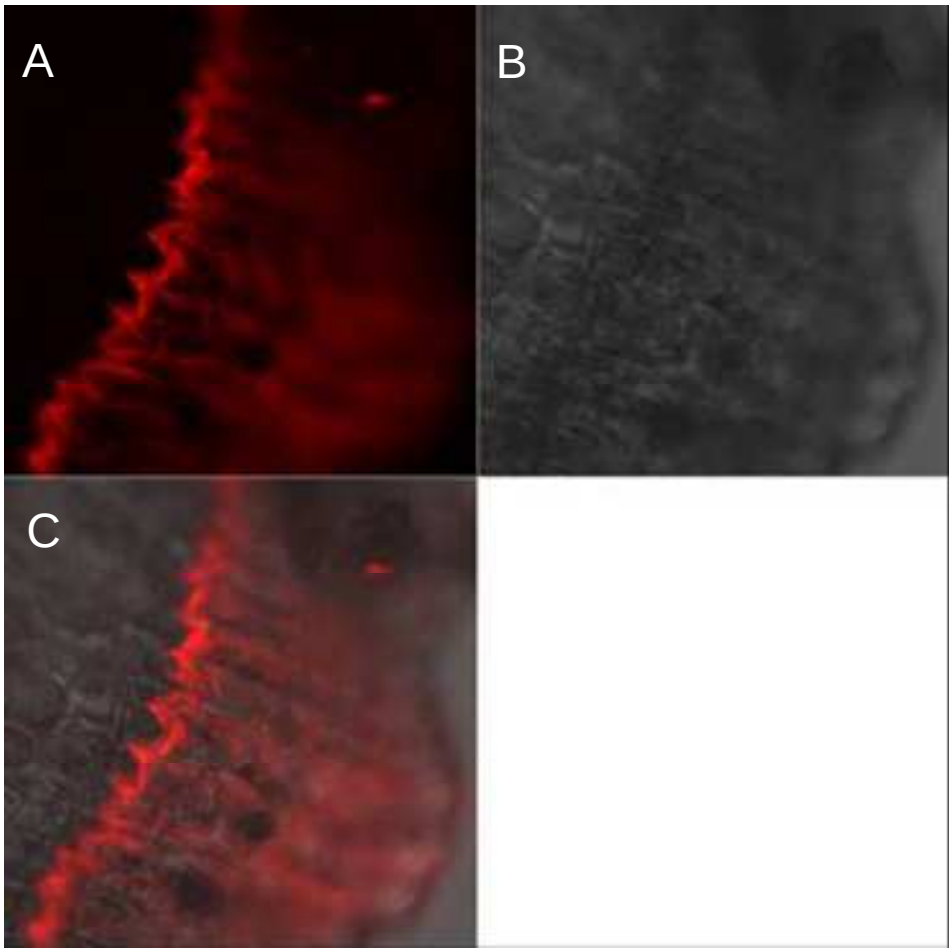


Fig 3.1_6 Fluorescence microscopy of nectary longitudinal-sections stained with FM4-64. (A) Nectaries labelled for 10 min at 4ºC with FM 4-64. (B) Bright field microscopy. (C) Merge of A and B. The dye is only internalized in the secretory cells and is unable to cross the apoplastic space between these and the neighbouring cells.

In *P.trichocarpa* the isodiametric nectary parenchyma cells (including the secretory cells) could be distinguished from ground parenchyma by the presence of a dense granular cytoplasm rich in ribosomes (Fig 3.1_7 A). A gradient in vacuole size and -formation was observed from the base of the tissue to the secretion site. Small vacuoles were present only in the pre-secretory phase. The cytoplasm is rich in mitochondria and chloroplasts, in line with the high energy requirements for nectar production (Fig 3.1_7 A and D). The vacuoles of the nectary parenchyma contained different types of inclusions (Fig 3.1_7 B).The nectary parenchyma cells have generally thin walls (D´Amato 1984), in *Populus trichocarpa* these cells possessed unusual thick walls with numerous pits and associated plasmodesmata connecting the protoplasts of adjacent cells (Fig 3.1_7 C and D). Around the symplastic connections one recognizes

numerous chloroplasts and mitochondria along with rough ER, dictyosomes (Golgi apparatus), and plastids containing numerous plastoglobuli.

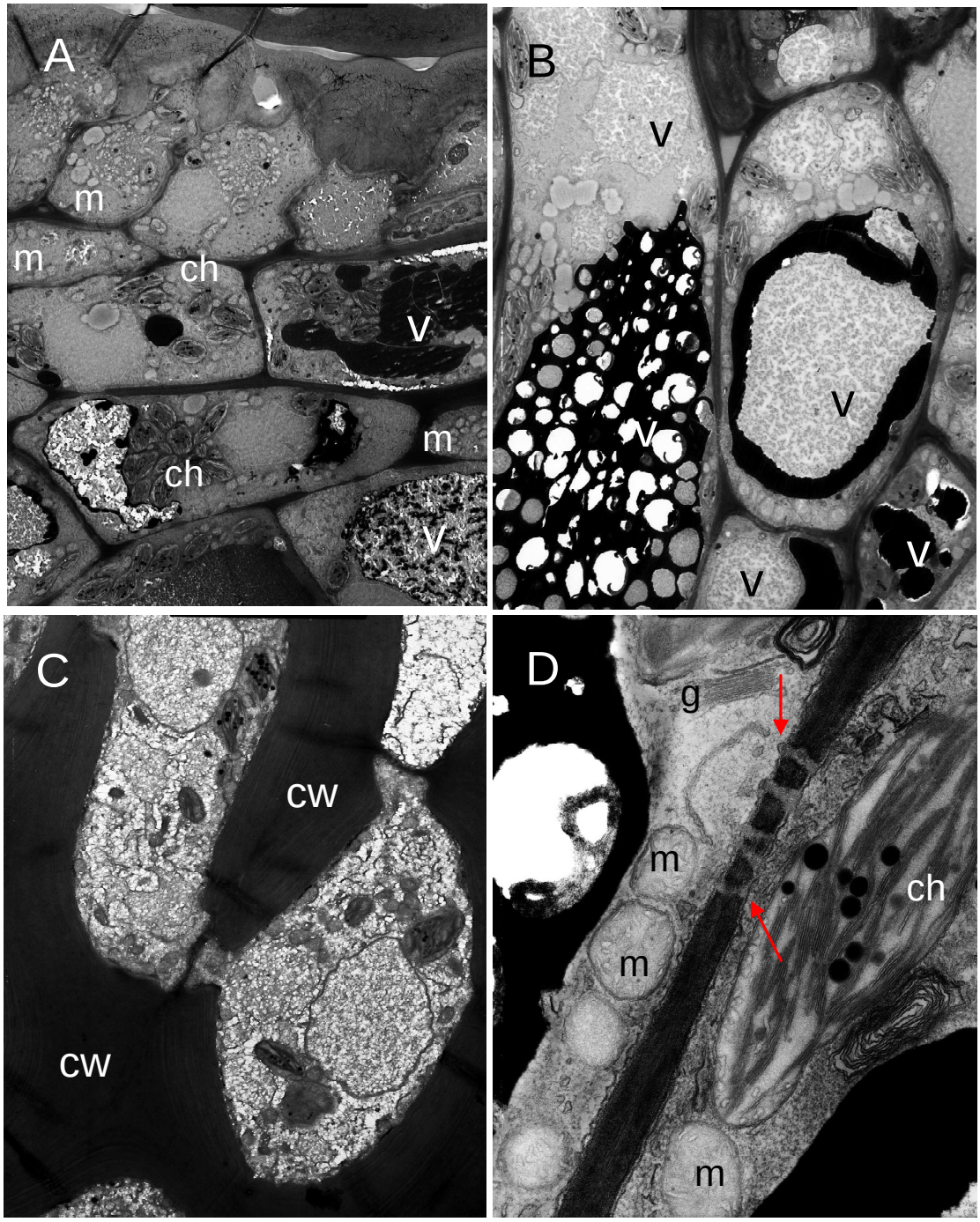


Fig 3.1_7 Nectariferous tissue of P.trichocarpa nectaries. (A) Overview. (B) Vacuoles containing different types of inclusions. (C) Unusual thick walls with symplastic connections. (D) Detailed view of plasmodesmata between cells (red arrows). Cw= cell wall; m= mitochondria; ch= chloroplast; g= golgi apparatus; v= vacuole.

Some publications suggest a correlation between the degree of vascularization of the secretory tissue and the concentration of nectar: nectaries that secrete very

concentrated nectar appear connected to the phloem only. Those, secreting low-sugar nectar, are attached to the xylem or both phloem and xylem (Wergin et al. 1975; Gunning and Hughes 1976; Sawidis et al. 1987). The nectar of *Populus trichocarpa* contained high sugar concentrations (Fig 3.1_9 B), although a number of xylem vessels innervate the nectary (Fig 3.1_8).

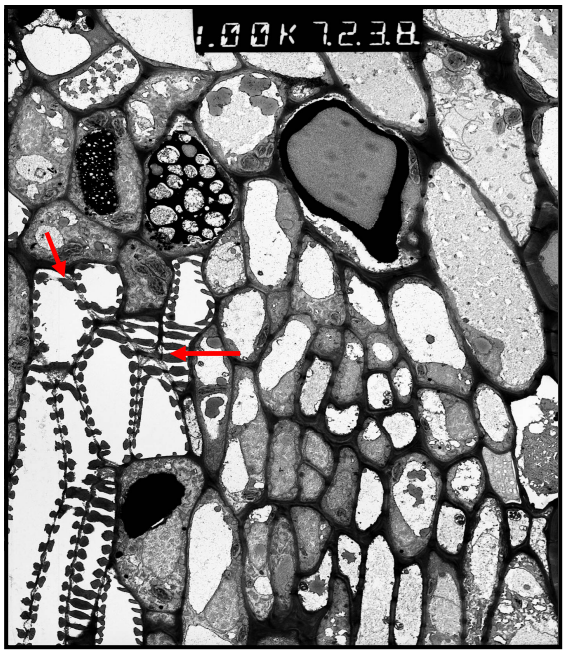


Fig 3.1_8 Vascular bundle in P.trichocarpa nectary. Xylem tracheides are prominent in the nectariferous tissue of *Populus trichocarpa.*

3.1.1.2. Nectar composition

With plants visited by the same kind of animals the nectar properties tend to be similar. The chemical composition of nectar strongly affects the interaction with the insects attracted (Bentley and Elias, 1983). Particularly important chemical factors are the amino acid and sugar content and ratio (Baker and Baker, 1973; Lanza, 1995). The nectar sugars are dominated by the disaccharide sucrose and monosaccharides fructose and glucose. The relative amount of the individual sugars very likely reflects invertase activity.

3.1.1.2.1 Sugars

Absolute nectar sugar concentration depends on the microclimatic conditions (humidity and evaporation) and/or removal by foragers. In the following, the individual sugar content is related to the total sugars amount.

The amounts of sucrose, fructose and glucose appeared to be relatively constant in the different samples from the same species (Fig 3.1_9), but differed remarkably between the two poplar species. In *Populus tremula x P. tremuloides* the ratio of glucose, fructose and sucrose is 1:1:1 (Fig 3.1_9 A), while in *P. trichocarpa* the percentage of sucrose in nectar was rather low. The glucose - fructose ratio was 1:1 (Fig 3.1_9 B). This result seemed to indicate that sucrose has been hydrolyzed (prior to secretion or thereafter).

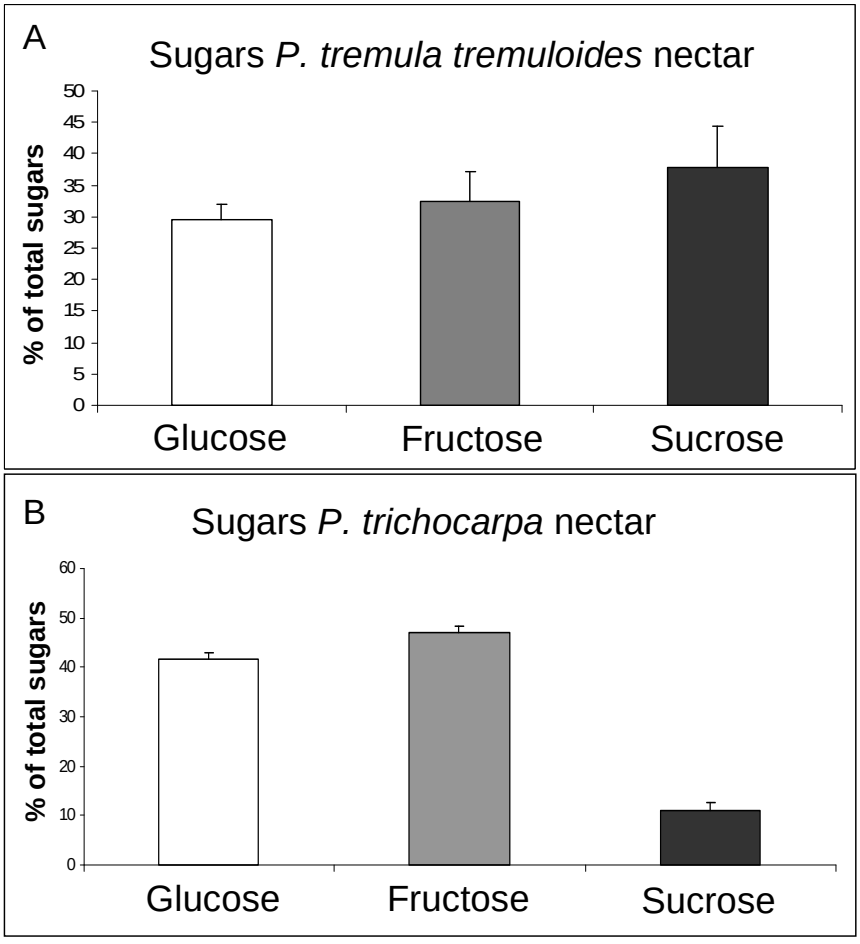


Fig 3.1_9 Sugar content in nectar. (A) *Populus tremula x P. tremuloides*. (B) *Populus trichoc*arpa. The ratio is constant within the single species but different for both poplar ecotypes. (Mean +/- SD, n=5).

3.1.1.2.2 Inorganic ions

So far little is known about the ion composition and concentrations in the nectar (Robards and Oates, 1986; Heinrich, 1989). Both nectars showed a similar ion composition, therefore only the composition of *P. trichocarpa* is shown. When analyzing the *P. trichocarpa* nectar potassium was the most abundant cation, followed by calcium, magnesium and sodium (Fig 3.1_10 B). High potassium and low sodium concentrations in nectar are in agreement with the relative concentration of these ions in xylem and phloem sap (Gerendás and Schurr 1999; Escalante *et al.* 2009). Likewise the anion spectrum in nectar reflects the situation in poplar phloem sap (Gerendás and Schurr, 1999). The nectar is dominated by Cl^- and SO_4^{2-}, followed by NO_3^- and PO_4^{3-} (Fig 3.1_10 A).

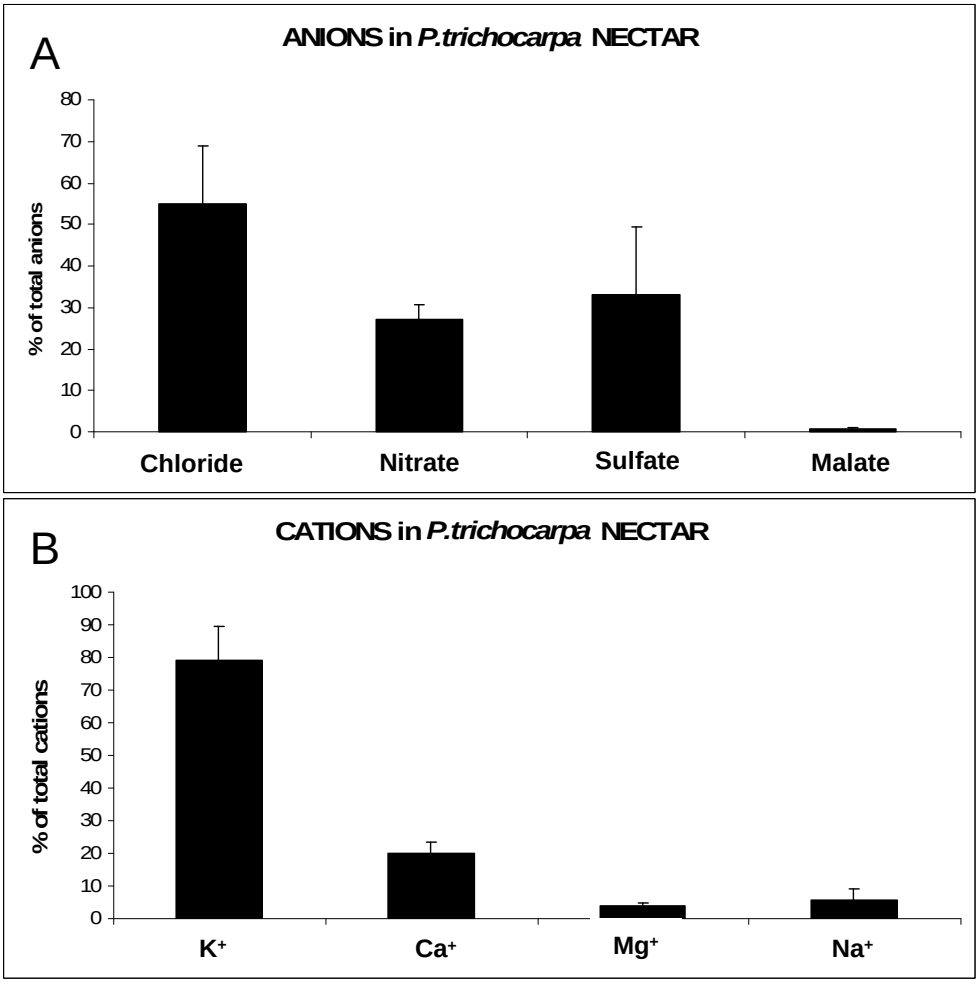


Fig 3.1_10 Ion content in *Populus trichocarpa* nectar. (A) Anion content. (B) Cation content. Mean +/- SD n=4

3.1.1.2.3 Amino acids

The amino acids in the nectars of *P.tremula tremuloides* and *P. trichocarpa* both showed a similar pattern. Nectars of both species contained similar relative amounts of all 20 protein-forming amino acids (data not shown). In the EFN of *P.tremula x P. tremuloides* phenylalanine and glutamine dominated, while in *P.trichocarpa*, asparagine, histidine and tyrosine were the most abundant (Fig 3.1_11).

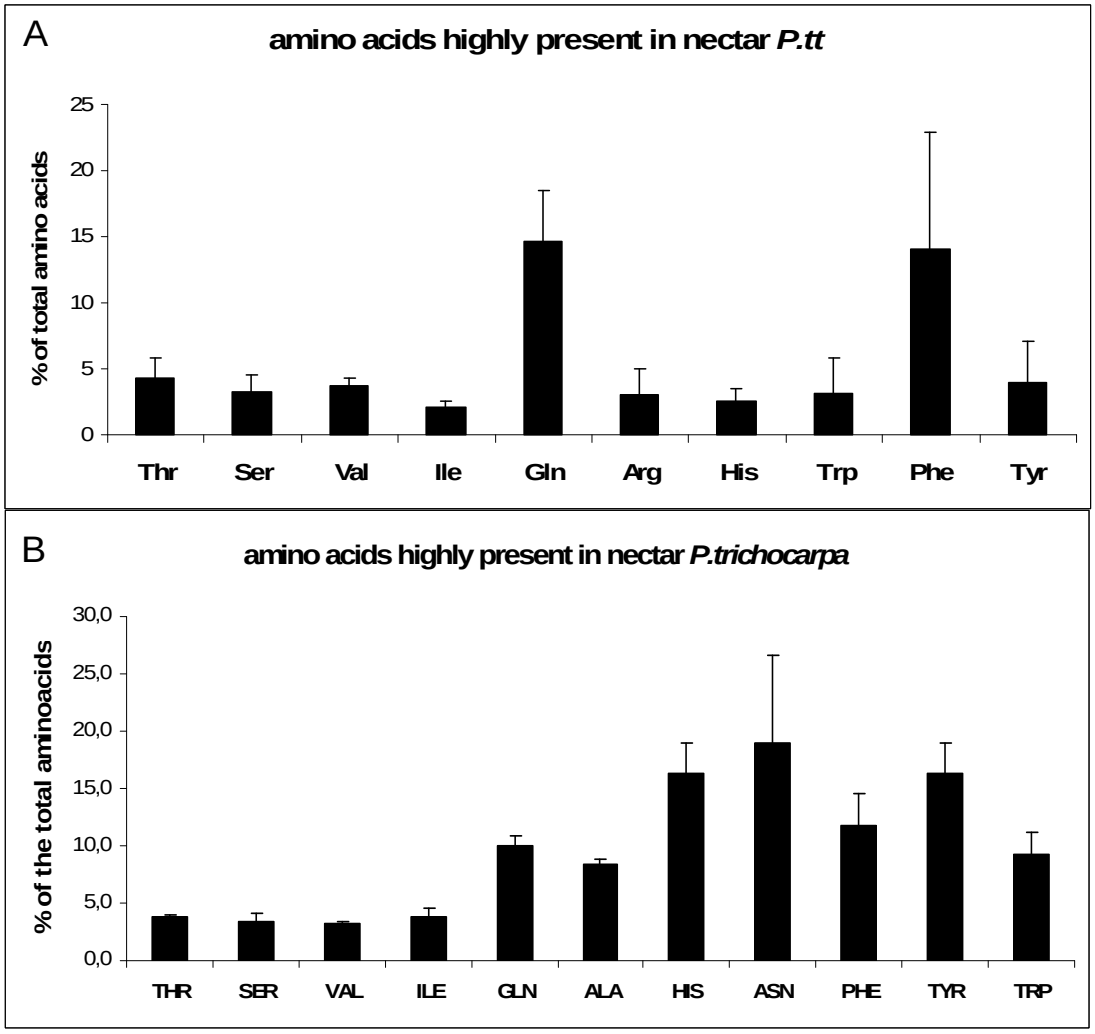


Fig 3.1_11 Amino acids highly present in nectar. (A) *Populus tremula x P. tremuloides*. (B) *Populus trichocarpa.* (Mean +/- SD, n=4). The aromatic amino acids particularly phenylalanine were present in higher quantities.

A few of the non-toxic non-proteinogenic amino acids like β-alanine and citrulline, were present in high amounts as well (not shown). Ornithine was found in lower amounts in samples of *P. trichocarpa* only. Interestingly, the aromatic amino acids such as phenylalanine appeared well presented in both samples, while in leaves they were

found only in small amounts (Data not shown). A likewise distribution was seen with amino acids required to create sweet taste like isoleucine, tryptophane and valine. Proline was found in high concentrations in nectar of *P.t.* (0.702 mM, SD+/- 0.26) and *P.t.t*, (4,78mM SD+/-2.9) while it was absent in leaves (not shown). GABA is a non-protein amino acid involved in various plant physiological responses (Bouche and Fromm, 2004). GABA is a neurotransmitter in vertebrates and invertebrates and was found only in traces in the nectar samples while it was highly abundant in leaves (data not shown). Thus nectar feeding insects do not ingest concentrations that would impair housekeeping-insects' development.

3.1.1.2.4 Proteins

The existence of proteins in nectar was recognized in the 40's of the last century (Pryce-Jones, 1944). Invertase, transglucosidase, transfructosidase represent common nectar proteins. In this study several completely different proteins were identified that all appeared to be pathogen defence related (Table 3.2). Among these proteins, known for their pathogen defence functions, a predicted chitinase together with two class IV chitinases and a chitanase II were found. These enzymes break down glycosidic bonds in the chitin polymer of e.g. the cell wall (Karasuda *et al.*, 2003; Ohnuma *et al.*, 2004; Yang *et al.*, 2008). Furthermore, an acidic endochitinase precursor belonging to the class I chitinases and a Hevein-like chitinase were identified. These chitinases might therefore represent antifungal products.

Some of the thaumatin family proteins, present in the nectar solution, are natural sweeteners and roughly 2000 times more potent than sugars (Temussi, 2002; Breiteneder, 2004; Kim *et al.*, 2005). Additionally, several members of this family display in various fungi significant *in vitro* inhibition of hyphal growth and sporulation (Ho *et al.*, 2007; O'Leary *et al.*, 2007). An SCP-like extracellular protein also known as "Ves allergen" was found, but the precise function of this protein class is still unclear. A wide number of evolutionary related eukaryotic proteins, including insect proteins like venom allergen 5 from vespid wasp, venom allergen 3 from fire ants and plant-pathogenesis proteins of the PR-1 family are known to be synthesized during pathogen infection or other stress-related responses (Henriksen *et al.*, 2001).

The other proteins found are a Proteinase inhibitor I3 with endopeptidase inhibitor activity, three lipolytic GDSL enzymes and a protein with a SGNH-plant-lipase like motif.

P. Gene Model ID	supposed function
826290	Class IV endochitinase
828956	Lipase/hydrolase, putative
673117	Lipase GDSL
673117	Lipase GDSL
586261	GDSL-lipase 1
751998	Glucan endo-1,3-beta-glucosidase
717157	Putative chitinase
826290	Class IV endochitinase
827727	GDSL-motif lipase/hydrolase-like protein
826290	Class IV endochitinase
652688	Beta-1,3 glucanase
746640	Acidic endochitinase SE2 precursor
571046	Hevein
763978	Proteinase inhibitor I3, Kunitz legume
669475	Thaumatin
550049	SCP-like extracellular protein

Table 3.2. Proteins found in nectar. Almost all proteins found in nectar are defence or pathogen related.

3.1.1.3 Anti-microbial properties of nectar

In order to test the anti-microbial activity of the nectar proteins, an antifungal assay was established first. Agar plates containing YEP medium with ampicillin to avoid bacterial growth were inoculated with epiphyllic microbes from field-grown *Populus tremula x P. tremuloides* leaves. Single fungal colonies appearing 24 hours after incubation at room temperature and were identified as *Fusarium solani*. This fungus causes infections in both humans and plants (Zhang *et al.*, 2006) and it is known to be broadly resistant to several drugs (O'Donnell *et al.*, 2008). The antifungal activity of the nectar was thus

assayed with *Fusarium solani*. Before the mycelial colony developed in the petri dishes, sterile blank paper disks, soaked with either the fungicide nystatin (positive control) or diluted nectar were placed on the plates. In fact halo like inhibition zones around the disk indicated the anti-fungal activity of the samples. The signal strength was found to depend on the stage of fungal developmental (Fig 3.1_12).

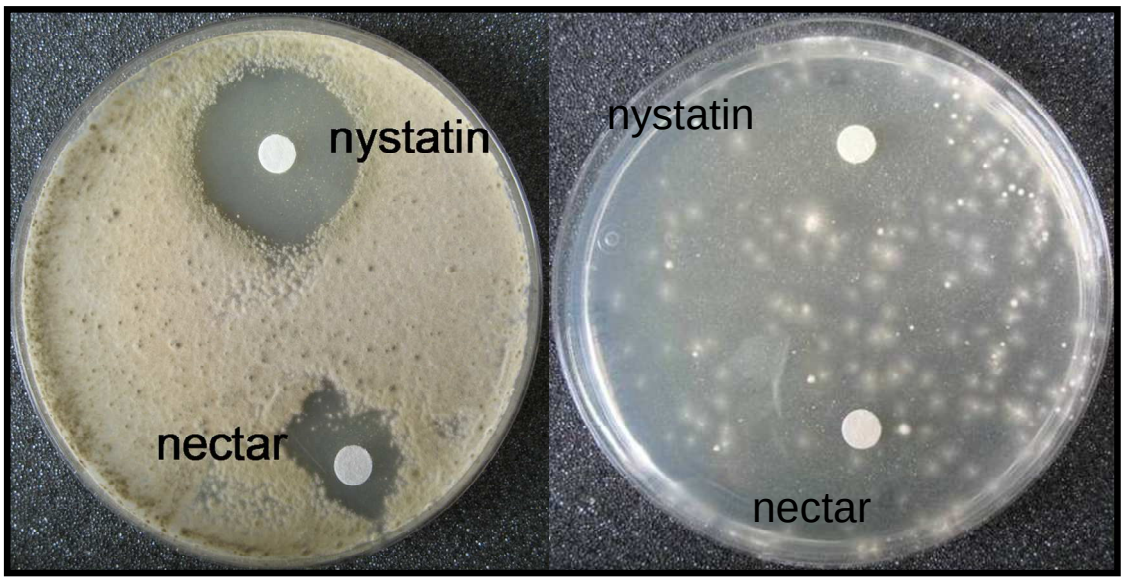


Fig 3.1_12 Antifungal assay. Left, nectar showing anti-fungal properties on *Fusarium solani* similar to those of fungicide nystatin (positive control). Right, the anti-fungal effect of nectar was reduced in a different developmental stage of the fungus.

3.1.1.4 Nectary protein analyses

To study the nectary-dominating protein composition, nectaries from field-grown *Populus tremula x P. tremuloides* were collected and proteins associated with this organ analysed. By mass spectrum analysis followed by database searches (National Centre for Biothechnology (NCBI) or in the poplar genome (http://genome.jgi.org) 100 proteins were identified and functionally classified (Figure 3.1_20).

A couple of proteins were found to be related to wounding or biotic stress response. The most representative group was associated with protein metabolism. Photosynthetic activity related proteins were also well represented, which correlated with the high number of chloroplast within the nectary tissues (Fig 3.1_4 and 3.1_7). Several proteins involved in carbohydrate metabolism were present, five of them from the glycolysis pathway, which might be directly related to the high energy requirement of nectaries.

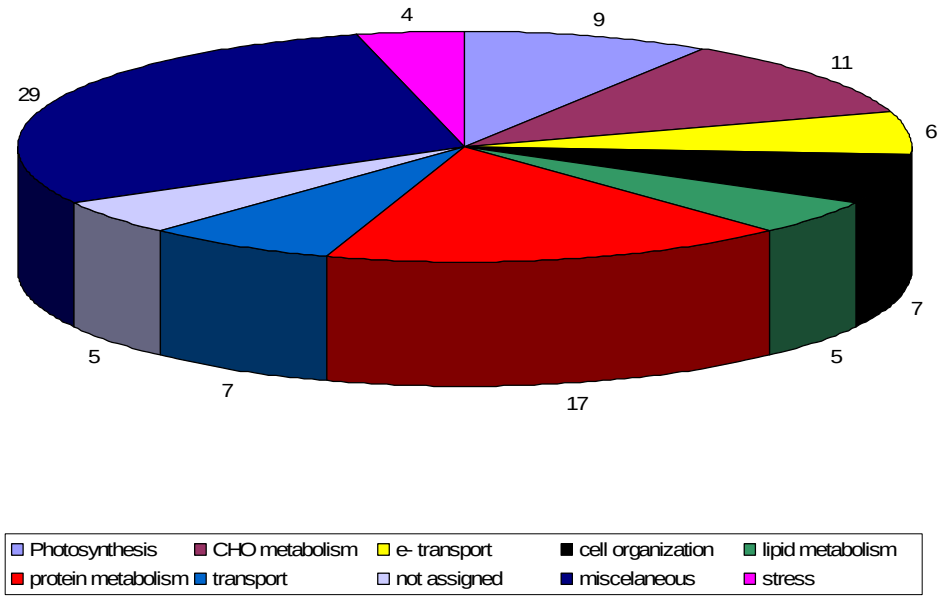


Fig 3.1_13 Functional classification of the major nectary proteins. Numbers quantify the individual groups. Proteins were classified according to the MapMan software 2.2.0.

3.1.1.5 Ecology of nectaries

Rudgers (2004) found that extrafloral nectaries morphology (size) and density in wild cotton (*Gossypium thurberi*) were heritable. To prove whether there is such a heritable genetic basis of EFN in the genera *populus*, EFNs density of field-grown *P.tremula x P. tremuloides* was determined. The population of leaves with and without nectaries was highly conserved among different trees: 62% of the leaves (n=12) grew nectaries.

About 80% of leaves wearing nectaries showed no visible symptoms of microbial infection or herbivore attack. About 15% were slightly damaged by herbivores and only about 2% had been severely attacked (Fig 3.1_14). All these results together pointed to a high effectiveness of the anti-herbivore defence by EFNs and the anti-microbial properties of nectar.

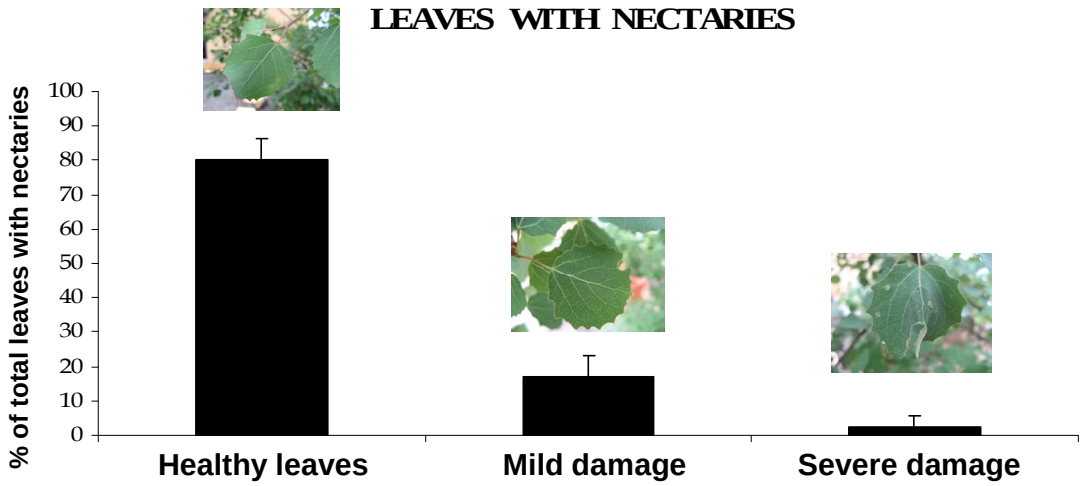


Fig 3.1_14 Efectiveness of the EFNs. About 80% of leave containing nectaries have not been attacked by herbivores. Mean +/- SD, n=12.

In spring , all emerging *Populus tremula x P. tremuloides* leaves (mid April) harboured nectaries (Fig 3.1_15). This followed predictions of the optimal defence theory, which claims that young tissues are proportionally more valuable for the tree than older ones. Presumably, producing EFNs in young tissues could increase the presence of natural enemies of herbivores around. This theory fails, however, with *Populus trichocarpa;* the growing young leaves were not equipped with nectaries (Fig 3.1_15). The latter variety developed EFNs after herbivory only. They thus invest into defence only when protection is required.

Fig 3.1_15 With budbreak emerging *P.t.t.* leaves grow nectaries, black arrows (left), while *P.t.* new leaves did not (right).

Several studies indicated that EFN secretion could increase in response to herbivory without herbivore-specific elicitors involved (Stephenson, 1982; Heil *et al*., 2001). In order to test if the poplar EFN production is induced by herbivore damage, leaves of *P.trichocarpa* were wounded. Herbivory was thus simulated by either puncturing the leaf blade with a needle or cutting the leaf tip. Irrespective of the sites or type of mechanical wounding, the outgrowth of nectaries was observed one day after leaves were treated (Fig 3.1_16 A). The nectarines, however, secreted no nectar within 4 days and eventually died (Fig 3.1_16 B).

These results led to the assumption that the nectary formation is induced by mechanical stress, but nectar production requires an additional elicitor. To test the hypothesis, plants were faced to herbivores. Two caterpillars were placed on *Populus trichocarpa* tree leaf blades. With limited caterpillar feeding nectar production was observed within the 24 hours after damage (Fig 3.1_17).

Fig 3.1_16 Induction of nectaries after artificial damage in *P.t.* (A) Undamaged leaves did not grow nectaries. Dry nectaries appearing 24 hours after the leaf was punctured with a needle. (B) 4 days after leaf damage nectaries died without having nectar produced.

To further test, whether nectar production is caterpillar specific or generally herbivore-induced, intact plants were infected with mealy bugs (Hemiptera: Pseudicoccidae). These insects in contrast of caterpillar feed on the phloem. After 4 days of infection about 50% of the nectaries produced nectar (data not shown). Thus induction of nectar formation seems not confined to a specific herbivore.

Fig 3.1_17 Nectar production following herbivory. Left, caterpillar feeding on *Populus trichocarpa* leaves. Right, nectar production after 2 days of feeding.

3.1.1.6 Microarray-analysis of EFN

To gain first insights into the transcript composition of nectaries, a genome-wide expression profiling was performed with *Populus tremula x P. tremuloides* in a differential approach. Therefore DNA chips were hybridized with RNA of extrafloral nectaries and nectary-free leaf sections at the “Microarray facility Tübingen”.

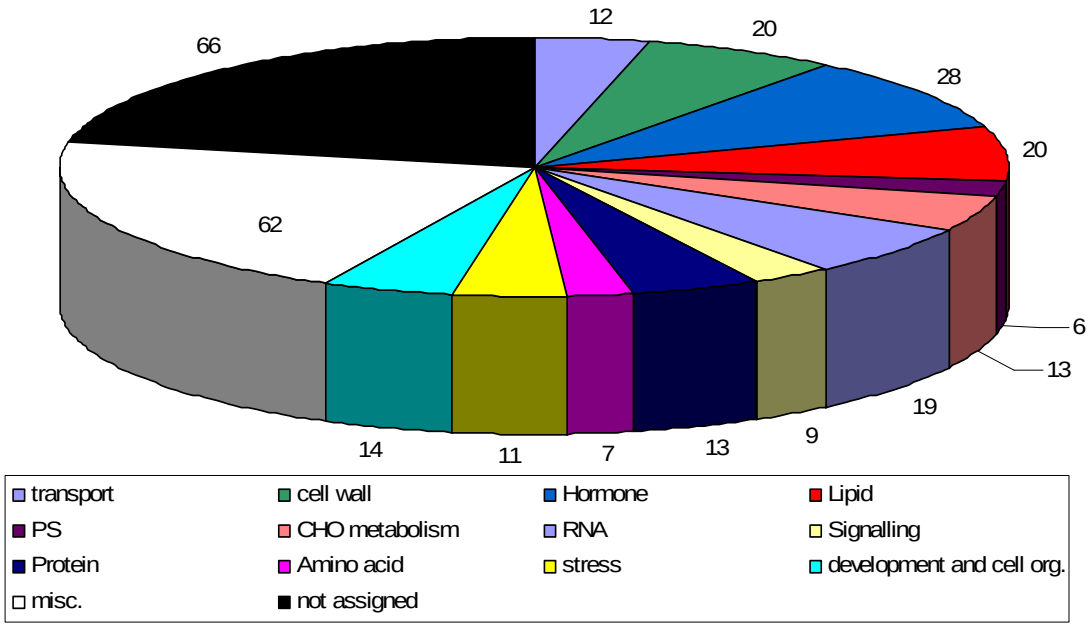


Fig 3.1_18 Distribution of 300 significantly differentially expressed genes in *P. tremula x tremuloides* nectaries. Numbers indicate genes representing individual groups.

The analyses was based on the comparison of two leaf mRNA samples to three gained from nectary. About 300 genes appeared to be differentially expressed and thus considered for further analysis. From these, 232 appeared up-regulated in nectaries. Highest levels of induction were observed for genes related to hormone action, cell wall and lipid metabolism. Several others were related to sugar metabolism, transport, wounding response, as well as pathogen interaction (Figure 3.1_18). Increased induction of genes from the latter two categories appeared to be in line with the protein composition of the nectar.

ID[a]	Bes hit (*A. thaliana*)[b]	Fold Change[c]	AGI match[d]
CV244899	UDP-D-APIOSE/UDP-D-XYLOSE SYNTHASE	3,784	At1g08200
CK093047	GATL1/GLZ1/PARVUS	4,890	At1g19300
pmrna27785	ATCSLC08 (Cellulose synthase-like C8)	4,175	At2g24630
CV236703	BGAL5 (beta-galactosidase 5)	6,381	At1g45130
CK109893	BGAL5 (beta-galactosidase 5)	6,722	At1g45130
CV230945	glycoside hydrolase family 28 protein	9,986	At3g61490
CX176311	glycoside hydrolase family 28 protein	8,730	At3g61490
CV230935	polygalacturonase-like protein	4,982	At4g23500
AI165969	pectate lyase family protein	4,217	At4g24780
CF227942	pectate lyase family protein	6,581	At4g24780
CV254082	PtEXPA3 (*P.tremula tremuloides*)	6,070	At1g26770
BU893810	PtEXPA4 (*P.tremula tremuloides*)	6,294	At1g69530
BP934428	ATEXPA8 (ARABIDOPSIS THALIANA EXPANSIN A8)	11,106	At2g40610
CX658121	PtrEXPA12 (*P.tremula tremuloides*)	7,757	At2g40610
AY435100.1	PtrEXPA2 (*P.tremula tremuloides*)	14,771	At2g40610
CN519614	Licheninase.xyloglucan:xyloglucosyl transferase	4,890	At3g23730
DN500537	pectinesterase family protein	0,036	At1g23200
pmrna29449	pectinesterase family protein	0,088	At1g23200
DN486441	ATPME3 encodes a pectin Methylesterase	4,904	At3g14310
CV268088	pectinesterase family protein	15.217	At4g33220
DN487944	SUS1 (SUCROSE SYNTHASE 1)	13.4	At5g20830

Table 3.3 Expression of cell wall metabolism associated genes differentially upregulated in nectaries of *P. tremula tremuloides*. [a] Probe ID number from the PICME 28K cDNA *Populus* microarray (http://www.picme.at/). [b] Best database match (if not otherwise indicated, *A. thaliana*) obtained with a BLAST query at http://genome.jgi-psf.org/Poptr1_1.home.htlm. [c] expression ratios calculated between nectaries and leaves. [d] AGI code of the best *Arabidopsis* homolog obtained at http://popgenome.ag.utk.edu/mdb/index.php

Genes encoding enzymes linked to the cell wall could be involved in wall modification/formation or endo-/exocytosis (Table 3.3). The major components of poplar cell walls are pectins (47% of dry weight, Mellerowicz *et al*. 2001). In the arrays genes/transcripts for enzymes involved in pectin metabolism as esterases or lyases are

up-regulated (Table 3.3). UDP-Glucose is the precursor for cell wall carbohydrates (Gibeaut and Carpita, 1994). A major player in this reaction: sucrose + UDP=UDP-D-Glucose + PP is SUS (sucrose synthetase). Three homologues of the *Arabidopsis thaliana* SUS1 were found to be significantly up-regulated in nectaries (underlined in table 3.3)

ID[a]	Best Hit (*A.thaliana*)[b]	Fold Change[c]	AGI match[d]
pmrna38847	PIN15, PIN3	6,741	At1g70940
pmrna3865	similar to axi 1 (Nicotiana tabacum)	10.963	At1g22460
pmrna8534	auxin-responsive protein, putative	5.189	At1g29460
CX658941	IAA19 (indoleacetic acid-induced protein 19)	5.035	At3g15540
pmrna19744	GH3-5, IAA-amido synthase	15,705	At4g27260
pmrna22231	GH3-4,GH3 family protein	10,730	At5g54510
CN524106	AA9 (indoleacetic acid-induced protein 9)	5.043	At5g65670
AJ306825.1	IAA9 (indoleacetic acid-induced protein 9)	4.930	At5g65670
CV233908	Amino Acid/Auxin Permease (AAAP) family	12.777	At5g09220
DN484561	Amino Acid/Auxin Permease (AAAP) family	5,411	At5g15240
BI125264	Amino Acid/Auxin Permease (AAAP) family	6,609	At5g15240
AJ778035	CABBAGE 1, CBB1, DIM	4.807	At3g19820
CA823181	CABBAGE 1, CBB1, DIM	6,349	At3g19820
CV280606	BRS1 (BRI1 SUPPRESSOR 1)	0.178	At4g30610
BP935619	2-oxoglutarate-dependent dioxygenase, putative	30.788	At1g06650
DN490862	oxidoreductase, 2OG-Fe(II) oxygenase family protein	0.090	At5g59540
pmrna659	ATMYC2 (JASMONATE INSENSITIVE 1)	5,664	At1g32640
CV231953	ETR2 (ETHYLENE RESPONSE 2)	11,150	At3g23150
DN492397	GASA1 (GAST1 PROTEIN HOMOLOG 1)	8,574	At1g75750
pmrna38875	JMT (JASMONIC ACID CARBOXYL METHYLTRANSFERASE)	22,769	At1g19640
CA928346	JAZ1/TIFY10A (JASMONATE-ZIM-DOMAIN PROTEIN 1)	8.961	At1g19180
DN494840	JAZ1/TIFY10A (JASMONATE-ZIM-DOMAIN PROTEIN 1)	6.217	At1g19180
CV234488	JAZ5/TIFY11A (JASMONATE-ZIM-DOMAIN PROTEIN 5)	6,156	At1g17380
CV264615	JAS1/JAZ10/TIFY9 (JASMONATE-ZIM-DOMAIN PROTEIN 10)	10,265	At5g13220
BU813493	JAS1/JAZ10/TIFY9 (JASMONATE-ZIM-DOMAIN PROTEIN 10)	24,752	At5g13220
CA929119	salicylic acid carboxyl methyltransferase (Clarkia breweri)	15,931	At1g68040
CV263317	BSMT1	30,715	At3g11480

Table 3.4 Expression ratios of hormone action related transcripts. A high number of genes related to hormone action were significantly up-regulated in the nectaries. [a] Probe ID number from the PICME 28K cDNA *Populus* microarray (http://www.picme.at/). [b] Best database match (if not otherwise indicated, *A. thaliana*) obtained with a BLAST query at http://genome.jgi-psf.org/Poptr1_1.home.htlm . [c] Expression ratios calculated between nectaries and leaves. [d] AGI code of the best *Arabidopsis* homolog at http://popgenome.ag.utk.edu/mdb/index.php .

Another group of genes highly expressed in nectaries are related to hormone action (table 3.4.). Auxins play an essential role in coordination of many growth processes in the plant life cycle. Within the hormone cluster 11 genes appear associated with auxin signalling. One PIN family member Pin 15/3 and its regulator KANADI 1 (KAN1) were found to be up-regulated in nectaries. PIN genes control auxin distribution and thereby regulate cell division, cell expansion and polar growth (Blilou *et al*. 2005; Petrasek *et al.* 2006). KAN1 is known to regulate expression of PIN1 and to promote adaxial-abaxial polarity in plants (Wu *et al*. 2008). BRI1 is a brassinosteroid transmembrane receptor kinase (Wang *et al*. 2001); the BR1 suppressor processes a protein involved in an early event of the BRI1 signalling (Li *et al*. 2001). Mutations in this gene severely affected plant growth and development (Clouse *et al*. 1996). Three genes involved in Brassinosteroid metabolism were found up-regulated in nectaries, while the BRI1 supressor was down regulated. Jasmonic acid- and salicylic acid-associated genes were up-regulated in nectaries. These phytohormones are known to play an important role in response to wounding and pathogen and resistance (Li *et al*., 2001; Kachroo and Kachroo, 2007; Turner, 2007).

As expected gene transcripts related to stress and pathogen interaction were up-regulated in nectaries. In agreement with proteins found in the nectar, genes for Thaumatin, classIV chitinase and SCP-like extracellular protein ("Ves"- allergen) and the four GDSL-lipases (Table 3.2) were induced.

Genes found in the nectaries belonging to lipid-metabolism were involved in floral differentiation and expansion stages. This fact might reflect similarities in the development and performance of floral and extrafloral nectaries. A lipid transfer protein gene upregulated in nectaries belongs to the PR-14 group. These genes encode small peptides related to defence against pathogens and are mainly expressed in flower organs (Garcia Olmedo *et al*., 1995). Secretory vesicles appeared to be very prominent in secretory cells from *Populus tremula x P. tremuloides* nectaries (Fig. 3.2_4).Two genes coding for proteins involved in vesicle trafficking, SEC14 and SHP1, were up-regulated in nectaries. *SEC14* encodes a phosphatidylinositol transfer protein essential for vesicle budding from the Golgi complex (Sha *et al*. 1998). *SHP1* is a protein involved in vesicle fusion with the plasma membrane. Finally an unspecific lipid transfer protein was found to be up-regulated in nectaries. This protein belongs to the group of PR-14, which are small peptides related to defence against pathogens and mainly expressed in flower organs (Garcia Olmedo *et al*., 1995).

About 19% of the proteins identified were also reflected as differentially up-regulated in nectaries when compared to leaves (see above).

3.1.2. Volatile compounds

In response to herbivore attack, many plants release volatile organic compounds (VOCs). These VOCs may attract predators or repel herbivores (Heil and Kost 2007). The outcome of a herbivore feeding experiments (Fig 3.1.1.5) suggested that in *P. trichocarpa* elicitors are required for the induction of nectar production and secretion. The fact that herbivory triggers nectar production systemically in leaves seems to ask for the involvement of VOCs in this process.

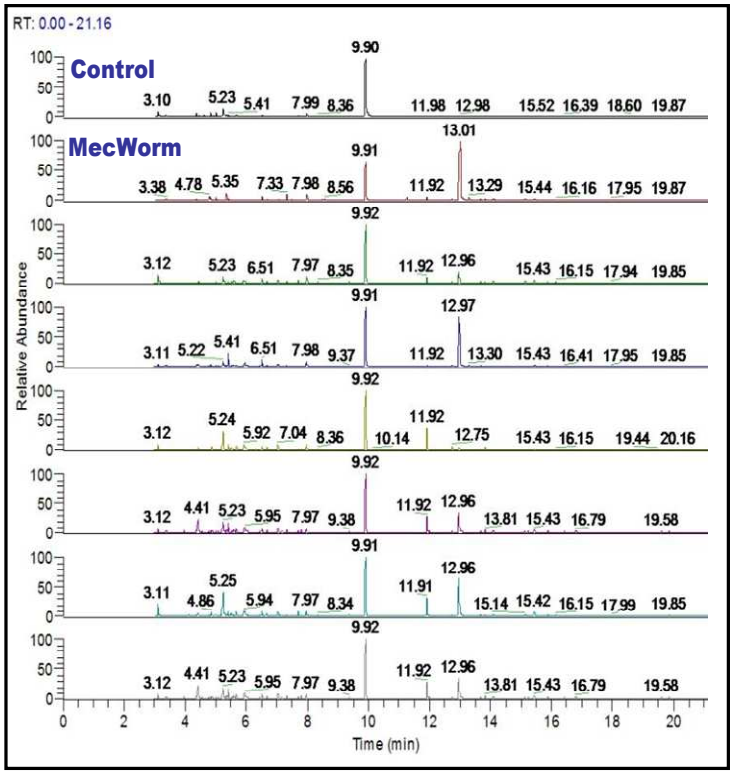


Fig 3.1_19 VOC emission induced by MecWorm. After 25 hours of punching only farnesene was released (11.92min). 12.97 and 13.01 min are artefacts.

To investigate the impact of leaf damage on VOCs release the mechanical caterpillar “MecWorm” was used. Automated wounding was supposed to mimic the mechanical damage caused by herbivores in terms of spatio-temporal pattern of leaf destruction (for detail see Mithöfer *et al*. 2005). A damage programme was applied that punched the leaf every 10 seconds over a period of 15 hours, resulting in a damage of about 80% of the leaf surface. In the volatile bouquet emitted by this treatment, Farnesene

(RT 11.92 min) appeared as the only induced compound (Fig. 3.1_19). As shown before with manual needles puncturing the leaf, MecWorm treatment did also not lead to nectar production (data not shown). This observation underlines the hypothesis that additional processes are required to induce nectar secretion.

Volatile compound synthesis and emission have been predominately investigated in response to feeding insects. The latter experiments indeed showed that nectar production and VOCs emission are not induced by mechanical damage. It rather requires a signal from the herbivores. To identify herbivore-derived/triggered, secretion-inducing VOCs, *Populus trichocarpa* were challenged with *Spodoptera littoralis* larvae.

After herbivore feeding again farnesene was found emitted together with other volatile compounds: Ocimen (5.68, 6.88, 8.07 min), TMTT (trimethyldecantetraen) (12.76 min) and a large quantity of sesquiterpenes characterized the herbivore modified gas phase (Fig 3.1_20), which clearly showed that in addition to damage another signal is provided by the herbivore.

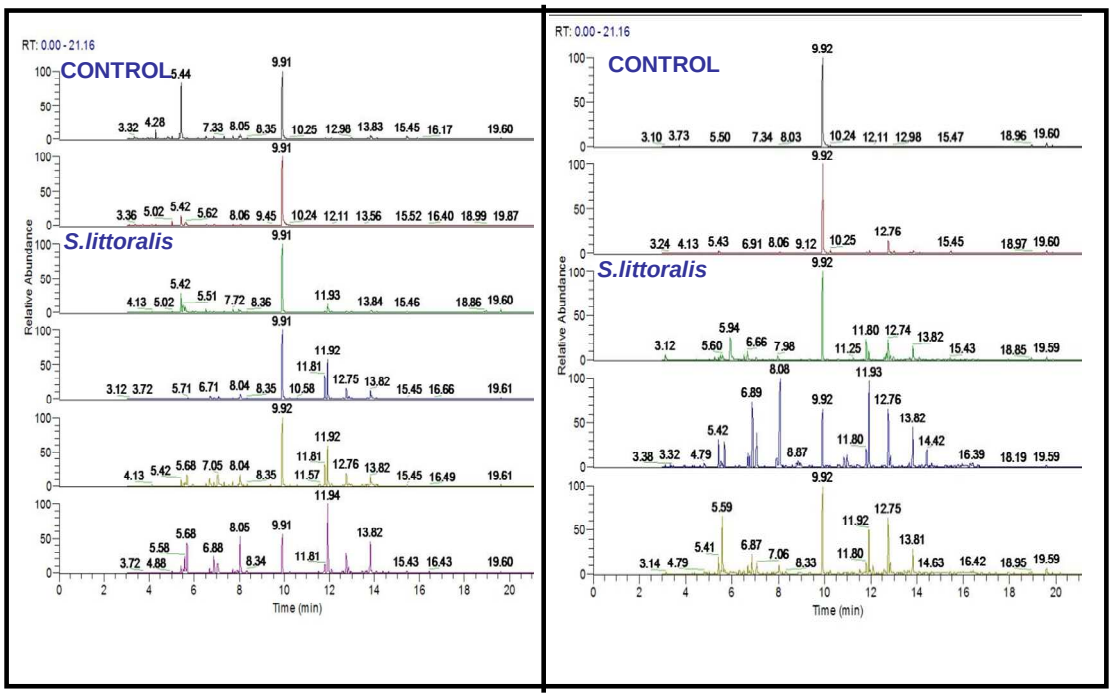


Fig 3.1_20 VOCs emission after feeding of *Spodoptera littoralis*. The headspace analysis showed high amount of Volatile release after herbivory attack.

Jasmonic acid has been shown in *Macaranga tanarius* to induce EFNs formation and EFN release after external application (Heil *et al*., 2001). It is known that herbivory elevates endogenous levels of jasmonic acid (Heil *et al*., 2001). To study the response

of poplar nectaries to this phythormone, plants were challenged with jasmonic acid. A dose dependent response was observed: less application of 0.5 mM jasmonic acid triggered weaker VOCs release as 1mM (Fig 3.1_21). Induced by the stimulus, ocimen together with other volatile compounds were emitted.

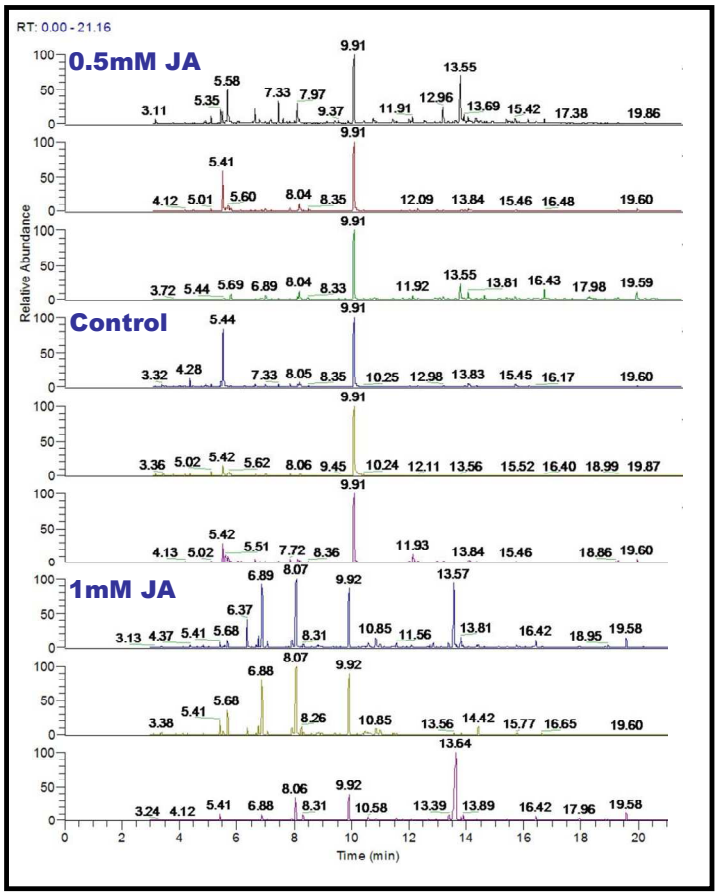


Fig 3.1_21 VOCs emission in leaves after external application of jasmonic acid. Less application of jasmonic acid leads to less VOC formation. Note that the JA induced peak at 13.57 min could not be identified so far.

Taken these results together, it is very likely, that herbivores release elicitors which are essential for jasmonic acid production. Jasmonic acid might in turn lead to VOCs emission and finally to production of nectaries and/or nectar in leaves sensing these VOCs.

3.1 ABIOTIC STRESS

Drought, salt and cold are the most common stress situations affecting plants worldwide and the response mechanisms are thought to overlap largely. Most studies on abiotic stress signalling have therefore been focused on salt stress. Salt stress can be imposed reproducibly under laboratory conditions.

3.1.1 Salt stress affects xylem differentiation of grey poplar (*Populus x canescens*)

To understand the effects of long term salinity on poplar growth and wood anatomy a two weeks salt experiment was performed on the salt sensitive *Populus x canescens*. TEM analysis revealed a reduced xylem differentiation zone. Vessels showed reduced lumina while the total number of vessel cells appeared to be increased. In search for the basis of these morphological changes in the wood region element and metabolite contents were analysed. Concomitantly to the increase of Na^+ and Cl^- content, potassium levels dropped uniformly. This was not observed in leaves, where the K^+ content increased by about 50%. To identify the molecular mechanism underlying the ion distribution processes the expression pattern of distinct poplar ion transport proteins were analysed. K^+ channel transcripts somehow followed the changes of potassium concentrations in the organ studied. The levels of the stress hormone ABA where elevated upon salt treatment in roots and leaves. ABA contents of roots ncreased continuously and coincided with the expression of its marker gene *PtKIN2*, while surprisingly, in leaves, ABA levels were up to 5-fold elevated without affecting *PtKIN2* expression.

These data have already been published (Escalante *et al.* 2009) and were summarized in order to shorten the result section of the present study. For further details see the attached publication. The author was mainly responsible for the experiments leading to figures 3, and 6 to 9 of this publication.

3.1.2 Long term salt stress response of *A.thaliana*

In addition to the published results above and in order to compare *A. thaliana* ABA dependent *KIN2* expression with poplar ABA independent *PtKIN2* levels in leaves, salt stress was applied to adult plants of *Arabidopsis thaliana* in the same manner as described for poplar.

3.1.2.1 ABA levels and expression of the ABA marker gene *KIN2* in *A. thaliana*

One month old *Arabidopsis thaliana* Col 0 plants were exposed to 100mM NaCl for up to two weeks. As a consequence, growth was found reduced dramatically (Fig 3.2_1).

Fig 3.2_1 Salt stress inhibits growth in *A. thaliana*. Left, plants grown under control conditions. Right, reduction in growth after 2 weeks of treatment with 100mM NaCl.

Control and salt treated plants were harvested before, after 1 and 2 weeks of salt stress. After 2 weeks of stress *KIN2* expression in leaves increased 5 fold (Fig 3.2_2a). At this time point the ABA content increased four fold (Fig 3.2_2b) thus *KIN2* expression seemed to be associated with the level of the stress hormone

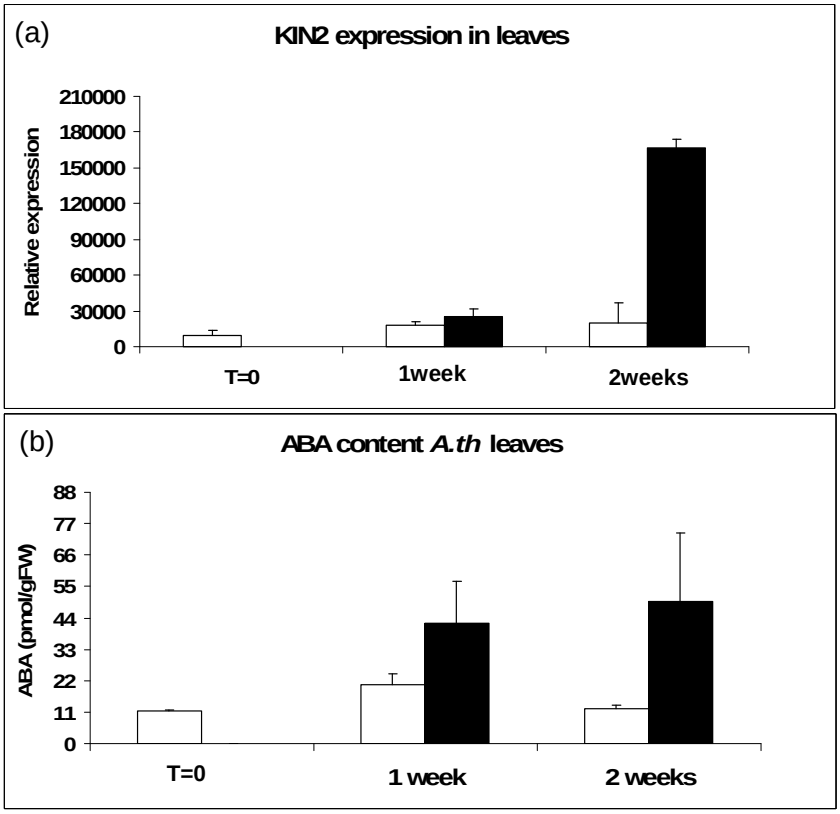
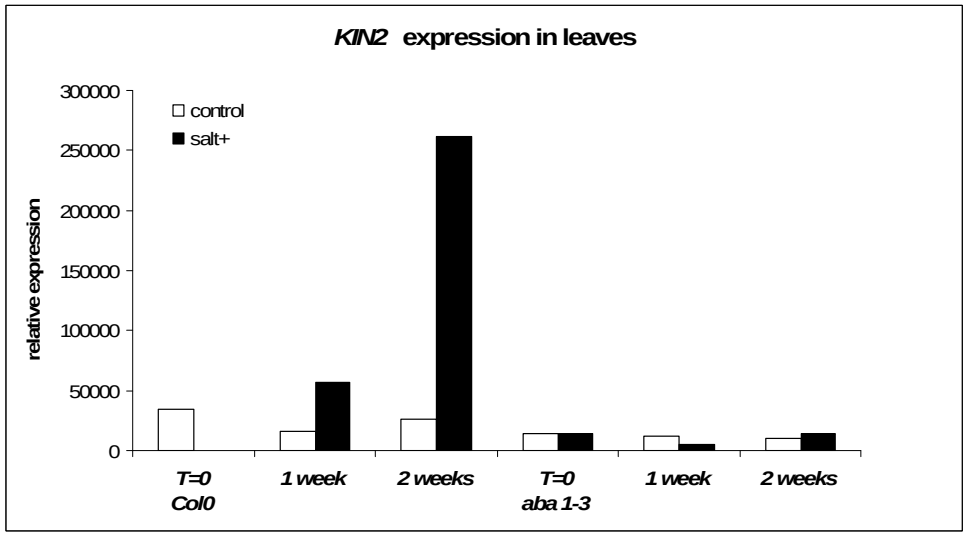


Fig 3.2_2 Expression of *KIN2* and ABA content in leaves, under salt stress (black bars) and untreated control (white bars). (a) After 2 weeks salt stress the expression of *KIN2* was 5 fold higher than in control. (b) ABA leves increased after the first week. (Mean +/- SD, n=3)

3.1.2.2 *A. thaliana KIN2* expression is strictly ABA dependent

The results from 3.1.2.1 indicated a direct correlation between *KIN2* expression and ABA levels. It has been described earlier that salt, cold or osmotic stresses also induce *KIN2* expression as well (Kurkela and Borg-Franck, 1992; Wang and Cutler, 1995; Webb *et al.*, 1996; Cheong *et al.*, 2002). ABA is involved in all these stress situations. Therefore it was tempting to speculate, that *KIN2* expression is exclusively controlled by ABA. To test this hypothesis *Arabidopsis thaliana* plants defective in ABA synthesis (*aba 1-3*) were challenged with the different stressors.



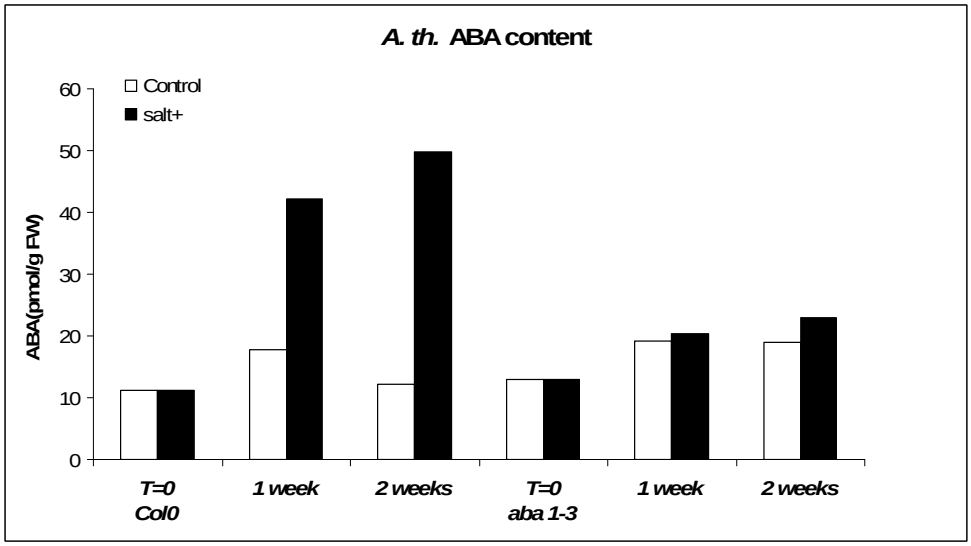


Fig 3.2_3 Expression of *KIN2* and ABA content in leaves from *Arabidopsis* WT plants and *aba 1-3* mutants. Under salt stress (black bars) and in untreated control (white bars). In WT plants as before the induction of *KIN2* is correlated with the amount of ABA, while in the mutants neither *KIN2*, nor ABA was increased (Mean n=2).

As expected, ABA levels and *KIN2* expression of WT plants increased upon salt treatment. In *aba1-3* mutants the amount of *KIN2* transcripts remained as in the controls (Fig 3.2.3). Thus *KIN2* expression in *A. thaliana* seemed to be controlled by ABA rather than by salt directly.

To test the predicted cold stress response, *A.thaliana* plants were subjected to low temperatures. After four hours of cold stress at 4ºC (Fig 3.2_4), in the wild type the ABA content and amount of *KIN2* transcripts increased.. In the *aba 1-3* transgenic line however, no increase in ABA content or *KIN2* gene expression was detected (Fig 3.2_4). The results of the cold experiment confirmed the hypothesis that *KIN2* expression in *A. thaliana* depends on ABA only.

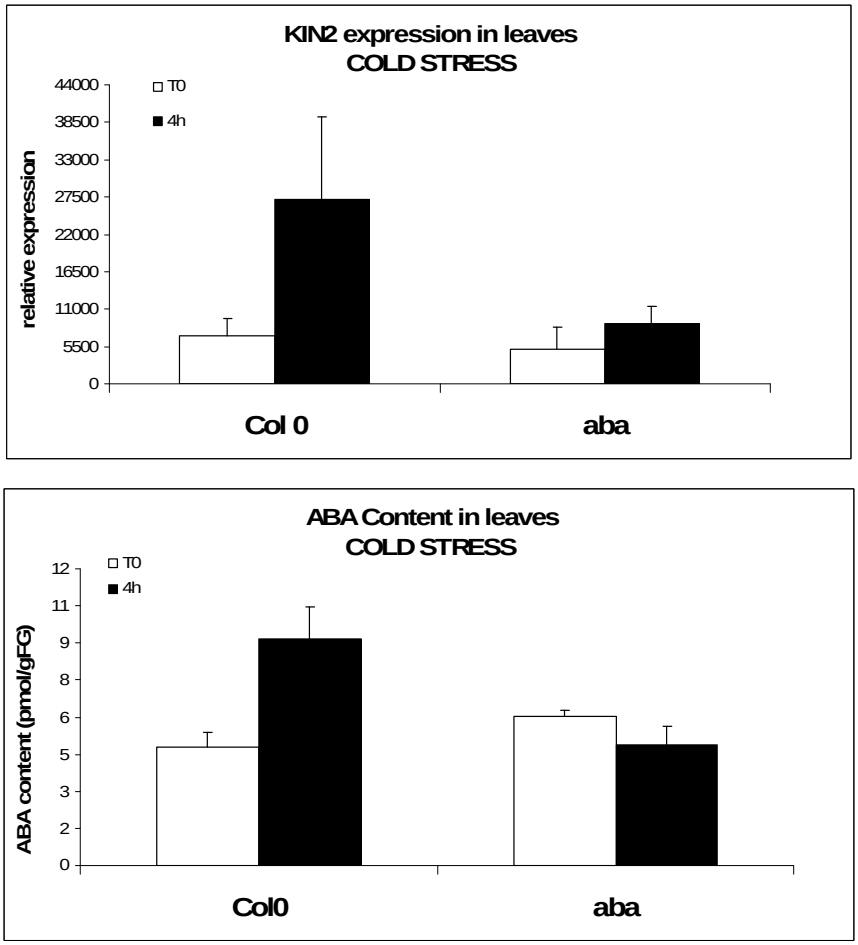


Fig 3.2_4 Expression of *KIN2* and ABA content under cold stress in WT plants of *Arabidopsis* and *aba 1-3* mutants. *KIN2* expression is directly regulated by ABA. (Mean+/- SD, n=3)

3.1.3 Osmotic stress

Plants are known to exhibit a "dual response" to salt stress: i) an early response to the osmotic stress caused by the more negative water potential of the salty soil solution, and ii) a later response due to the Na^+ toxicity resulting from the relative entry of Na^+ ions into the plant (Munns, 1993). Most studies on *A. thaliana* have been performed with short term (few days) salt exposure (Donaldson *et al*., 2004; Huang *et al*., 2005; Liu *et al*., 2007; Liu *et al*., 2008). To study the salt response in more detail a short term stress experiment was performed with both *Populus canescens* and *Arabidopsis thaliana*. Six weeks old plants of *Arabidopsis thaliana* Col 0 were stressed for three days and, leaves and roots were harvested separately. After three days treatment with 100mM NaCl *KIN2* expression in leaves increased 4 fold (Fig 3.2_5a). In roots, however, a transient rise in *KIN2* transcripts appeared within the first 24 hours (Fig 3.2_5b).

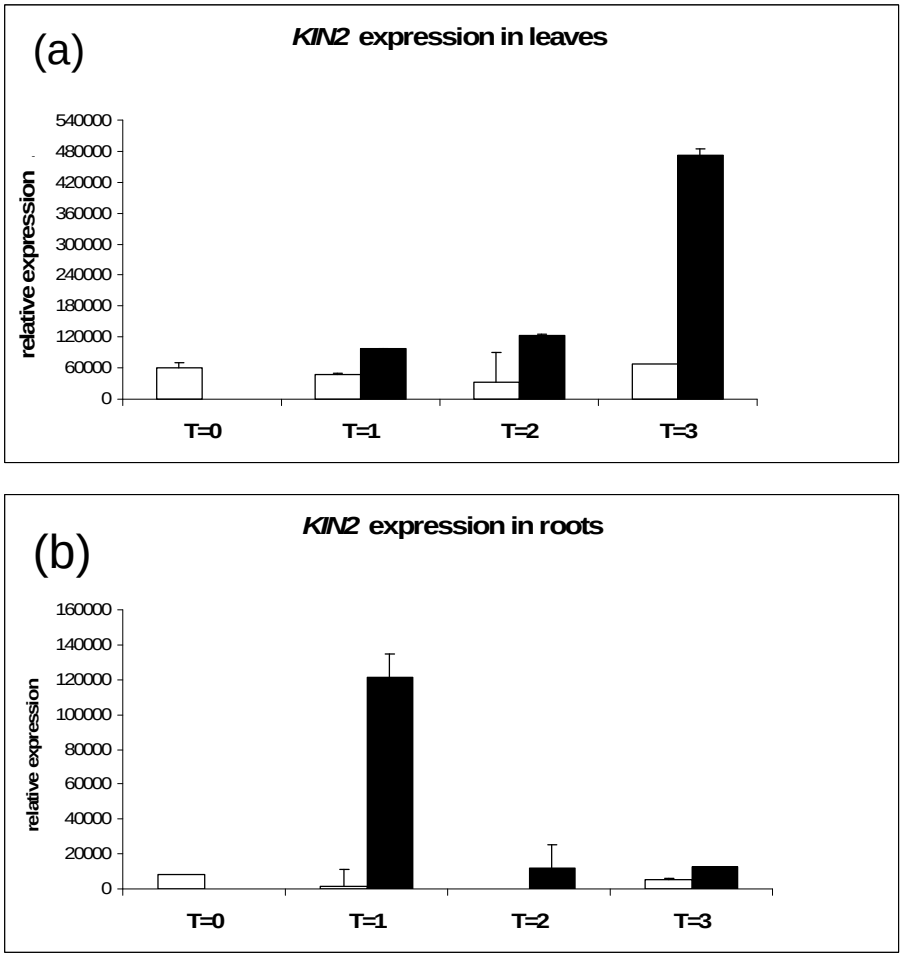


Fig 3.2_5 Short term (3 days) salt stress in *Arabidopsis thaliana*. Under salt stress (black bars) and in untreated controls (white bars) (a) *KIN2* expression in leaves. (b) Transient

increase of *KIN2* expression in roots. T=0, start of the experiment, T=1 and T=2 after 1 or 2 days of 100 mM salt application (Mean +/- SD, n=3).

Under the same conditions, in *Populus canescens* ABA levels in leaves increased strongly after 24 hours and high levels persisted until the end of the experiment . Although delayed by one day, leaf *PtKIN2* expression followed the signal. In roots in contrast *PtKIN2* levels appeared directly associated with the ABA synthesis (Fig 3.2_6).

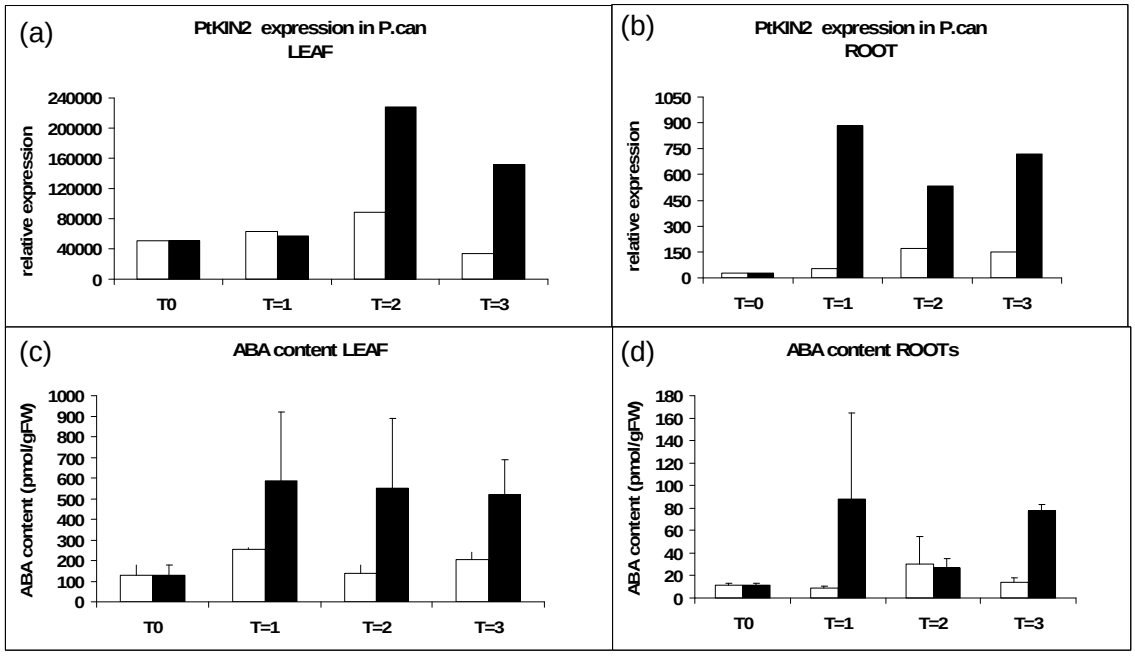


Fig 3.2_6 Short term salt stress in *P. canescens*. Under salt stress (black bars) and in untreated control (white bars). T=0 at the beginning of the experiment, T=1 and T=2 after 1 and 2 days of salt application. (a, b) *PtKIN2* relative expression in leaves and roots (mean, n=2), the relative amount of *PtKIN2* reached the maximum after 2 days of salt exposure in leaves while the induction in roots occured after only 24 hours. (c, d) ABA content of leaves and roots (mean +/- SD, n=3). Note that the ABA content in roots is parallel to the expression of *PtKIN2*.

4. DISCUSSION

In the present study poplar trees were examined under salt (abiotic) and herbivory (biotic) stress. Salinity and drought are the most common abiotic stresses plants on earth experience. A crucial player in abiotic stress accommodation is the phytohormone ABA. In contrast to these abiotic stresses, feeding by herbivores in the most cases cannot be controlled by the plant. Experiments focussed on ABA action on one side and indirect mechanisms against herbivore attack on the other. Among others, genome-wide expression profiles provided the molecular basis for analysis of poplar root, shoot/bark, leaves and EFNs. Salt stress-dependent gene expression as well as ABA biosynthesis and action in poplar was compare to the situation in *A. thaliana*.

4.1 BIOTIC STRESS

This part of the present study was aimed to gain insights into indirect biotic stress control by extrafloral nectaries. To reach this goal the morphology, development, secretory activity, and nectar composition of extrafloral nectaries from *P. trichocarpa* and *P. tremula x tremuloides* was analyzed.

4.1.1 Nectary morphology and ultrastructure and its possible repercussions on nectar secretion

Extrafloral nectaries morphology and nectar secretion are highly correlated items. It has been demonstrated that the gland morphology influences the volume of secretory tissue and that vascular supply determines secretion rates (Diaz-Castelazo *et al*., 2005). Two types of secretion have been described for plants and animals: the holocrine type, which leads to cell death and the merocrine type, in which the secreting cells survive and continue their secretory activity. The latter involves either, eccrine secretion via carrier molecules, or granulocrine secretion using transport vesicles.

P. trichocarpa nectaries consist of a single layered epidermis and several layers of sub epidermal secretory cells (Fig 3.1_2). The latter are small compared to subsecretory parenchyma cells. The secretory cell layer of these nectaries exhibited a number of unique features (Fig 3.1_7). Cells are covered by a remarkably thick wall (Fig 3.1_3), which probably impeded the free flow of nectar even in the absence of cutinized layers.

Numerous plasmodesmata between cells in the complex likely provide for symplastic transfer of nectar between neighbouring cells. Secretory vesicles were not observed in this nectary type. Instead large numbers of mitochondria were found. These features may point to an active transport of nectar through the plasma membrane. The outer epidermal cell wall of *P. trichocarpa* nectaries are covered by a cuticle. This cuticle harbour many microchannels likely representing a potential low resistance pathway for nectar secretion (Fig 3.1_3). Following the release of large volumes of nectar, secreting nectaries eventually died (Fig 3.1_1). Thus merocrine secretion seemed to be followed by a self-destructing holocrine secretion.

Nectaries from *P. tremula tremuloides*, in contrast, possessed only one layer of clearly separated large secretory cells (Fig 3.1_2). These cells did not show any homology to other tissue types described for plant nectaries. The absence of symplastic connections between this cell layer and the cells from the parenchyma tissue suggest apoplastic loading of the secretory cells (Fig. 3.1_4). Thereby phloem-derived nectar might further be modified. Equilibration between the secretory cells is facilitated by a large number of plasmodesmata in their periclinal/anticlinal cell walls (Fig. 3.1_4). The presence of numerous secretory vesicles characterizes this nectary type as granulocrine (Fig. 3.1_4). In contrast to *Populus trichocarpa*, secretory cells of *Populus tremula tremuloides* nectaries appeared not covered by a cuticle (Fig 3.1_5).

Nectar production is an expensive investment for the plant. In order not to waste energy, floral nectaries of some plants are able to reabsorb the unconsumed nectar by pollinators (Nepi and Stpiczynska, 2008). A similar situation may exist with extrafloral nectaries and their bodyguards. Substitution of nectar secreted from flowers by diluted solutions of vital stains (e.g. neutral red) and subsequent accumulation of the stain in the nectary have been commonly used to monitor solute reabsorption (Nepi *et al*., 1996). In the present study the use of the membrane soluble chromophore FM4-64 demonstrated that this kind of reabsorption is also possible via the secretory cells of *Populus tremula tremuloides* extrafloral nectaries (Fig. 3.1_6).

4.1.2 Nectar chemistry

4.1.2.1 Sugars

Nectar is a complex mixture of metabolites. The nectar substances are dominated by the three sugars sucrose and its monosaccharide components, glucose and fructose.

Nectar chemical properties tend to be similar in plants visited by the same animals. A difference in nectar sugar ratios points to different visitors. For instance, floral nectaries visited by hummingbirds produce sucrose-dominant nectar, whereas bee-pollinated flowers tend to produce nectar with a predominance of hexose (Baker and Baker, 1983). Myrmecophyte (or ant plants) are plants which live in association with a colony of ants in a deep symbiotic relationship. A study of the sugar composition in relation to visiting animals revealed that extrafloral nectar of non-myrmecophyte plants always contain sucrose as well as varying amounts of glucose and fructose. The extrafloral nectar of the myrmecophyte Acacia in contrast contains only glucose and fructose. The addition of sucrose to this nectar significantly increased its attractiveness to generalists (animals that show no preference for a special nectar composition) and on the contrary made the nectar less desirable for specialized ants (Heil *et al*., 2005). Nectar of *P. tremula tremuloides* showed a similar ratio between sucrose, glucose and fructose, and therefore might be considered as nectar for generalists (Fig 3.1_9A). In contrast *P. trichocarpa* nectar contained only low amounts of sucrose and is mainly composed of glucose and fructose. Thus *P. trichocarpa* seems to be ant specialized (Fig 3.1_9B).

The nectar source is the phloem sap, which mainly consists of sucrose. This led to the conclusion that most of the glucose and fructose present in poplar nectar, derived from phloem sucrose, has been processed by an invertase activity associated with the nectary. Assuming that partial hydrolysis leads to mixed sugar compositions, it is difficult to determine how the ratio between the three main sugars did not vary among nectar samples from nectaries of individual trees collected under various external conditions. The maintenance of a fixed ratio of sugars could be explained by the ability of the cell to control secretion and resorption of the individual sugars. Sugars are able to act as central signalling molecules (Rolland *et al*., 2002) interacting with hormones, light and stress signals (Rolland and Sheen, 2005). Membrane bound receptors from yeast, which are closely related to sugar transporters, have been demonstrated to act as sensors for external sugar concentrations. In plants the same kind of proteins are involved in sugar sensing and transduction (Loreti *et al*., 2001). It will be a future goal to test whether similar mechanisms allow nectaries to control the nectar composition in a bodyguard dependent manner.

4.1.2.2 Nectar inorganic ions

In floral nectar the most prominent inorganic ion is potassium (Ziegler, 1975; Pate *et al*., 1985). In agreement with the consideration that phloem sap is the “raw” material of

nectar, the ion composition of poplar extrafloral nectar appeared to be similar to the relative concentrations in phloem sap (Fig. 3.1_10, and Escalante *et al*., 2009). In the nectar chloride was found as the highest representative anion (Fig. 3.1_10). Secretion by glands of other systems is driven by the export of chloride and cell type-specific counter ions. Release of water from the cells is achieved by transport of osmotically active ions (eg. K^+, Cl^-) and organic metabolites (eg. sugars) (Bleich *et al.*, 1998; McManaman *et al*., 2006). Recently an anion channel associated with the plasma membrane, SLAC1 (slow anion channel), and 4 homologs, SLAHs (SLAC homolog's), have been characterised. These channels are essential for the ion homeostasis in plant cells (Negi *et al*., 2008; Vahisalu *et al*., 2008). In order to gain reasonable aqueous nectar solutions, additionally to the main nectar compounds, ions and water have to be released from the secretory cells to the nectary surface. In poplar nectaries one of the SLAC1 homologues, SLAH3, was found to be highly expressed (Data not shown). This channel might be involved in chloride release and thus secretion of the nectar. The characterization of this channel is in progress.

4.1.2.3 Nectar amino acids

During the early 1970s, Herbert and Irene Baker revised the hypothesis that (floral) nectar is simply a sugar-water solution. They found that nectar contained different amounts of amino acids, and that the quantity and composition varied among plant species according to the visitor (Baker and Baker, 1973). Amino acids are the second most abundant class of solutes in nectar (Baker and Baker, 1987) and their composition is important for the nectar taste. Insects possess different classes of chemosensory receptors that respond to water, salt or sugar (Shiraishi and Kuwabara, 1970). Phenylalanine, tryptophan and valine are amino acids with the ability to stimulate the sugar cell, when either of these amino acids is applied to the sugar cell it produce the same response as glucose or fructose (Shiraishi and Kuwabara, 1970). The extrafloral nectars from both species studied were rich in these "sweet taste" amino acids (Fig. 3.1_11). Proline and hydroxyproline, have the unique ability to stimulate the salt cell (Wacht *et al*., 2000). When the salt receptor cell was stimulated by a low concentration of proline, both the salt and water receptor cells discharge impulses (Shiraishi and Kuwabara, 1970). Proline was found in poplar extrafloral nectars (particularly high in comparison with the amount detected in leaf extracts). Stimulation of the salt cell results in an enhanced elicitation of the feeding behaviour (Hansen *et al*., 1998). Proline is by far the most abundant amino acid present in honeybee haemolymophe, and is also important for egg laying (Hrassnigg *et al*., 2003).

Moreover honeybees use an invertase to convert nectar into honey. Proline regulates the secretion of this enzyme (Davis, 1978). Finally proline is used as an energy source in the initial stages or lift phase of insect flight (Micheu *et al*., 2000). Phenylalanine also stimulates the sugar cell and is the most abundant amino acid in nectars of bee pollinated plants. It is also known to be a phagostimulant for these insects (Inouye and Waller, 1984; Pentanidou *et al*., 2007). The composition of P. *tremula tremuloides* and P*. trichocarpa* nectars seemed therefore to be suitable for honeybees. At the end of the 19th century Trelease published the first study about extrafloral nectaries in the genera *Populus*. In this study it was suggested that honeybees are the visitors of the poplar nectaries. Parasitic flies (Tachinidae) and parasitic wasps (Ichneumonidae) have been reported to feed on aspen extrafloral nectaries, too (Wooley *et al*., 2007). Field observations in the present study could not confirm any of these hypotheses.

Wasps are predators, using other insects, usually caterpillar as food source. In addition, adult male wasps sometimes visit flowers to obtain nectar in the same manner as honeybees. Flying wasps create airborne vibrations that stimulate sensory hairs of many caterpillars (Tautz, 1977). As a reaction, caterpillars stop moving or drop off the plant (Tautz, 1978). Flying honeybees produce air disturbances similar to those of wasps that also stimulate these sensory hairs. A recent study showed that bees flying around plants inhibit the feeding intensity of herbaceous caterpillars, resulting in a reduction of leaf damage (Tautz and Rostas, 2008). Attracting honeybees with tasty nectar might therefore be an effective strategy to reduce poplar leaf damage by herbivore infestation.

4.1.2.4 Anti-microbial properties of *P. trichocarpa* nectar proteins.

The composition of nectar makes it an excellent microbial growth medium, however no bacterial or fungal growth was observed on the extrafloral nectaries. When the nectaries terminate releasing fresh nectar, abundant fungal growth was observed (data not shown). In line with an anti-microbial activity (Fig.3.1_12), most of the proteins identified in poplar nectar appeared somehow to be related to pathogen defence (table 3.2).

Among the proteins identified in *P. trichocarpa* nectar five different chitinases (chitin-degrading enzymes) were found. Since chitin is the major component of the fungal cell wall, these enzymes may act as potent fungicides (Ye and Ng, 2005). Transgenic

plants which over-express chitinases exhibited improved fungal resistance (Kaomek et al., 2003; Hammel and Bellemare, 1995).

Four different GDSL lipases were also highly represented in nectar. These enzymes catalyze the cleavage of ester bounds, particularly those between fatty acid and other functional groups. It has been suggested that GDSL lipases may have a role in biotic defence. Indeed, AtGLIP1, an *Arabidopsis* GDSL lipase, has direct antimicrobial activity against the fungus *Aternaria sp*. Moreover, mutants lacking the gene for this protein are much more susceptible to infection (Oh *et al*., 2005). A recent study suggested that JNP1, a GDSL lipase from *Jacaranda mimosifolia* floral nectar, may also serve as an antimicrobial agent. Growth of *E. coli* expressing JNP1 was largely reduced in comparison with controls (Kram *et al*., 2008).

4.1.3 Induction of nectaries and nectar.

In wild cotton (*Gossypium thurberi*) the morphology (size) and the density of extrafloral nectaries have been demonstrated to be heritable (Rudgers and Strauss, 2004). One study has suggested a possible genetic component in the poplar extrafloral nectaries induction, showing that *Populus tremuloides* nectaries are more abundant on young leaves than on older ones. Moreover the number of leaves bearing nectaries was constant among the plants (Wooley et al. 2007). In the present study with *P. tremula tremuloides* similar results were found. Patterns of extrafloral nectaries on these plants tend to follow predictions of the optimal defence theory (McKey, 1974).

The extrafloral nectaries appeared in all new leaves in spring (Fig 3.1_15), these tissues are particularly photosynthetically valuable for the tree and it was demonstrated that bearing nectaries protect them from herbivore attack (Fig 3.1_14). On the contrary, *P. trichocarpa* did not present extrafloral nectaries on intact leaves (Fig 3.1_15). Many plants are known to induce nectary or nectar production only after herbivory, to protect the plant only when needed. In addition, *P. trichocarpa* is known to have several direct defence mechanisms against herbivores like condensed tannins, phenolic glycosides and salicortin (Hwang and Lindroth, 1998; Osier *et a*l., 2000; Donaldson and Lindroth, 2007). Both, direct and indirect defences are energy expensive, the inducible expression of nectaries on *P. trichocarpa* could be a way to avoid trade-offs between both defence systems.

Several studies indicate that extrafloral nectar secretion may increase in response to herbivory and that this reaction occurs in response to mechanical damage without further need of an herbivore-specific elicitor (Heil *et al*., 2001). For example extrafloral nectar flow of *Macaranga tanarius* can be induced under field conditions in response only to artificial damage. The present study with *P. trichocarpa* showed that nectary formation occurred 24 hours after mechanical damage, but nectar flow was missing, unless the damage was performed by herbivores (Fig 3.1_16, 3.1_17). The induction of nectar not only occurred after caterpillar attack; also leaf damage produced by other insects activated the nectar release, pointing to the involvement of a further elicitor. Recent studies indicated that mechanical tissue damage is often, but not always, sufficient to trigger jasmonic acid production and the subsequent defence responses. These studies suggested that chemical elicitors present in insect oral secretions play an important role in the nectary response (Mithöfer and Boland, 2008).

Herbivory induces release of volatile organic compounds (VOCs). These VOCs may be used by carnivorous arthropods as cue for host localization or as plant-plant signalling mechanism (Heil *et al*., 2008). In lima beans (*Phaselous lunatus*) the release of VOCs from damaged plants resulted in an increase of extrafloral nectar secretion in undamaged plants (Arimura *et al*., 2000; Kost and Heil, 2008). Upon herbivory, VOCs release by *P. trichocarpa* showed the same time course as nectar release (Fig 3.1_20 and 3.1_21). External application of jasmonic acid also induced volatile compounds release (Fig 3.1_22). These results further supported the theory that artificial mechanical damage of *P. trichocarpa* leaves is not sufficient to trigger endogenous jasmonic acid production and subsequently VOCs and nectar release. Understanding the biology of the factors triggering defence responses against herbivores remains a challenging task for the future.

4.1.4 *P. tremula x tremuloides* nectary specific genes.

It is likely that specialized structural and functional properties of nectaries are based on the expression of a nectary-specific set of genes. Therefore the nectary transcriptom identified in this study represents a suitable field for respective data mining. The initial analysis of functional categories was performed taking advantage of homology-based information of the *Arabidopsis* genome *(*www.arabidopsis.org, and Fig 3.1_18).

Hormones

The hormone category displayed the most pronounced induced group with 28 genes (Table 3.4). Many of these genes are involved in jasmonic acid metabolism. This is in line with the hypothesis that the release of volatile organic compound (VOCs) and the secretion of extrafloral nectar (EFN) are both induced by herbivory damage and that this response is mediated by jasmonic acid signaling (Heil *et al*. 2001). Auxins play an essential role in many growth and behavioral processes in the plant life cycle (Vanneste and Friml, 2009). In nectaries auxin related transcripts like PIN3/15 and several auxin permeases were up-regulated. It is possible that auxin is also important in growth or polarity of nectaries. Also *GH3* was found to be up-regulated, in rice this protein is known to enhance defence responses and resistance to fungal pathogen (Domingo *et al*., 2009). In this category highest induction compared to leaves was found for BSMT1 transcript. This gene is known to be important in the biosynthesis of salicylic acid and directly involved in the biotic defence (Chen at al., 2003). Taken together these results pointed to nectary specific induced defence against biotic aggressors.

Cell wall metabolism

The pool of nectary-specific up-regulated genes was also found enriched in transcripts associated with cell wall metabolism (Table 3.3). The plant cell wall architecture and composition undergoes dramatically changes during cellular differentiation. Plant cell wall dynamics is the result of membrane trafficking, fusion and secretion of cytosolic components. The plant cell wall consists of cellulose microfibrils that are embedded in a complex mixture of matrix polysaccharides and structural proteins (O Lerouxel *et al*. 2006). This architecture and composition dramatically changes during cell differentiation. In recent years insights into the secretory pathway regulation of deposition and metabolism of the cell wall polysaccharides was gained (Nergard *et al*., 2006). The cell wall polymers are integrated into existing structures, and undergo extensively remodelling. These processes involve a wide array of modifying agents like expansins, xyloglucan transglycosidases/hydrolases, or peroxidases, all of which appeared up-regulated in nectaries. Since *Populus tremula x P. tremuloides* nectaries secreted nectar via membrane vesicles, secretory cells seem to run well-controlled exo- and endocytosis cycles.

It should be mentioned that transcript pattern similarities to secretory organs like stigma were found. In both organs a strong polarity within the cells can be found. In line with the polar architecture and the specialized secretory nature of the apical cell layer(s),

increased expression of genes involved in cell wall/cuticle formation, lipid metabolism as well as exo- and endocytosis was found.

In future studies the major nectary specific proteins need to be localised and functionally analyzed to gain a model on nectar development on one hand and polar organization of the sectretory complex on the other. Concerning the latter it will be interesting to see how a plant epithelium works. By answering the following questions new insights into this weakly studied but very important organ will be gained:

i) How is uptake of `raw´ nectar from the phloem oriented basolateral side accomplished?

ii) How is the `mature´ nectar released on the apical side?

iii) Which transport processes are involved?

iv) Is there a common principle in animal and plant epithelia functional organisation and action?

4.2 ABIOTIC STRESS

4.2.1 Salts stress affects xylem differentiation of grey poplar (*Populus x canescens*)

The impact of up to two weeks salt stress on the morphology of grey poplar was investigated. Thereby a reduced wood differentiation zone and reduced vessel lumina occurred. A possible reason for this observation was a reduced nutrient supply of the cambial zone.

The rise of Na^+, Cl^- and osmolarity in leaves appeared to be delayed by one week compared to roots and shoots, pointing to a prevention of the negative impact of salt on leaves and thereof on photosynthesis. In line with this hypothesis sodium accumulates first within the roots, subsequently in the shoots and finally in the leaves.Additionally in roots and shoots a strong reduction in potassium was observed but not in leaves. The expression analyses of poplar potassium transporters were consistent with the potassium changes under salt stress. ABA concentrations in leaves appeared to be about 20fold compared to roots and were further elevated upon salt exposure. The resulting ABA induced permanent stomatal closure led to reduced carbon assimilation which in turn affected the photosynthetic machinery. This might be a possible

explanation for the observed reduced wood growth. For details of these summarized conclusions see the attached publication (Escalante et al 2009).

4.2.2 Different roles of ABA in salt stressed Poplar and *Arabidopsis*

One of the first effects upon salt exposure is a severe disturbance of the plant water homeostasis. The phytohormone abcisic acid (ABA) plays a major role in the regulation of the water status. Salinity in general causes increased ABA biosynthesis and accumulation (Chang *et al*., 2006; Chen *et al*., 2001; Karmoker and van Stevenick, 1979). ABA induces stomatal closure, but may also systematically adjust the water balance (Christmann *et al*., 2005). At the molecular level the responses are primarily mediated by regulation of ion channel activity and changes in gene expression (Zhu, 2002). In the present study different salt stress treatments, long and short term, were investigated focusing on the effect of ABA with the salt sensitive tree *P. canescens* and the glycophyte *Arabidopsis thaliana*. Most of the studies on salt responses of *Arabidopsis* are performed over short periods (several days) and with seedlings. In the present study, in contrast, adult plants were used to obtain comparable conditions.

Studies with different poplar species hypothesized that persisting high levels of ABA trigger salt resistance (Chang et al., 2006). *P. euphratica* plants submitted to 50mM NaCl increased root ABA concentrations (Chen et al, 2001 and 2002). Salt resistant *P. Popularis* and sensitive *P. Euramericana, however,* exhibited a different timing and pattern of ABA production. *P. Popularis* reacts faster and produces more ABA in roots at the early stages of stress (Chen *et al*. 1997). In roots of *P. canescens* the induction of ABA did not occur until the second week of stress, thus delayed with respect to salt tolerant species like *P. euphratica* or *P. populuaris* (Escalante *et al.*, 2009 and Chen *et al*. 1997). Under this conditions salt sensitive *P. euphratica* under long term stress maintained higher ABA concentrations (Chen *et al*., 2001). The ratio of ABA increase of salt stressed grey poplar was similar in roots and leaves; however ABA basal levels in leaves appeared about 20-30 fold higher than that of roots (Escalante *et al.*, 2009). In contrast to other salt sensitive species investigated before, *P. canescens* leaf throughout all the experiment gained similar ABA levels as measured in the salt tolerant species. Therefore these data were not in line with the previous hypothesis predicting that continuously high ABA levels are important for salt tolerance but support the hypothesis that an early ABA synthesis in roots might trigger adjustments in the shoot and thus could confer salt resistance.

After 2 weeks of salt exposure leaves of *P.canescens* showed necrotic lesions (Escalante *et al*., 2009). Under similar conditions *Arabidopsis* plants showed a severe growth reduction (Fig 3.2_1). Leaves from *P. canescens* showed maximal ABA induction after two weeks of salt exposure (Escalante *et al*., 2009). In Arabidopsis, ABA peak levels appeared already after one week (Fig 3.2_2). Small plants like *Arabidopsis*, unlike trees, seem to be unable to compensate a pronounced salt load via buffers within the stem.

Short term salt stress, however, regarding of ABA induced genes resulted in similar patterns in both plants. *P.canescens* and *Arabidopsis* after only 24 hours of salt application in roots showed a strong and transient up-regulation of ABA levels and corresponding *KIN2* expression (Fig 3.2_5 and 3.2_6). However, this early response in roots might induce the subsequent responses in leaves with a delay of 2 days in *Arabidopsis* and only one day in Poplar supporting the hypothesis that root ABA triggers ABA production in leaves.

4.2.4 Different regulation of an ABA marker gene in poplar and *Arabidopsis*

AtKIN2 (also called COR6.6) is a small peptide known to be induced by ABA, cold, drought and salinity (Kurkela and Borg-Franck, 1992). Experiments with *Arabidopsis abi1-1* mutant plants, which are defective in ABA biosynthesis, showed a dramatic reduction in *AtKIN2* gene expression compared to wild type plants (Wu *et al*., 2003). In the present study using the ABA deficient mutant *aba1-3* no induction of the *AtKIN2* gene under salt or cold stress was observed, demonstrating the direct control of *AtKIN2* expression by ABA (Fig 2.1_3 and 3.2_4).

While in roots of *Populus canescens*, *PtKIN2* induction appears associated with ABA content (Escalante *et al*., 2009 and Fig 3.1_6), in leaves *PtKIN2* expression did not change, even when the ABA levels arose after two weeks of salt application (Escalante *et al*., 2009). When the plants were submitted to short term salt stress the levels of *PtKIN2* were also not found to be linked to the ABA content in this organ (Fig. 3.1_6), while again in roots the induction coincided with ABA levels. Thus in poplar leaves *PtKIN2* is either controlled by other factors, or the ABA levels were above the *PtKIN2* induction threshold from the beginning of the experiment.

In conclusion, salt stress responses differ between small annual and large perennial plants. With e.g. *Arabidopsis thaliana* gene regulation in response to salt stress is likely to be dependent on the ABA concentration. Trees like poplar, however, seem not to translate salt stress into an ABA signal expierenced by the photosynthetic tissue. In grey poplar ABA seems rather to close the stomata permanently. As a matter of fact reduced photosynthetic carbon intake likely feeds back on wood anatomy (Escalante et al., 2009).

SUMMARY

In this study poplar trees have been examined under different stress conditions. Apart from the detailed descriptions above two main conclusions might be drawn:

i) A small plant like *Arabidopsis thaliana* is highly susceptible to stress situations that might become life-threatening compared to a tree that has extremely more biomass at its disposal. Such an organism might be able to compensate severe stress much longer than a smaller one. It seems therefore reasonable that a crop like *Arabidopsis* reacts earlier and faster to a massive threat.

ii) In poplar both tested stress responses seemed to be regulated by hormones. The reactions to abiotic salt stress are mainly controlled by ABA, which also has a strong impact upon cold and drought stress situations. The term commonly used for ABA is “stress hormone” and is at least applicable to all abiotic stresses. In case of herbivory (biotic stress), jasmonic acid appears to be the key-player that coordinates the defence mechanism underlying extrafloral nectary and nectar production.
Thus the presented work has gained a few more insights into the complex network of general stress induced processes of poplar trees. Future studies will help to understand the particular role of the intriguing indirect defence system of the extrafloral nectaries in more detail.

ZUSAMMENFASSUNG

In dieser Arbeit wurden Pappelbäume unter verschiedenen Stressbedingungen untersucht. Zusammenfassend und zusätzlich zu den obigen Beschreibungen lassen sich zwei Schlussfolgerungen ziehen:

i.) Eine kleine Pflanze wie Arabidopsis ist viel empfindlicher für Stresssituationen, die möglicherweise lebensbedrohlich werden könnten, im Gegensatz zu einem Baum mit wesentlich grösserer Biomasse . Solch ein Organismus kann schwerwiegendem Stress viel länger kompensieren als ein kleinerer Organismus. Es erscheint daher sinnvoll, dass eine Pflanze wie Arabidopsis viel früher und schneller auf eine massive Bedrohung reagiert.

ii.) In Pappeln scheinen beide untersuchten Arten von Stressreaktion durch Hormone reguliert zu werden. Die Reaktionen auf abiotischen Salzstress werden hauptsächlich durch ABA kontrolliert, welches auch einen starken Einfluss auf Kälte- und Trockenstressszenarien hat. Üblicherweise wird für ABA der Ausdruck "Stress-Hormon" verwendet, was zumindest für abiotischen Stress zutreffend ist. Im Fall von Herbivorie (biotischer Stress) scheint Jasmonsäure die Schlüsselrolle zu spielen, die die Abwehrmechanismen koordiniert, die den extrafloralen Nektarien und der Nektarproduktion zu Grunde liegt.

Demzufolge hat die vorliegende Arbeit ein paar neue Einsichten in das komplexe Netzwerk der Stress-induzierten Prozesse der Pappel ermöglicht. Zukünftige Studien werden dazu beitragen die besondere Rolle des faszinierendem indirektem Abwehrmechanismus der extrafloralen Nektarien en detail zu verstehen.

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6. Supplement

6.1 Chemical suppliers

Sigma-Aldrich
3050 Spruce st.
St.Louis, MO 63103
USA

AppliChem Inc.
505 Highlan Avenue
Cheshire, CT 06410
USA

Carl Roth GmbH
Schoemperlenstr. 1-5
76185 Karlsruhe
Germany

Duchefa Biochemie B.V.
A. Hofmaweg 71
2031 BH Haarlem
The Netherlands

Tib Molbiol GmbH
Eresburgstr. 22-23
12103 Berlin
Germany

Qiagen Sciences
19300 Germantown Rd
MD 20874
USA

Promega Corporation
2800 Woods Hollow Road
Madison, WI 53711
USA

La Roche Ltd.
CH-4070 Basel
Switzerland

Eppendorf AG
Barkhausenweg 1
22339 Hamburg
Germany

Thermo Fischer Scientific Inc.
81 Wyman st.
Waltham, MA 02454
USA

Agar Scientific Ltd.
66a Cambridge Road
Stansted, Essex CM248DA
England

Leica Microsystems GmbH
Ernast-Leitz str. 17-37
35578 Wetzlar
Germany

Keyence
1-3-14 Higashi-nakajima
Osaka
Japan

Wescor Inc.
459 South Main Street
Logan, Utah 84321
USA

Dionex Corporation
1228 Titan Way
Sunnyvale CA 94088
USA

Omega Bio-Tek Inc.
1850 E Beaver Ridge Cicle
Nordcross GA, 30071
USA

BD Bioscience
2350 Qume Drive
San Jose, CA 95131
USA

Invitrogen Corporation
5791 Van Allen Way
Carlsbad, CA 92008
USA

BIO-RAD laboratories
1000 Alfred Nobel Drive
Hercules, CA 94547
USA

6.2 primer sequences

Name	sequence	Tm (ºC)
PtACT2 fwd	5´-ccc aga agt cct ctt-3´	48-54
PtACT2 rev	5´-act gag cac aat gtt ac-3´	
PtKIN2 fwd	5´-ctg aca ata ccc aga ag-3´	48-58
PtKIN2 rev	5´-cgt aga cat cac ctg tt-3´	
KPT1 fwd	5´-tat cca cag gca gct tca-3´	48-56
KPT1 rev	5´-ttg cgc ttt ttg tta ata gtt c-3´	
PTK2 fwd	5´-atg cga tat ac acct g-3´	48-52
PTK2 rev	5´-tgc tca ccc taa tac a-3´	
PtKUP fwd	5´-ccc aaa ctt tac agg a-3´	48-52
PtKUP rev	5´-tcg cct taa tat gag agt-3´	
PTORK fwd	5´-tga tga agc tcg tat tg-3´	48-54
PTORK rev	5´-gta acc acc tga aga tt-3´	
PTORK2 fwd	5´-cat ggg gtg caa aag aac-3´	48-54
PTORK2 rev	5´-aac ttc tgg cca tca tcg-3´	
PTORK3 fwd	5´-aca ctc cac ttg ac gag-3´	50-58
PTORK3 rev	5´-gcc acc atc aat cat gtt-3´	
PKT1 fwd	5´-ccc aaa aca gtc ata at-3´	54-56
PKT1 rev	5´-tca gcg aca aac ata at-3´	
PKT1b fwd	5´-aac caa cta ttc gac ct-3´	48-56
PKT1b rev	5´-cgg gtg aga tgt cga a-3´	
PtTUB fwd	5´-gat ttg tccc ctc gcg ctg t-3´	48-62
PtTUB rev	5´-tcg gta taa tga ccc ttg gcc-3´	
PttDT fwd	5´-tcc aaa ttg cga aga cg-3´	50-56
PttDT rev	5´-ctc cta gtg tag gca tga-3´	
PTKC1 fwd	5´-tct cat tgt cca tgc tg-3´	48-50
PTKC1 rev	5´-caa tac ctg atc tgt tcc-3´	
PTKC2 fwd	5´-cct tgt tgt ccg ttc c-3´	50-60
PTKC2 rev	5´-ggg tgg cat acc act g-3´	
PKT6 fwd	5´-aaa tag gat gca gac ga-3´	48-56
PKT6 rev	5´-cag aag cgt tct tac ca-3´	
PtSOS1 fwd	5´-agt gtg gtc tta tga gc-3´	48-56
PtSOS1 rev	5´-acg atg cgg ttc tta aa-3´	
KIN2 fwd	5´-tca gag acc aac aag aat-3´	50-56
KIN2 rev	5´-cga tat act ctt tcc cgc-3´	