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I. INTRODUCTION

This work will deal with the interaction between two different organisms, one small and moving and one big and sessile. The interaction described here is of parasitic nature and forces one of the two organisms to change its way of being in a great deal. We're talking about the interaction of the aphid *Pemphigus spirothecae* Passerini (*Aphidoidea*, *Pemphigidae*) and the tree *Populus nigra* var. *italica* Muenchh. (*Malphigiales*, *Salicaceae*). During this interaction that will be described here almost solemnly on the plant side, dramatic morphological changes like the formation of a gall on the infested leaves petioles are induced, phytohormone levels change in adaptation to this stressor and the transcriptional pattern is adapted to the challenge and to mount a defense suitable for defending the plant or at least allowing for co existence.

I.1 GENERAL REMARKS ABOUT PLANT-INSECT INTERACTIONS

In general, there are three different possible outcomes for an interaction between plant and insect. The simplest case is a neutral interaction which basically means no effect on either individual like, for example, a bee landing on a tree. As it is easy to imagine, neither organism is harmed by this. If this specific tree was a flowering one and the bee would collect honey from it, this interaction would turn into a mutualistic positive one as both organisms benefit from it. Following this logic, a negative interaction would be one that had negative effects on at least one of the two interacting organisms. When considering plant–insect interactions with negative outcomes for one partner, there are different reasons for this negative outcome. On the plant side, the reason for this might be that the plant is not well enough defended against the attacker or that the attacker has learned to deal with the specific defense. On the insect side, this interaction could turn out to be negative for it if the plant had suitable defenses against it. Generally, defense in plants can either be constitutive (like, for example, a thick cuticular) or induced (like, for example, glycosinolates). But what kind of attacks does a plant need to deal with? In the broadest sense, two different classes can be discriminated: herbivores that feed on live plant material without causing further

disease and pathogens that do so. Both kinds of attacks lead to a specific, diverse response of the tree. This is in big parts due to the specific pattern of damage inflicted by the animal as well as due to specific elicitors like pathogen associated molecular patterns (PAMPs) that are recognized by the plant. The specific pattern of damage evoked leads to the differentiation into different feeding guilds, ranging from leaf chewing insects to stem boring ones and from insects to elephants. In this work, the interaction of a phloem feeding, colonial gall forming aphid and a tree is detailed examined and next, the tree is introduced.

I.2 THE TREE

In science, the genus *Populus*, consisting of 29 different species (Eckenwalder 1996), developed into a model species to study woody trees due to some reasons. First of all, it is a fast growing tree that makes it easy and feasible to cultivate when it comes to generation times in the lab and in the field. Because it can be propagated by cuttings, clonal lines are easily established and maintained. Furthermore, *Agrobacterium tumefaciens* readily infects *Populus* (Han, Meilan et al. 1999) enabling transgenic work without transfection problems; a fact that is further aided by its relatively small genome. Combined, these specifics led to the development of a wide range of genetic and molecular tools and in 2006, the draft sequence of the genome of *Populus trichocarpa* was published (Tuskan, DiFazio et al. 2006). Taken all this together, *Populus* is indeed a good model organism well suited to study processes of long lived plants such as defense aimed at survival of the individual rather than for propagation of the species like in herbs. Because of this, *Populus* was chosen for this work.

The molecular phylogeny of *Populus* shows that it is a relatively homogenous group with *Populus trichocarpa* belonging to the *Leucoides* section of the genus, whereas the study organism used in this study, *P. nigra* var. *italica*, groups into the most recently evolved *Aigairos* section of the family. Together with *Populus nigra*, it forms an own group distinct from all others within the *Aigairos* section (Cervera, Storme et al. 2005).

The morph “*Italica*” used in this study is thought to originate from Italy (and is hence also called Lombardy poplar), from where it was brought to France in the 17th century. It was

then radiated to the neighboring countries and also reached Germany as a fashionable tree representing a certain longing for Italian lifestyle comparable to the takeover of classic style in that time in literature and fine arts. This is most likely due to the fact that its shape mimics the fashionable cypress from Italy. Due to its characteristic fast growth, it was suitable to be used for the accentuation of parks and as a tree planted along alleys. As it became apparent after some time that the Lombardy poplar has certain drawbacks such as becoming bare branched when it ages, its use chased in the 18th century; however, due to its fast growing nature it was still widely used as a plantation tree for matchstick production in the 18th and early 19th century (*sensu* (Wimmer 2001)). It is a dioecious plant but is propagated only clonally by cuttings and only males form the morph *Italica*. Its growth form is of stretched nature without much expansion towards the sides, in that being different from the original wild type *Populus nigra*, from which it stems. Its spread throughout the northern hemisphere and **Fig. I.2.1** shows the distribution of *P. nigra* on the Eurasian continent.

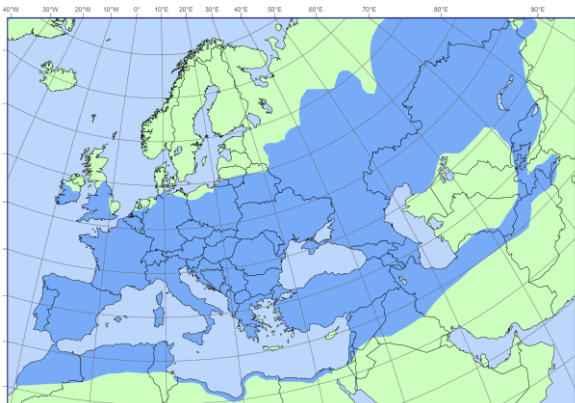


Figure I.2.1: Distribution of *Populus nigra* on the Eurasian continent (EUFORGEN, 2006, http://www.euforgen.org/distribution_maps.html, accessed 27.8.2010)

I.3 PLANT DEFENSE IN *POPULUS*

In general, defense of plants falls into two different classes: constitutive defenses and induced defenses. The former is rather unspecific and can be seen as a first line of defense, whereas the latter is more specialized and forms a second line of defense. *Populus* has a massive repertoire of defense mechanisms that will be outlined in this section. Before going into too much molecular detail, the most obvious and still often overlooked mechanism of

(constitutive) protection against harmful influences is a physical one that abrogates most attempts of opportunistic invaders. The poplar leaf, for example, is protected quite well against non specialized or opportunistic herbivores and pathogens due to its thick cuticula. Besides this palpable point *Populus* also has a wide array of chemical defenses which range from phenolic compounds to protease inhibitors. Among the former, the most prominent are phenolic glycosides (PGs) which, together with condensed tannins, make up up to 35% of the leaf's dry weight (Lindroth and Hwang 1996) and some of which were shown to be deterrent to generalist herbivores and to have a negative impact on larval development (Lindroth 1988; Lindroth and Bloomer 1991). It is thought that this is due to the toxic breakdown products that are formed once the compound is either ingested by the feeding animal or broken down by the plant's glycosidases upon tissue damage. The different side chains have different toxicity, which also explains the effects observed for the different phenolic glycosides. To date, at least 20 PGs have been identified (Tsai, Harding et al. 2006) and conventionally, they are seen as constitutive defense, but their inducibility seems to be an option (Stevens and Lindroth 2005).

On the biochemical side of defense, *Populus* employs a wide range of enzyme inhibitors, especially protease inhibitors to prevent effective digestion of its tissue and lowering its digestive value. It seems that there is an adaptation of different protease inhibitors to specific proteases found in different herbivores as there was a large gene family of nearly 30 different inhibitors found in poplar (Philippe and Bohlmann 2007) which are mostly inducible defenses. Besides those, other enzymes found in poplar can either specifically target the attacking animal or aim at the production of toxic products. Among them are endochitinases like WIN6 and WIN8, which genes are strongly upregulated upon herbivory (Parsons, Bradshaw et al. 1989) but some are also expressed constitutively as it is the case for a polyphenoloxidase (Wang and Constabel 2004).

I.4 PLANT HORMONES

I.4.1 GENERAL REMARKS

Plant hormones have long been known for their ability to regulate different processes in plant development, ranging from long lasting changes induced such as regulation of longitudinal growth, seed germination, induction or maintenance of senescence or apical dominance as well as the establishment of cell polarity to regulatory events happening often, such as stomatal closure.

Historically, the first ones to postulate compounds that have the functions described above were Sachs and Darwin around 1885. They postulated identity determining or movement inducing substances. These hypothesis led to the design of elegant experiments by Went in the Netherlands and to the discovery of the first phytohormone (PH), auxin (*sensu* (Davies 2010)).

Subsequently, a lot of different plant hormones have been identified and a lot of effort has been made to characterize their functions and mode of action. They are as chemically diverse, ranging from lipid derivates to small peptides, as are their functions and effects. A lot of the current understanding about the signaling of the different compounds has been obtained from mutant strains of *Arabidopsis thaliana* and the details of the key signaling steps of different phytohormones begin to emerge, drawing a complex picture of this elaborate network of regulation that seems to integrate various environmental cues in complex blends of PH.

I.4.2 HORMONAL SIGNALING IN DEFENSE

A lot of work in the past decade helped to unravel the key function of jasmonic acid (JA) in the regulation of defense responses against herbivory and, at least partly, to pathogen attack. The octadecanoid pathway has been implicated to a lot of defense responses against herbivory by chewing insects and necrotrophic pathogens in plants. Gene regulation by this pathway leads to the upregulation of genes encoding protease inhibitors (Doares, Syrovets et al. 1995), cell wall synthases (Creelman, Tierney et al. 1992) or the accumulation of secondary metabolites such as nicotine. It seems that the activation of this pathway is an

unfavorable event for the aphid as it seems to be able to effectively reduce its fitness (Ellis, Karafyllidis et al. 2002).

The major active signaling compound derived from the Jasmonate pathway is the jasmonic acid isoleucine conjugate (+)-7-iso-Jasmonoyl-L-isoleucine (which is mimicked by carotinoate of *Pseudomonas syringiae*) (Fonseca, Chini et al. 2009), but other so called jasmonates are also thought to have signaling function. On a gene regulation basis, JA signaling means the de-repression of genes by proteolytical degradation of a repressor (the JAZ-proteins) after poly ubiquitination of this repressor by the COI-1 protein (Chini, Fonseca et al. 2007; Thines, Katsir et al. 2007). This event leads to various changes in the transcriptome of the corresponding cell, such as upregulation of JA biosynthetic enzymes like lipoxygenase 2 (LOX2) creating positive feedback as well as a negative feedback by upregulating the jasmonate ZIM domain (JAZ) proteins mRNAs.

The main functions of the SA defense pathway, which is used by the plant to fight mainly pathogens, as well as its signaling will be outlined briefly. Salicylic acid plays a crucial role in the establishment of systemic acquired resistance (SAR) against biotrophic and hemi-biotrophic pathogens. The mobile signal for that is not known for sure and different points are discussed: the mobile signal could be a lipid derived from the plastids (Chaturvedi, Krothapalli et al. 2008) or a volatile produced from SA, MeSA (Park, Kaimoyo et al. 2007). Further, it aids in the defense against pathogens by driving the expression of a specific subset of the so called Pathogenesis Related (PR) genes. The only common feature among this extremely heterogeneous class of proteins is their induction upon pathogen challenge. Their functions range from lipid transfer proteins to proteinase inhibitors and they are grouped into 17 different families according to biochemical properties; however, some of them were also found in healthy, noninfested tissue (van Loon, Rep et al. 2006). Upon pathogen challenge, SA also induces the so called hypersensitive cell death to prevent spreading of the disease. But how is this action achieved on a molecular level? The key protein for the regulation of SA signaling as well as its crosstalk to the JA is the nonexpressor of PR1 protein, NPR1 (Spoel, Koornneef et al. 2003). NPR1 functions as a redox sensor, changing its oligomerisation state according to the cells redox status. In unchallenged tissues, the protein forms an oligomeric complex in the cytosol via disulfide bonds. Upon SA

stimulation, the reduction state of the cell changes, NPR1 monomers form and enter the nucleus (Tada, Spoel et al. 2008), where they serve as transcriptional coactivator for PR genes (Despres, DeLong et al. 2000). Furthermore, they induce the expression of several WRKY transcription factor genes (Wang, Amornsiripanitch et al. 2006). It has been shown that NPR1 is phosphorylated in the nucleus and that this phosphorylation is necessary for the onset of SAR (Spoel, Mou et al. 2009).

Besides the two briefly discussed phytohormones, there are many others associated with defense. It is, for example, known that Abscisic Acid (ABA) and SA antagonize in pathogen-plant interactions (Zabala, Bennett et al. 2009) and also Ethylene, a gaseous phytohormone, has been shown to be part of a defense response in concert with JA (Bari and Jones 2009). It becomes increasingly clear that the reaction of a plant towards stress is governed by a complex blend of PH, rather than just one (Wu and Baldwin 2009; Zabala, Bennett et al. 2009).

I.5 THE APHID

Pemphigus spirothecae (Passerini) is an gall inducing aphid specialized to *Populus nigra* and its subgenera (Stresemann 1994). It is an obligate phloem feeder and doesn't change its host during one life cycle, which is not unusual for an aphid as only 10% of all aphid species alternate their host during their life cycle (Peccoud, Simon et al. 2010). In spring, during the first leaf flush, the fundatrix hatches from the egg on the bark of the tree. The egg is its overwintering form and was laid there in fall of the previous year. The aphid then, by a not completely understood mechanism, selects a leaf suitable for its purpose and starts the induction of the gall by probing the petiole with its stylet. This ultimately enables it to suck phloem juice out of the petiole of the leaf and feed on the rich variety of compounds transported in it. It is thought that this repeated probing, together with other mechanisms stimulated by the presence of the aphid, leads to the formation of the gall. The formation of this highly unusual and characteristic feature takes about six to ten weeks depending on the general conditions (see **Fig. I.5.1**). It is brought about by two to three rotations of the leaf around its vertical axis which, in a way, twists the petiole around the aphid. The completed

gall is of knob like appearance with a durable haptic impression and a size between 0.7 to 1.5 cm along the petiole and an expansion of approximately 0.7 cm in diameter. The plant tissue that forms the gall is amorphic. The gall is completely closed and opens only in fall, when the winged progeny of the fundatrix leaves it through a flight hole on the apex of the gall. **Fig.I.5.1** shows the development of the gall at the different stages that were sampled.

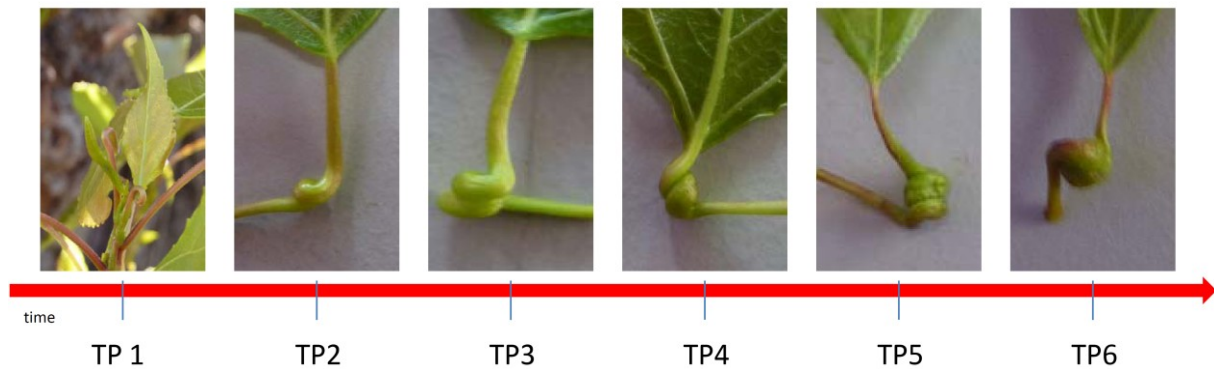


Figure I.5.1, showing the different developmental stages of the gall induced by *Pemphigus spirothecae* on *Populus nigra* var. *Italica* leaf petioles. Although the figure suggests otherwise, the time elapsed between the different pictures is not equal (see text for further details). TP = Time point.

A big part of the life cycle (see **Fig. I.5.2**) of *P. spirothecae* happens within the gall. The inducing fundatrix moults four times inside the gall and then gives rise to the next generation and forms the so called virginoparae. Whereas only one fundatrix is found per gall, up to 80 virginoparae can be found inside one gall. They hatch at different time points during the year and some of them are specialized soldiers to protect the gall. These soldiers can't moult themselves and hence can't grow or produce offspring; the normal virginoparae can and after 4 moulting cycles those lay ovarioles that give rise to the sexuparae. When fully grown these are winged and leave the gall through the flight hole to lay ovarioles onto the bark of the tree that bring on the sexually reproducing generation of the sexuales, the fourth generation. The sexuales mature with four moulting cycles and represent the only sexually differentiated stage. After the 2 sexes have mated, the female lays six to eight eggs onto the bark or into clefts and cracks on the tree. These eggs overwinter and from them, the fundatrix hatches in spring. The hole life cycle was summarized after (Toth 1937) and is shown again in **Fig. I.5.2**.

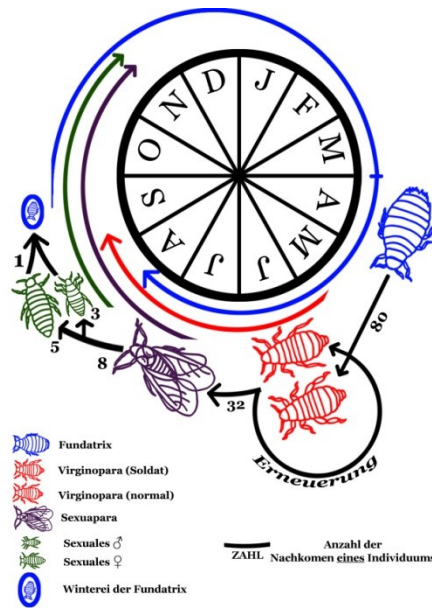


Figure I.5.2: This figure summarizes the life cycle of *P. spirothecae*. The figure was taken from http://de.wikipedia.org/wiki/Pemphigus_spirothecae, accessed 14.12.2010.

I.6 APHID PLANT INTERACTION

The interaction between aphids and plants has caught considerable interest in the past few years due to its dynamic nature. As there is neither an agreed on plant nor an established phloem feeder to study this system, comparisons between studies are challenging, especially those working with either specialists or generalists. Furthermore, the studies that will be described here were mostly not done with colonial gall forming aphids on long lived trees, but rather on seasonal plants, mostly with *Arabidopsis thaliana*. Some of the general principles established in these reports will however hold true for the interaction between *P. spirothecae* and *Populus nigra* var. *italic* studied in this work as well and are outlined briefly below.

When the aphid has selected on a suitable plant and part of it (what it apparently doesn't do in a random fashion, at least for some species (Zucker 1982)), it begins probing the tissue with its stylet to find the phloem of the plant. Interestingly, no plant cells are destroyed as the stylet progresses into the plant because punctured cells are sealed by gelling saliva (Tjallingii and Esch 1993; Tjallingii 2006). During the whole process of intruding the plant, saliva of varying composition is constantly secreted and taken up again by the

aphid to navigate towards the sieve elements of the plant (Giordanengo, Brunissen et al. 2010).

This saliva is worth looking at in more detail as it contains some interesting enzymes thought to be partly responsible for enabling plant responses or preventing defense. Some of the enzymes described in the saliva such as cellulases, amylases or polygalacturases might have the mere function of aiding stylet progression by digestion of cell walls (Cherqui and Tjallingii 2000; Harmel, Letocart et al. 2008). Others might be involved in the detoxification of defense compounds such as polyphenoloxidases and peroxidases (Urbanska, Tjallingii et al. 1998; Harmel, Letocart et al. 2008) by altering the redox state of the corresponding compound, making it less toxic to the aphid. Other important components of aphid saliva are Ca^{2+} -chelating proteins, that are able to suppress the calcium waves normally elicited when the phloem is damaged and that induce and lead to clogging of the sieve elements (Harmel, Letocart et al. 2008).

It is, however, not only beneficial to the aphid to secrete all of those enzymes as the plant is able to recognize and react to many of the breakdown products created. It is known, for example, that the plant can perceive oligogalacturonides produced by cell wall degradation. This leads to different results in different plant species: in tomato, this can, as a response, activate the octadecanoid pathway (Doares, Syrovets et al. 1995; Will and van Bel 2008). In *Arabidopsis*, however, these breakdown products are perceived by wall associated kinase 1 (WAK1) and this activates the SA-regulated defense (Walling 2009).

The plant reaction towards aphids is also commonly associated with salicylic acid (SA), a phytohormone normally connected to pathogen resistance. This is interesting to note as it had been shown that a jasmonic acid (JA) driven response is much more effective in fighting the aphid (Ellis, Karafyllidis et al. 2002). Because of this, the idea arose that the insect itself can modulate the defense response of the plant and this was termed the decoy strategy (Zarate, Kempema et al. 2007). One has to keep in mind that the activation of JA based defenses has been shown to be closely correlated to the amount of tissue damage and different phloem feeders show a high degree of variability on the activation (Kempema, Cui et al. 2007).

But why would aphids favor the SA response over the JA response? There are different lines to think along when answering this question. First of all, as stated above, does the JA elicited defense have a more negative effect on the aphid's fitness than does the SA pathway. Second, different mutations in the SA pathway have an effect on aphid progeny and this leads to the conclusion that SA signaling does not have a detrimental influence on survival of the aphid. One example would be that the *npr1* mutant and the *NahG* transgenic, both of which reduce SA signaling significantly, showed decreased aphid reproduction (Mewis, Tokuhsa et al. 2006). This hints towards a role of the SA pathway in repressing another, more potent response. The picture that evolves is that the defense responses elicited by aphids are a mixture of both the SA and the JA response (Moran and Thompson 2001).

Besides the obvious shortcomings in the understanding of phytohormonal coordination of the defense mounted, there are numerous studies showing massive genetic regulation upon aphid feeding, most of them dealing with the early steps of the interaction. Genes identified to be regulated range from just 78 when aphid salvia was introduced (De Vos and Jander 2009) to more than 1000 (Kusnierczyk, Winge et al. 2008), after the specialized aphid *Brevicoryne brassicae* was allowed to feed on *A. thaliana*. The authors of the latter study proposed a model for the early steps of the interaction that is summarized in **Fig.I.4.1**. Whereas some of these induced responses appear to be localized (e.g. the production of ROS) others (like the up regulation of the SA biosynthesis) seem to be systemic.

Furthermore, different gene expression studies indicated that phloem feeding leads to massive upregulation of cell wall modifying enzymes such as O-methyltransferases both locally and systemically. This is thought to reinforce the cell wall and introduce a mechanical barrier (Thompson and Goggin 2006). Another point worth mentioning is the induction of synthesis of secondary metabolites such as Glycosinolates in *Arabidopsis* (de Vos, Kim et al. 2007) after aphid feeding.

All of the responses outlined here till now only dealt with the very first steps of the establishment of an interaction that can last much longer, in the case of gall forming aphids longer than the life of the inducing insect.

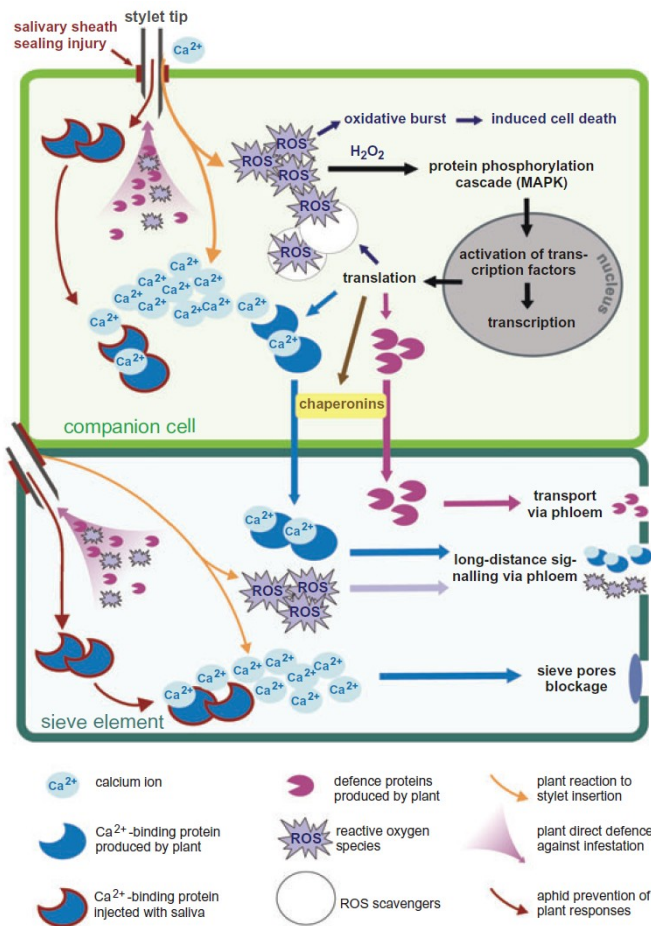


Figure I.6.1: An illustration showing the early steps of the interaction between aphid and plant. The top part shows the events happening after companion cell has been penetrated by the stylet and the bottom part shows events after sieve element infiltration (taken from Kusnierczyk, Winge et al. (2008)).

I.7 GALL FORMING APHIDS AND PLANTS

On the long run, the infection with gall forming aphids leads to massive changes in the physiology of the infested plant part. For one, galled tissue can reduce the photosynthetic activity of at least the leaf they sit on (Larson 1998) and also shows increased invertase activity (Allison and Schultz 2005). As the sugar loading of the phloem is a signal for either sink or source in nutritional trafficking (Dinant and Lemoine 2010), this effectively makes the gall a sink tissue, which has also been shown (Larson and Whitham 1991; Larson and Whitham 1997). Following the nutrition hypothesis (Price, Waring et al. 1986), the induction of the gall is performed by the aphid, creating a favorable, nutritionally superior rich niche in which it can live. This has also been proven empirically by transplantation experiments

(Diamond, Blair et al. 2008) and galls are indeed richer in soluble carbohydrates and lipids as well as amino acids (Motta, Kraus et al. 2005; Suzuki, Fukushi et al. 2009). Another aspect, somehow following up on this hypothesis is the fact that the inside of galls (the so called nutritive tissue) found on different willow species induced by different gall formers is generally lower in phenolic defense compounds than is the outside of the gall or the leaf on which the gall sits. This is possibly because the aphid might potentially be able to selectively block certain endpoints of the phenylpropanoid pathway (Nyman and Julkunen-Tiitto 2000), although the function of the nutritive tissue for an aphid that feeds on phloem sap exclusively is discussable. Another fact worth mentioning is that the influence of gall forming insects reaches much further than just towards the tree it infests as it significantly decreases the rate of decomposition of the corresponding tissues and by doing so also reduces the quality of the litter at any given time (Schweitzer, Bailey et al. 2005). This has massive influence on the shape their whole ecological community. It seems that in general, galls behave as a new organ induced and are maintained by the aphid by a yet elusive mechanism and extending its phenotype. Furthermore, it seems as if aphids in general are able to circumnavigate most defenses by their way of feeding or by tricking their hosts (Peccoud, Simon et al. 2010).

I.8 THE AIM OF THIS WORK

The goal of this work is to shed more light onto the interaction between the Lombardy poplar (*Populus nigra* var. *italica*) and the spiral gall aphid (*Pemphigus spirothecae*) and its implications for the secondary metabolites and gene transcript levels in the leaf blade. As this interaction is long lasting, the focus is put onto the early phase of this interaction until the time points (TP) when the first progeny of the aphid is visible inside the gall, which is considered to be the phase of establishment of this interaction. The progression of development will be judged on the morphological changes of the growing gall and on the development of the aphid colony.

But what exactly will be done within the framework of this work? The following phenotypic parameters will be measured:

- Levels of different phytohormone in the leaf blade
- Levels of different phenolic glycosides will be determined.

From the information obtained in this first section, time points showing the most distinct pattern of difference will be selected for transcriptional profiling. This will be done using custom designed single color arrays which represent all predicted genes of *P. trichocarpa* as well as all known expressed sequence tags of *P. nigra*.

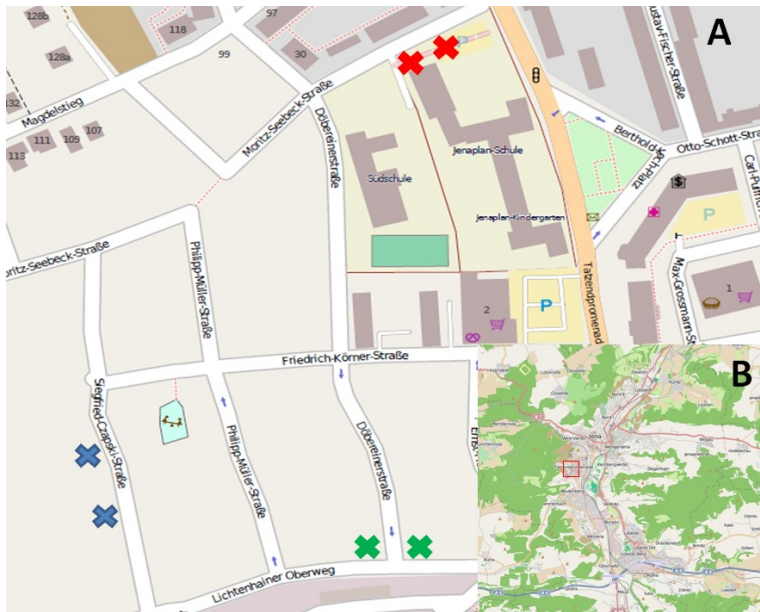
This work will lay the framework to qualify the following hypothesis.

- The phytohormone levels of *P. nigra var. italica* will be responsive to aphid infestation. The most pronounced response (due to reasons outlined above) will be seen in salicylic acid (SA) levels, whereas jasmonic acid (JA) will not be responsive.
- The amount of phenolic glycosides will be different in leaf blades that harbor an aphid gall compared to non-infested control leaves.
- The transcriptional profile of infested and non-infested leaves will be different. Here, the activation of SA responsive genes will be more pronounced than JA responsive ones.

II MATERIAL AND METHODS

II.1 PLANT MATERIAL

All plant material used in this experiment was harvested from *Populus nigra* var. *italica* trees growing in Jena, Germany. Leaves from six different trees (**Map II.1.1**) were harvested following a given scheme detailed in **Fig. II.1.1**. Trees were about 50 to 60 years old and reached a diameter at breast height of approximately 1 m. Before the first harvest, ten randomly selected branches infested with *P.spirothecae* situated in the lower canopy region between 50 cm and 2.5 m above ground were labeled for later identification with colored tape. All trees were sampled at six time points between April and June 2010 (see below for dates and **Fig. I.4.1**). Samples collected at the different time points were always taken from the same branches and the timing of sampling was kept as constant as possible to avoid circadian effects. The time points were corresponding to the development of the gall; the first samples were taken when the first kink in the petiole appeared (TP1, sampled at the 21.4.2010) and the second when the first twist of the petiole was finished (TP2, sampled 26.4.2010). The third sample collection was done after another twist (TP3, 30.4.2010) as was the fourth (TP4, 7.5.2010). The fifth sampling was performed when the galls were closed completely (TP5, 22.5.2010) and the last after the first progeny of the aphid was visible inside the gall (TP6, 10.6.2010); the development of the aphid colonies was constantly monitored by inspecting non target galls. As the development of the gall was not observed as a linear process (see **Fig. 1.4.1**), the time between sampling varied.



Map II.1.1: Map A shows the exact position of the different trees from which samples were taken, Map B details the position of the sample area in Jena. In map A, green crosses correspond to trees A and B (from right to left), blue crosses to trees C and D (from top to lower on) and red crosses mark the former position of trees E and F (from left to right). The red rectangle in Map B shows the position of the sample site within the Jena city limits. (maps from www.openstreetmap.org).

Samples were taken in the following manner: each leaf was cut off separately using a scalpel, the leaf was cut into half along the midrib and the petiole was separated from it. This was done for all leaves harvested from all ten branches on each tree and for each sample class separately. The samples obtained were pooled according to treatment (“Pemphigus”=P and “Control from the same leaf swirl as “Pemphigus””=CS) and position (1=more basal, 2=more apical on the branch) and flash frozen immediately in liquid nitrogen. Thus three different samples were obtained (for example left halves, right halves, and the petioles of all leaves of P1) from each of the four different sample classes (P1, P2, CS1, CS2). From each labeled branch, a total of four leaves were harvested per time point, one pair coming from a lower part of the branch and the other from more apical. In case of *P. spirothecae* infested branches, out of the four leaves harvested from two different positions two carried a gall (P1 and P2) induced by *P. spirothecae* on their petiole, whereas the other (CS1 and CS2) two did not. The leaves were processed as detailed above and care was taken

not to mix leaves from different positions on a branch. The sampling schema is detailed in **Fig. II.1.1**. After leaves had been collected they were stored at -80°C .

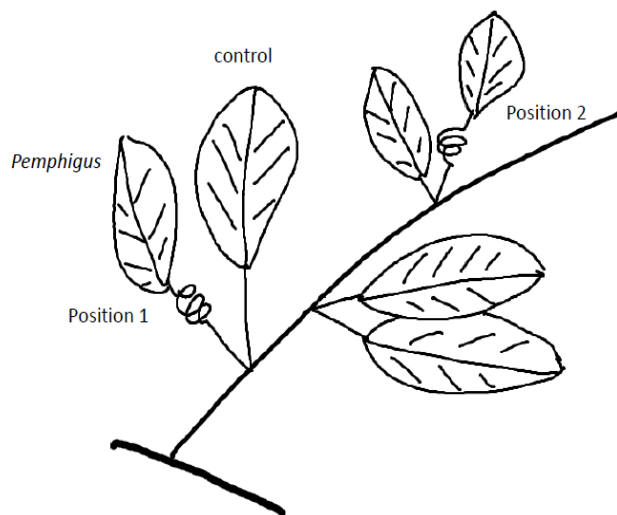


Figure II.1.1: Schematic *P. nigra italic* branch infested with *P. spirothecae*. From each branch the two leaves from position one and two leaves from position two were harvested. Aphid infested and control leaves from ten branches per tree were pooled according to their position on the branch.

II.2 METHOD DEVELOPMENT

II.2.1 EXPERIMENTS TO DETERMINE PARAMETERS OF PHYTOHORMONE DYNAMICS

In order to optimize and validate the extraction method for phytohormones as well as the sample preparation used, the following experiments were carried out. As salicylic acid (SA) was commercially available as a standard compound, this phytohormone was used for method evaluation primarily.

II.2.1.1 SALICYLIC ACID RECOVERY EXPERIMENTS

In order to find out whether there was a difference in the recovery of SA from fresh and freeze dried material, the following experiments were carried out. Briefly, leaves from *P. nigra var. italic* trees other than the trees for the main study were harvested fresh from different positions on the tree, cut in half and 20 half leaves were pooled per sample.

Immediately after this, samples were flash frozen in liquid N₂. This created two equal samples with leaf material of identical origin.

The identical samples were then ground under liquid N₂ and one pool was freeze dried using a Christ Alpha 1-4 CDplus freeze drier (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) set to -40°C and a pressure of 0,37mbar. The other was kept at -80°C as a fresh sample. Aliquots from this material were used in all further experiments.

In order to see if there was a difference in SA recovery, defined amounts of SA were added to samples and the amount recovered after the extraction procedure was determined by mass spectrometry as described below.

II.2.1.2 SALICYLIC ACID STABILITY EXPERIMENTS

As the recovery experiments (detailed above) showed a difference in recovery of SA, the question occurred whether this was due to the enzymatic breakdown of SA in the fresh extracts. To test if this enzymatic breakdown really occurred, SA was extracted from fresh plant material for 0,5h, 1h, 2h or 22h. Additionally, for each time point, a defined amount of SA was added to half of the samples to get a more precise measure of a possible breakdown.

II.3 PHYTOCHEMICAL ANALYSIS

All chemicals used were obtained from Sigma-Aldrich (Sigma-Aldrich, Munich, Germany).

II.3.1 EXTRACTION OF PHYTOHORMONES AND PHENOLIC GLYCOSIDES

Phytohormones and phenolic glycosides were extracted from plant material following a previously established protocol (Reichelt, M. and Boeckler, A., unpublished). All plant material was ground to fine powder using a mortar and pestle under liq. N₂. Samples were never allowed to thaw from the time of harvest till being in extraction solvent. After grinding, the material was stored at -80°C until further processing. In order to be able to give exact relative contents of the different compounds later, all samples were weighed prior to extraction and about 100 mg of each sample was used for extraction purposes.

Directly after weighing the samples on a fine balance (Mettler Toledo, Giessen, Germany) into a pre cooled 2 ml Eppendorf tube, 1 ml of pure methanol supplemented with 4 µl phytohormone Standard (deuterated salicylic acid, ¹³C-labeled JA, ¹³C labeled JA-isoleucine conjugate: 10ng/µl; abscisic acid (ABA) 50 ng/µl; all standards obtained from the lab of Ian Baldwin at the Max-Planck-Institute for Chemical Ecology in Jena, Germany) was added to each sample and the tube was inverted several times to allow good mixing of the extraction medium with the finely ground plant tissue. To standardize the extraction procedure, the tubes were flash frozen and kept in liquid nitrogen to avoid different extraction times and the possibly associated degradation processes. When the weighing process was finished, all samples were placed into Eppendorf Thermo mixers (Eppendorf, Hamburg, Germany) and shaken at 1400 rpm for 2 hours at 4°C. Afterwards, samples were spun down in an Eppendorf 5415R centrifuge at 13200 rcf; the centrifuge was pre-cooled to 4°C. From the supernatant, 800 µl were transferred carefully avoiding to disturb the pellet and put into a separate 1.5 ml Eppendorf tube. 500 µl of pure methanol were added to the pellet afterwards and the pellet was disturbed by vortexing and pipeting up and down. The tube was placed into the thermo mixer again to extract for 30 min at the same conditions. After centrifugation, 500 µl of the supernatant were collected and unified with the first collection. To avoid any contamination that would clog the HPLC column, the combined supernatants were spun down for 2min at 13200 rcf in a centrifuge pre cooled to 4°C and the complete supernatant was transferred to a GC-vial. Extracts were stored at -20°C.

II.3.2 ANALYSIS OF PHYTOHORMONES BY MASS SPECTROMETRY

Phytohormones were identified and quantified using a Bruker Triple Quad (Bruker Daltonics GmbH, Bremen, Germany) mass spectrometer in the MRM (multiple reaction monitoring) mode coupled to an Agilent 1200 HPLC system. The column used was a XDB-C18, 1,8 µm particle size, 4,6x50 mm (Agilent, Böblingen, Germany). The solvents and percentages used for the chromatographic separation are detailed in **Table II.3.2.1**, the masses corresponding to the phytohormones are detailed in **Table II.3.2.2**. The mode used for the ionization of the different compounds was a negative one.

Table II.3.2.1: Solvents and corresponding relative amounts used to separate the different phytohormones with liquid chromatography; absolute flow was set to 800 μ l/min.

Time [min]	Solvent [%]	
	Acetonitrile	0,05% Formic Acid in H ₂ O
0	5	95
0,5	5	95
9,5	58	42
9,52	100	0
12	100	0
12,1	5	95
15	5	95

Table II.3.2.2: Retention times and MRMs of the different phytohormones and their corresponding standards analyzed.

Analyte	Retention Time [min]	M1 [Dalton]	M2 [Dalton]
salicylic acid	6.71	136,8	93,1
D4-salicylic acid	6,65	140,8	97,1
jasmonic acid	8,19	209	59,1
D2-jasmonic acid	8,95	213	59,1
jasmonic acid-Ile1	9,30	322,1	130,1
jasmonic acid-Ile2	9,38	322,1	130,1

JA-C13-ile-Std	Depending on isomer	328,1	136,1
ABA	8,15	263	153,2
D6-ABA	8,18	269	159,2

After the stability of the retention times was established, the compounds were identified and quantified semi-automatically using the Analyst 1.5 software (Applied Biosystems, Life Technologies, Carlsbad, California, USA). Each integration event was, however, inspected visually afterwards and corrected if necessary.

II.3.3 ANALYSIS OF PHENOLIC GLYCOSIDES BY HIGH PERFORMANCE LIQUID CHROMATORAPHY AND UV-DETECTION

Preliminary experiments showed that a 1:3 dilution led to a linear absorption range of the phenolic glycosides on the DAD detector and thus all samples were diluted 1 : 3 (100 µl extract : 300 µl biH₂O) and 10 µl were injected into the Agilent 1100 HPLC system (Agilent, Böblingen, Germany). The gradient and solvents used for the analysis are given in Table II.3.3.1. For separation, a Nucleodur Sphinx RP column, particle size 5 µm, 4.6 mm x 250 mm (Macherey-Nagel, Düren, Germany) was used and the detection of the compounds was done on a DAD detector, using the 200 nm wavelength only. The quantification was done semi-automatically using the HP Chemstation for LC software (Agilent, Böblingen, Germany). As the retention time varied between runs and not all peaks were identified correctly by the computer, all integrations were checked before the data was used.

Table II.3.3.1: Solvents and gradients at given times used for phenolic glycoside analysis on the HPLC system; the flow was kept constant at 1ml/min.

Time [min]	Solvent A (Acetonitrile, [%])	Solvent B (0,2% Formic acid in H ₂ O, [%])
0	86	14
22,0	42	58
22,1	0	100
25,0	0	100
25,1	86	14
30	86	14

II.4 RNA-EXTRACTION AND PURIFICATION

In order to find an optimal method for the isolation of total RNA from *Populus nigra* var. *Italica*, different methods detailed below were evaluated and it was decided to use the kit provided by Invitex (Invitex, Berlin, Germany). All extracted RNA was analyzed for integrity and purity using the 2100 Bioanalyzer system (Agilent, Böblingen, Germany) described below.

II.4.1 METHOD ONE

Total RNA was isolated from ground leaf material using a protocol kindly provided by Almuth Hammerbacher developed in the Forestry Genome lab of Jörg Bohlmann at the Michael Smith Laboratory at the University of British Columbia in Canada based on methods from Wang (Wang, Hunter et al. 2000) and Chang (Chang, Puryear et al. 1993). The buffers given in table II.4.1.1 were used for extraction. In order to be able to work in an RNase free environment, all materials used were either autoclaved, heat sterilized at 200°C or sprayed with disinfectant (Metasys bench disinfectant, Metasys, Rum bei Innsbruck, Austria).

Mortar and pestle were pre-chilled to -20°C before use to minimize heat tension upon chilling with liq. N_2 . Tissues were homogenized under liq. N_2 in the mortar until a fine powder remained. 7.5ml of extraction buffer were added to the powder which froze immediately. More liq. N_2 was added and the mixture was ground till powdery again and transferred into a 50 ml Falcon tube. The tube was then vortexed to ensure homogeneity and snap frozen in liq. N_2 and left there for 10 min.

The homogenate was then allowed to thaw at 37°C and the tube was inverted several times to ensure homogeneity. It was placed into a Sorvall centrifuge cooled to 4°C equipped with a RTH-750 rotor and spun for 20min at 4000 rpm (this centrifuge and setup was used throughout the experiment). The tubes were transferred on ice and the supernatant was filtered of using a kimwipe (Kimberly-Clark GmbH, Koblenz, Germany) in a funnel to avoid particles in the supernatant. The volume of the supernatant was determined visually and 1/30 of this volume of 3,3M NaAc-buffer and 1/10 of 100% EtOH were added to it to remove polysaccharides. The mixture was incubated on ice for 10min and subsequently centrifuged for 30min at 4000 rpm. The supernatant was filtered of through a kimwipe in a funnel into new 50ml tube. At this stage, samples were stored at -80°C over night.

The following day, tubes were taken out of the freezer and centrifuged for 40min at 4000 rpm at 4°C . The supernatant was poured off, and the pellet was re-suspended on ice in 2ml TE buffer and 2ml 5M NaCl buffer until it was no longer visible, which took approximately 30min. The suspension was transferred into a 15ml Falcon tube and taken off the ice. 1ml of 10% CTAB solution was added and a homogenous solution was ensured by vortexing. The sample was subsequently incubated at 65°C for 5min to warm it up to this temperature and then extracted two times with 5ml of Chloroform : Isoamylalcohol (24:1 v/v); after each extraction, the sample was centrifuged for 20min at 4000 rpm at 4°C with the equipment described above. The supernatants were combined and $\frac{1}{4}$ volume of those of 10M LiCl was added to the samples. On the way to the -20°C freezer, samples were mixed by inverting them and then left at -20°C over night to precipitate the nucleic acid.

Samples were retrieved from the freezer the next day and placed into a centrifuge (setup described above) and centrifuged for 30min at 4000 rpm at 4°C . The supernatant was poured off carefully, all remaining supernatant was removed with a pipette and the milkish-

white pellet was re-suspended in 0,9ml TE buffer on ice. Samples were then transferred into 2ml Eppendorf tubes and 0,9ml of chilled isopropanol, together with 0,1ml of 3,3M sodium-acetate buffer were added to precipitate out the RNA at -80°C for a minimum of 30min. Samples were thereafter centrifuged at 4°C at 13200 rpm in an Eppendorf 5415R Centrifuge to pellet the RNA. After removal of the supernatant, the pellet was washed once with 1ml of 70% EtOH while spinning for 10 min under the same conditions. Then, the remaining supernatant was removed and the remaining pellet was of glass like appearance. It was dried for no longer than 10 min on air and resuspended in 0,5ml DEPC-treated H₂O on ice with occasional vortexing.

Table II.4.1.1: Composition of the different buffers used for RNA-extraction in Method One.

<u>Buffer</u>	<u>Contents</u>	<u>Quantity</u>
Extraction Buffer (500ml) (autoclaved before use)	1M Tris-HCL (pH 8.5) Lithium dodecyl sulfate (w/v) Lithium chloride (10 M) Disodium salt EDTA (0,5 M) Sodium deoxycholate (w/v) Tergitol NP-40 (w/v) DEPC-treated H ₂ O	200ml 7,5g 15ml 10ml 5g 5ml to 500ml
Working Extraction Buffer (200ml) (made fresh from autoclaved Extraction Buffer)	1 mM aurintricarboxylic acid 10 mM Dithiothreitol 5 mM Thiourea 2% PVPP (w/v)	0,0844g 0,3084g 0,0763g 4g
TE Buffer (1l) (autoclaved before use)	10 mM Tris (pH=8) 1 mM EDTA (pH=8) DEPC-treated H ₂ O	10ml of 1M stock 2ml of 0,5M stock To 1l

5 M Sodium Chloride buffer (500ml) (stirred for one hour at room temperature after active DEPC was added; autoclaved thereafter)	Sodium Chloride Milli-Q H ₂ O Active DEPC	146,1g to 500ml 500µl
3,3 M Sodium Acetate buffer (500ml) (pH=6,1) (stirred for one hour at room temperature after active DEPC was added; autoclaved thereafter)	Sodium acetate DEPC-treated H ₂ O Active DEPC	224,5g to 500ml 500µl
10% CTAB buffer (500ml) (not autoclaved, heated to 65°C before use)	CTAB DEPC-treated H ₂ O	50g to 500ml
10 M Lithium Chloride buffer (filter-sterilized)	Lithium Chloride DEPC-treated H ₂ O	211,9g to 500ml

II.4.2 METHOD TWO. DIFFERENT KITS

In a strive to time optimize the extraction of RNA from *Populus* tissue, two kits from different companies were tested against the results of method one. Both kits tested were used according to the manufacturers given protocol without any changes to it. All tissue used was snap frozen in liquid nitrogen upon collection, stored at -80°C and only allowed to thaw in extraction buffer after being ground under liquid nitrogen.

The two kits compared were on the one hand InviTrap® Spin Plant RNA Minikit manufactured by Invitex (Invitex, Berlin, Germany) and on the other hand the Spectrum™ Plant Total RNA Kit by Sigma (Sigma Aldrich, Munich, Germany). As outlined above, both kits were used according to the manufacturers' protocols. Whenever given a choice, the treatment promising purer RNA was chosen.

II.4.3 DNASE DIGESTION AND SECOND CLEAN UP OF RNA

In order to yield highly pure RNA for later analysis, possibly contaminating DNA was digested using the Ambion Turbo™DNase (Applied Biosystems/Ambion, Texas, USA) according to the manufacturer's protocol. After digestion, the enzyme wasn't inactivated but the RNA was further purified using Quiagen's RNeasy Mini kit (Quiagen, Hilden, Germany) in the clean-up only mode.

II.4.4 QUALITY CONTROL OF ISOLATED RNA

Quality of isolated RNA was monitored after each step to avoid working with degraded RNA using an Agilent 2100 Bioanalyzer (Agilent, Böblingen, Germany) together with the Agilent RNA 6000 Nano Kit (Agilent, Böblingen, Germany) according to the manufacturers protocols. The data was analyzed using the 2100 Expert software (Agilent, Böblingen, Germany). Only RNA without any visible contamination or degradation was used for further analysis. Concentration of corresponding samples was determined using the NanoDrop (Fisher Scientific, Schwerte, Germany).

II.5 MICROARRAYS

In this experiment, only single color arrays were used for better comparability between slides. This is because in dual color arrays, competition of the differentially labeled probes for the same spot occurs. As concentrations of the individual probes vary, those arrays can't be compared to each other in a quantitative way.

II.5.1 MICROARRAY DESIGN

The microarrays used in this work were custom designed arrays produced by Agilent with a maximal number of 128.000 genes per array. For the design of the arrays used, two different datasets were combined and 60mer oligonucleotides corresponding to each gene were generated. These were designed in a way that each oligomere was specific to only one gene sequence by using Agilent's eArray online platform (<http://genomics.agilent.com/CollectionSubpage.aspx?PageType=Product&SubPageType=ProductDetail&PageID=1455>). The spots were then randomly distributed on the array. The

data used was obtained from two publicly available sources: <http://www.phytozome.net/poplar.php> and http://www.populus.db.umu.se/proj_downl.php. The first source contained information on the *P. trichocarpa* genome and was created based on the work of Tuskan et al.(2006). From this resource, 45033 different possibly protein coding sequences were obtained and specific 60 nucleotide long oligomers were designed semi automatically with the above mentioned tool; the same was done with the sequences from the second source which represents 24912 different expressed sequence tags from *Populus nigra*. In order to increase stringency, oligonucleotide design was biased towards the 3'untranslated region of the gene for sequences from *P. nigra*. As the sequences for *P. trichocarpa* were generated using automated gene searching, it is possible that not all predicted coding sequences are in the right orientation in this database. To account for this, oligonucleotides against sequences that had no annotation to it were designed double; one in the given direction and one against the reverse complement.

II.5.2 LABELING OF RNA AND MICROARRAY HYBRIDISATION

RNA was isolated and purified as described above and was then labeled with Cy3 using Agilent's Low Input Quick Amp Labeling Kit (Agilent, Böblingen, Germany). Briefly, total RNA was first reversely transcribed into cDNA. This template was then transcribed to generate so called cRNA; this cRNA was labeled stochastically by adding cyanine-3-cytosin-tri-phosphate to the reaction. cRNA was then isolated using Quiagen's RNeasy Mini kit (Quiagen, Hilden, Germany) and the amount of label incorporated as well as the concentration of cRNA was determined using the NanoDrop (Fisher Scientific, Schwerte, Germany). From this, specific activity was calculated (specific activity= {(Concentration of Cy3 [pmol/μl]) / (Concentration of cRNA [ng/μl])} x1000). Only cRNA with specific activity above eight was used.

From each sample, 1.65 μg of labeled cRNA was taken and fragmented using NaOH according to the manufacturers protocol supplied with the Gene Expression Hybridization Kit (Agilent, Böblingen, Germany) used. This fragmented cRNA was hybridized onto an array as soon as possible after the fragmentation had been stopped using supplied buffers. Samples were hybridized onto the arrays in a hybridizing oven at 65°C at 10 rpm for a minimum of 17h. Samples were hybridized to the array in a randomized fashion (see table II.5.1.1). Care

was taken not to introduce any stationary bubbles into the hybridization chamber. After that, microarray slides were washed and coated as detailed in the manual to avoid ozone related bleaching of the dye.

Table II.5.2.1: Identification number of the individual microarray slides and the samples that were hybridized to it. Please note that each slide carries two arrays; array one denotes the one closer to the bar code.

Agilent slide identifier	Sample hybridized to array one	Sample hybridized to array two
252829910001	D-CS2-391	C-P-G1-364
252829910002	C-CS1-564	B-P-G1-543
252829910003	D-CS1-588	C-P-G2-146
252829910004	D-CS1-171	B-P-G1-346
252829910005	B-P-G2-143	A-P-G2-334
252829910006	D-P-G2-388	B-CS1-349
252829910007	B-CS1-547	A-P-G1-525
252829910008	D-P-G2-586	C-P-G1-561
252829910009	C-CS1-367	A-CS1-117
252829910010	A-CS1-337	A-CS1-529
252829910011	B-CS2-140	A-P-G1-110
252829910012	D-P-G1-164	C-CS2-151

II.5.3 ANALYSIS OF MICROARRAY DATA

To identify possible genes correlating to the different spots, blast searches were performed for each sequence connected to each spot by using the primary sequence of either *P. trichocarpa* or *P. nigra* from which the oligonucleotide defining the spot was obtained and the blast results were used to annotate the dataset. This was done using the free software Blast2Go (B2G) (http://www.blast2go.org/start_blast2go) developed at the Bioinformatics and Genomics Department, Centro de Investigación Príncipe Felipe, Valencia, Spain by (Conesa, Götz et al. ; Gotz, Garcia-Gomez et al. 2008). This software assumes that the Gene Ontology (GO) terms assigned to the blast results it obtains for individual sequences are likely to broadly represent the function of the individual sequence that was blasted. These GO term annotation of the sequences can be controlled using the Annex tool as well as the GOslim module implemented into B2G, which was done. Using these Gene Ontology terms, it then extensively mines publicly available databases for different information like EC (enzyme code) numbers. Those are then used to retrieve Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway information like maps. B2G orders these maps in a way that the

most significantly hits are displayed on top of the list. These maps were subsequently used to identify regulated pathways. As B2G does not take into account the direction of regulation (up or down relative to controls), all identified genes of interest were manually checked for direction of regulation using Excel (Microsoft, Unterschleißheim, Germany).

II.6 STATISTICAL ANALYSIS AND DATA PRESENTATION

All statistical assumptions such as normal distribution and heteroscedasticity were checked. Means are always displayed with standard errors (SE). All statistical analysis for phenotypic data were carried out using the freely available statistics package R (<http://www.r-project.org/>). Graphs were created using either SPSS 17.0 (IBM, Munich, Germany) or Microsoft Office Powerpoint or Excel 2007 (Microsoft, Unterschleißheim, Germany).

Microarrays were scanned using Agilent's High-Resolution C Scanner (Agilent, Böblingen, Germany), together with the Feature Extraction software provided by the manufacturer. Raw data was further processed using the commercial Genespring GX software (Agilent, Böblingen, Germany). All microarrays were normalized with the percentile shift method (shift to 75th percentile) over all slides, thereby globally adjusting values of all spots. The baseline was transformed to the mean of all samples. Significantly differentially regulated spots were identified using a Student's T-Test to compare individual spots and only spots significantly differentially regulated across all replicates were considered. A multiple testing correction according to Benjamini and Hochberg's False Discovery Rate model was performed. Both tests were performed within Genespring GX. Graphs presenting microarray data were made using either Genespring X or Blast2Go (freely available from www.blast2go.org).

III RESULTS

III.1 METHOD DEVELOPMENT AND EVALUATION

For this work, different tools needed to be evaluated and compared. This is documented in the following section.

III.1.1 SALICYLIC ACID RECOVERY AND STABILITY EXPERIMENTS

After the first set of extractions, it became obvious that there were differences in the recovery of salicylic acid (SA) when freeze dried material was compared to fresh material. To see whether matrix effects or enzymatic breakdown in the fresh material were the reason for this, spiking experiments were conducted. One set of experiments aimed to find out whether the recovery of SA added was depending on the concentration and on the state of the material and the other whether there was any enzymatic break down of SA taking place.

It can be concluded that a) the recovery of artificially added SA is lower from fresh leaves than it is from freeze dried leaves (see **Fig.III.1.1.1 A**, top panel) and b), because time had no effect on the recovery rate, that the difference is not attributable to enzymatic breakdown (**Figure III.1.1.1 B**, bottom part).

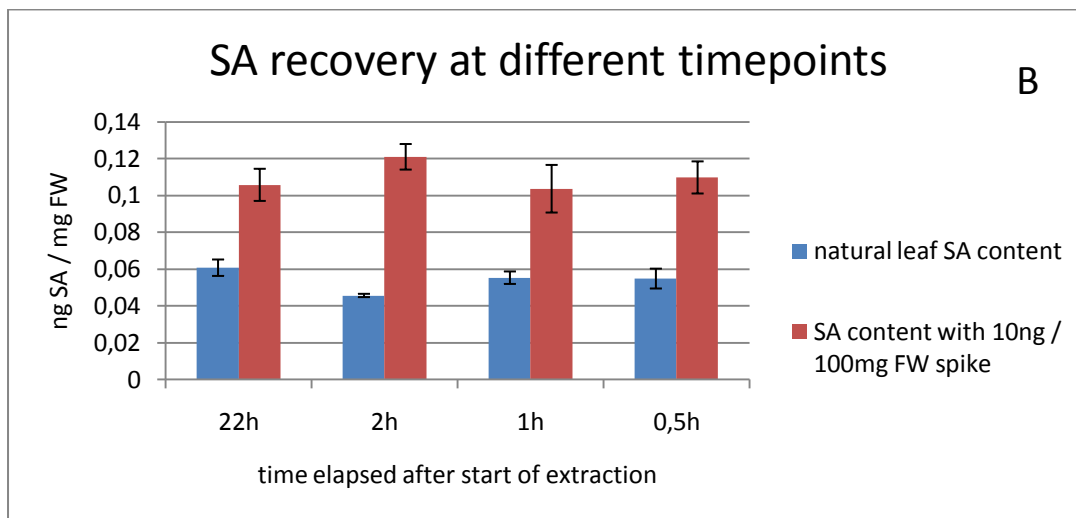
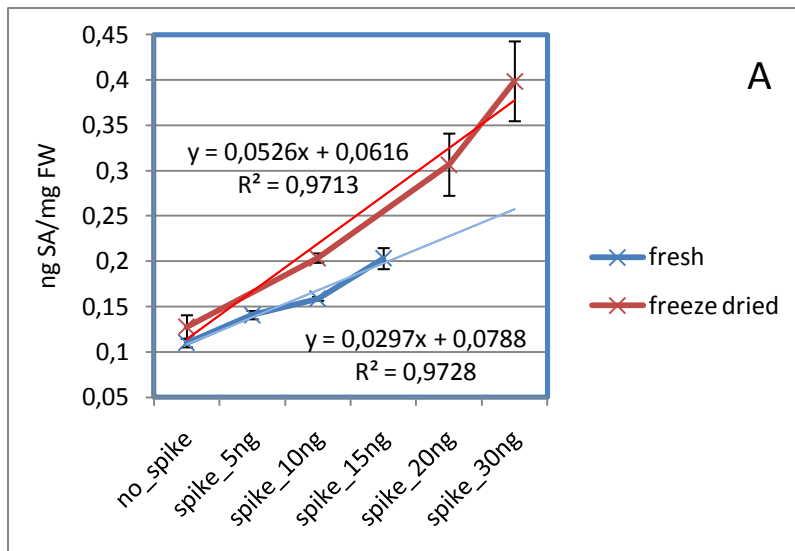


Figure III.1.1.1: Panel A shows the differences of recovery of SA when comparing fresh (blue) and freeze dried (red) material after spiking. Panel B shows SA recovery after different time points after start of extraction either without (blue) or with a spike of 10ng/100mg FW added at the start of the extraction.

III.1.2 RNA EXTRACTION

Different methods for extracting RNA from *Populus* have been tested; as leaves of Poplar contain a lot of possibly interfering phenolic compounds, the results were rather different for the different methods used.

As illustrated in **Fig. III.1.2.1**, both the long lasting protocol developed Forestry Genome lab of Jörg Bohlmann at the Michael Smith Laboratory at the University of British

Columbia in Canada based on methods from Wang (Wang, Hunter et al. 2000) and Chang (Chang, Puryear et al. 1993) and the Kit from Invitex showed comparable results in terms of quality of RNA. Both methods extracted mostly intact total RNA without much degradation as can be seen on the low baseline of the signal and the sharp peaks of the different plastid and ribosomal RNAs. Both methods also showed only minor traces of contaminating DNA as the signal from 50 sec onwards is hardly above baseline. None of the above can be said for the kit provided by Sigma-Aldrich and it was not considered for further applications. After evaluating these results, I decided to use the Invitex kit for isolation of RNA as it provides a relatively non toxic and quick method.

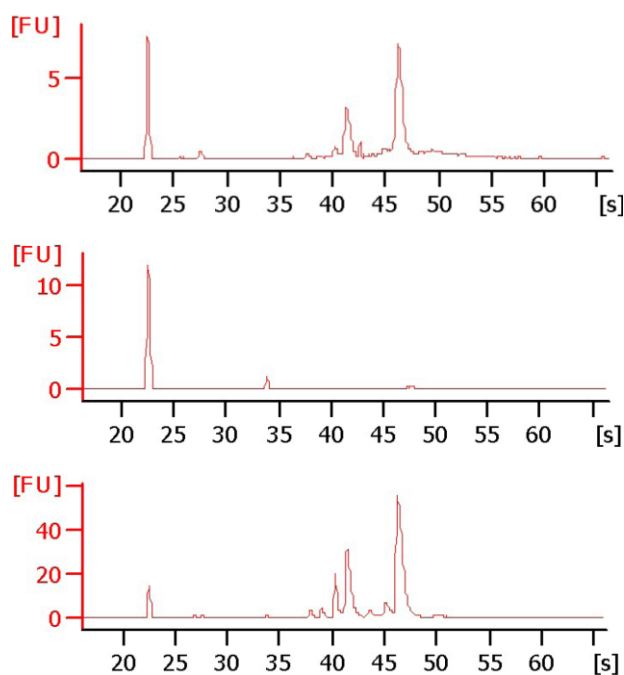


Figure III.1.2.1: Comparison of RNA extracted by different extraction methods on the Bioanalyzer. From top to bottom: Extraction using the method termed “all by hand”, the Sigma Plant total RNA kit and Invitex’s InviTrap® Spin Plant RNA Minikit. Results of the methods displayed on top and bottom are largely similar, whereas the result displayed in the middle panel is clearly different. Please note that neither 18s nor 28s rRNA (big peaks at 42sec and 47sec, respectively in the top and bottom diagrams) have been isolated by Sigma’s kit and that the absolute amount of RNA isolated is not correlated completely to the fluorescence units (FU) given in the graphs.

III.2 EXPERIMENTAL RESULTS

All collections and results presented here were initially obtained from the six trees mentioned in material and methods, as long as all of them were standing. Due to an administrative decision by the city council of Jena, 2 trees were cut down before the end of the experiment. Where ever necessary, this is indicated.

III.2.1 PHYTOHORMONES AND *PEMPHIGUS*

Within the framework of this thesis, levels of different phytohormones were analyzed. Whereas most phytohormones that were attempted to be analyzed were clearly detectable on the mass spectrometer (SA, JA, JA-Ile), ABA was always below reliable detection irrespective of treatment and thus will not be dealt with here.

III.2.1.1 SALICYLIC ACID

From all compounds analyzed, salicylic acid (SA) showed the clearest correlation to aphid feeding in all trees and for almost all time points. When looking at the SA content in leaf blades over time, the levels of SA are clearly elevated in leaves infested with *P. spirothecae* relative to non infested ones (see **Fig. III.2.1.1.1**). Statistically, aphid infestation alone is not sufficient to explain the overall levels (N=4, $p=0.2145$). Another fact worth mentioning is the steady decline seen for Salicylic acid over the first four time points, but with the pattern described above still prevalent. When following the idea of an antagonism between SA and JA, this is also in nice concert with JA levels described and discussed below. If the sampling had stopped at this early time point, TP6 and its challenging to interpret results would have been missed. Among the six time points, TP6 shows the highest levels of SA irrespective of the treatment and no differences can be found any more between infested and control tissue. When analyzing the influence of the two factors varied in this experiment over all time points, only time has a significant influence on SA levels (N=4, $p<0.0001$), whereas treatment does not (N=4, $p=0.2145$). The interaction of both is also not significant (N=4, $p=0.4007$). When TP6 was excluded from the analysis, the picture changes and both aphid infestation as well as time have a significant influence on SA levels (N=4; $p=0.0216$ and $p=0.0001$, respectively), but not the interaction (N=4; $p=0.6933$).

As the analysis of SA showed the clearest and most reproducible pattern of all analyzed compounds, these results were used mainly for the decision on which time points to use for microarray analysis.

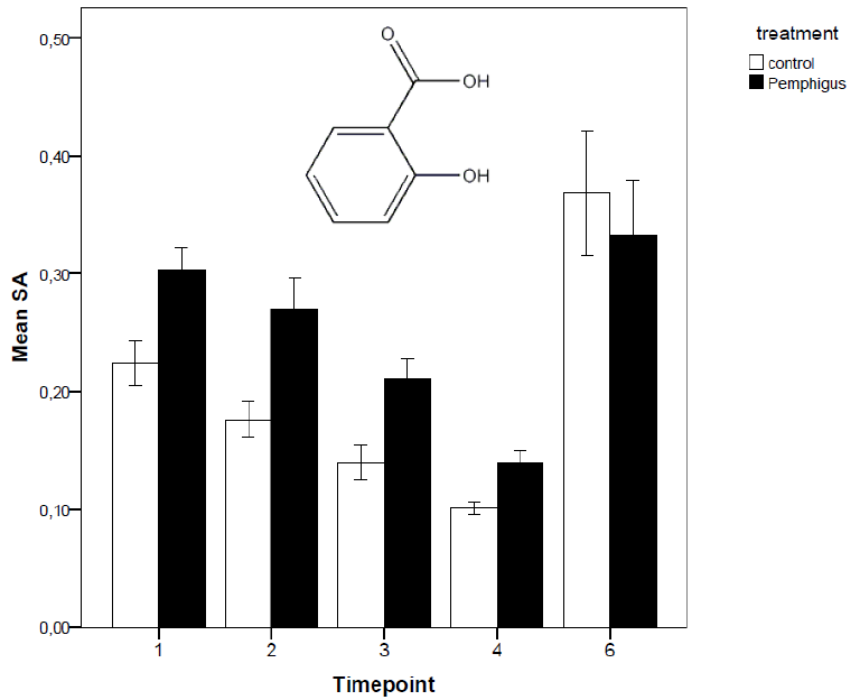


Figure III.2.1.1.1 shows the time course of salicylic acid (SA) levels in ng SA / mg FW in leaf blades with either a gall induced by *P. spirothecae* on its petiole (black bars) or without (empty bars). Numbers 1-6 represent chronologically ordered time points (see text); bars are means of either 6 (time points 1-3) or 4 biological replicates (time points 4,6) and error bars are +/- 1 standard error.

III.2.1.2 JASMONIC ACID

As already mentioned above, the observed pattern for jasmonic acid (JA) (see **Fig. III.2.1.2.1**) is in a way the negative of SA for the first time points. Whereas SA declines, JA rises steadily for the first three time points and for the first two time points, no pattern and no discrimination between infested and non infested leaves can be made based on JA contents. At TP3, however, JA levels clearly mirror those of SA, maybe indicating the onset of effective defense as JA levels are significantly higher in treated than untreated leaves. JA then follows the trend of SA as the absolute levels are the lowest at TP4 and increase again at TP6 but

remain relatively low. Also, the correlation between *P. spirothecae* infestation and JA levels seen at TP3 is gone and no clear patterns attributable to infestation can be found. When carrying out statistical analysis, it becomes evident that only time has a significant influence on JA levels (N=4; $p < 0.0001$) but treatment has not (N=4; $p = 0.3376$). The exclusion of time point 6 does not reduce overall variability as treatment remains non significant (N=4; $p = 0.2330$) and time does (N=4; $p < 0.0001$). The interaction is not significant for either case.

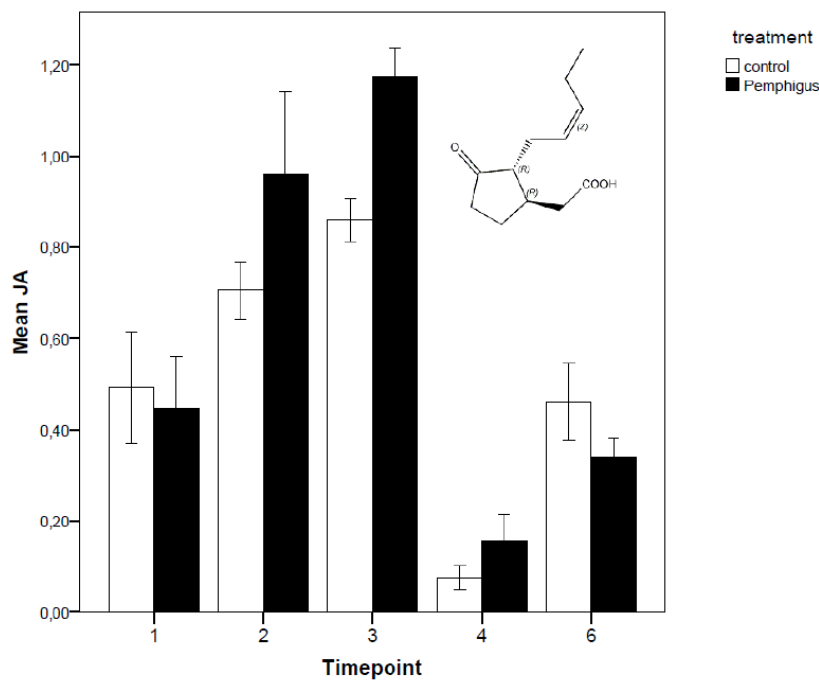


Figure III.2.1.2.1: Black bars represent mean jasmonic acid (JA) levels (in ng / mg FW) in leaf blades with *P. spirothecae* infestation on their leaf blade. Empty bars show mean JA levels in non infested leaves. Numbers are time points in chronological order, error bars are +/- 1 standard error and means were generated using either 6 (TPs 1-3) or 4 (TP 4,6) biological replicates.

III.2.1.3 JASMONIC ACID-ISOLEUCINE CONJUGATE

In this section, levels of two different isoforms (out of 4) of JA-isoleucine are shown (**Fig. III.2.1.3.1**). Here, the inactive isomer (–)-JA-L-Ile is named JA-Ile1 and the active form (+)-JA-L-Ile is called JA-Ile2.

When looking at levels of JA-Ile1, TPs 1-3 and the last time point seem to be relatively uniform, with only time point 4 being clearly different. For the active isomer, the pattern seen is more pronounced and somehow reflects that of JA with a steady increase over TPs 1-3 and a sharp decrease on time point 4. Levels rise again for the 6th time point, reaching their initially measured amounts. As the ratio of the two isoforms might better reflect possible regulation events triggered by the aphid, these were formed and are shown in **Fig. III.2.1.3.1 C**. From this graph, it can be clearly seen that there is no shift towards the active form in infested leaves relative to controls at any time point and that time is the main reason for the differences seen. This is also supported by statistical analysis as treatment does not have an influence (N=4; p=0.4712) on the pattern seen and only time can be used to explain the differences seen (N=4; p<0.0001).

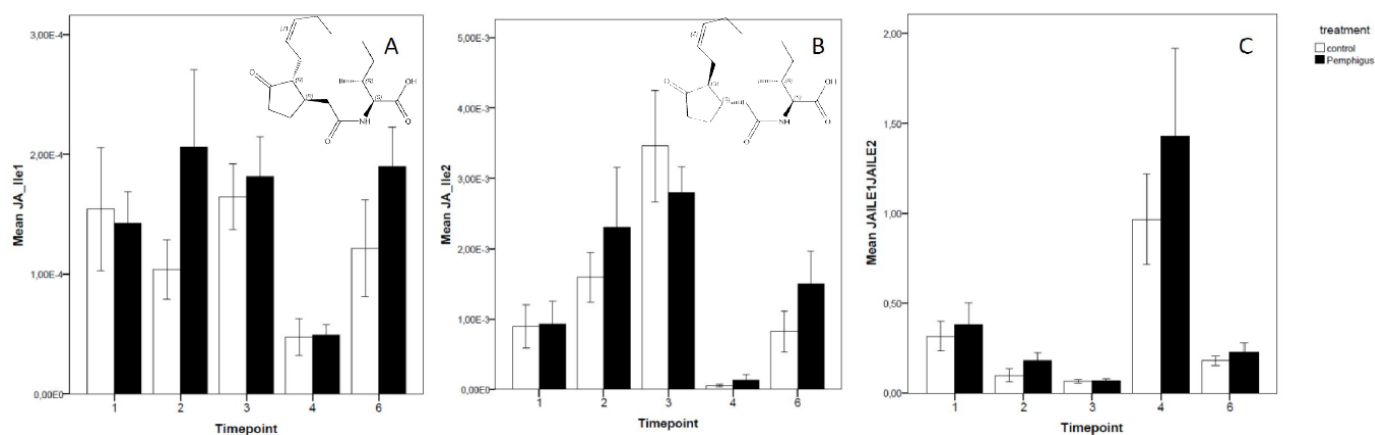


Figure III.2.1.3.1: Panels A and B show levels of either different isomers of JA-Ile (A shows JA-Ile1, B shows the active isomer JA-Ile2) in leaf blades from either aphid infested (black bars) or uninfested (empty bars) tissue in ng / mg FW. C depicts a ratio of the two isomer concentrations (inactive/active diastereomer), using the same color schema as in A and B. Bars represent means of either 6 (time points 1-3) or 4 (time points 4,6) biological replicates, each consisting of ten pooled leaves. Error bars are +/- 1 standard errors.

III.2.2 PEMPHIGUS AND THE ANALYSIS OF PHENOLIC GLYCOSIDES

This section will describe the results obtained for different phenolic glycosides (PGs), which were clearly induced by *P. spirothecae* infestation in the leaf blades analyzed.

III.2.2.1 SALICIN

Salicin, which represents one of the simplest PG, showed a clear pattern of induction with levels in treated leaf blades above those of untreated controls at all time points besides TP1 (a pattern also seen for all other PGs). Over time, means decrease slightly but steadily without showing massive differences in concentrations when comparing two neighboring time points but clearly when comparing TP1 and TP6 (see **Fig. III.2.2.1.1**). This decrease is especially pronounced in the controls, whereas infested leaves show more modest alterations. It is, however, important to note that *P. spirothecae* has an influence on Salicin levels (N=4; p=0.0765) as well as time (N=4; p=0.002). The pattern seen for the phytohormones with drastic decreases for time point 4 is not seen for with this PG.

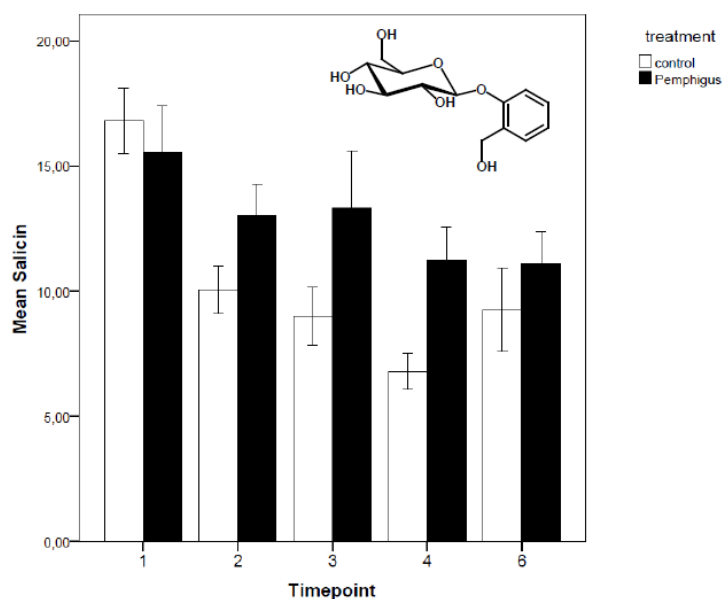


Figure III.2.2.1.1: This graph shows absorption units of salicin, normalized to mg FW. Black bars represent means of salicin in leaf blades sitting on petioles infested with *P. spirothecae* whereas empty bars represent means of non infested leaves. Error bars show +/- 1 standard error. Means on TPs 1-3 were calculated based on 6 biological replicates; those of TPs 4 and 6 use 4 replicates.

III.2.2.2 SALICORTIN

The levels of salicortin showed an interesting pattern in the tissue of interest. Different to salicin, it increased over the first three time points and decreased sharply thereafter. Control leaves and treatment tissue showed an overall similar trend in contents of this defense compound, but overall content was higher for all except the first time point in infested tissue. The overall effect of *P. spirothecae* as a factor influencing the levels of salicortin was tested and found to have a significant influence (N=4, $p=0.0068$). The same is true for time as a factor (N=4, $p=0.0001$). The interaction of time and treatment was marginally non-significant (N=4, $p=0.0546$); as it becomes obvious from the graph (Fig. III.2.2.2.1), these two factors have a close relation.

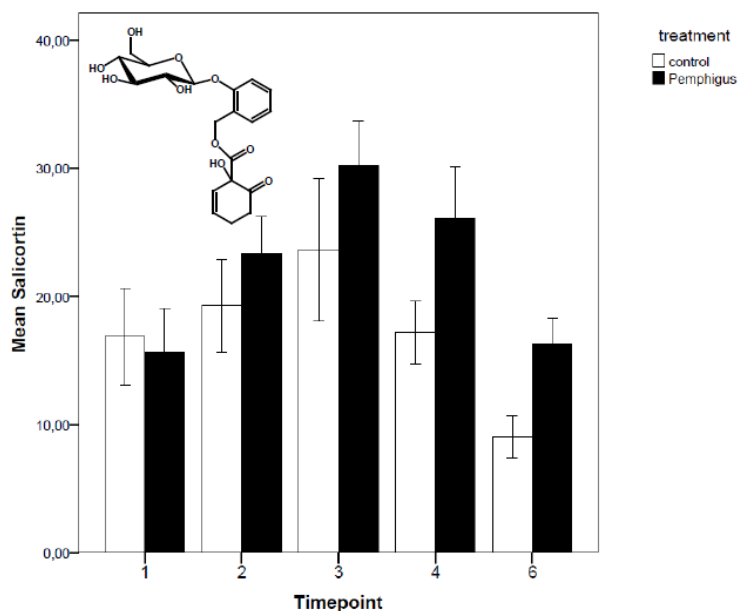


Figure III.2.2.2.1: Black bars represent means of absorption units of salicortin normalized to mg FW of leaf blades with *P. spirothecae* infection. Empty bars are controls having no infection. Time points 1-3: N=6; time points 4,6: N=4. Each replicate consists of 10 pooled leaf blades and error bars show +/- 1 standard error.

When looking at individual time points, *P. spirothecae*'s influence on levels of salicortin becomes obvious, especially for the two last time points, whereas the pattern seems to be evolving over the first three time points. As stated above, the first time point seems to be too early for showing differences.

III.2.2.3 NIGRACIN

The PG nigracin shows a pattern different to salicortin described above: whereas the former shows an abstracted bell curve, nigracin decreases steadily. Further, the pattern diversification seen for the two above described PGs with a relatively clear difference between treatment and control is only seen for TP2, TP3 and TP4, but not for the first or last one. This is also reflected in the statistical analysis. When asked whether galling aphids have a significant effect on the levels of this defense compound, the answer is no ($N=4$, $p=0.4148$). As can be inferred from the pattern seen in **Fig. III.2.2.3.1** already, time is the main factor influencing the levels of nigracin ($N=4$, $p \leq 0.0001$). It is again observable for this PG that at the first time point the levels of the control are higher than of the infested leaf and absolutely the highest of all time points. The levels then decline until TP4 and rise slightly to the last time point. For TP2, TP3 and TP4, levels in controls are always lower than treated leaves and only for TP1 and TP6, the levels are equal. This behavior is also contrary to the other PGs described here.

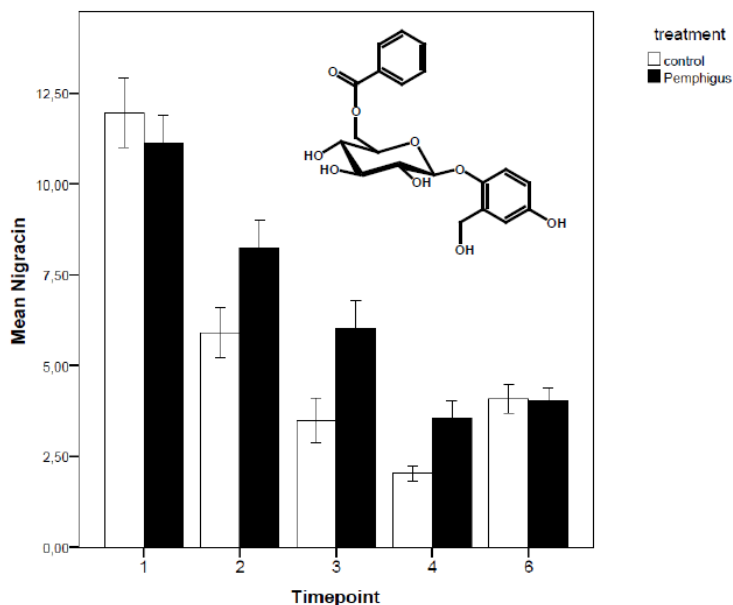


Figure III.2.2.3.1: This figure compares levels of nigracin in either *P. spirothecae* infested leaves (black bars) or in control leaves (empty bars). Bars represent means of relative absorption units (absorption / mg FW) of either six (TP1 to TP3) or four (TP4, TP6) biological replicates. Error bars: +/- one standard error.

III.2.2.4 HOMALOSIDE D

The general pattern seen for homaloside D resembles the one of salicortin shown above as well as of tremulacin in that it also is closer to a bell curve when abstracted than to a linear decrease as are the other two PGs described here (see **Fig. III.2.2.4.1**).

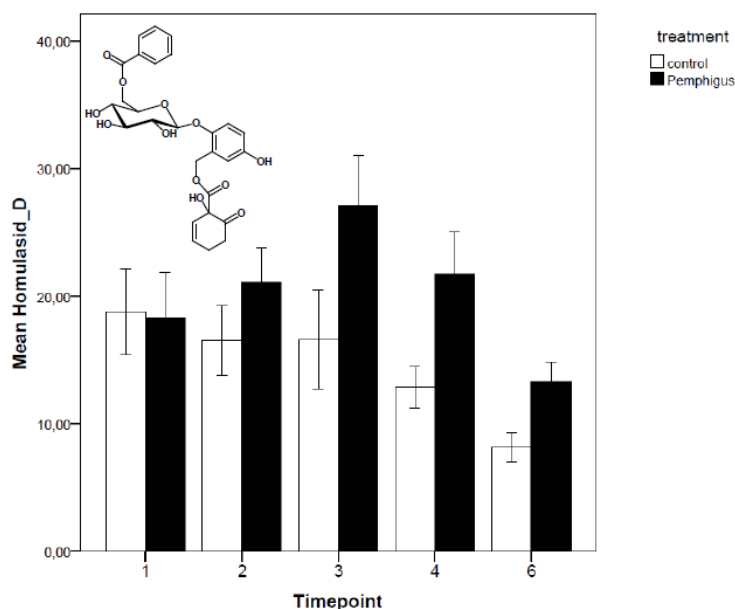


Figure III.2.2.4.1: This chart shows means of absorption units of homaloside D normalized to mg FW. Black bars represent data derived from *P. spirothecae* infested leafs and empty bars represent controls without infection. TPs 1,2 and 3 have 6 biological replicates, 4,6 have 4. Each replicate consists of 10 pooled leaf blades, error bars represent +/- 1 standard error.

It is, however, dissimilar from the other two in that time is not the factor having the most significant influence on the levels of this compound ($N=4$, $p=0.0109$) but the infestation with the aphid ($N=4$, $p=0.0008$). Further, only the treated leaves show the bell curve described above, whereas the controls show a more linear decrease.

III.2.2.5 TREMULACIN

Tremulacin, the last of the five PGs analyzed in this study, showed the most variable pattern of all derivatized glycosides (see **Fig. III.2.2.5.1**). Whereas it, like all other compounds of this class, shows no induction on the first TP, its levels are significantly responsive to both aphid infestation ($N=4$, $p=0.0052$) as to time ($N=4$, $p=0.0001$). This is also true for the interaction of

these two factors (N=4, p=0.0252). The bell shaped pattern seen for this substance is much more pronounced for the infested leaves and only hardly visible for control tissue. Here, values remain relatively constant and only drop at the last time point.

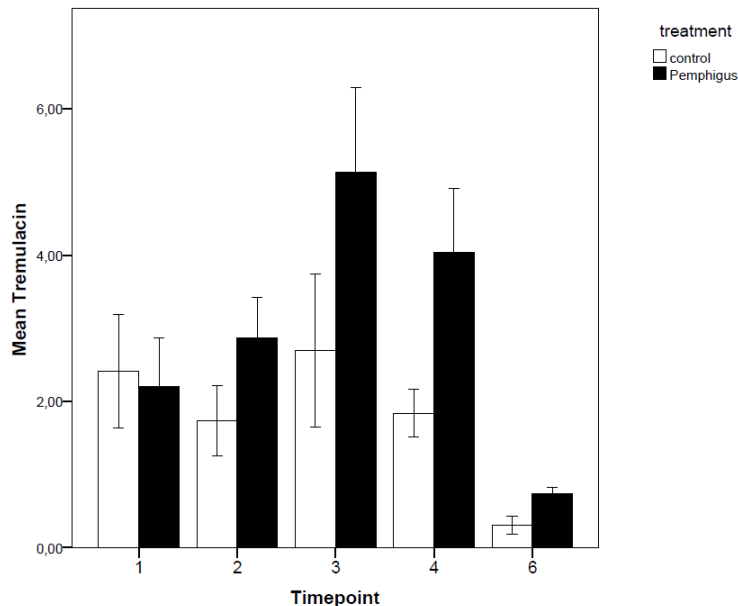


Figure III.2.2.5.1. This Graph shows the relative absorbance (absorbance / mg FW) of tremulacin at different time points and in leaves with either *P. spirothecae* infestation (black bars) or of corresponding controls (empty bars). N=6 for TP1 to TP3, N=4 for TP4, TP6. Bars are +/- one Standard error.

III.2.2 MICROARRAY RESULTS

After the evaluation of the phenotypic data presented above, TPs 2, 4 and 6 were chosen for transcriptional profiling by microarrays. The first two mentioned time points yielded significantly regulated genes across all four replicates (trees A, B, C, D) and only those TPs were further analyzed. The idea that time plays an important role in the character of the results observed in this experiment was further hardened by the outcome of the general microarray properties. When a clustering analysis across all time points and treatments was performed, always time was the main determiner of groups but not treatment (see **Fig. III.2.2.1**). Further, the amount of significantly regulated genes decreased drastically with time; it halved from time point one to time point two profiled (3738 vs. 1735, with p=0,05 as a cutoff) and no transcriptional difference was found for the last time point analyzed.

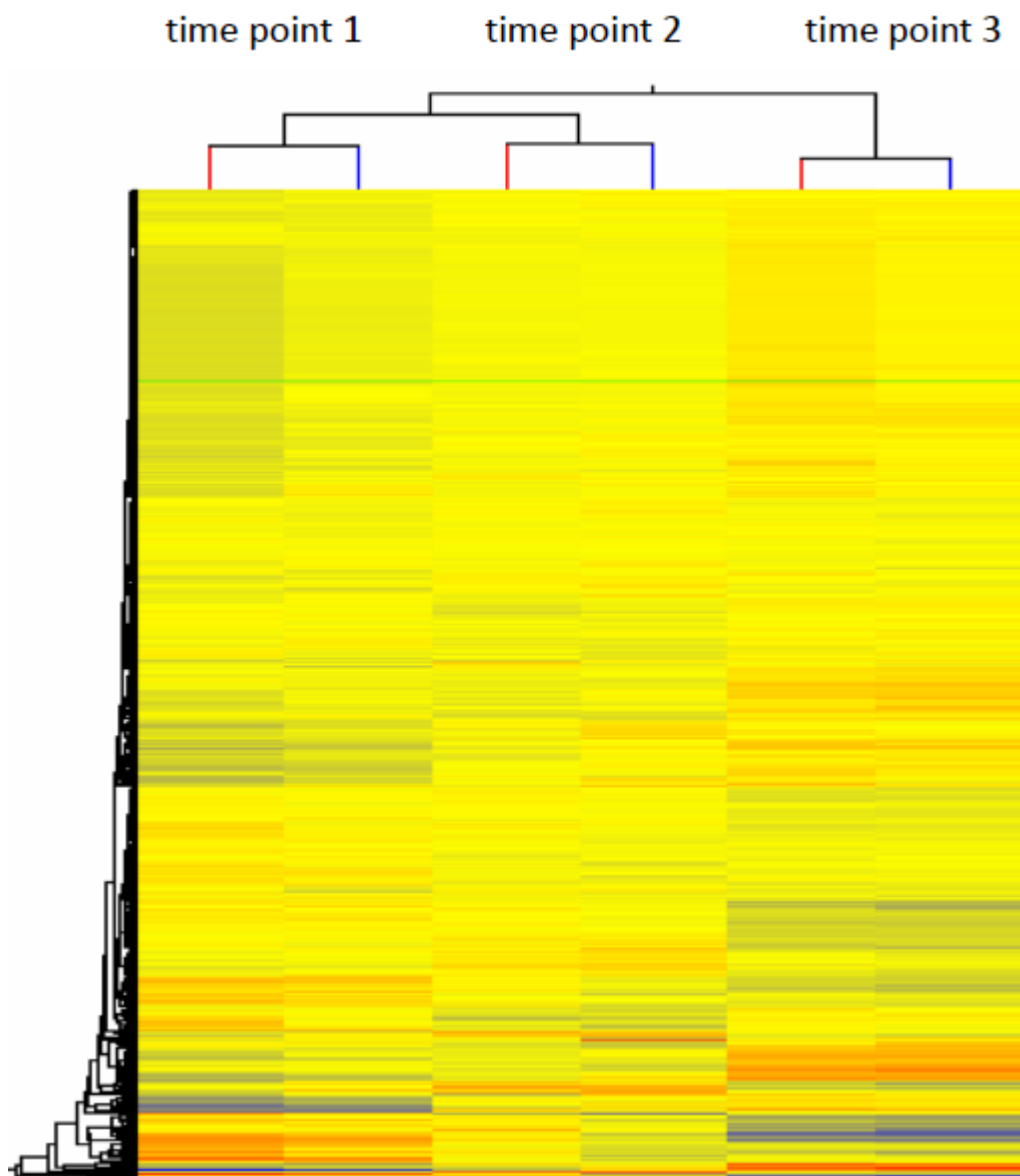


Figure III.2.2.1: This figure shows the result of a hierarchical clustering analysis of all entities on the array over all time points. On the left, the different genes are clustered according to similarity of regulation and on top whole arrays are clustered hierarchically according to similarity. Red and blue ends of the tree on top denote similar treatment class (red = *P. spirothecae* infested, blue = control). On the picture corresponding to the arrays, a shift towards the red spectrum correlates with a high transcript abundance and a shift towards blue with low abundance. Each column represents the average of four biological replicates.

When the question was asked which genes show differential regulation at both time point one and two on a low significance level ($p=0.05$), 332 genes showed up. Of those, all but 6 showed the same regulation at both time points relative to control samples. A list of those

genes can be found in the Appendix. A Venn diagram of this data can be found in **Fig. III.2.2.2.**

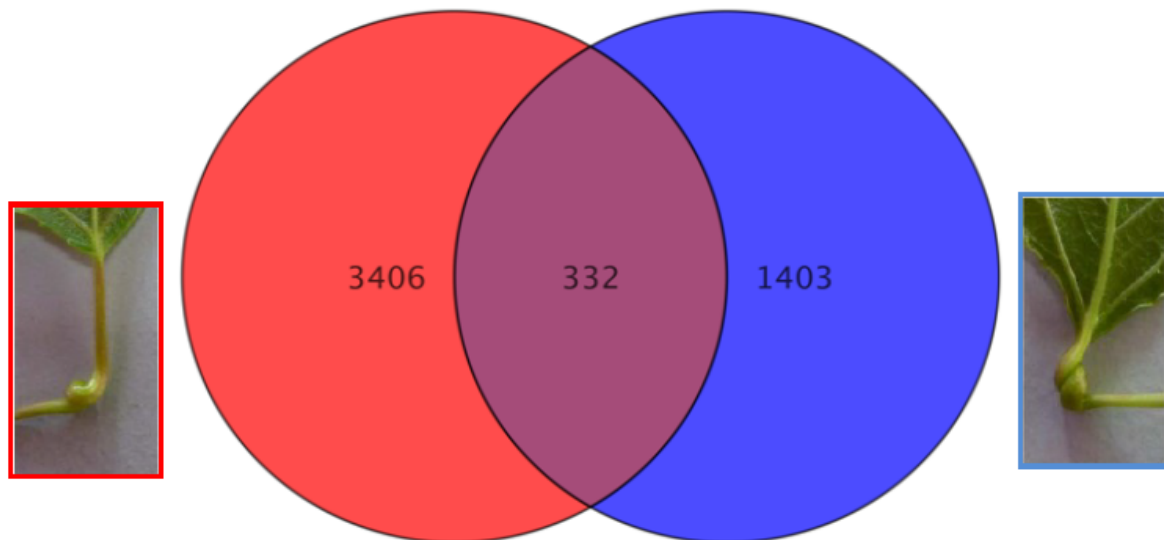


Figure III.2.2.2: Venn diagram of all differentially regulated entities and their overlap. In red, time point one is given and blue denotes time point two. The pictures show the condition of the gall at the respective time points. Numbers represent absolute regulated entities. Please note that the absolute number of genes regulated per time point is 3738 for time point one and 1735 for time point two profiled.

III.2.2.1 THE FIRST TIME POINT ANALYZED

The first time point for which transcripts were profiled was the one with samples taken on the 26.4.2010, which was the second sample time point. This time point was selected based on phenotypic data presented above as a clear pattern was visible there. A total of 3738 genes were differentially expressed when infested tissue was compared with non infested on a low significance level ($p=0.05$, $N=4$) and 596 genes were found to be differentially regulated when the cutoff for significance was set to $p=0,02$ with $N=4$. Of the genes differentially regulated with $p=0.05$, a total of 2194 genes were up regulated relative to controls (of those, 882 showed a change in transcript abundance > 2 relative to controls). 1639 genes were down regulated in infested leaf blades relative to controls; of these, 434 genes showed > 2 fold downregulation in infested tissue relative to controls. These genes were identified using Genespring X software; almost all further analysis like annotation and

interpretation was done using Blast2Go. In order to shed more light onto the annotation process done by Blast2Go (B2G), some key facts of this process will be detailed here briefly for the dataset obtained for differentially regulated genes on the first time point at the low significance level. First of all, B2G managed to annotate almost all sequences and of the given data set. Interestingly, most blast hits came from *Vitis vinifera* (11511), but most top blast hits came from *Populus trichocarpa* (3322). Based on these BLAST results, GeneOntology terms were assigned and only about one seventh of all sequences failed to obtain any GO terms (an overview of the assigned GO terms is given in **Fig. III.2.2.1.1**). The average level of GO-annotation possible was level 5. Using these GO terms, less than half of all sequences (1340) obtained an EC annotation and a total of 1949 EC numbers were distributed. These EC numbers led to the identification of 127 differentially regulated pathways after *P. spirothecae* infestation; all pathways are summarized in the appendix. Here, an overview of the different GO-terms on level three assigned is given for molecular function and for the biological processes involved. Among the pathways regulated are the pathways for the synthesis of phytohormones, which will be dissected in the following paragraph. It is noteworthy that a recently discovered stress induced transcription factor, MYB134 (Mellway, Tran et al. 2009), which is thought to regulate proanthocyanidin synthesis in poplar is also highly induced in infested tissue and this goes along well with the upregulation of the phenylpropanoid pathway.

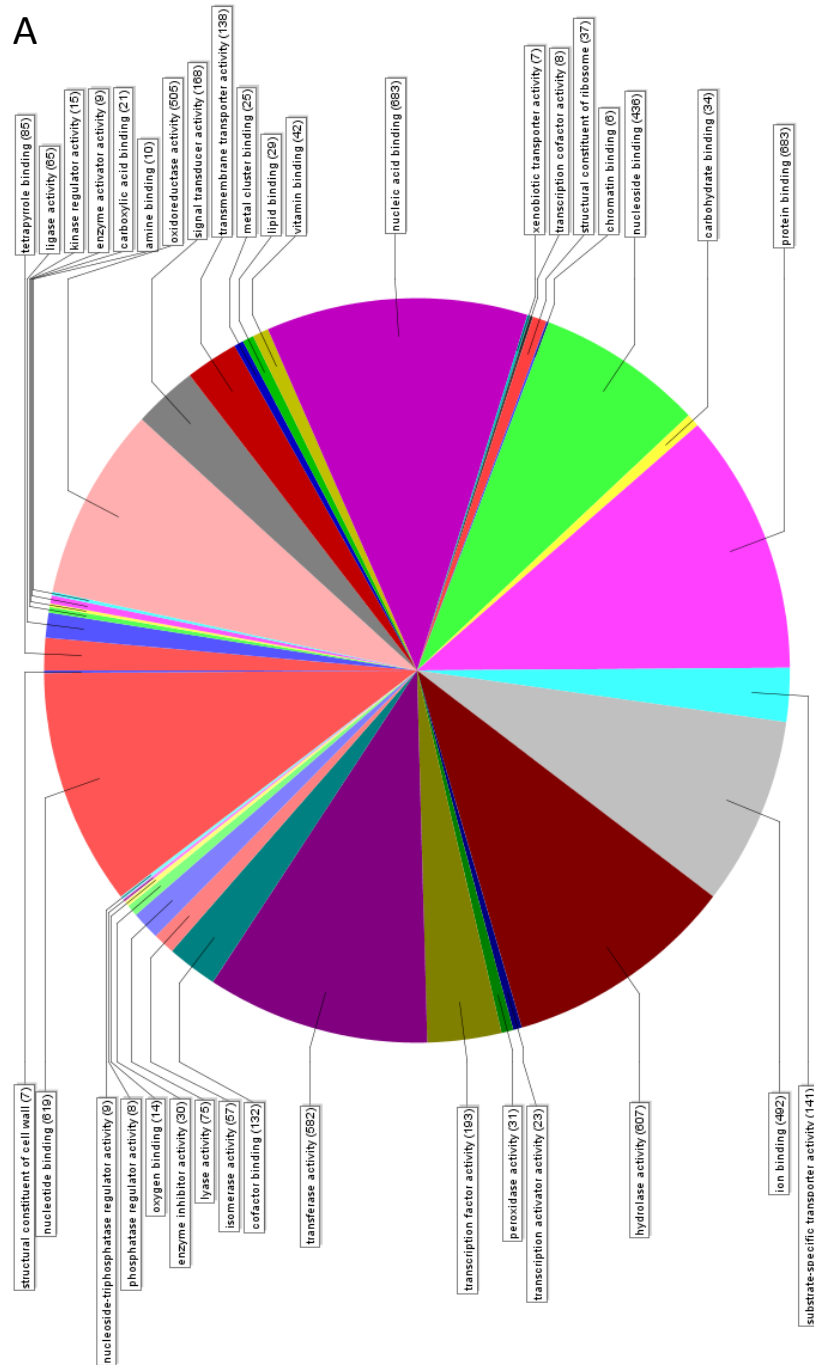


Figure III.2.2.1.1: Legend see next page

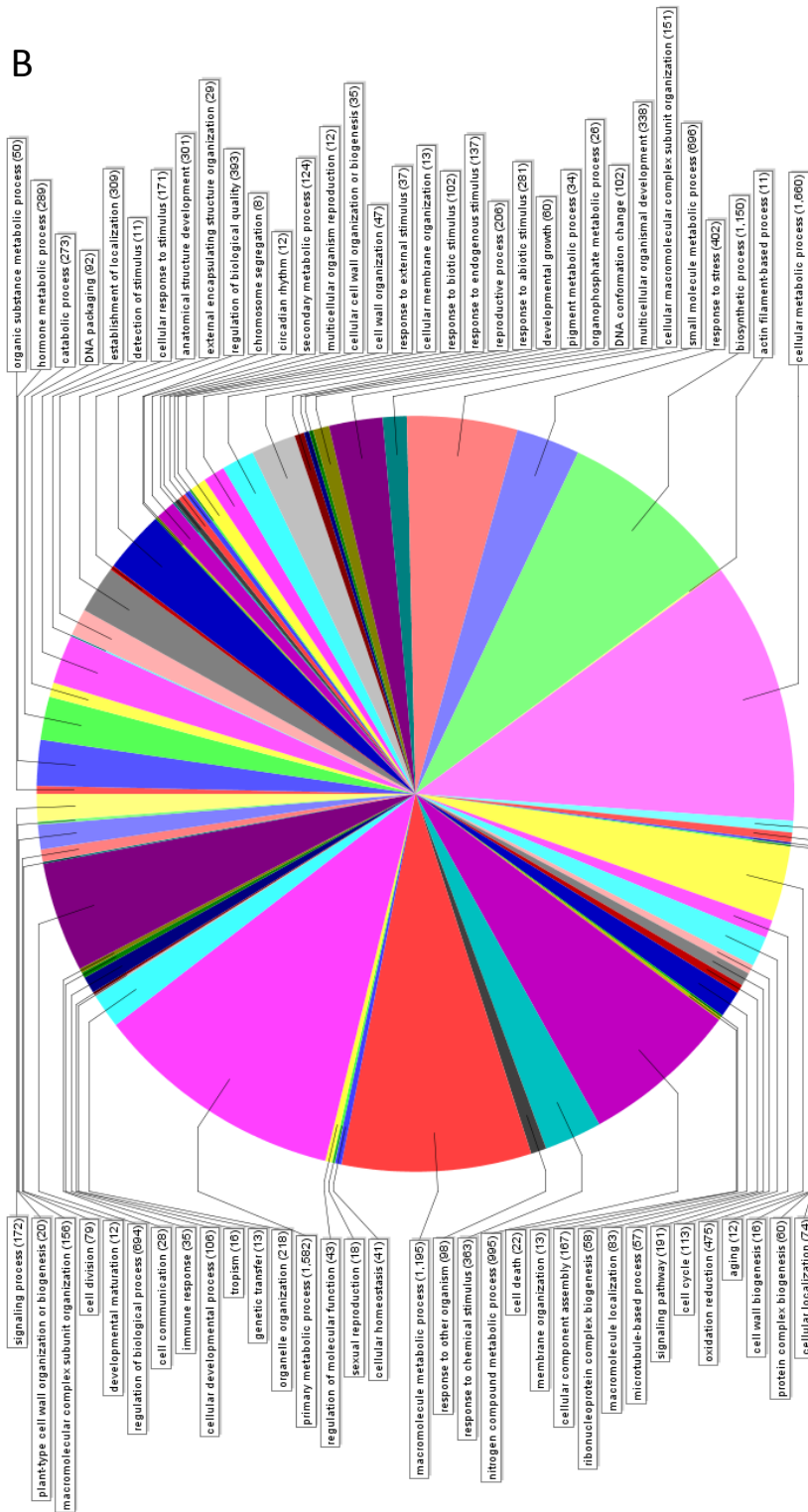


Figure III.2.2.1.1: This figure shows the GO-terms on level 3 assigned by B2G to the different genes differentially regulated at TP1 with $p=0.05$ and $n=4$. The pie chart A on the previous page shows molecular function whereas the chart B on this page shows biological processes regulated.

III.2.2.1.1 DISECTION OF THE TRANSCRIPTIONAL REGULATION OF THE PHYTOHORMONE BIOSYNTHETICAL PATHWAYS AND OTHERS AT TP1

As phytohormonal levels were one of the two points that were used to select time points for microarray analysis, it is interesting to see whether the pathways that lead to the regulation of phytohormone levels show transcriptional response to aphid feeding. The phytohormone pathways that could be transcriptionally profiled were the ones leading to auxin, gibberelin (GA1), cytokinin, abscisic acid, the brassinosteroid brassinolid, salicylic acid, jasmonic acid and ethylene. **Fig. III.2.2.1.1.1** details the pathways leading to the individual endpoints and selected examples will be given in the text. A list of all spots annotated for these processes is given as well in **Table III.2.2.1.1.1**.

Table III.2.2.1.1.1: This table summarizes all transcripts that were found to be regulated differentially and that have a connection to the biosynthesis of plant hormones. For better evaluation of the annotation, some key numbers are given. The primary accession given allows easy identification of the spot in the supplemental material.

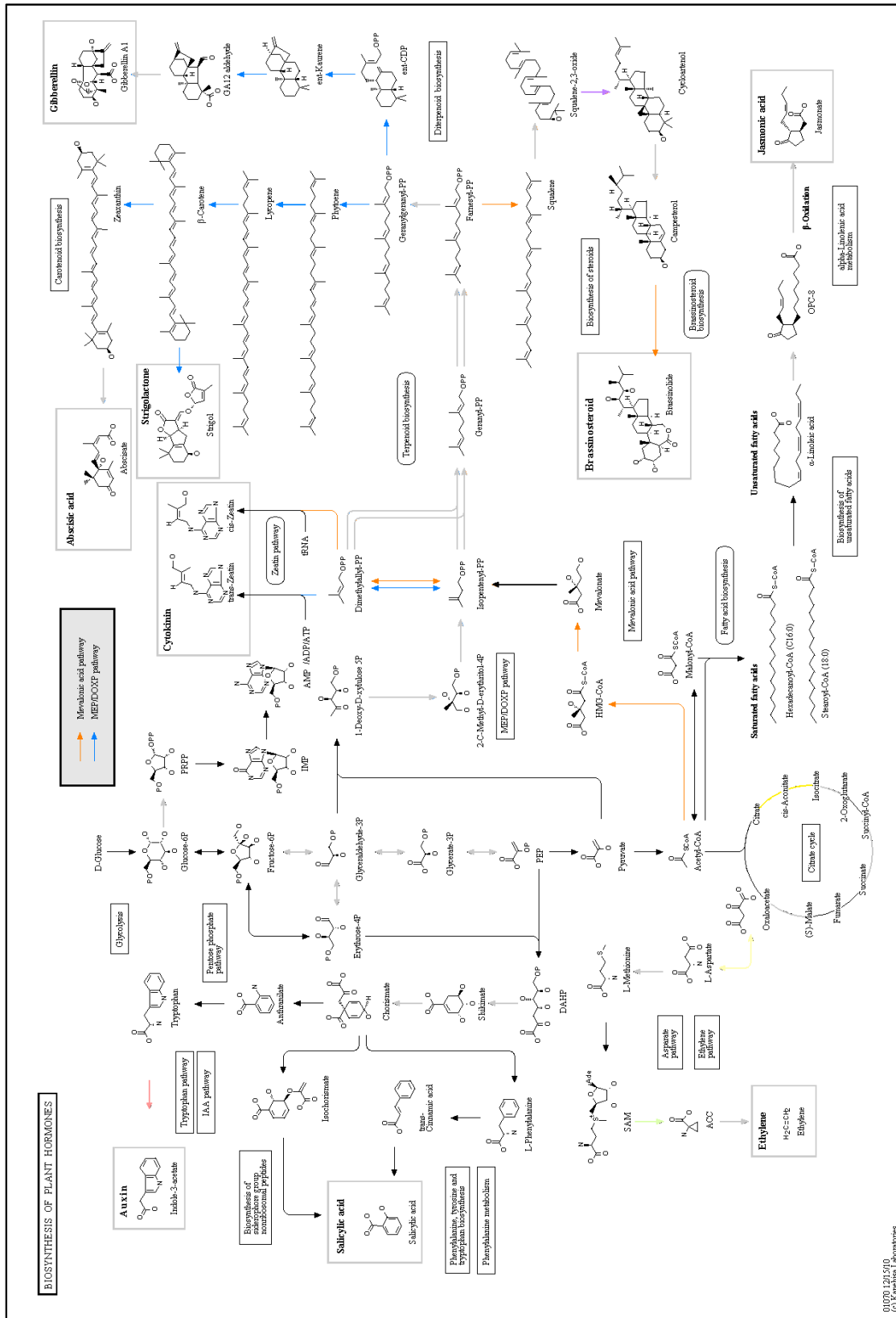
Plant hormone affected	Transcript level of Enzyme regulated	Regulation of corresponding spot (down = aphid infested tissue less than control)	E value; species of determining blast hit; % similarity	Spot identification number and primary accession
Abscisic Acid	Zeaxanthin epoxidase	Down	0,0; <i>Ricinus comunis</i> ; 86%	POPTR_0007s10980.1
	Xanthoxin Dehydrogenase	down	1.52916E-41; <i>A. thaliana</i> ; 92%	Populus_nigra26497

Table III.2.2.1.1.1 continued from page before

Plant hormone affected	Enzyme regulated	Regulation of corresponding spot (down = aphid infested tissue less than control)	E value; species of determining blast hit; % similarity	Spot identification number
Auxin	Amidase	Up	2.22382E-12; Nictotiana tabakum; 81%	Populus_nigra31007
Ethylene	Homocystein-S-Methyltransferase	Up	4.82683E-60; Populus trichocarpa; 100%	POPTR_0001s21590
	1-Aminocylopropane 1-carboxylase	Up	0; Populus trichocarpa; 100%	POPTR_0007s05880
	1-Aminocylopropane 1-carboxylase	Up	1.36109E-65; Populus trichocarpa; 98%	Populus_nigra23427
	1-Aminocylopropane 1-carboxylase	Up	5.36991E-42; Populus trichocarpa; 97%	Populus_nigra27677
	Aminopropanecarboxylate oxidase	Up	1.17299E-79; Populus trichocarpa; 98%	Populus_nigra15610
Gibberelin	Gibberelin-3 β -dioxxygenase	down	0; Populus trichocarpa; 100%	POPTR_0018s01190.1

Table III.2.2.1.1.1 continued

Plant hormone affected	Enzyme regulated	Regulation of corresponding spot (down = aphid infested tissue less than control)	E value; species of determining blast hit; % similarity	Spot identification number
Jasmonic Acid	Lipoxygenase	Down	0; Populus trichocarpa; 97%	POPTR_0014s17550.1
	Enoyl-CoA-Hydratase (Pasticcino 2)	down	1.18569E-98; Arabidopsis thaliana; 90%	Populus_nigra-30724
	Acyl-CoA-Oxidase 2 (ACX2)	down	0; Arabidopsis thaliana; 91%	POPTR_0007s05710.1
	3-Ketoacyl-CoA-thiolase	Down	5.72133E-21; Arabidopsis thaliana; 86%	POPTR_0035s00250.1
	3-Ketoacyl-CoA-thiolase	Down	1.78803E-30; Arabidopsis thaliana; 86%	Populus_nigra-20686
Salicylic acid / shikimate pathway	Chorismate synthase	Up	3.40465E-61; Populus trichocarpa; 95%	Populus_nigra-15667
	Shikimate dehydrogenase	Up	0; Populus trichocarpa; 100%	POPTR_0013s03070.1



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Figure III.2.2.1.1.1: This figure highlights differentially regulated steps in the biosynthesis of different plant hormones with either light grey or colored arrows. The coloring was done using the B2G program and the map was derived from the KEGG database. Details about the differentially regulated enzymes can be found in table III.2.2.1.1.1 and in the text.

As it can be seen above, the infestation of *P. nigra* var. *italic* with *P. spirothecae* has a massive influence on the transcriptional profile of the leaves sampled. As the levels of SA and JA have also been determined, it is interesting to note that only JA biosynthesis seems to be regulated on a transcriptional level. Another quite interesting result is that, when looked at globally, terpene biosynthesis is transcriptionally down regulated in infested tissue. In contrast, lignin biosynthesis is up-regulated: one of the key enzymes involved, cinamoyl-CoA-reductase (Kawasaki, Koita et al. 2006) shows an increase on the transcript level by 1.41 fold relative to controls. This picture continues as transcript levels of coniferyl-alcohol-glucosyltransferase are also increased 1.83 fold. There is also evidence for an increase in peroxidase transcript levels; this class of enzymes is thought to link the monomers to form Lignin. It was, however, impossible to unambiguously identify a specific peroxidase involved in this process.

As an induction of the transcript levels of the transcription factor MYB134 (4.27 fold increase relative to controls) could be seen and because this transcription factor was shown to be associated with protoanthocyanidin biosynthesis, curiosity arose whether this change was also reflected in the transcripts of genes of the biosynthetic pathways controlled. Among the genes strongly induced was anthocyanidin synthase (3.45 fold increase relative to controls, sequence similarity 100%), as was flavonoid-3',5'-hydroxylase (4.68 fold increase, 99% sequence identity) and chalcone isomerase (2.16 fold increase, 100% sequence similarity) given in reverse order of appearance in the pathway.

III.2.2.2 THE SECOND TIMEPOINT ANALYZED

The second TP which had been chosen for global expression analysis was the one unifying all samples from the 7th of May 2010. On a low significance level ($p=0.05$; $N=4$), a total of 1735 genes were found to be differentially regulated; this number decreased by more than one order of magnitude when a significance level of smaller or equal than $p=0.02$ was asked and only 104 genes were found to be differentially regulated. At $p=0.05$, genes showing a fold change >2 were 519 which were up-regulated in infested leaf blades relative to controls (of 875 up-regulated in total) and of the down-regulated genes, 491 were found to be regulated > 2 fold (out of 964 total down regulated ones). In order to not miss any results, all results presented in the following section are obtained from the lower significance level. Again,

most top blast hits came from *Populus trichocarpa* (1601) and almost all sequences could be annotated (1578 out of 1735). About 1/3 of the sequences obtained an EC number (712) and those yielded a total of 112 pathways in which at least one enzyme was differentially regulated. To get a better overview of the molecular functions and processes involved, again pie charts representing the GO terms assigned on level three are shown in **Fig. III.2.2.2.1**. In the following section, selected pathways will be highlighted.

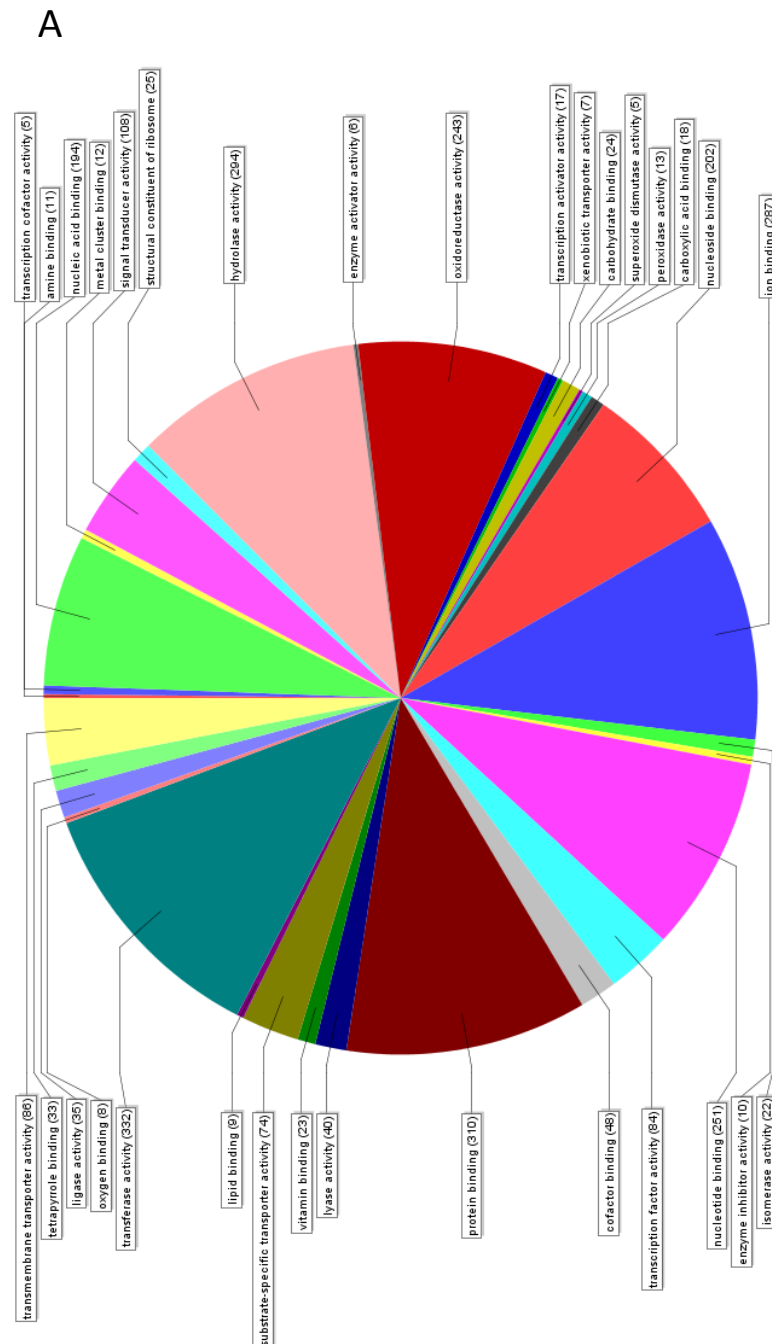


Figure III.2.2.2.1: legend on next page

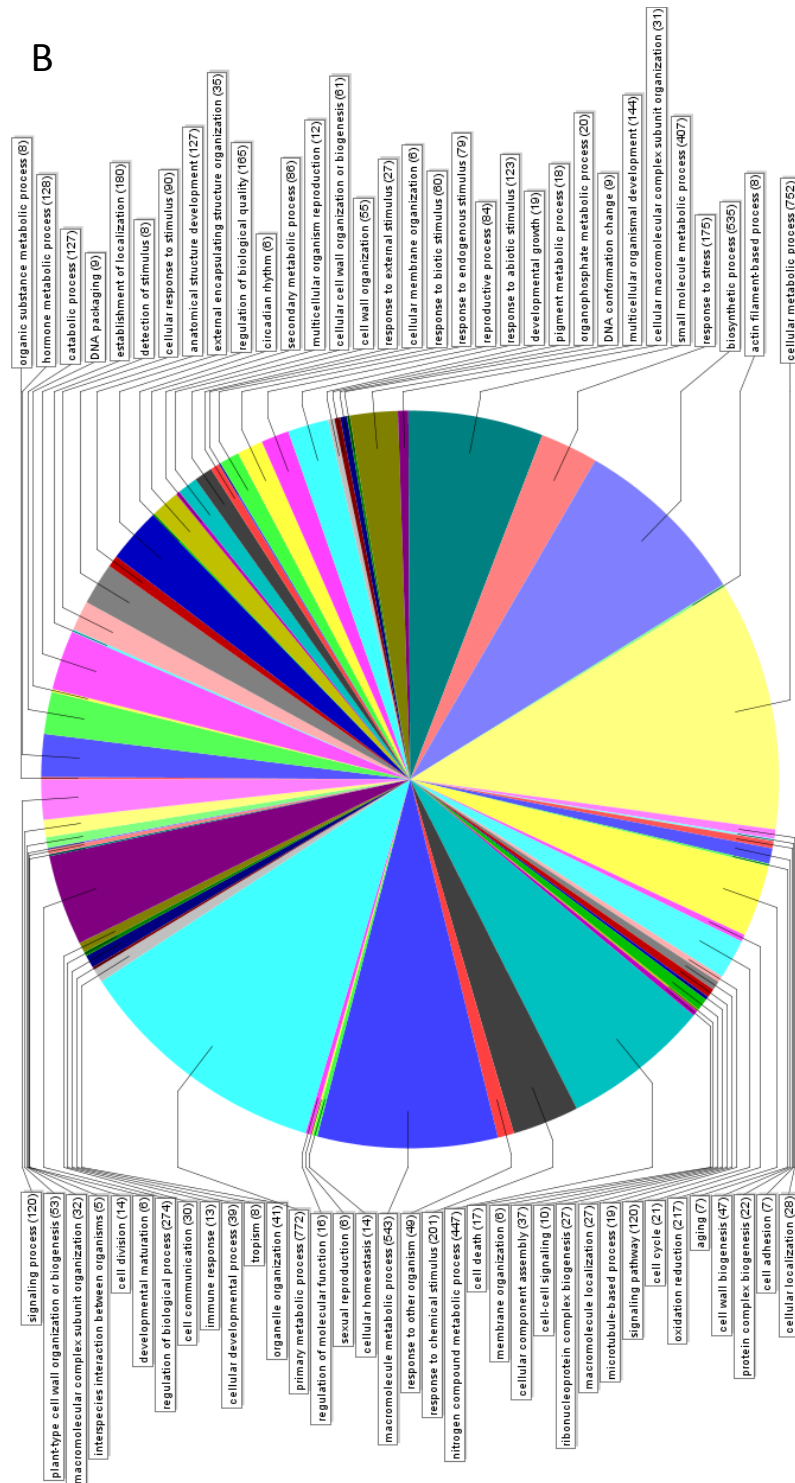


Figure III.2.2.1: The pie charts depicted here shows the level three GO terms assigned to the genes that were found to be differentially regulated at TP2 (with $p=0.05$ and $N=4$). Chart A on the previous page shows molecular function whereas chart B on this page shows biological process terms.

III.2.2.3 DISECTION OF DIFFERENT PATHWAYS DIFFERENTIALLY REGULATED AT TP2

As the phytohormonal pathways (and their outcomes) are of crucial importance to this work, this pathway will first be analyzed in depth. The pathways that showed differential regulation upon *P. spirothecae* infestation were the ones for ethylene, jasmonic acid and the brassinosteroid brassinolid. None of the other pathways showed regulation that could have been unambiguously assigned to one phytohormone. For example, phytoene desaturase, which catalyses the conversion from phytoene into lycopene was also found to be differentially expressed (down regulated 1.32 fold in infested tissue relative to controls for TP2 and the same can be said for TP1 (1.33 fold down regulated)); as many different products result from the terpenoid pathway, this was not considered to be regulation of phytohormone production. **Table III.2.2.3.1** shows the transcripts of different enzymes regulated in the pathways for phytohormone production.

Table III.2.2.3.1: This table shows some of the differentially regulated genes whose products are involved in biosynthesis of different phytohormones indicated.

Plant hormone affected	Enzyme regulated	Regulation of corresponding spot (down = aphid infested tissue less than control)	E value; species of determining blast hit; % similarity	Spot identification number
Ethylene	1-aminocyclopropanecarboxylate oxidase	down	8.77977E-180 <i>Populus trichocarpa</i> ; 100%	POPTR_0002s07880.1
	1-aminocyclopropane-1-carboxylate synthase	up	0; <i>Populus trichocarpa</i> ; 100%	POPTR_0002s08650.1

Table III.2.2.3.1 continued

Plant hormone affected	Enzyme regulated	Regulation of corresponding spot (down = aphid infested tissue less than control)	E value; species of determining blast hit; % similarity	Spot identification number
Ethylene	1-aminocyclopropane-1-carboxylate synthase	down	9.94198E-180 <i>Populus trichocarpa</i> ; 97%	POPTR_0010s05530.2
	5-methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase	down	6.45438E-33; <i>Populus trichocarpa</i> ; 92%	Populus_nigra-1860
Jasmonic acid	Lipoxygenase	up	1.72209E-47; <i>Populus trichocarpa</i> ; 95%	Populus_nigra-23271

Table III.2.2.3.1 continued

Plant hormone affected	Enzyme regulated	Regulation of corresponding spot (down = aphid infested tissue less than control)	E value; species of determining blast hit; % similarity	Spot identification number
Jasmonic acid	Allele oxide cyclase	Up	3.94566E-99; <i>Ricinus communis</i> ; 83%	POPTR_0004s10240.1
	Allele oxide cyclase	Up	3.00666E-139; <i>Populus trichocarpa</i> ; 99%	Populus_nigra-31197
	Enoyl-CoA-Hydratase	up	0; <i>Ricinus communis</i> ; 89%	POPTR_0018s02570.1
	Acyl-CoA-oxidase	up	0; <i>Glycine max</i> ; 93%	POPTR_0001s15530.1

When looking at the same pathways as the ones analyzed for TP1, the picture that evolves is less complex at first sight as a lot of differential regulation has ceased to exist, what can also be seen when looking at blank numbers (3738 genes differentially regulated for TP1 but 1735 for TP2 at $p=0,05$ and $n=4$). The terpenoid pathway now only shows regulation with the 1-deoxy-d-xylulose 5-phosphate reductoisomerase (1.3 fold down relative to controls), which is by far less than at TP1. The picture continues to simplify when looking at the protoanthocyanidin biosynthesis. Here, the inducing transcription factor MYB 134 does not show differential regulation anymore and this goes along with the fact that from the above mentioned enzymes, only flavonoid-3',5'-hydroxylase showed up to be regulated and this in the inverse way as for TP1 (2.422 fold down regulation relative to controls, 100% sequence similarity within alignment). Although lignin biosynthesis is still massively regulated, this is not the case for the key enzyme mentioned above with a total of 6 enzymes differentially

regulated (vs. 8 for TP1). Among those, again peroxidase comes up with 13 spots being annotated for this GO term (vs. 31 for TP1).

IV. DISCUSSION AND OUTLOOK

IV.1 SUMMARY

In the course of this work, the interaction of *Populus nigra* var. *italica* and *Pemphigus spirothecae* was examined over a time span of eight weeks and the consequences of this interaction on a phenotypical as well as on a transcriptional level in the leaf blade are described here. When comparing tissues by looking at the different phytohormone levels analyzed (either salicylic acid (SA), jasmonic acid (JA) or jasmonic acid-isoleucine conjugate (JA-Ile)), it becomes obvious that *P. spirothecae* infestation definitely affects the levels of those. For SA, the effect of aphid infestation is clearly visible with infested leaves showing an increase in SA content. This difference in levels decreases over time (as do the absolute levels) and the pattern is inversed for the last TP where also the absolute levels are the highest. For JA, the absolute levels increase over the first three TPs, drop to the lowest level at the fourth TP analyzed and increase again for the last one to almost reach levels of TP1. The effect of aphid infestation is less clear cut than for SA as there is no difference attributable for TP1, TP4 and TP6 in between the different groups and only TP2 and TP3 show higher levels in aphid infested than in control leaves. The picture seen for JA with no clear influence continues for the signaling conjugate of JA, JA-Ile. Here, no differences can be found at any time point for either isomer examined.

This cannot be said for the phenolic glycosides, the second class of compounds analyzed. Here, almost all compounds looked at show a clear and statistically significant positive correlation with aphid infestation. However, time again plays an important role in the level development and determines the overall content. The patterns that evolve when the levels are plotted are diverse. For salicin and nigracin, a clear decrease of content over time can be seen in both controls and treatment, with infested leaves carrying more of those two glycosides from TP2 onwards but levels seem to converge for the last TP. On the other hand, salicortin, homaloside D and tremulacin show a bell shaped pattern with maximum levels measured at TP3 and again a rapprochement for the last time point, also not as pronounced as for salicin and nigracin. Taken together, these results show clearly that aphid

infested tissue is better defended than is non-infested and that PG levels are not static, but responsive to challenge.

For the transcriptional profiling, a lot of interesting results have been found. First of all, the regulation of the different phytohormone pathways did not always reflect the levels measured. For example, JA levels are clearly higher for *Pemphigus* infested leaves at the first profiled TP (TP2). Still, the transcriptomics data suggests a down regulation of the genes of the corresponding enzymes of the pathway. This is also the case at TP4 (the second time point analyzed with microarrays) where the up regulation observed does not go along well with the level change. The different regulatory pattern of the transcripts for the ethylene biosynthesis was also interesting to note as for the first analyzed time point, the pathway is clearly upregulated in *P. spirothecae* infested leaves and clearly down at the second time point analyzed. The most puzzling result was, however, that the pathway that produces SA, the phytohormone that had been implicated in driving defense responses in plants in response to aphid infestation (Mohase and van der Westhuizen 2002; Zarate, Kempema et al. 2007), showed no differential regulation for either time point.

Another fact worth mentioning here is the transcriptional induction of many enzymes leading to lignin biosynthesis in infested leaves for both time points analyzed, again speaking for the fact that infested and non infested leaves clearly differ. This work also showed nicely a clear correlation between the MYB134 transcription factor and the induction of Protoanthocyanidin biosynthesis genes in aphid infested leaves relative to controls, thereby confirming the results of others (Mellway, Tran et al. 2009).

IV.2 DISCUSSION OF THE RESULTS OBTAINED FOR PHYTOHORMONES

It is known that the two phytohormones classically associated with defense, SA and JA, lead to different phenotypes when the pathways are activated. SA is normally seen as a response towards biotrophic and hemi-biotrophic pathogens such as bacteria and leads to hypersensitive cell death, the induction of the synthesis of pathogenesis related proteins and the onset of systemic acquired resistance (see, for example (Vlot, Dempsey et al. 2009)). SA responses are also conotated to aphid infestation (Howe and Jander 2008). In contrast, JA is

thought to mediate defense responses against herbivorous insects like caterpillars and necrotrophic pathogens. This response mainly occurs through the induction of JA responsive defense genes (Howe and Jander 2008; Bari and Jones 2009). Based on these findings, the hypothesis that was created initially about the levels of the phytohormones (see Section 1.8) had been that SA would be clearly responsive and infestation would correlate positively with the levels. Further, it had been proposed that levels of JA would be negatively correlated with aphid infestation due to a possible decoy caused by the aphid and antagonistic crosstalk between the two pathways. The first part of this idea can be clearly accepted, whereas the second one needs to be rejected as JA levels are also higher in infested than non infested leaves at TPs 2, 3 and 4. This was unexpected as both pathways are thought to largely be antagonistic with the SA pathway possibly overriding the JA pathway (Koorneef and Pieterse 2008). There is, however, a possible explanation to this. In a study performed by Zarate et al. (2007), the authors showed that the feeding of the larvae of Silver leaf whitefly (*Bemisia tabaci* type B), which is a phloem feeding insect as well, strongly induces the transcripts of SA responsive defense genes both locally and systemically (a fact that might also, at least partially, explain the synchronous level changes in both control and treatment in my experiments) whereas the ones responsive to JA seem to be repressed in *Arabidopsis thaliana*. By using mutant *Arabidopsis thaliana* plants with either impaired JA signaling or over activation of the SA pathway, Zarate et al. (2007) showed that either constellation led to a drastic acceleration in larval development. The opposite picture evolved with plants that had either constitutive JA signaling or impaired SA signaling with larval development being drastically slowed. A similar picture had also been obtained by others as well (Ellis, Karafyllidis et al. 2002). The authors of the former study conclude that the insect itself is able to shift the response of the plant towards the SA driven and less effective defense. For this, the term decoy was coined and this also seems to be an adequate explanation for the positive correlation of JA and SA with *P. spirothecae* infestation in that respect that JA levels are the “real” response of the tree towards the challenge whereas, in a way, the SA response is imposed on the tree. When following the line of thought that the levels seen are rather a product of the aphid actively perturbing the hormonal signaling, one needs to ask the question how this might happen. An interesting clue comes from different works that analyzed the proteome of the salvia the aphids secrete. Here, a lot of cell wall degrading

enzymes are found (Harmel, Letocart et al. 2008) and it is known that the break down products of this are potent elicitors of plant defense responses (Will and van Bel 2008). In *Arabidopsis*, WAK1 perceives oligogalacturonides and activates the SA dependent response (Walling 2009). This effect will probably be seen mostly in the phase when the aphid starts probing with its stylet but should be weaker when a solid phloem-stylet-“pipeline connection” is established. When looking at the data and the developmental stages of the gall, this hypothesis might well be able to explain, at least in part, the levels seen: as it is thought that the feeding pattern with changes in the position of the aphid on the petiole leads to the twists of the petiole, this first phase should be the one with relatively high SA levels. Those should then reach a minimum (the “pipeline point” with a stable connection established and hence, this hypothesis created here will be termed pipeline hypothesis) and the increase in population inside the gall would again trigger an increase do to more aphids probing the tissue. Further, it would be tempting to speculate that JA levels increase gradually because the tree accumulates information about the invader and also because the antagonistic effect of SA might become less prominent as its levels decrease and by this, the decoy becomes less effective. When a stable connection is established (which should be around TP4), it is conceivable that the tree can’t recognize the invader any more, a fact that is also supported by the observation that phytohormone levels don’t differ that drastically at TP4 compared to others. At this time point, the aphid would have succeeded in its strategy to influence the defense response in a way not suitable to fight of an aphid infestation; this is a strategy that has been reported by others as well (Ellis, Karafyllidis et al. 2002; Zarate, Kempema et al. 2007; Howe and Jander 2008). Levels would thereafter increase again when the aphid’s first progeny tries to establish a phloem connection and this can be seen in the data as well. The hypothesis has one shortcoming to it as well as it can’t really explain the drastic increase in levels of both SA and JA for the last time point analyzed and also not the inverse pattern with controls slightly higher than treatment for this time point. It is however tempting to speculate that other infestations like fungal pathogens or other insects active then could have massively influenced levels of those hormones by increasing levels of either JA or SA as a response to them. Another possible point for interfering with the early signaling of the plants defenses is the reduction of the Ca^{2+} signal. This is, at least for *Mycus persicae* feeding on *Arabidopsis*, achieved by the aphid by the delivery of calcium chelating proteins

that effectively block this signaling route and also prevent sieve element clogging (Will, Tjallingii et al. 2007), which is of crucial importance for a phloem feeder.

Another fact that needs to be evaluated here is that development in time is also a factor influencing phytohormone levels as can be seen in the global statistical analysis where time was always a highly significant factor, whereas the aphid was not irrespective of the particular phytohormone analyzed. This is most likely due to the fact that the developmental processes the flushing leaf undergoes are coordinated by phytohormones that act globally rather than just locally limited; following this idea, the pattern seen would rather be a fine tuning of the responses of the plant to the specific local condition of aphid infestation. Further, it is known that vascular connections between leaves are also a mean of the tree to communicate and signal stress. As no samples from non infested branches are available, the possible hypothesis that both levels in control and treated leaves are the product of *P. spirothecae* infestation that changes levels in this stratum globally by inducing systemic signaling can't be tested vigorously. Unfortunately, it has also been missed to sample material that had no aphid infestation and compare those results to the ones already obtained, as this would have provided valuable information for verifying this idea.

One more fact that was not discussed yet but that is of great importance for this dataset is the notion that all data obtained here comes from old grown, possibly polyclonal trees growing under natural conditions. This might on the one hand of course have introduced a good bit of variability into the data but on the other hand we should remind ourselves of the fact that nature doesn't grow in a green house.

One of substance class possible for defense could be the PGs that will be discussed next.

IV.3 DISCUSSION OF THE RESULTS OBTAINED FOR THE PHENOLIC GLYCOSIDES

In the light of the ambiguous results I obtained for the phytohormones, the outcome of the analysis of the leaf phenolic glycoside contents showed a clearer pattern. The levels found in the leaves show a clear positive correlation to *P. spirothecae* infestation and these

correlations found are highly significant for all of those compounds except for nigracin, which levels fail to be correlated globally (that is, over all time points analyzed together rather than just looking at individual time points) (see **Fig. III.2.2.1-III.2.2.5**). It is further interesting to note that the levels of all compounds are not seen to be different at the first time point analyzed, differ for the next 3 time points and then either start to converge again (as seen for salicin, nigracin and tremulacin) or still differ (as with salicortin and homaluside D). The finding that levels don't differ for the first time point is in clear accordance to the idea that these compounds are mobilized during the first leaf flush to protect the vulnerable young leaves (Lindroth, Hsia et al. 1987) and also provide evidence for the idea that PGs stored in the bark over winter (Foerster, Ulrichs et al. 2010) and are then mobilized in spring to full fill the above mentioned function. It is interesting to note that the overall decline of levels seen in the controls is initially not found in the infested leaves in the beginning of the sampling period and only occurs at the last time point analyzed. Before that, levels are either relatively constant (which is surprising, given the fact that the data was obtained using a possibly polyclonal setup which should produce huge inter-clonal variability with this substance class (Julkunentiitto and Meier 1992)) or even rise above the levels seen initially. It is, however, clear to see that there is an induction of PGs in response to galling aphid infestation.

These results inevitably lead to questions of the ecological meaning of the induction seen in the leaf blade. One possibility would be that the PGs are a mean of the tree to defend itself against aphids, possibly lowering either its reproductive success or slowing its growth rate. In accordance with this idea is the finding by Zucker et al. (1982) that lower concentrations of PGs mean a higher aphid infestation rate on an individual tree as well as that those levels seen are important for the selection of a suitable leaf to establish the colony for the aphid. When thinking of the feeding style of the aphid, one requirement for fulfilling this function is definitely that the compounds in question need to be phloem mobile; this has been shown for salicin (Gould, Jones et al. 2007). Unfortunately, no studies have been performed to date that addressed the question of toxicity of PGs for *P. spirothecae* and also, the mobility of other PGs in the phloem has not been shown yet. It is known that at plant galls contain higher absolute amounts of phenolic compounds than do

non-galled tissues (Hartley 1998) but the exact distribution of PGs within the gall, especially in the nourishing tissue and within the phloem juice is unknown.

It should also be noted that some different hypothesis might as well explain the differences seen. One would be that the aphid itself is able to manipulate the levels of PGs for its own benefit on different levels. The idea of an active manipulation seems to be far fetched at first, but there is some evidence for this hypothesis from other works and also within the scope of this work. First of all, it has been shown that gall forming aphids have profound influence on the physiology of their hosts by altering sink source dynamics (Larson and Whitham 1991) and also are able to change the rate of photosynthesis in galled leaves (Larson 1998). Within my work, transcriptional profiling has been performed and a huge number of GO terms associated with the biological function “primary metabolic process” have been obtained when differentially regulated genes were analyzed (1582 for the first TP analyzed and 772 for the second). Taken together, this provides strong evidence for the ability of the aphid to influence within plant processes. But why would it than be beneficial for the insect to increase levels of PGs within the leaf blade adjacent to its gall? One idea would be that it is somehow able to sequestrate those compounds and use them as a defensive mean against its own enemies, a phenomena not unheard of for other species (Pasteels, Daloze et al. 1986). This would of course facilitate survival of the aphid, which would also be the case if the leaf it sits on is better defended and less prone to be disintegrated by other feeders as the integrity of this leaf is of crucial importance to the insect. This would definitely be the case when PG levels are higher. Another possible explanation would be that the aphid is able to use the glucose moiety of the PGs as an additional energy source, but given the high sugar content of the phloem sap, this seems to be unlikely.

This reasoning also brings one to the question of ultimate nature of why are galls there at all. Two opposing ideas are possible for this. One would be that the gall is a defensive mean of the tree to encapsulate the enemy, which is unlikely given the fact that galls of a specific species are unique in its shape compared to others which would not be the case if this process was a mere encapsulation system. It is by far more likely that the insect itself is the driving force of the gall formation itself. Because of this, galls have been termed

the “extended phenotype” of the insect that is brought about by the aphid actively manipulating plant morphology (Stern 1995; Whitham, Young et al. 2003).

In the next section, the methodology used to obtain the data will be evaluated.

IV.4 EVALUATION OF THE METHODS USED

IV.4.1 PHYTOHORMONE EXTRACTION AND ANALYSIS

In the context of this work, phytohormones were extracted from fresh, flash frozen leaf blades. The method was chosen for different reasons. First of all, it enabled to use the same material for RNA extraction, thereby getting an accurate idea of the processes happening in the corresponding tissues. Further, as raw material was considered to be a limiting factor especially for the early time points, this procedure allowed for back up material to be saved. In small test experiments, it could be established that no enzymatic break down for SA (and most likely not for other phytohormones as well) took place over the entire extraction time in MeOH (see **Fig. III.1.1.1**, lower panel). Further, it also showed that the extracted amount of SA is constant even when the extraction period is lengthened, hinting at the fact that all SA is extracted. It is, however not the case that the extraction produces a saturated extraction medium as the amount of SA, that can possibly be extracted is much higher. This can be seen in **Fig. III.1.1.1** in the upper panel. Here, SA was artificially added in different amounts and even a fourfold increase (from 0,1ng/mg FW to 0,4ng/mg FW) was taken up by the medium. One back draw of the decision to take fresh material for the reasons outlined above is that one possibly underestimates the real SA content of the tissue in a systemic fashion as the material seems to be able to hold back at least artificially added SA. This can be seen in **Fig. III.1.1.1** in the upper panel: Whereas freeze dried material (red graph) showed a linear increase of recovered SA, matching the additively expected values of SA after spiking, fresh material (blue graph) failed to do so for higher added amounts tested. This is most likely due to matrix effects in the still water containing tissues. As this effect can be considered systematically, it however does not question the results obtained here.

IV.4.2 PHENOLIC GLYCOSIDE EXTRACTION AND ANALYSIS

The extraction of PGs was performed the same way as was the one for phytohormones. As the crude extracts were found to be too highly concentrated, these were diluted to meet the linear range of the detector system used. As no internal standard was available, all data presented here is only normalized to plant tissue weight; nevertheless, the indications obtained from the dataset give valuable hints and allow interesting conclusions. This issue will, however, need to be solved in follow up experiments by either using a labeled internal standard, performing a calibration curve for each set of runs with an unlabeled pure standard or by the use of a chemically akin internal standard.

IV.4.3 RNA EXTRACTION AND MICROARRAY HYBRIDISATION

For this work, different extraction methods for RNA from plant material had to be evaluated for two reasons: first of all, leaves of *Populus* species contain a high amount of phenolic compounds, which possibly interferes with the extraction process. Further, as quite a high number of samples had to be processed, an efficient way for extraction was necessary. It was found that both the “traditional” method described in section II.4.1 and the kit provided by Invitex (see section II.4.2) provided comparable results in terms of quality of isolated material (see **Fig. II.1.2.1**) as neither degradation nor contamination was visible on the Bioanalyzer blots. It was finally decided to use the kit as it provided an invaluable time advantage (2 h as compared to 2.5 d for one sample), the materials used are less toxic and as the starting material needed is much smaller (about 100 mg of ground tissue compared to 1.5 g).

In all further steps performed, the integrity of the RNA used was monitored continuously and carefully. The hybridizations to the arrays were performed in two successive sessions to avoid handling difficulties. It should be noted that small air bubbles were found on some slides after the hybridization which were probably due to small leaks in the sealing of the hybridization chambers. After the arrays were scanned, visual inspection of the raw pictures showed that those did not lead to areas that could not be reached by the hybridization solution and can therefore be considered irrelevant.

IV.4.4 EVALUATION OF THE MICROARRAY STUDY DESIGN

This part of the work aimed at the identification of differentially regulated genes after *P. spirothecae* infestation in *P. nigra* var. *italica*. In theory, there would be two different approaches to this: either labeling of RNA extracted from control or treatment with two different dyes and hybridization onto the same array or labeling of both subsets with the same color and hybridization to different arrays. Due to possible cross sample interaction and competition for the same binding sites, only the second method allows for inter array comparison as it eliminates those factors and because of this, this method was pursued. Due to limitations in the slide availability caused by financial constraints, the second decision that had to be made was whether to replicate the experiment technically (that is, only using one sample but hybridizing this to replicated slides) or to replicate biologically. As this work aimed at getting a broad view of the biological processes happening upon aphid infestation rather than to give an exact description of the processes in just one tree individual, I decided to replicate biologically, a way also taken by others (Babst, Sjodin et al. 2009). Further, this decision has been taken because of the sample material used, which came from a field site with possibly varying conditions for each tree. This random variations can be considered to equal out in the study design used but would, in the worst case, produce artificial results in case only one tree was used.

IV.5 DISCUSSION OF THE RESULTS OF THE MICROARRAY EXPERIMENT

For this work, a time course based upon the early development of the gall as a defining mean for sampling time has been done. After evaluating the obtained phenotypical results detailed above, three time points were chosen for further transcriptional profiling. As it can be seen in the both the phytohormonal as well as in the phenolic glycoside data, at TP2 a clear pattern seems to be evolved, which is not the case for TP1 as the PGs are induced with a time lack and also many early developmental processes are happening at this TP. For these reasons, TP2 with the first twist in the petiole finished was chosen over TP1. When further proceeding in time, TP3 does not seem to be strikingly different from TP2, but TP4 is as phytohormone levels reach their lowest values here, arguing for a transition happening around this time in both leaf development and also in the establishment of the interaction

and it was chosen for these reasons. The remaining TP6 then seems to bring about something new as phytohormone levels increase again whereas levels of PGs start to decrease in both control and infested leaves. This TP was consequently picked as well.

When the analysis of these three TPs was performed, some striking patterns could be found. First of all, the number of differentially regulated genes decreased in an almost linear fashion with 3738 spots differentially regulated at the first profiled TP, 1735 at the second and none at TP3. The first two analyzed TPs share 332 genes that are differentially regulated but the regulation of most genes seems to be a unique event (see **Fig. III.2.2.2**). Also, when comparing the results found to different other studies, the number of genes affected is quite huge. A study performed by Babst et al. (2009) compared gene expression changes in response to both the application of JA and gypsy moth feeding in *Populus nigra* in either systemic or local leaves and found that 781 genes were differentially regulated upon *Lymantria dispar* feeding on local leaves and expression levels of 185 genes were found to be changed in systemic fashion. JA treatment did influence the expression pattern of 684 genes locally and changed the expression of 338 genes systemically. There are, however, major differences in the study design used: whereas Babst et al. worked with a monoclonal cuttings grown under standardized conditions in a green house, my study was performed with free standing individuals of the approximate age of 50a. Furthermore, only one time point 22h after beginning of the treatment was analyzed by Babst et al. and this might well underestimate the transcriptional changes happening over a longer period of time. Another big difference is the array platform used as Babst et al. only used a relatively small chip which represents only about 16500 genes, which is not even half of the more than 45000 reported possible gene coding sequences in the *Populus trichocarpa* genome (Tuskan, DiFazio et al. 2006), thereby possibly missing differentially regulated genes. To avoid this, the study discussed here used a customized 120K chip representing both the entire predicted coding sequences of the *Populus trichocarpa* genome and all EST known of *Populus nigra*, together most likely representing the entire possible transcriptome. Furthermore, it is known that transcriptional patterns seen differ greatly among different feeding guilds on the same plant as well as between different clones of the same species and this as well might explain the huge differences (*sensu* Babst et al. (2009)).

It is worthwhile mentioning that the number of genes regulated at the first profiled TP is likely to be close to the maximum number of genes possibly regulated in response to aphid infestation as it has been reported that, at least in *Arabidopsis*, the number of genes regulated in response to aphid feeding increased continuously in a period of 48h post infection that this study looked at and more than doubled in that time window (Kusnierczyk, Winge et al. 2008). The decrease seen in the dataset presented here might be attributable to the fact that the longer the interaction lasts, the more attenuation of signaling might take place, thereby reducing the number of genes regulated. Another possible explanation would be that either the behavior of the aphid does not elicit further signaling at the last TP analyzed because a stable connection is established, that by integration of the various stimuli, a decision for co-existence is reached somehow or that the transcriptional changes are shifted into the more proximate petiole area once the leaf matures. It would be interesting to test this by profiling either leaves that harbor galls with offspring in it (this would allow to test the development of co-existence due to less elicitation) or profiling the petioles itself at the same TPs. Furthermore, when keeping in mind the drastic morphological changes induced by the aphid at the first TPs analyzed (repeated twisting of the petiole, massive extension of the petiole at the place of the gall), it is conceivable that this also requires transcriptional reprogramming and that this is less pronounced when the gall formation is finished.

When looking in more detail into the results obtained for the analysis of biosynthetic genes associated with phytohormone production, it is striking to note that the phenotypical results obtained are not mirrored in the transcriptomic dataset completely. When looking at the levels of phytohormones, one would expect to see a decline in the biosynthesis of SA and JA when comparing the two earlier time points profiled and an up-regulation of both in infested tissue at each individual TP. Indeed, the shikimate pathway that could potentially feed into the SA biosynthesis is up-regulated partly (chorismonate synthase and shikimate dehydrogenase) in infested tissue at TP1, but no enzymes exclusively committed to SA synthesis were regulated. All genes found to be differentially regulated for JA biosynthesis were down regulated at TP1 in aphid infested tissue relative to controls, which clearly is contradictory to the postulated ideas and the phenotypic results. Still, it is known that JA biosynthesis is also strongly regulated on a post transcriptional level as levels increase

already before transcriptional up regulation of the corresponding biosynthetic genes (Delker, Stenzel et al. 2006; Wu and Baldwin 2009). When studying the KEGG maps created by Blast2Go, it became apparent that the biosynthetic pathway leading to the volatile phytohormone ethylene was strongly up-regulated at TP1 as were ethylene-responsive transcription factors and it is known that ethylene can work in concert with JA in plant defense (Zarate, Kempema et al. 2007). Unfortunately, it was not possible to measure ethylene directly. At the second TP analyzed, no differential regulation could be found for SA biosynthesis, JA biosynthesis was up regulated throughout the pathway, which again does not reflect the phenotypic measurements that show no difference in infested and non infested tissue. Further, ethylene biosynthesis was down regulated in infested tissue. Taken together, these results again argue for two things. For one, that there is massive post transcriptional regulation within the biosynthetic pathway for phytohormones happening and two, that transcriptome data should be used to rather give a first direction than a final information.

This idea should also be followed for some other transcriptomics results that are, by itself, highly promising. One is that the MYB134 transcription factor, that had recently been shown to positively regulate the whole pathway leading to protoanthocyanidins (Mellway, Tran et al. 2009), is upregulated at the first profiled TP (but not at the second) as are many of the genes involved in the synthesis of the Protoanthocyanidins. This is another hint for a role of condensed tannins in the defense of *Populus* as Unsicker et al. (unpublished data) could also show massive increase in condensed tannins in response to aphid infestation in a dataset obtained from a field site in eastern Germany. This increase has also been shown in other systems that show gall induction (Nyman and Julkunen-Tiitto 2000; Allison and Schultz 2005). In the future, the levels of condensed tannins will be tested in the corresponding tissues to see whether this transcriptional change also translates to phenotypic effects.

Taken together, the results of the transcriptomic profiling performed here lead to interesting conclusions. First of all, it seems that the leaf blades that harbor a gall on its petiole are prepared to be better defended than are those without. Secondly, the decline seen over time in differential regulated genes makes a strong point for the idea of co-existence that develops over time. The reasons that trigger the development of this remain

partly elusive but a cue could come from the determination of the time point when the sink-source transition is happening. It is conceivable that this transition also changes the flux in the phloem, thereby mechanistically abrogating the possibility of detection of the aphid, which would then lead to the observed results. This idea is also supported by the observation that only early galls are abandoned by the aphid, but not later ones. This could be due to more effective defense in the beginning of the interaction and a lack thereof in later stages.

IV.6 CONCLUDING REMARKS AND OUTLOOK

The results of this work shed light onto the interaction of the gall forming, colonial aphid *Pemphigus spirothecae* and the dieucous tree *Populus nigra* var. *italic*. Based the results outlined above, it can be said now that the influence of the aphid on the adjacent leaf blade is huge and leads to major changes in the phytochemistry. The clear induction seen for the phenolic glycosides make a strong point for those compounds not only being constitutively present in poplar but are induced upon challenge. This also opens new perspectives for the elucidation of the biosynthetic pathway involved and will help to answer the question of translocation and *de novo* synthesis. If the promising results obtained for the regulation of the pathway leading to the protoanthocyanidins are also mirrored on a phenotypical level, the same is true for these compounds. Further, it was shown nicely that the infestation creates a complex phytohormonal pattern that clearly argues for the fact that it's the interplay of different hormones that orchestrate the response, not just one major one driving it. This picture will most likely diversify even further when ethylene is measured in spring as part of the volatile bouquet, which, by itself, will be interesting to see as it might show a completely new level of defense employed by the tree.

Keeping all this in mind, the interaction of the aphid with the tree will be evaluated in a closing section. As this study aimed at a better understanding of the changes happening in the leaf blade which were shown to be massive on every level looked at. It has been shown nicely that *P. spirothecae* has an influence on phytohormone levels in the leaf blade it sits on. The pattern seen at the different time points can be explained globally by the "pipeline

hypothesis” developed and outlined above. It remains unclear however, whether the tree is able to sufficiently fight the aphid as a consequence of these changed phytohormone levels. This question could be addressed in different ways. One way would be to establish a greenhouse culture that could be modified genetically to test the effects of the individual phytohormones and their contribution to defense. Another possibility would be to analyze the phloem juice of the respective petiole as this is probably the only way to effectively deliver a defensive mean to the aphid once it is attached to the phloem.

It is tempting to speculate that the aphid is somehow able to actively create an environment that is better protected from damage both at the petiole and at the leaf this sits on than is the non-infested leaf. This is highly beneficial for it as the crucially important leaf is indeed it’s backyard it needs to keep neat and clean for its own survival. Future work will test these ideas by looking in more detail into physiological changes happening by measuring photosynthesis rates and by this being able to determine the time point of sink source transition. Further, performance tests with specialized and generalist herbivores will allow the testing of the idea of a better defended leaf and extend the results of this complex to higher trophic levels. The results of this work are definitely a good motivation for this as it has been shown that the PGs are clearly higher in infested leaves and the results obtained here open different new perspectives for follow up experiments. First of all, it would be interesting to analyze the phloem sap above and below a gall for changes in PG composition to get an idea whether those compounds are taken up by the aphid and whether those are phloem mobile at all. As it has been shown that the levels of PGs are drastically influenced by aphid infestation, this provides a completely new and simple approach towards the question of biosynthesis of PGs *in planta* for feeding studies to answer the long standing question of *de novo* synthesis vs. re-location. Another relatively simple way of measuring possible uptake and possible sequestration would be to extract aphids and analyze those extracts for PGs. When addressing the question of defense on a higher trophic level, it should be tested if parasitoids of the aphid are selectively attracted to the infested leaves by means of volatiles synthesized by the tree. As SA is clearly induced by aphid infestation, it would also be interesting to correlate *P. spirothecae* infestation with the occurrence of pathogenic pests like fungi and see whether this infestation prepares the tree for those; this would also support the idea of the aphid as an far reaching engineer.

To address the question of the mechanistic cause for the gall induction, it would be interesting to see how an antibiotic treatment that would kill the insects endosymbiotic bacteria would affect this process. One possibility of how this turning of the petiole is induced would be the delivery of auxin (probably synthesized by its endosymbionts) or close analogues by the aphid. If this was true, one should be able to induce gall like processes by repeated application of auxin on one side of the petiole.

Taken all together, this work paved the road towards understanding the processes leading to insect gall formation in a great deal by creating new questions and supplying numerous hypothesis, whose vigorous testing will allow for a even deeper understanding of this puzzling phenomena.

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VII. APPENDIX

VII.1 ABBREVIATIONS USED

PH	phytohormone
PG	phenolic glycoside
SA	salicylic acid
JA	jasmonic acid
JA-Ile	jasmonic acid – isoleucin conjugate
ABA	abscisic acid
NPR1	non-expressor of PR1
PR X	pathogenesis related protein X (X=any Number)
TP	time point
Ca ²⁺	calcium ion with two positive charges
WAK1	wall associated kinase 1
var.	variance
Fig.	Figure
CS	control form the same leaf swirl
P	<i>Pemphigus spirothecae</i> infested sample
N ₂	Nitrogen
liq.	Liquid
rpm	rapids per minute
rcf	relative centrifugal force
min	minute
h	hour
a	annum (year)
μl	micro liter

HPLC	high performance liquid chromatography
GC	gas chromatography
MRM	multiple reaction monitoring
EtOH	ethanol
CTAB	Hexadecyltrimethylammonium bromide
v/v	volume per volume
RNA	ribonucleic acid
DNA	desoxy ribonucleic acid
DEPC	Diethylpyrocarbonate
cDNA	copy DNA
cRNA	copy RNA
Cy3	cyanine-3-cytosin-tri-phosphate
NaOH	Sodium hydroxide
B2G	Blast2Go
GO	Gene Ontology
EC	enzyme code
KEGG	Kyoto Encyclopedia of Genes and Genomes
d	dies (day)
JA-Ile1	(-)-7-iso-Jasmonoyl-L-isoleucine
JA-Ile2	(+)-7-iso-Jasmonoyl-L-isoleucine

VII.2 RESULTS FOR INDIVIDUAL TREES

In this section, all individual results obtained for the different trees are given as graphs. These results are ordered according to compound, rather than tree to allow better comparison. The original data can be viewed on the supplemented CD. This CD does further contain all files related to the microarray analysis (B2G files, all excel sheets created and used) as it was found to be impossible to squeeze those onto paper.

VII.2.1 PHYTOHORMONES

VII.2.1.1 SALICYLIC ACID

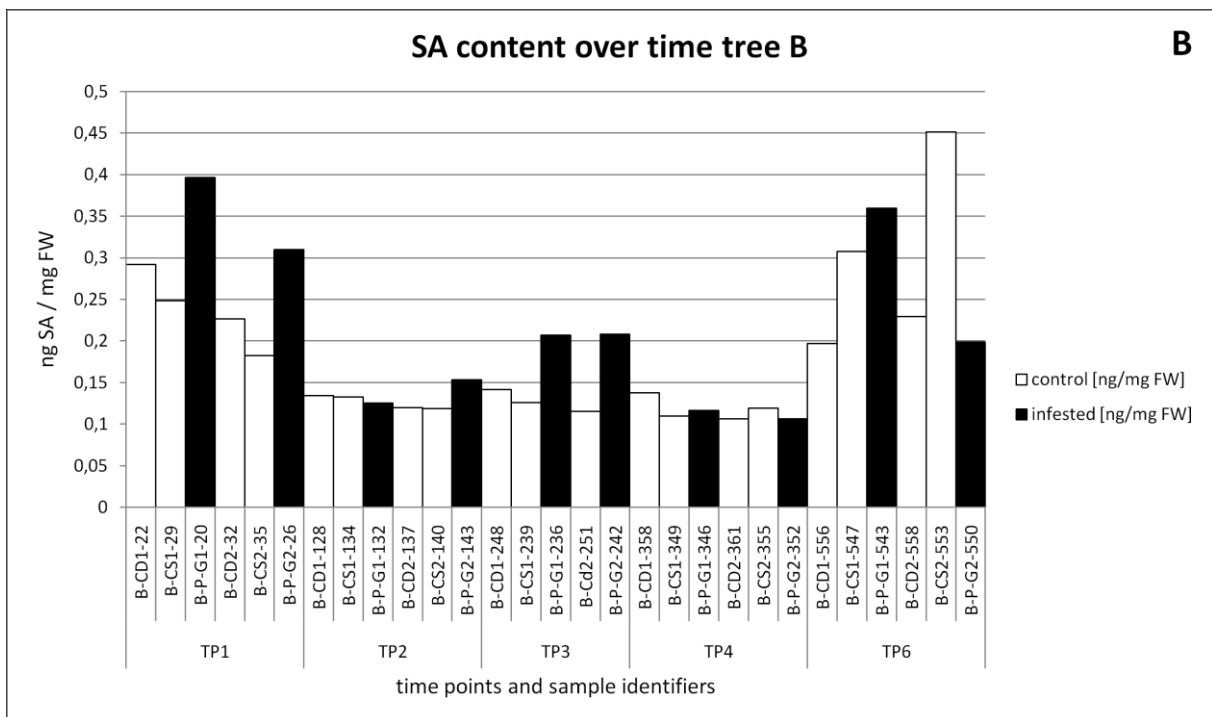
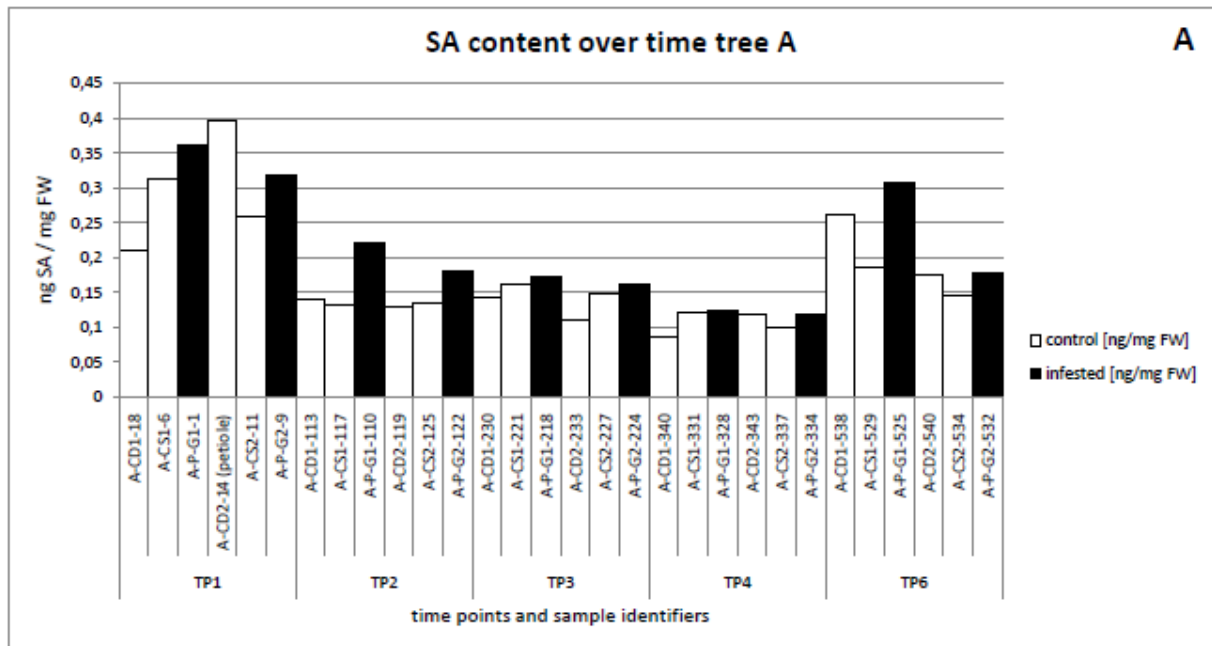


Figure VII.2.1.1.1: legend see next page

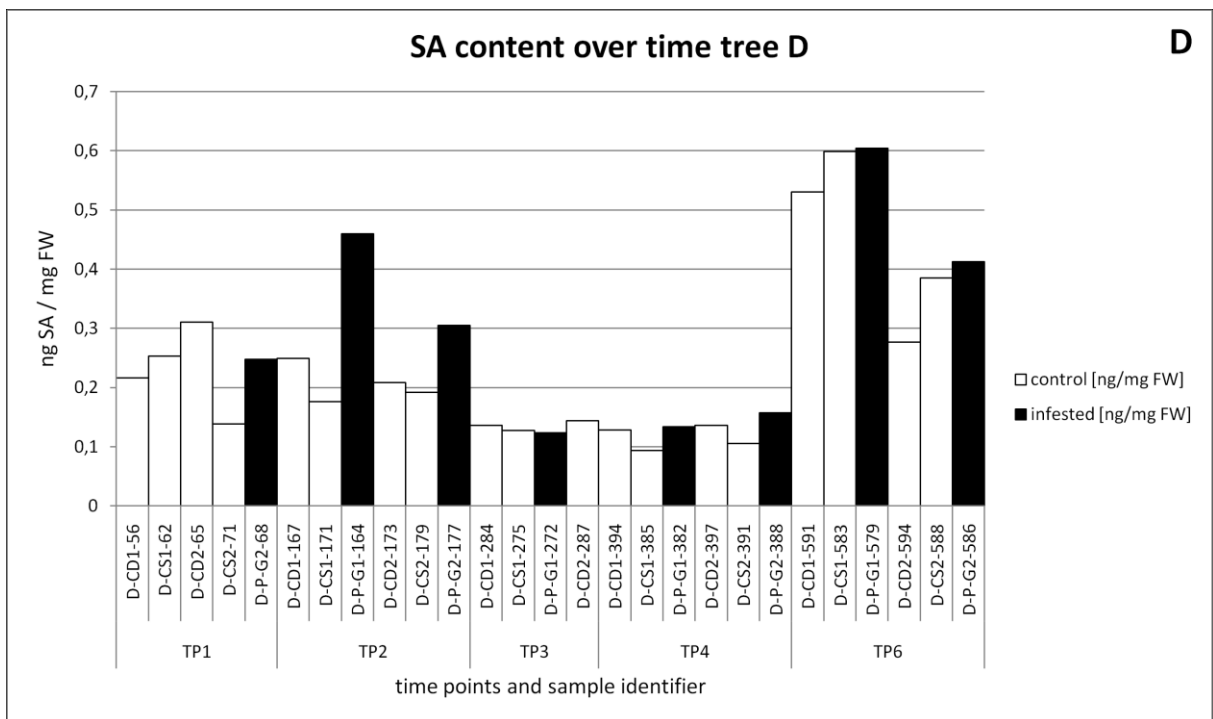
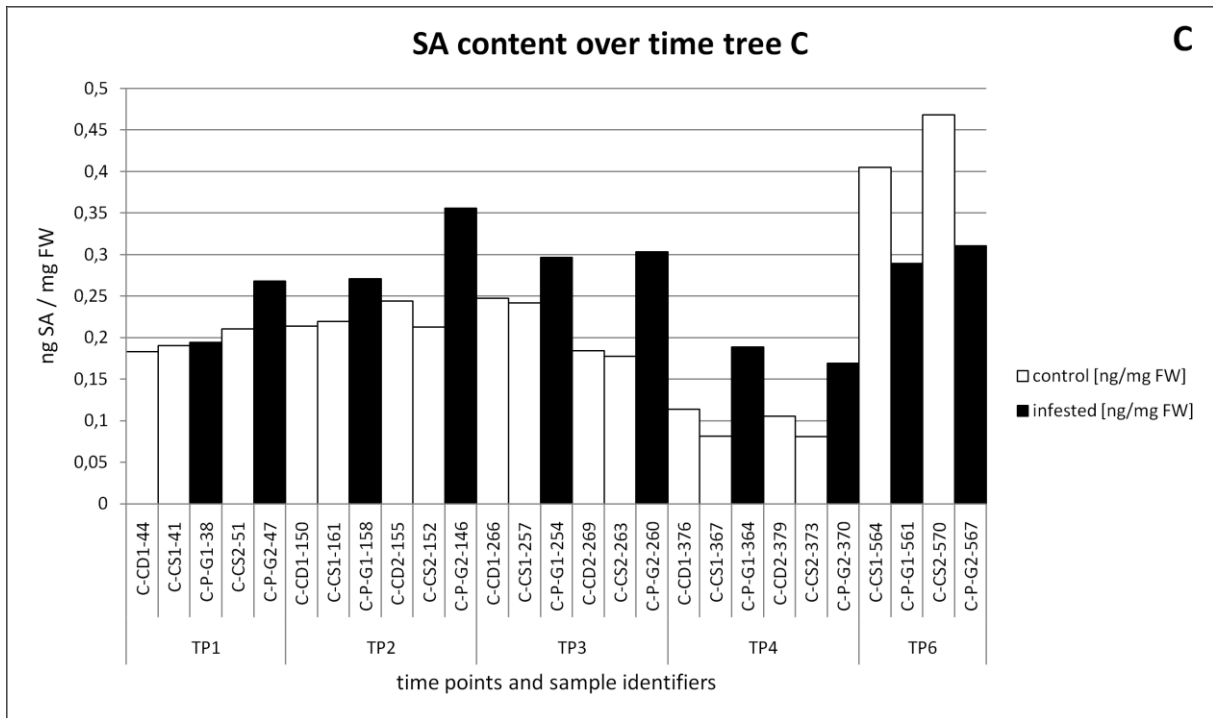


Figure VII.2.1.1.1: legend see next page

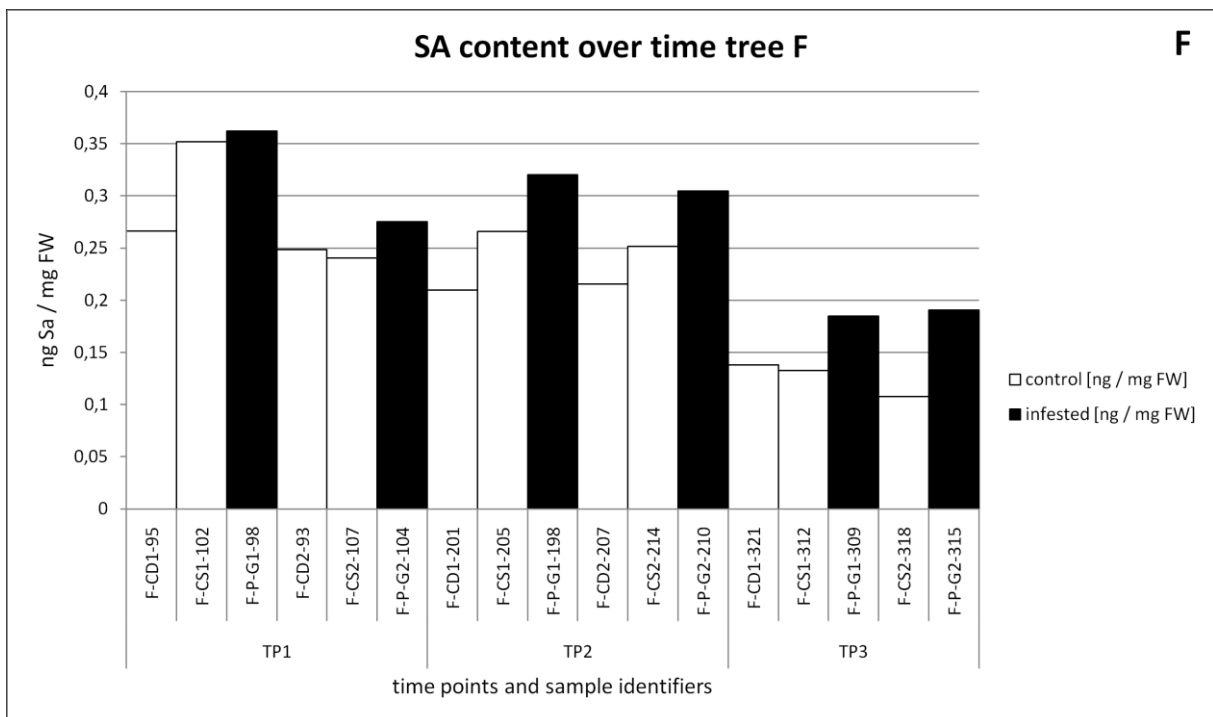
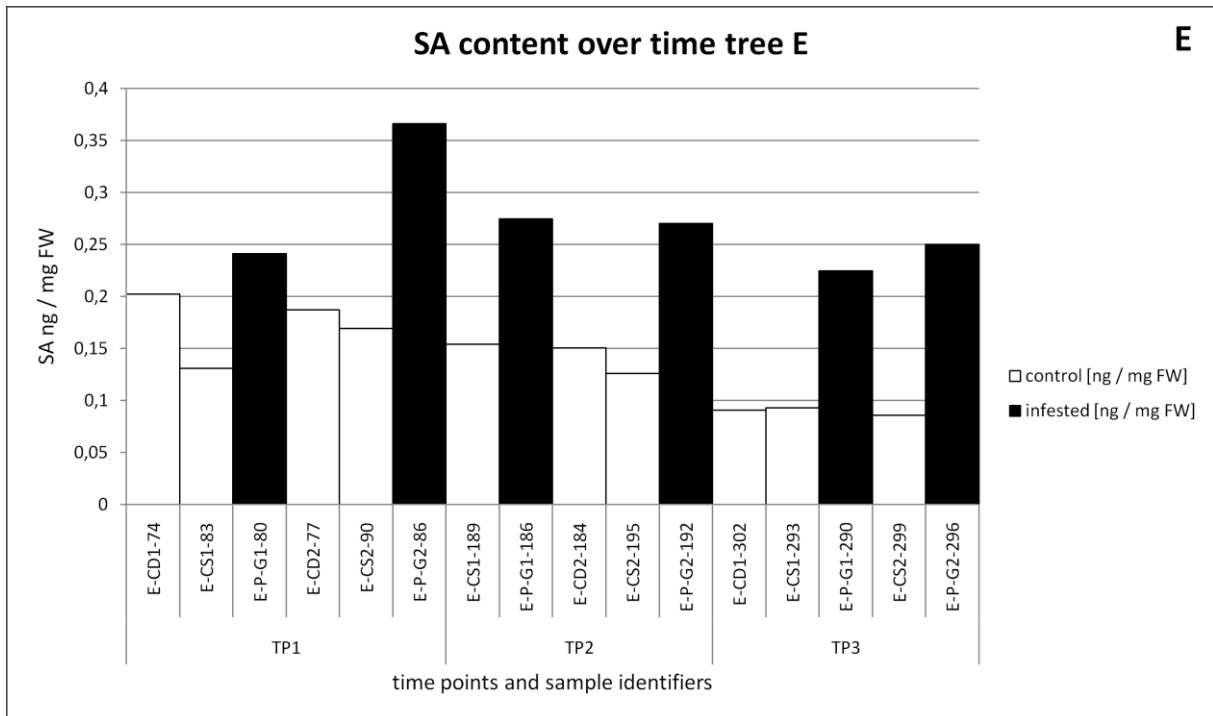


Figure VII.2.1.1.1: This figure shows the different levels of SA in infested (black bars) and non-infested (empty bars) leaf blades in the individual trees; panel A shows tree A, panel B shows tree B, C tree C, D tree D, E tree E and F shows SA levels of tree F. Abbreviations: CS = control same, CD = control different and P-G = Pemphigus gall. All data obtained from CD leaves was not considered in the main body of the work and positions 1 and 2 were pooled in the main work as no statistical differences were observed.

VII.2.1.2 JASMONIC ACID

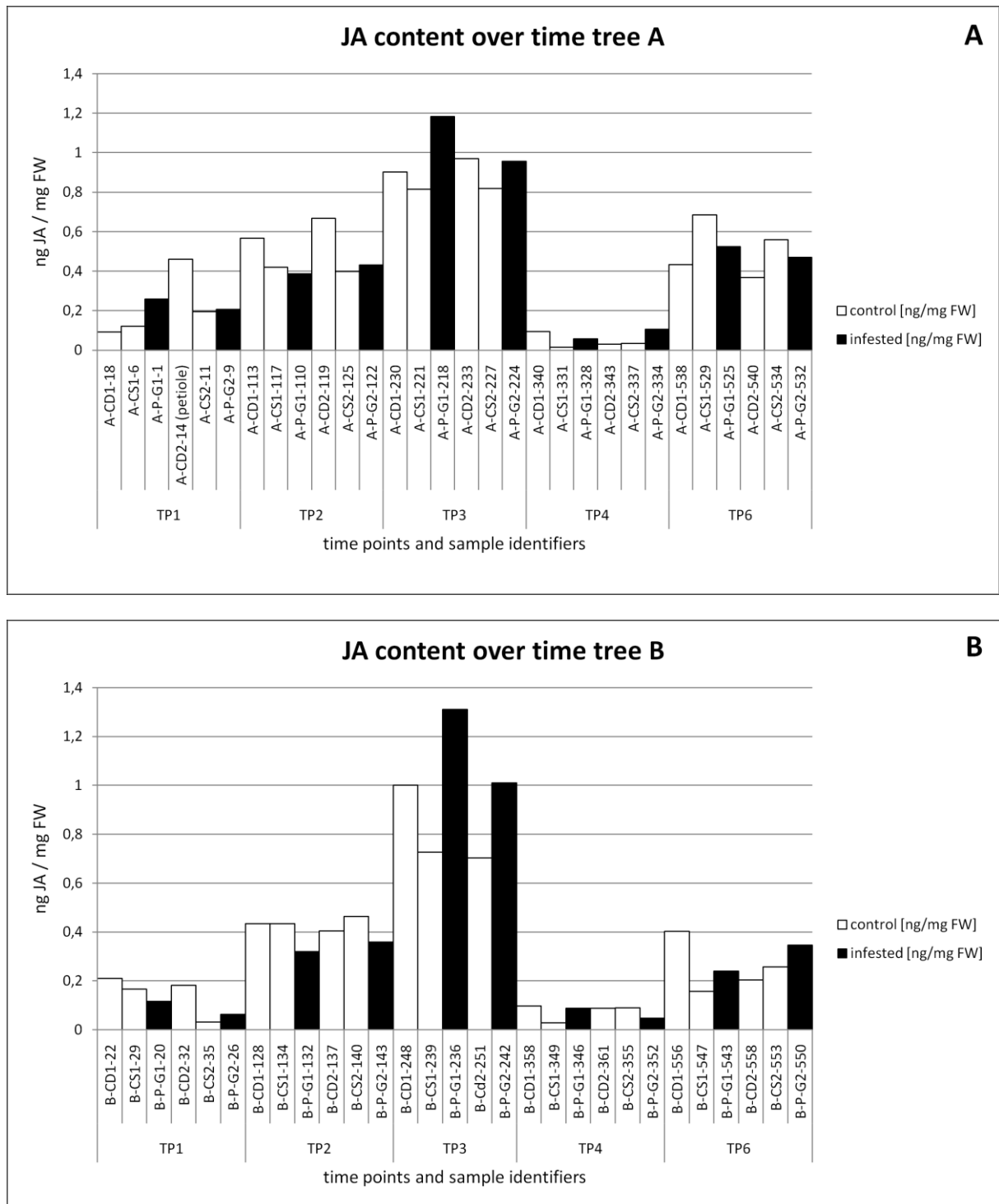


Figure VII.2.1.2.1: legend see next page

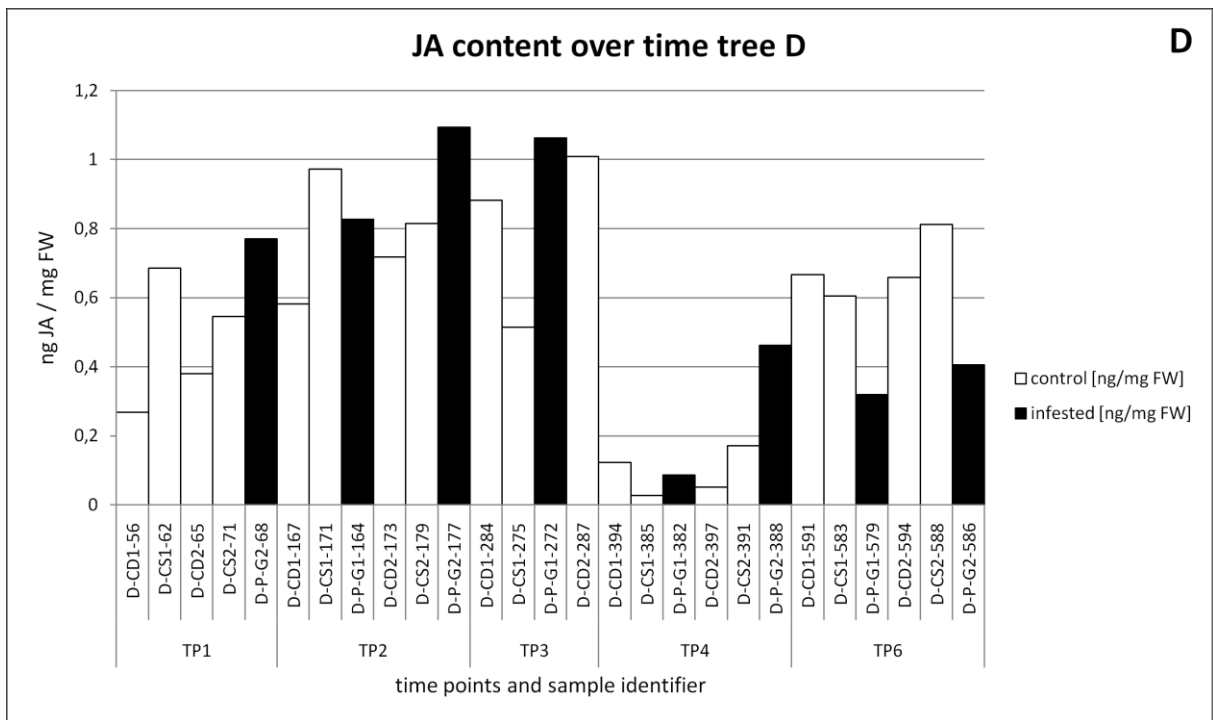
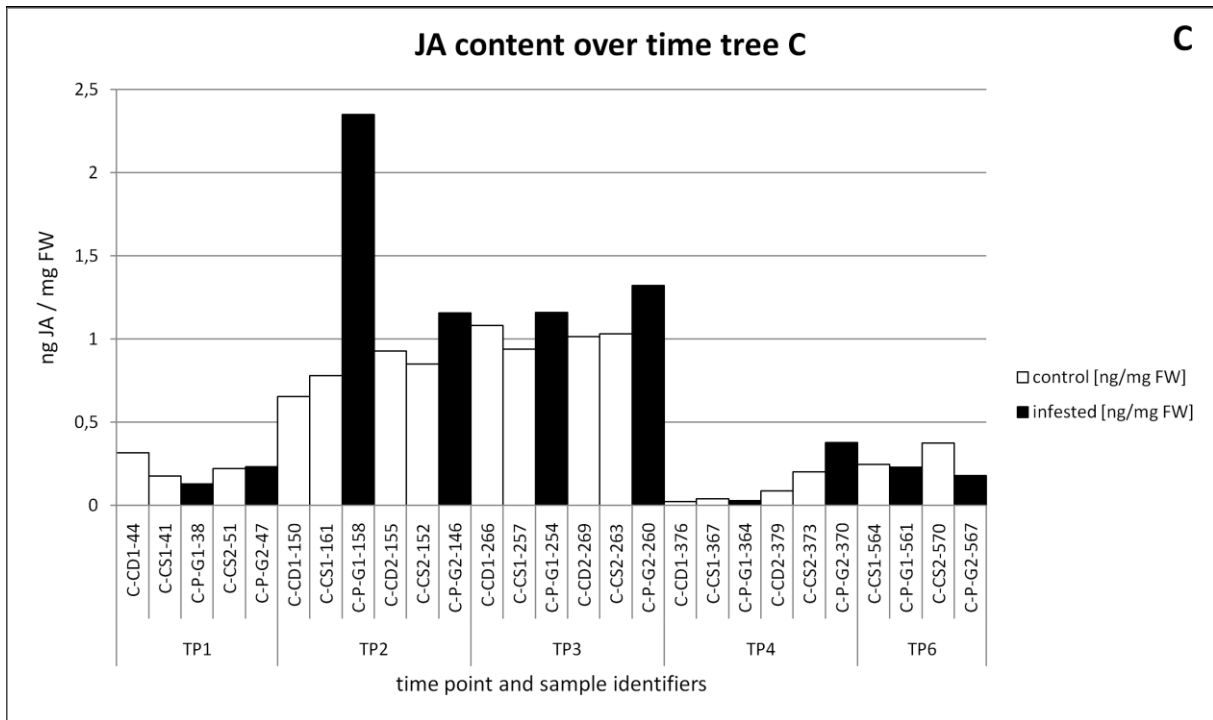


Figure VII.2.1.2.1: legend see next page

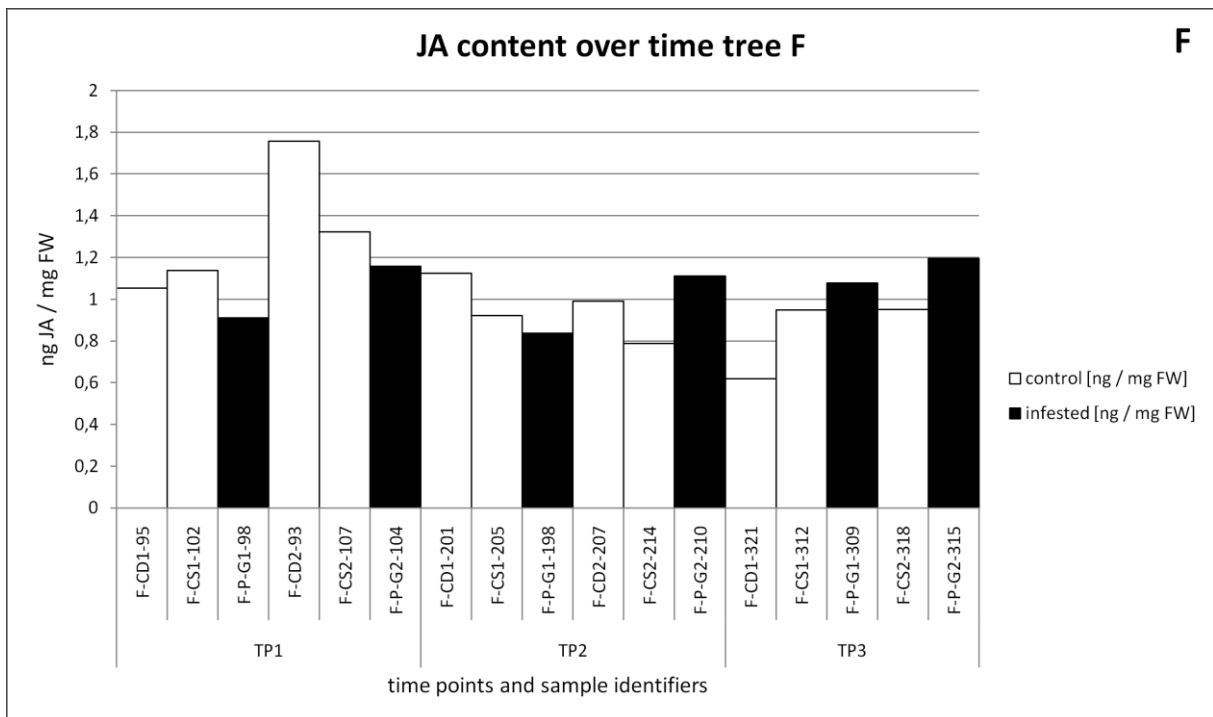
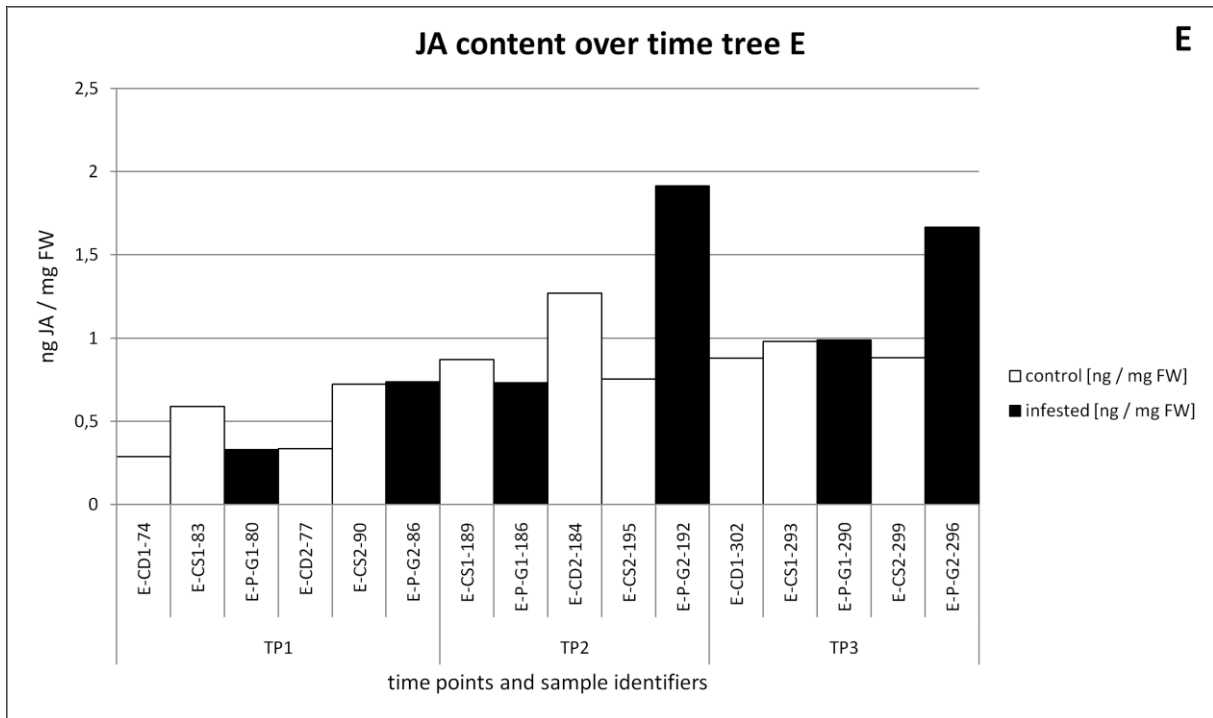


Figure VII.2.1.2.1: This figure shows the levels of JA in infested (black bars) and non-infested (empty bars) leaf blades of the individual trees; panel A shows levels in tree A. B in tree B, C in tree C, D in tree D, E in tree E and F shows JA levels in leaf blades collected from tree F. Abbreviations: CS = control same, CD = control different and P-G = Pemphigus Gall. All data obtained from CD leaves was not considered in the main body of the work and positions 1 and 2 were pooled in the main work as no statistical differences were observed.

VII.2.1.3 JASMONIC ACID – ISOLEUCIN CONJUGATE

VII.2.1.3.1 (-)-7-ISO-JASMONOYL-L-ISOLEUCINE (JA-ILE1)

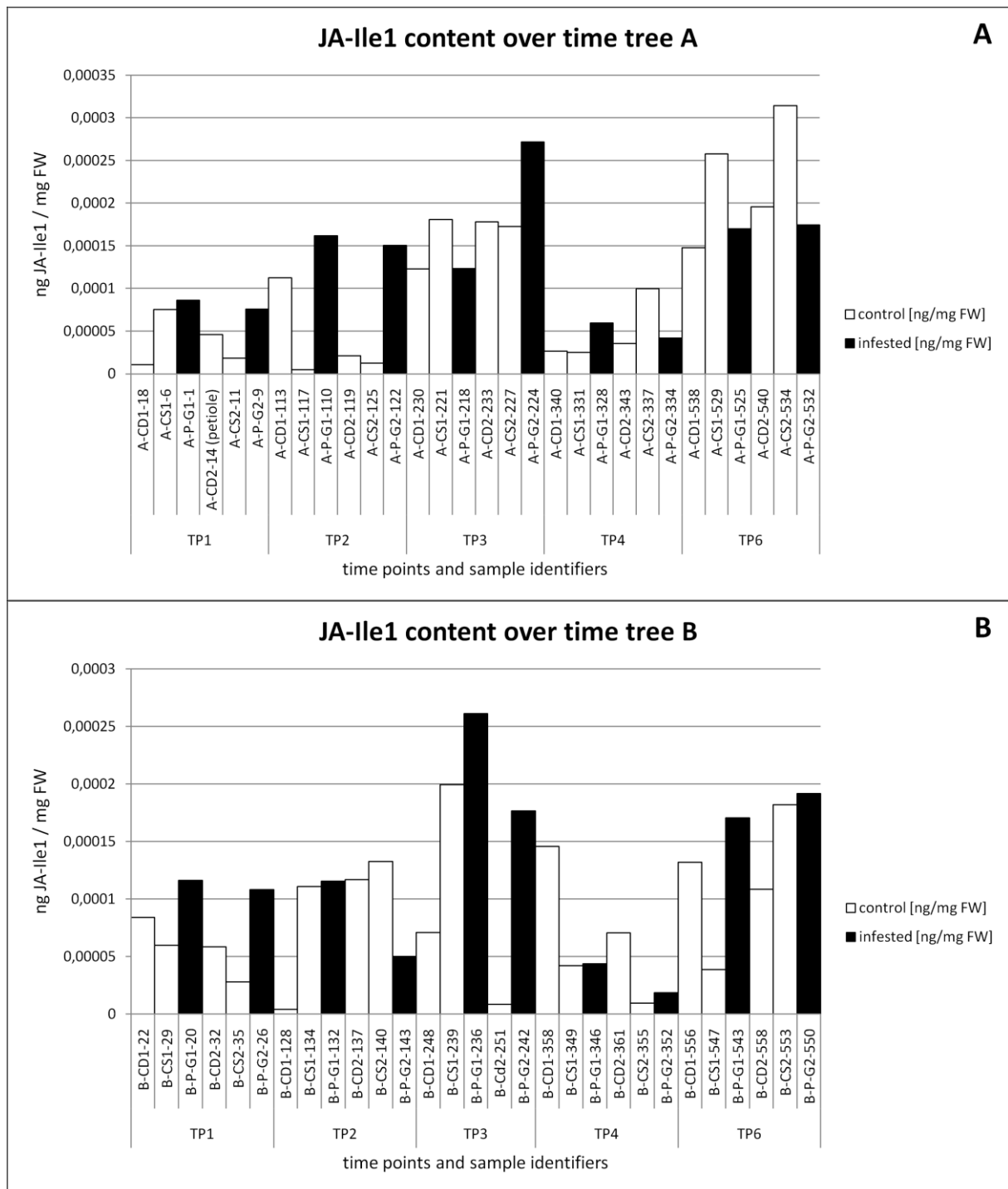


Figure VII.2.1.3.1.1: legend see next page

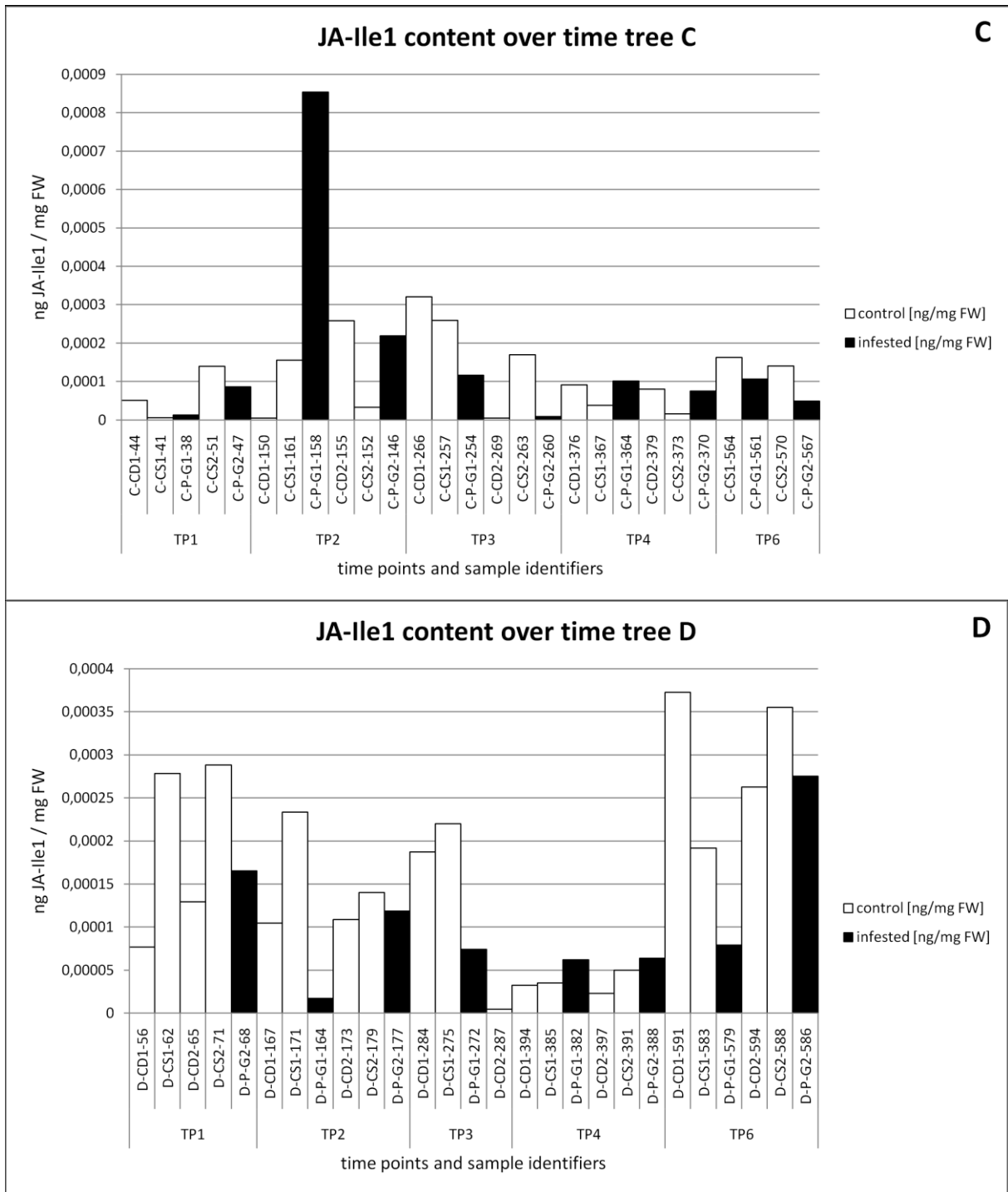


Figure VII.2.1.3.1.1: legend see next page

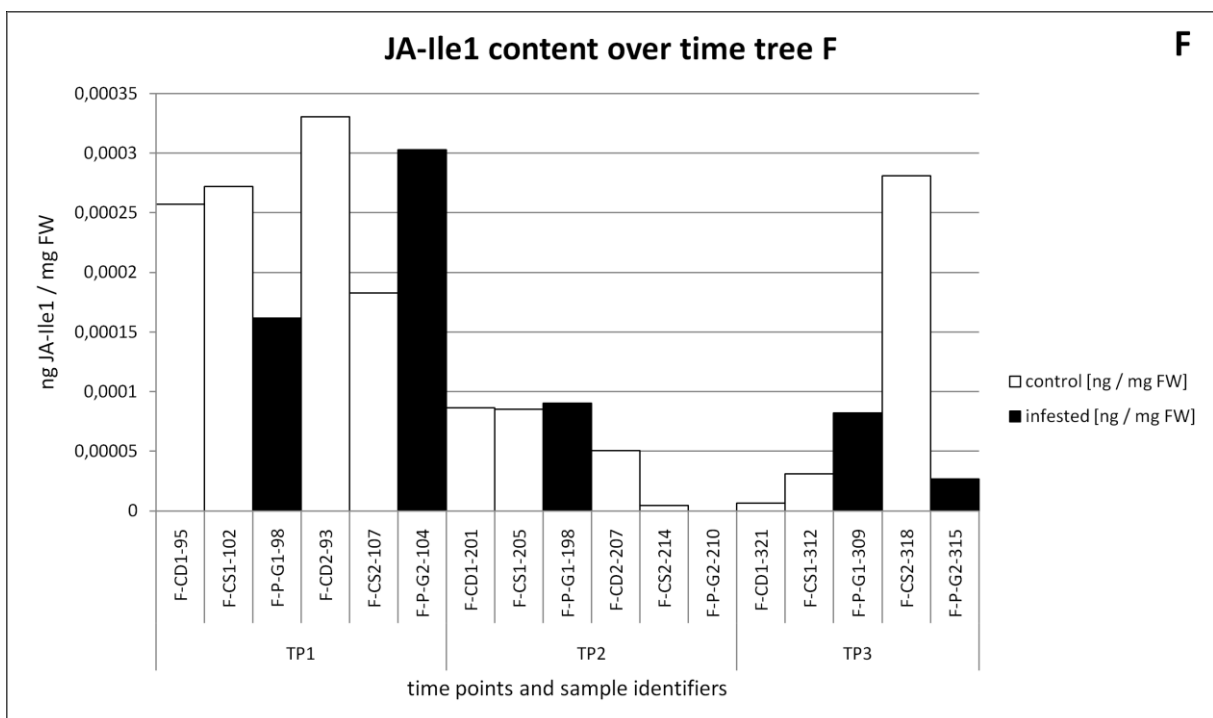
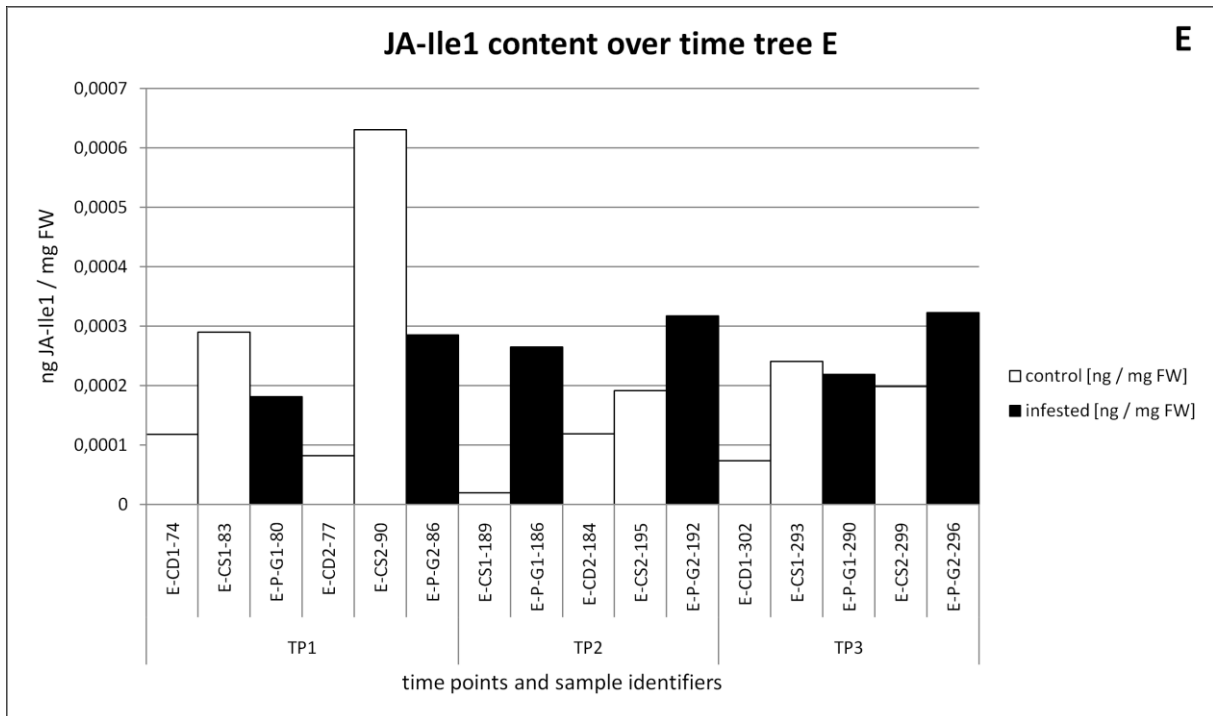


Figure VII.2.1.3.1.1: This figure shows JA-Ile1 levels in infested (black bars) and non-infested (empty bars) leaf blades of tree individuals. The panels show (from top to bottom) levels of JA-Ile1 in tree A (panel A), tree B in B, tree C in C, tree D in D, tree E in E and levels of JA-Ile1 in leaf blades of tree F are shown in panel F. Abbreviations: CS = control same, CD = control different and P-G = Pemphigus gall. All data obtained from CD leaves was not considered in the main body of the work and positions 1 and 2 were pooled in the main work as no statistical differences were observed.

VII.2.1.3.2 (+)-7-ISO-JASMONOYL-L-ISOLEUCINE (JA-ILE2)

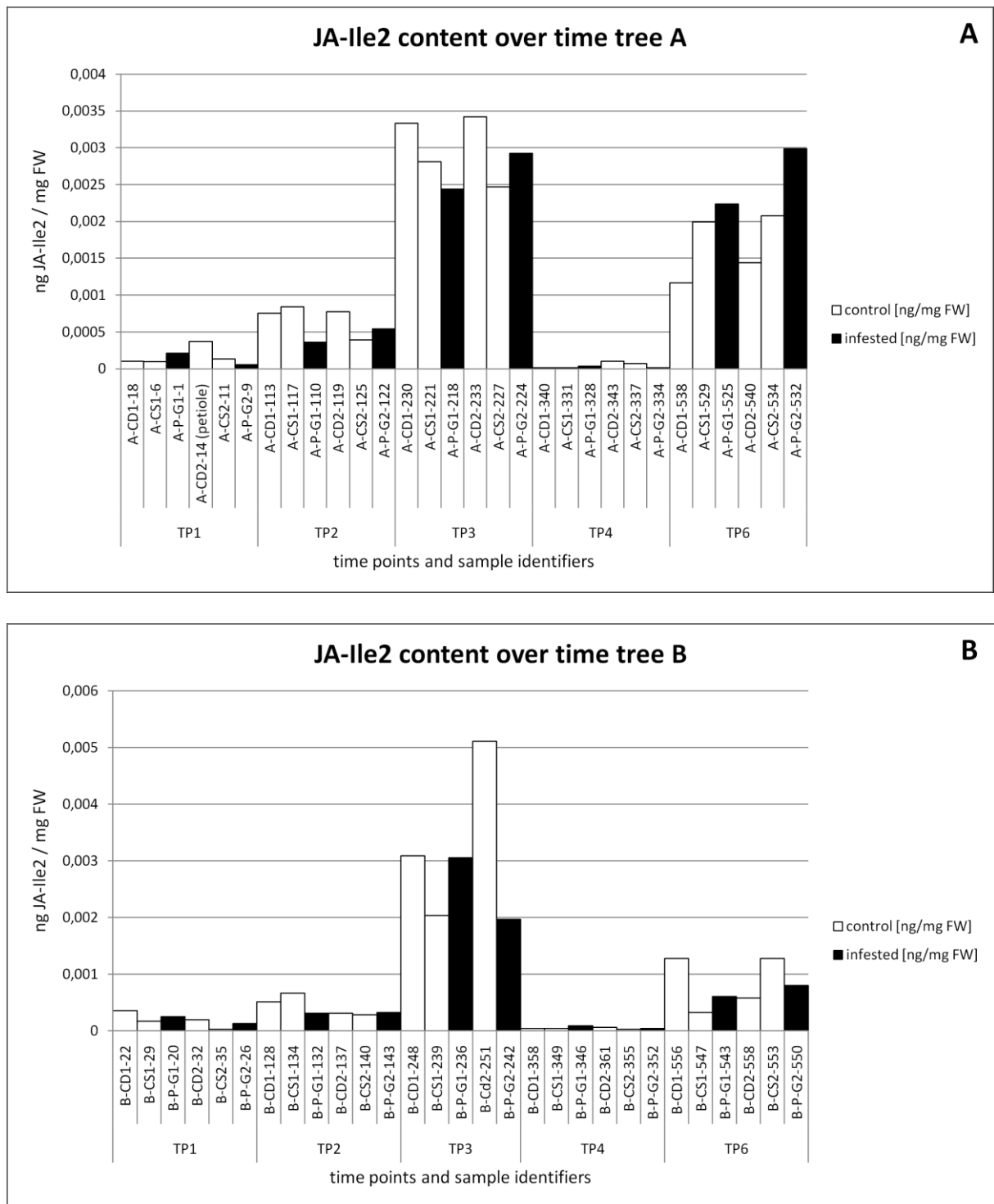


Figure VII.2.1.3.2.1: legend at the end of the figure

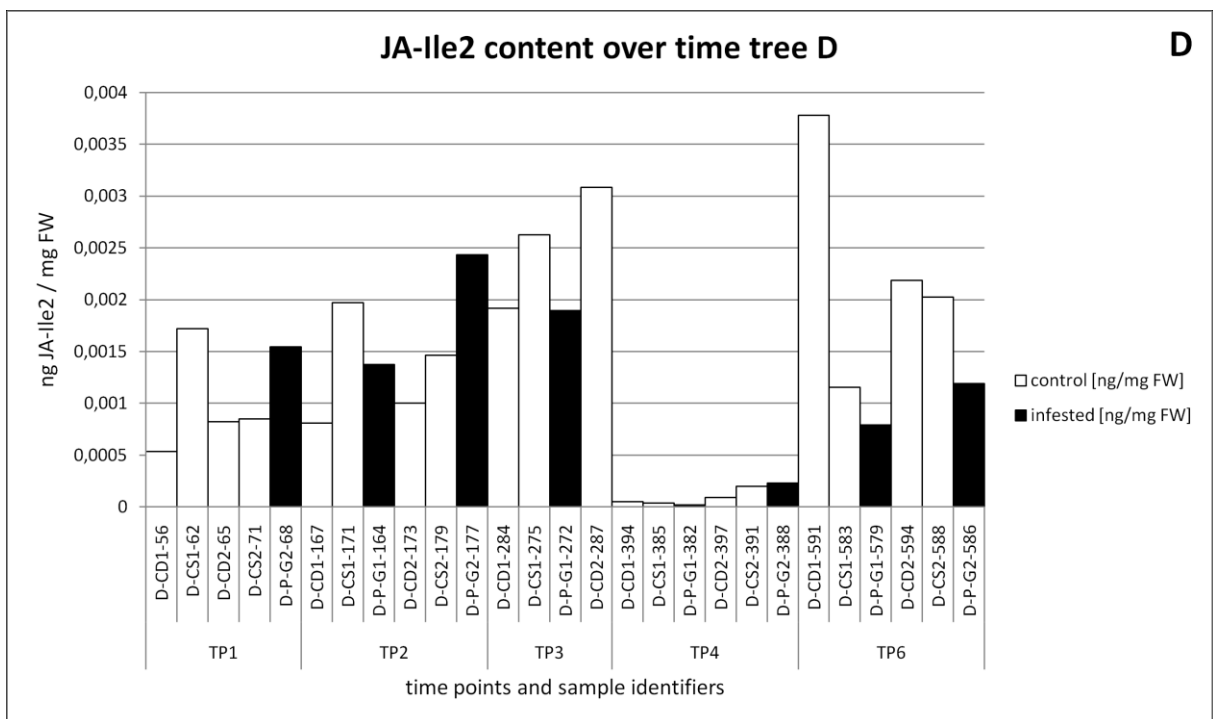
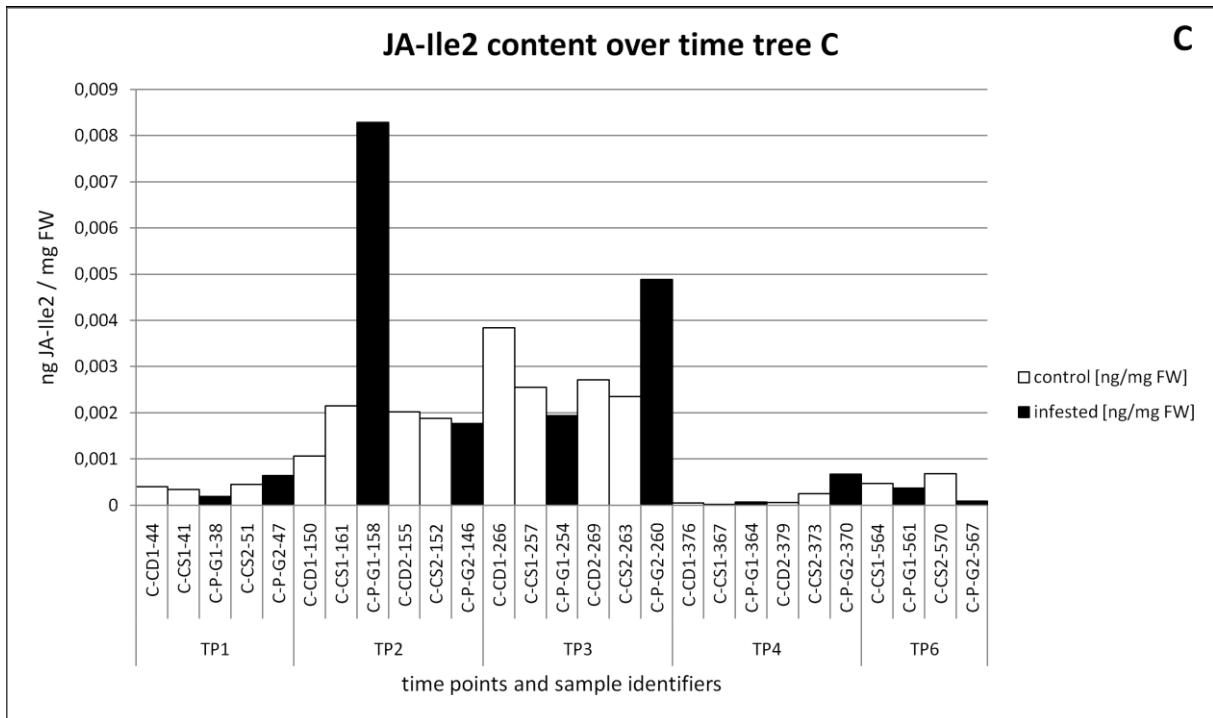


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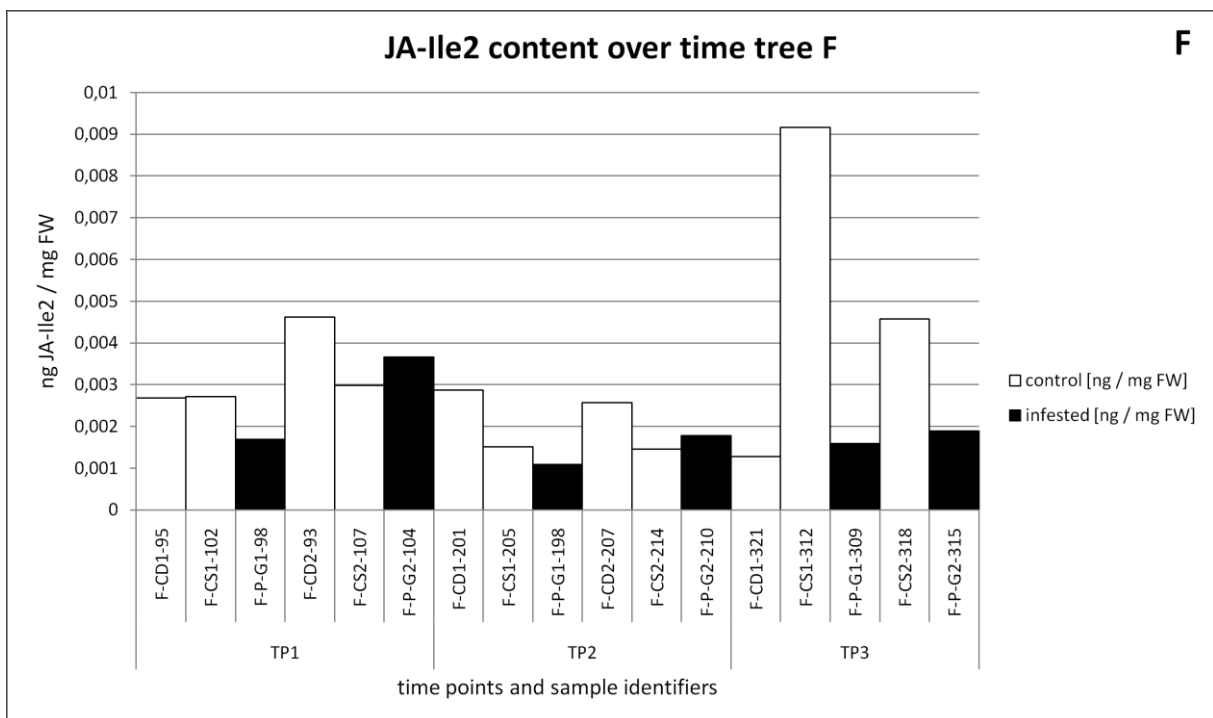
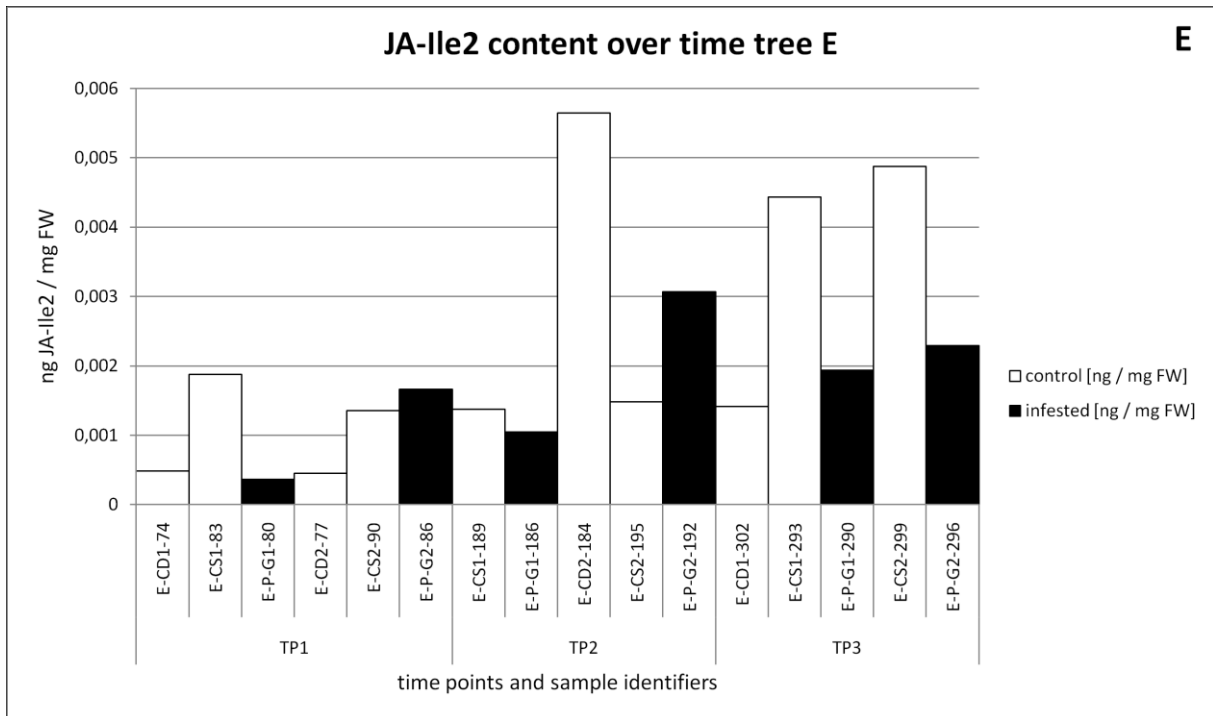


Figure VII.2.1.3.2.1: This figure shows the levels of JA-Ile2 in infested (black bars) and non-infested (empty bars) leaf blades of the individual sample trees. Panel A shows levels in leaf blades of tree A, B in tree B, C in tree C, D in tree D, E in tree E and F shows the levels in leaf blades of tree F. Abbreviations: CS = control same, CD = control different and P-G = Pemphigus Gall. All data obtained from CD leaves was not considered in the main body of the work and positions 1 and 2 were pooled in the main work as no statistical differences were observed.

VII.2.1.3.3 RATIO OF JA-ILE1 / JA-ILE2

This ratio was formed to get a better idea whether the changes seen in levels of JA, JA-Ile1 or JA-Ile2 correspond to changes in the ratio of the active signaling compound, JA-Ile2 and the inactive form JA-Ile1.

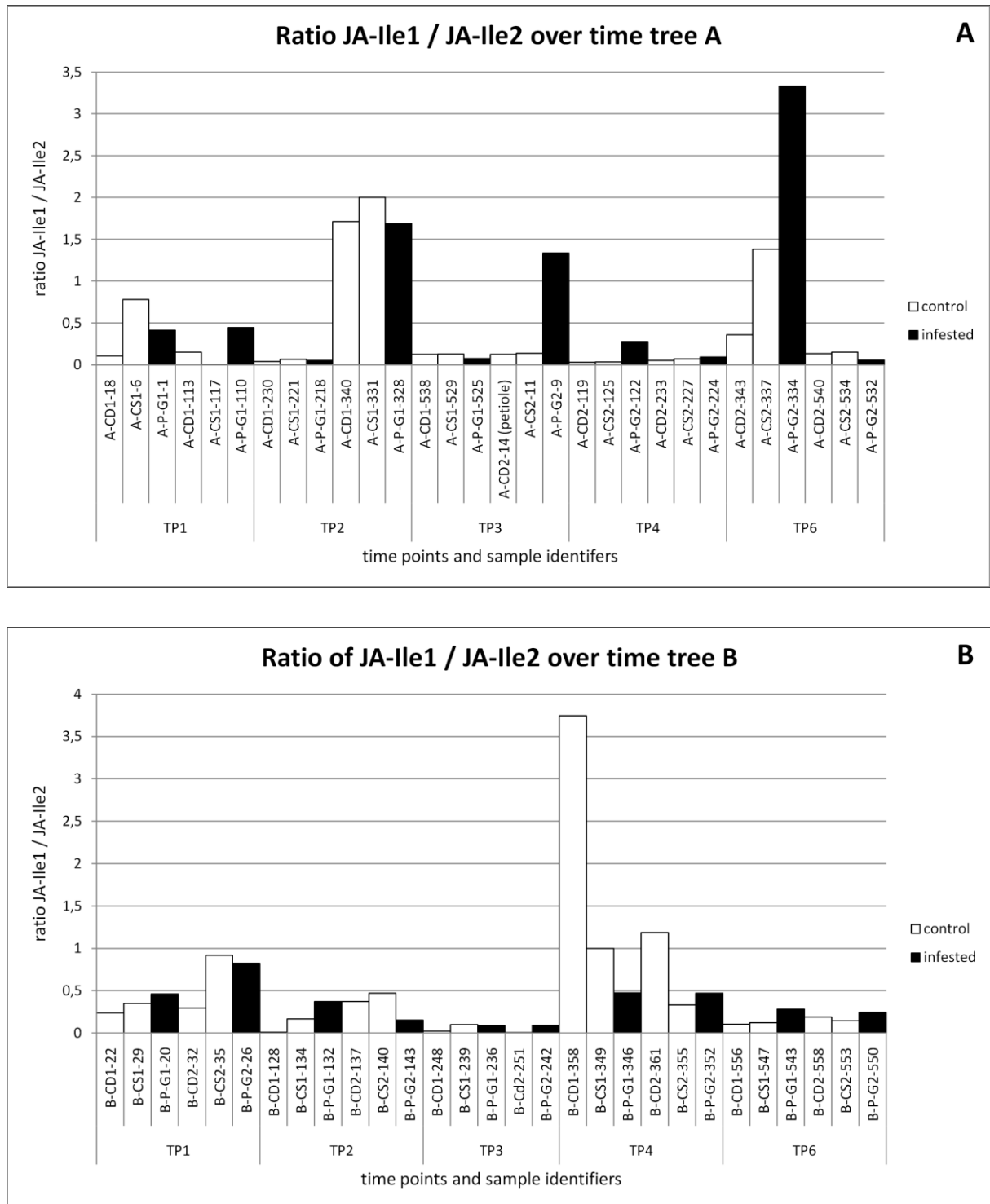


Figure VII.2.1.3.3.1: continued on the next page, see legend at the end of this figure

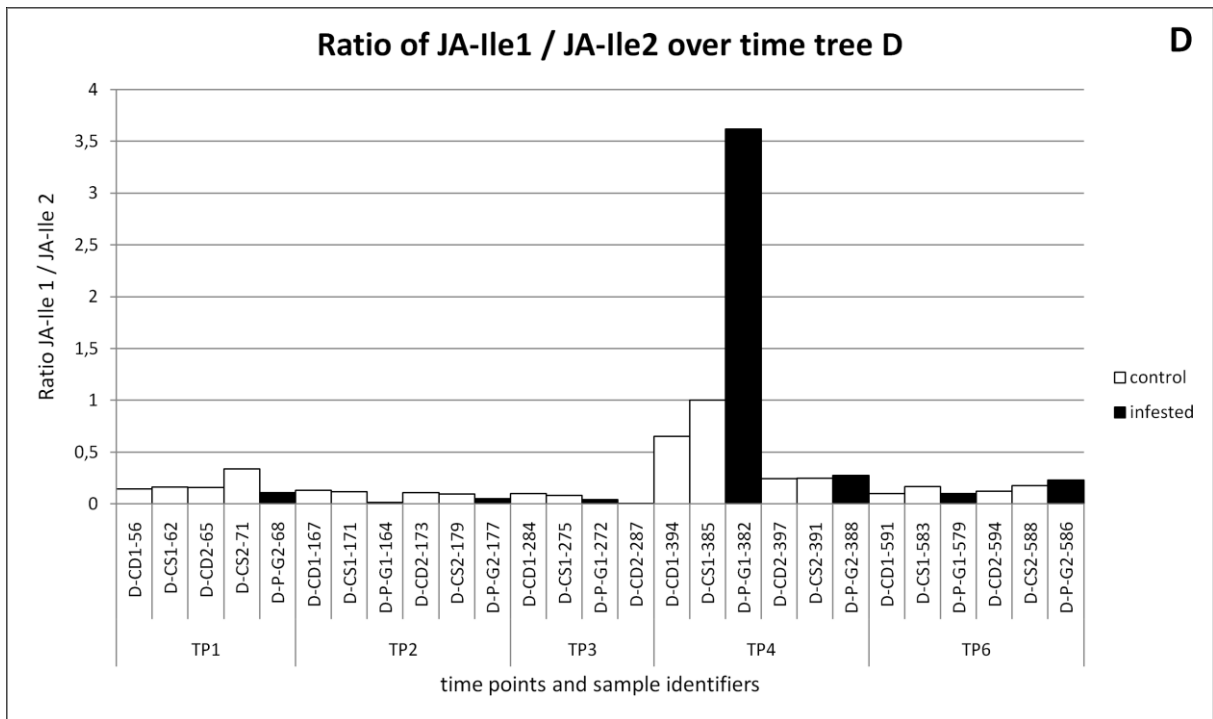
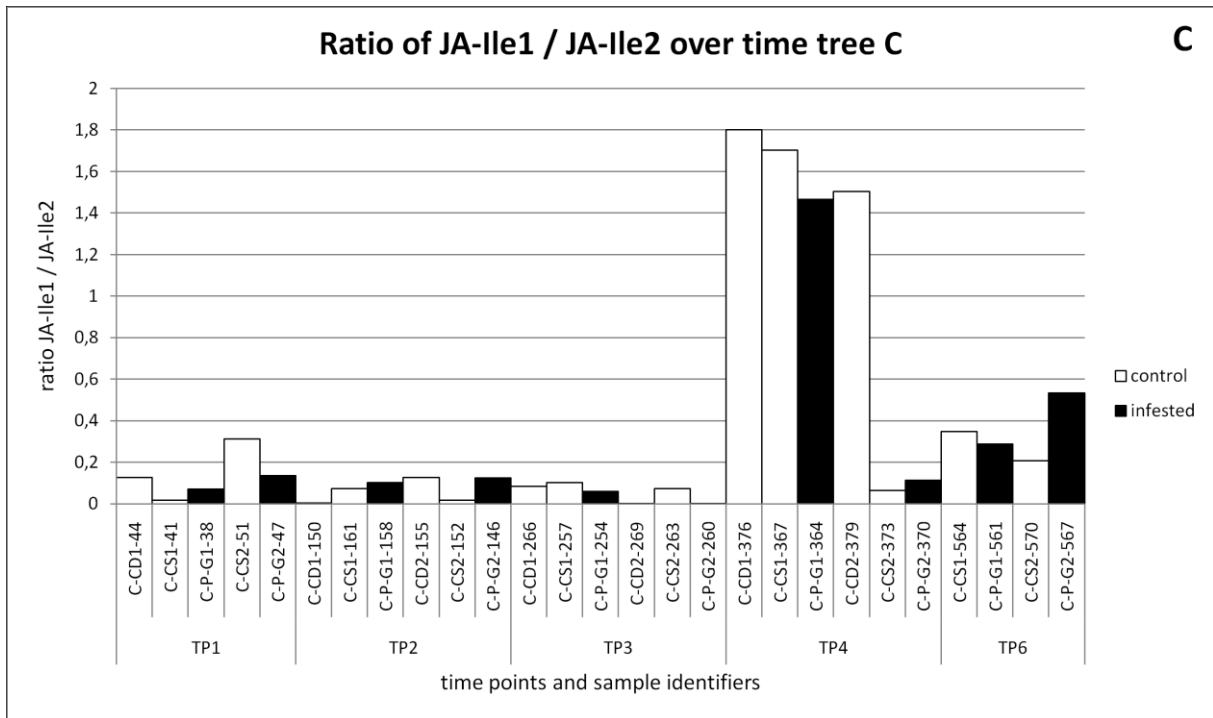


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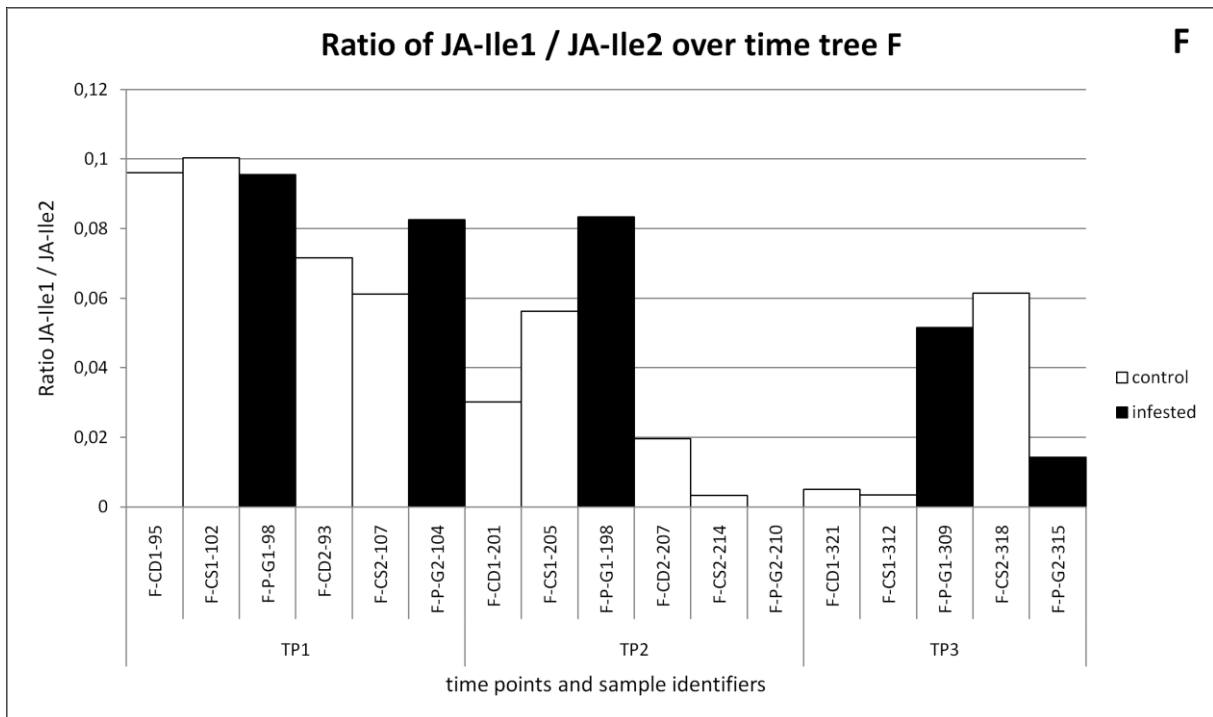
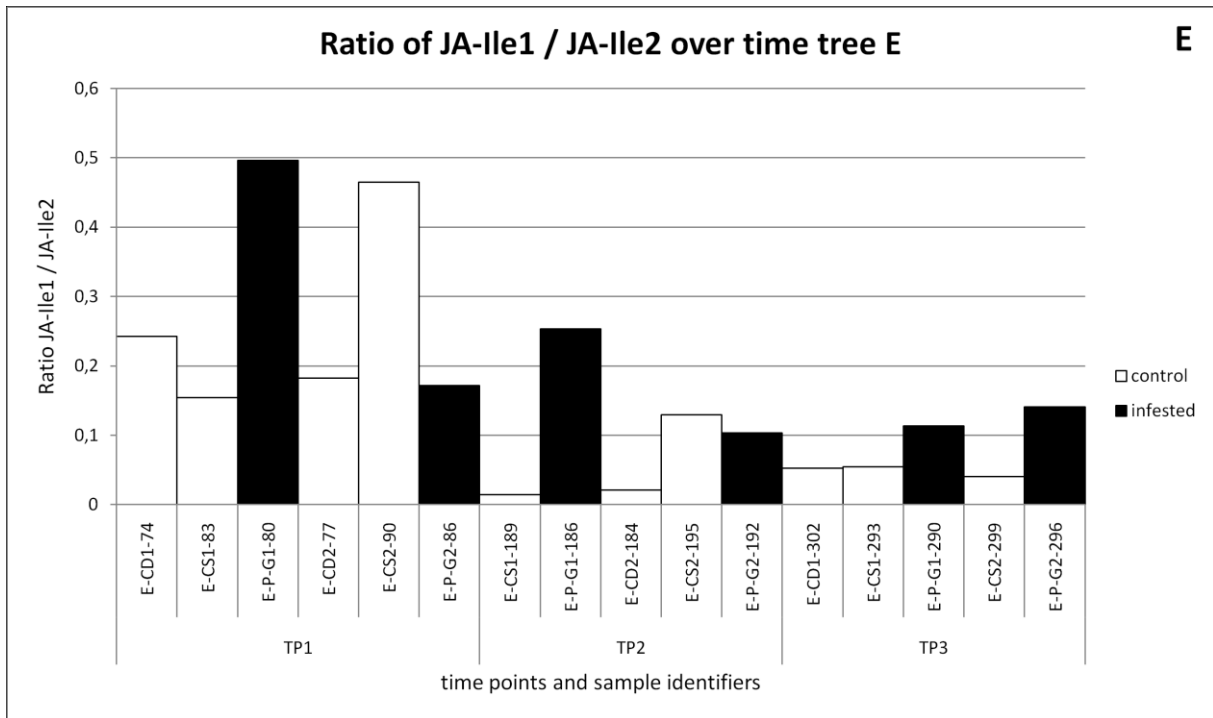


Figure VII.2.1.3.3.1: This figure shows the levels of JA-Ile2 in infested (black bars) and non-infested (empty bars) leaf blades of individual trees; levels found in tree A are shown in panel A, of tree B in B, of tree C in C, of tree D in D, of tree E in E and of tree F in F (from top to bottom). Abbreviations: CS = control same, CD = control different and P-G = Pemphigus Gall. All data obtained from CD leaves was not considered in the main body of the work and positions 1 and 2 were pooled in the main work as no statistical differences were observed.

VII.2.2 PHENOLIC GLYCOSIDES

VII.2.2.1 SALICIN

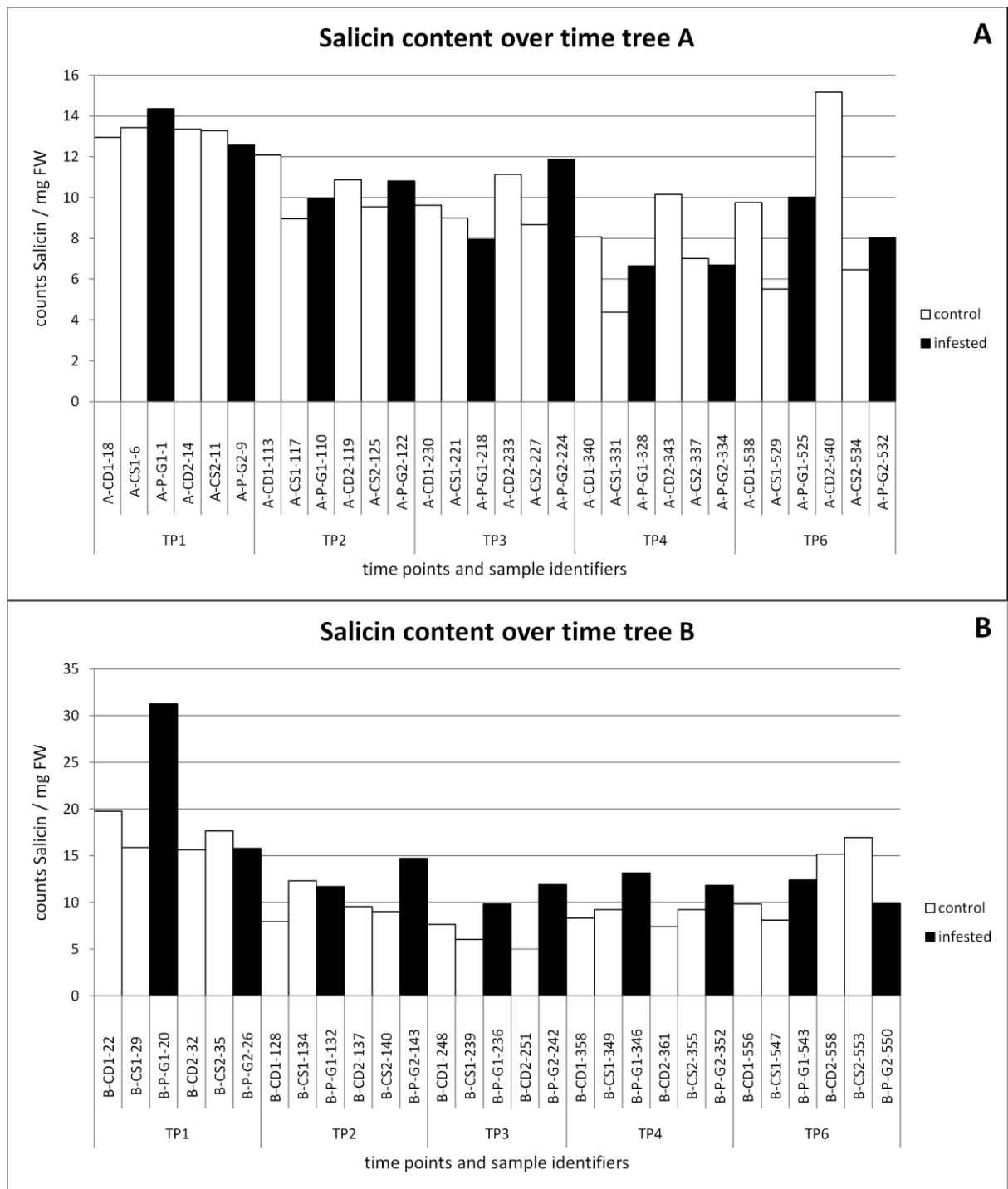


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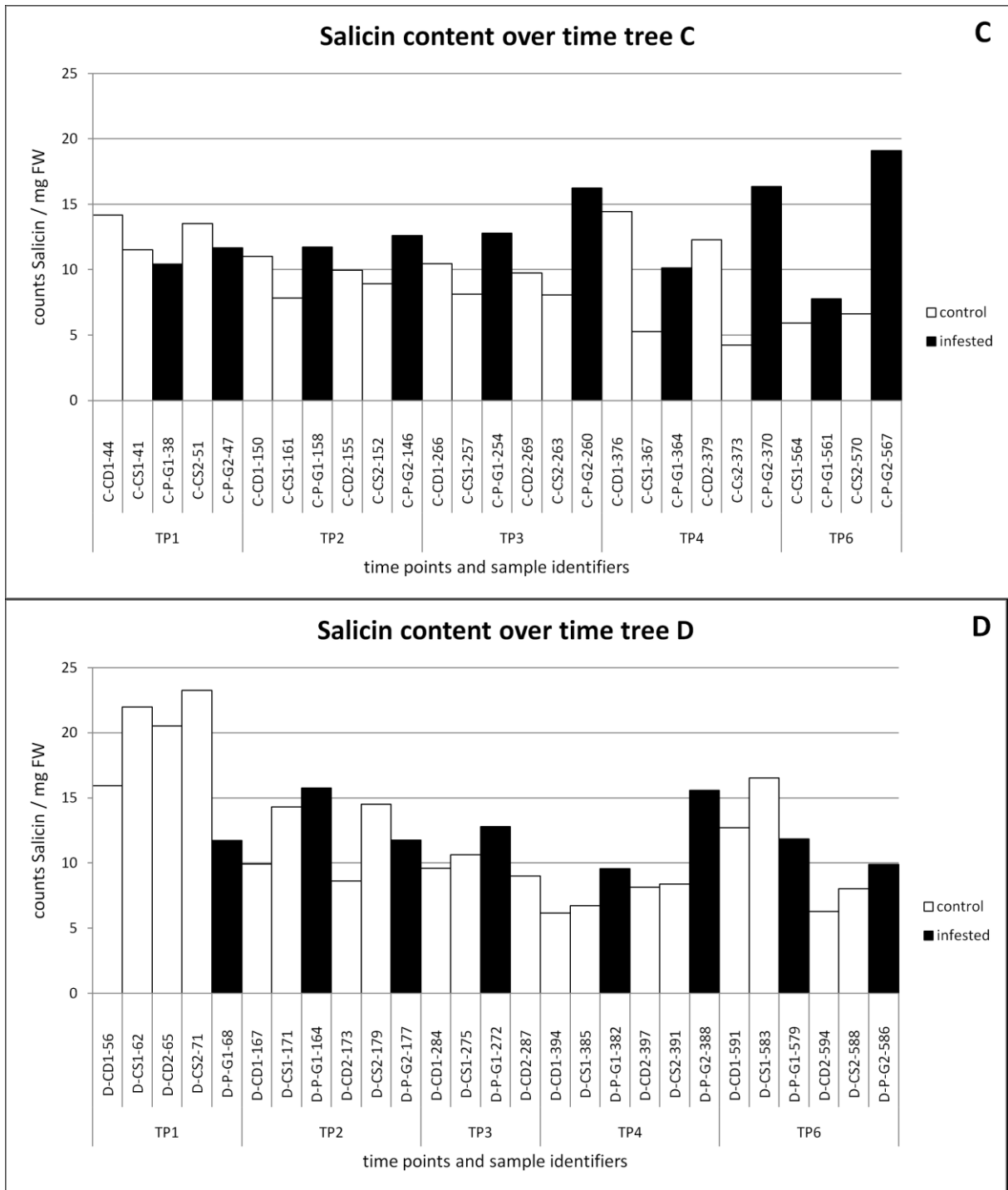


Figure VII.2.2.1.1: continued on the next page, see legend at the end of this figure

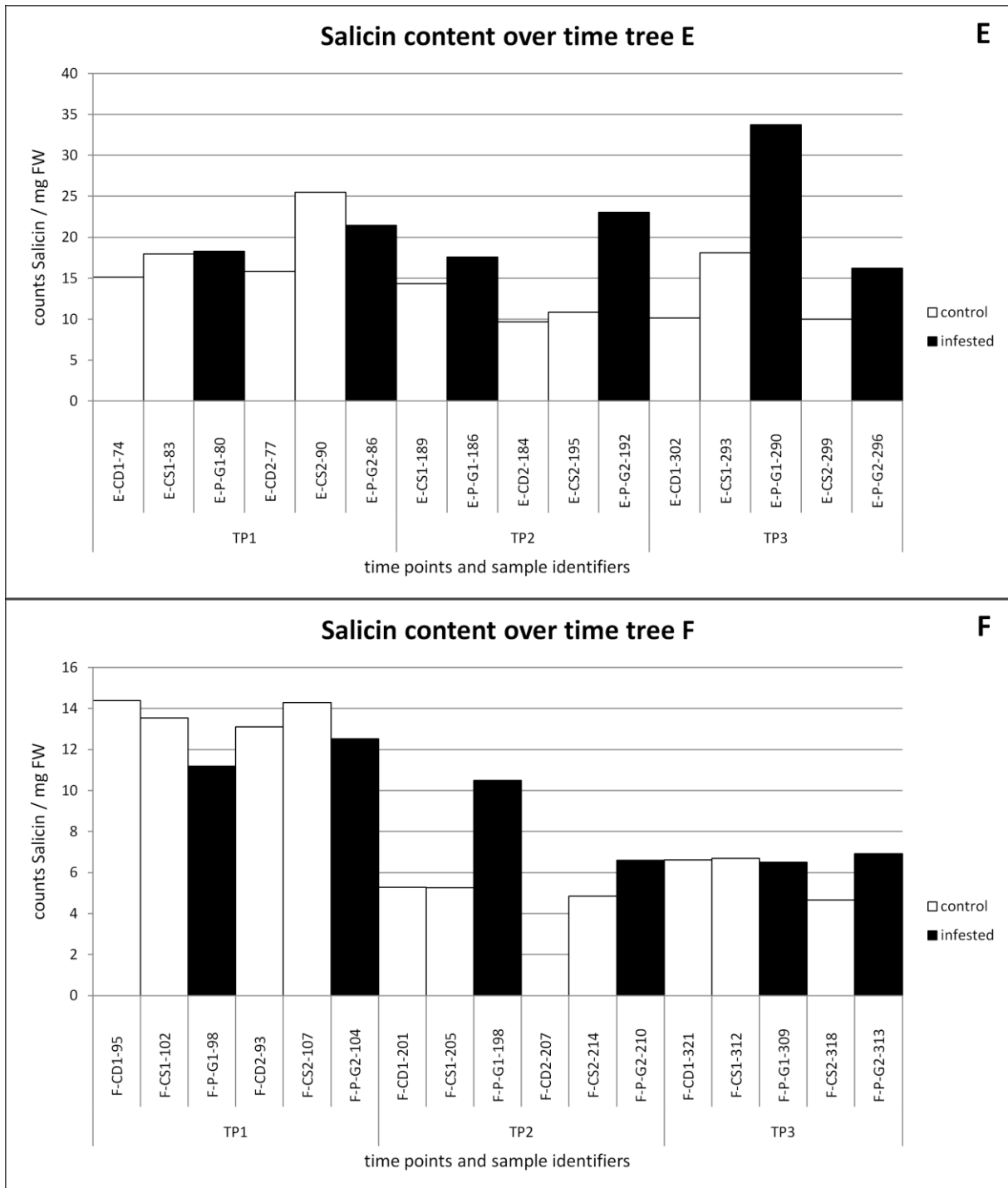


Figure VII.2.2.1.1: This figure shows the levels of Salicin measured in leaf blades of the indicated individual tree. Panel A shows levels in leaf blades of tree A, panel B those of tree B, C of tree C, D of tree D, E of tree E and F shows Salicin levels of leaf blades in tree F. Levels are shown in infested (black bars) and non-infested (empty bars) leaf blades. Abbreviations: CS = control same, CD = control different and P-G = Pemphigus gall. All data obtained from CD leaves was not considered in the main body of the work and positions 1 and 2 were pooled in the main work as no statistical differences were observed.

VII.2.2.2 SALICORTIN

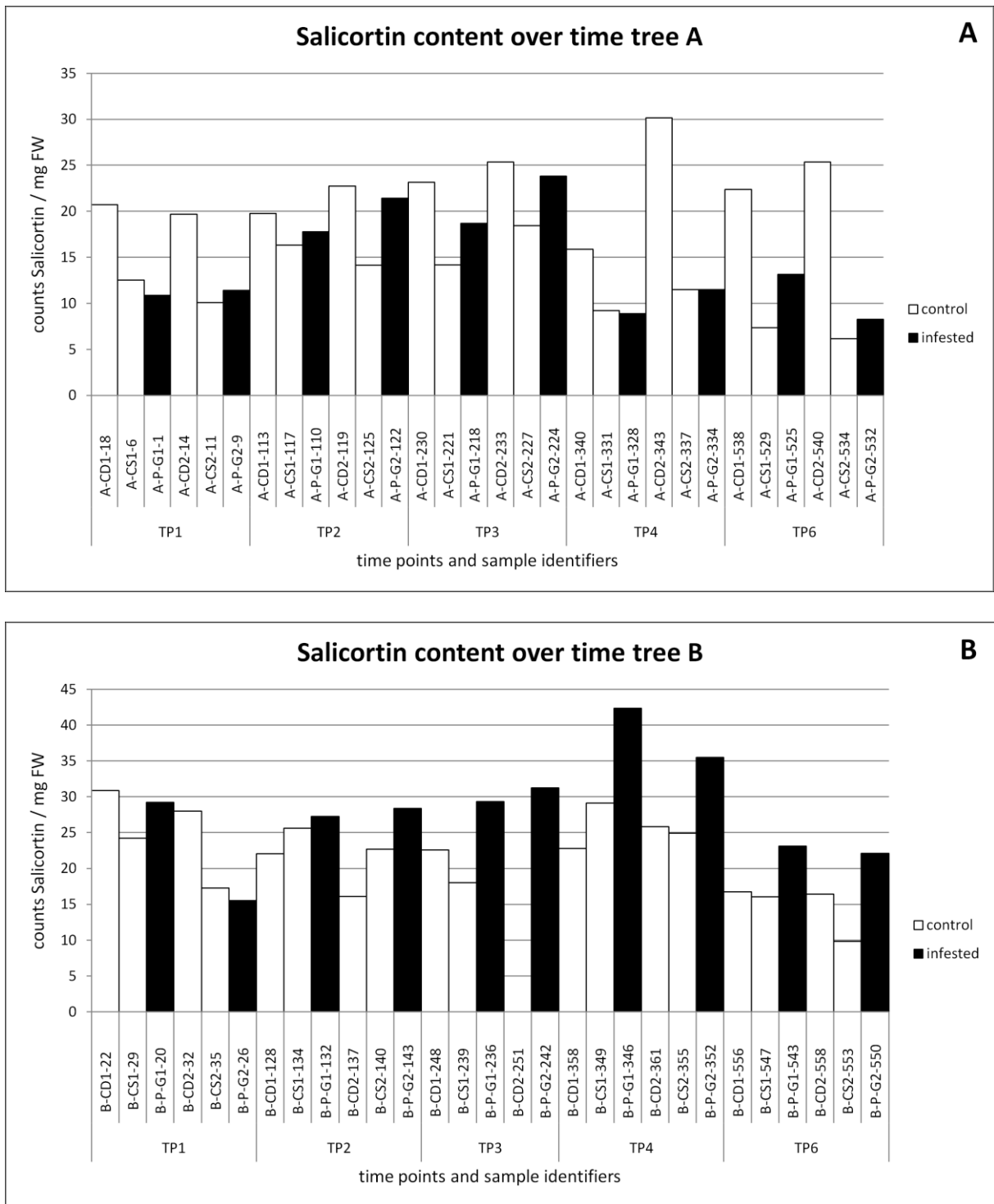


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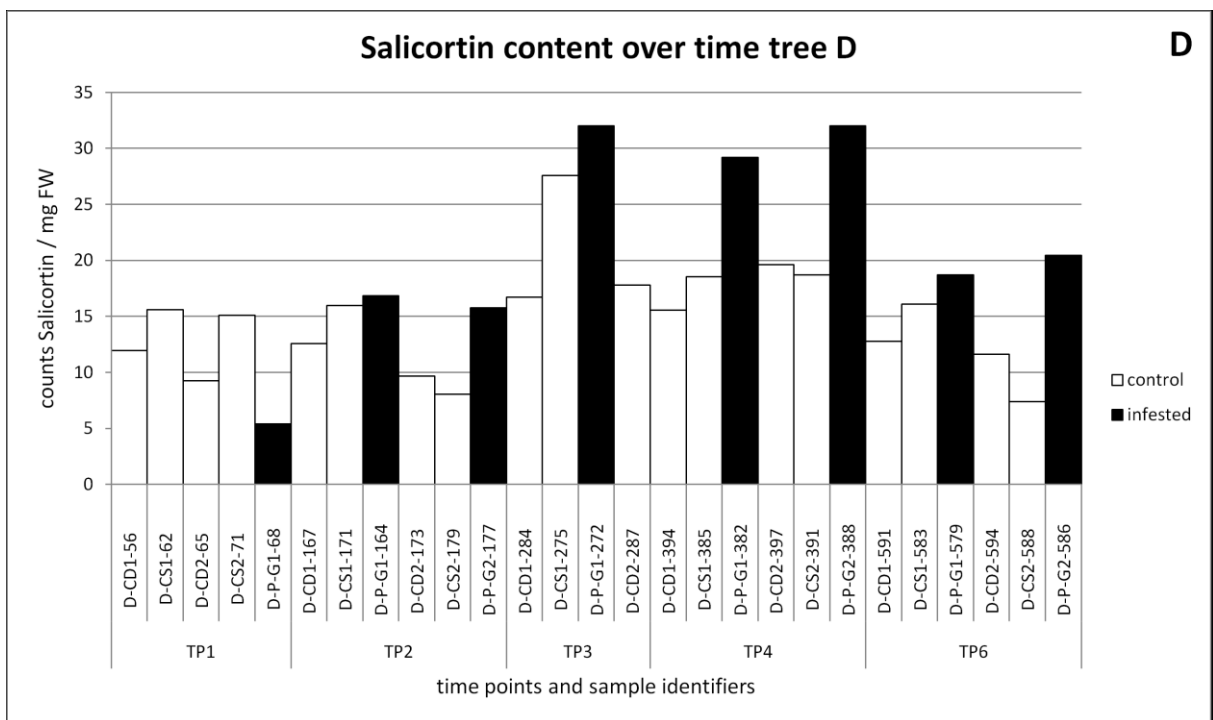
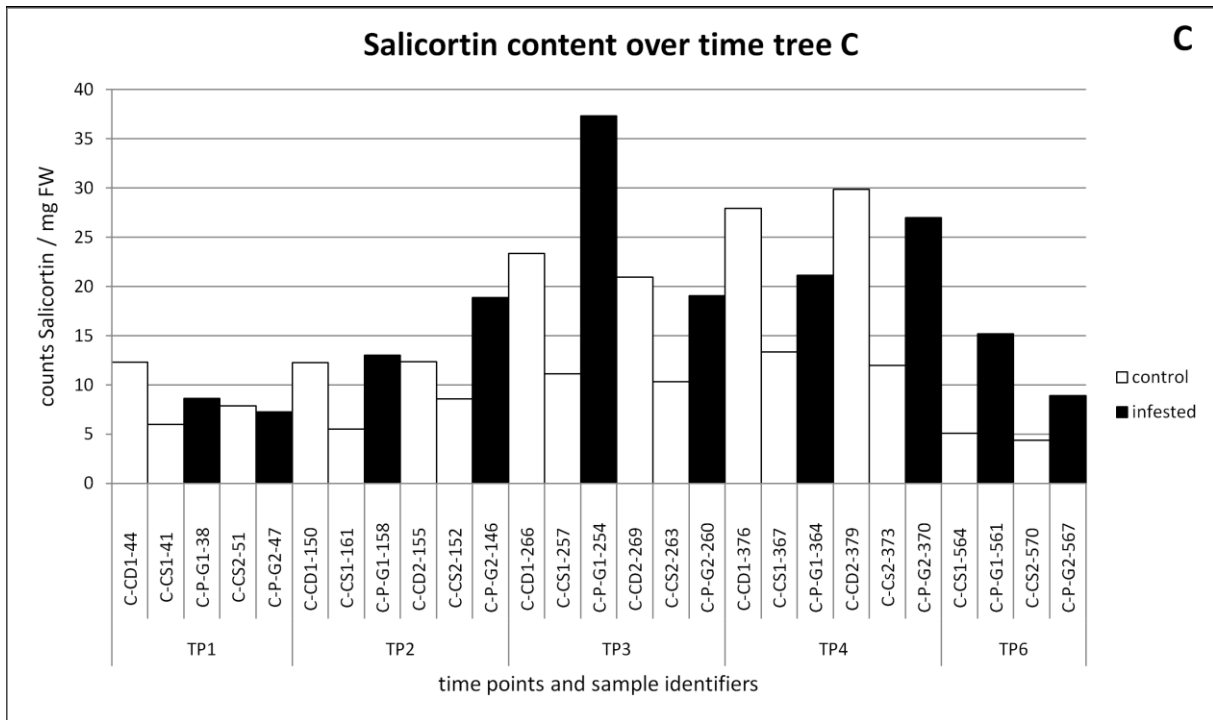


Figure VII.2.2.2.1: continued on the next page, see legend at the end of this figure

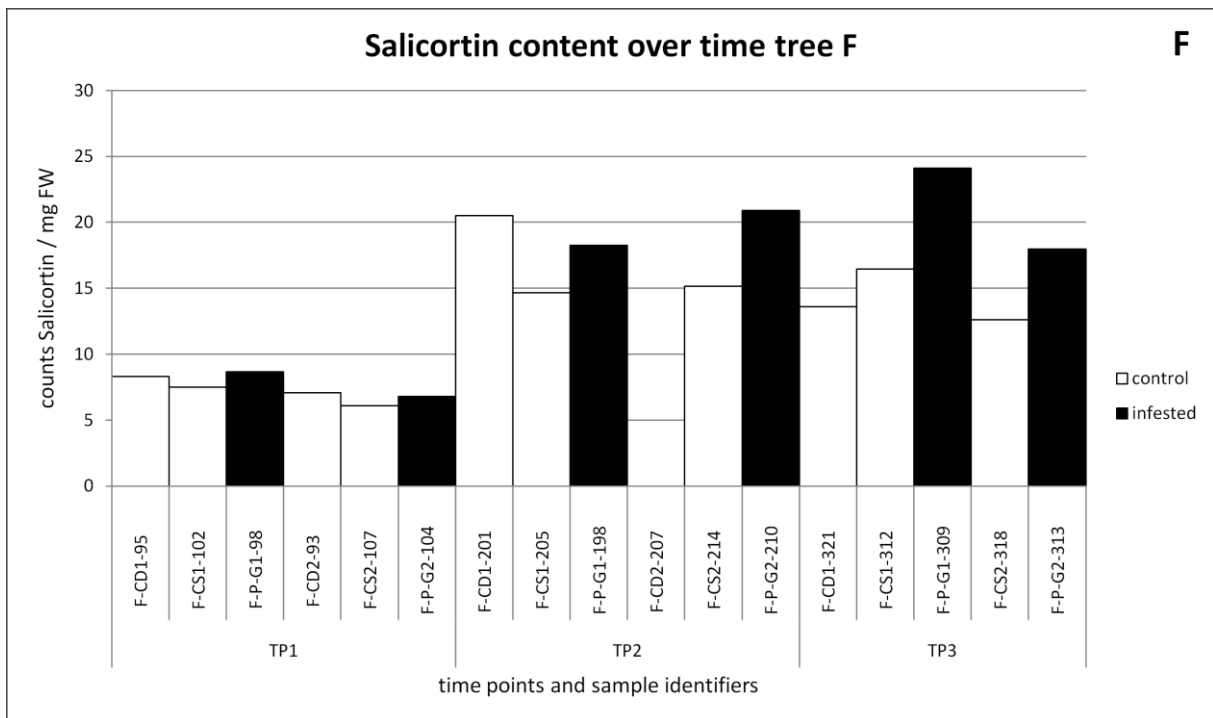
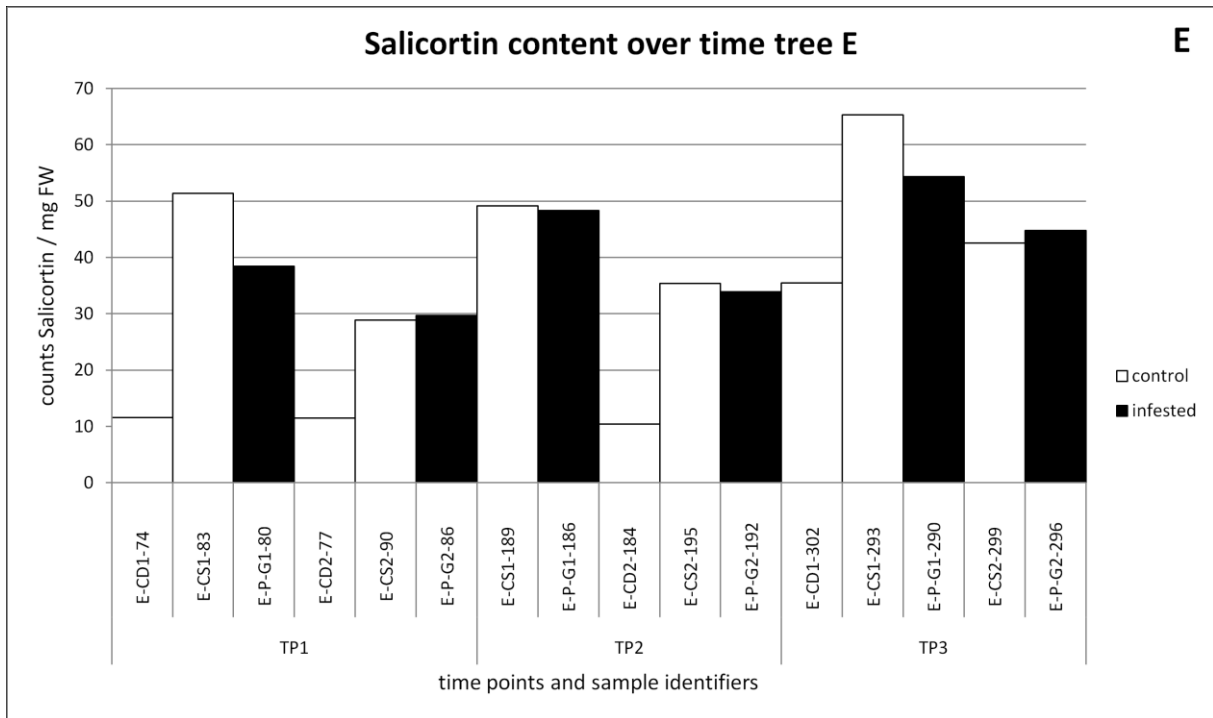


Figure VII.2.2.2.1: The graphs depicted here show levels of Salicortin in individual trees. The panels A-F show levels of Salicortin in infested (black bars) and non-infested (empty bars) leaf blades of trees A-F from top to bottom. Abbreviations: CS = control same, CD = control different and P-G = Pemphigus gall. All data obtained from CD leaves was not considered in the main body of the work and positions 1 and 2 were pooled in the main work as no statistical differences were observed.

VII.2.2.3 NIGRACIN

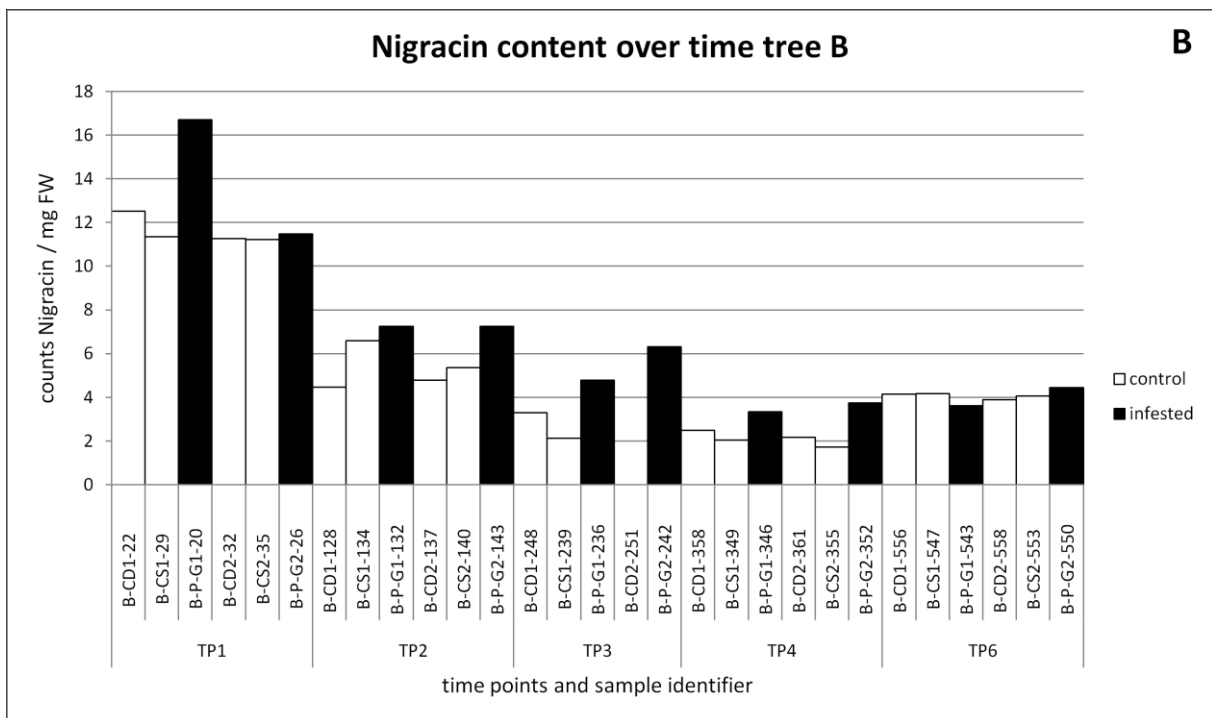
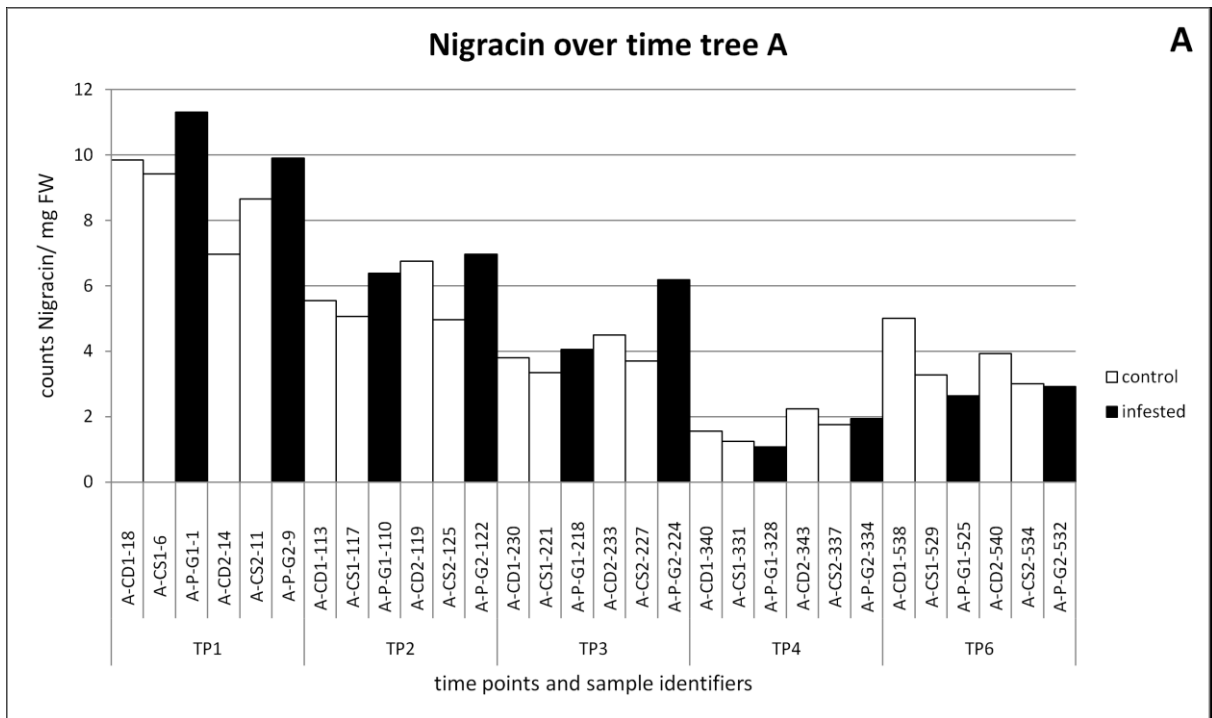


Figure VII.2.2.3.1: continued on the next page, see legend at the end of this figure

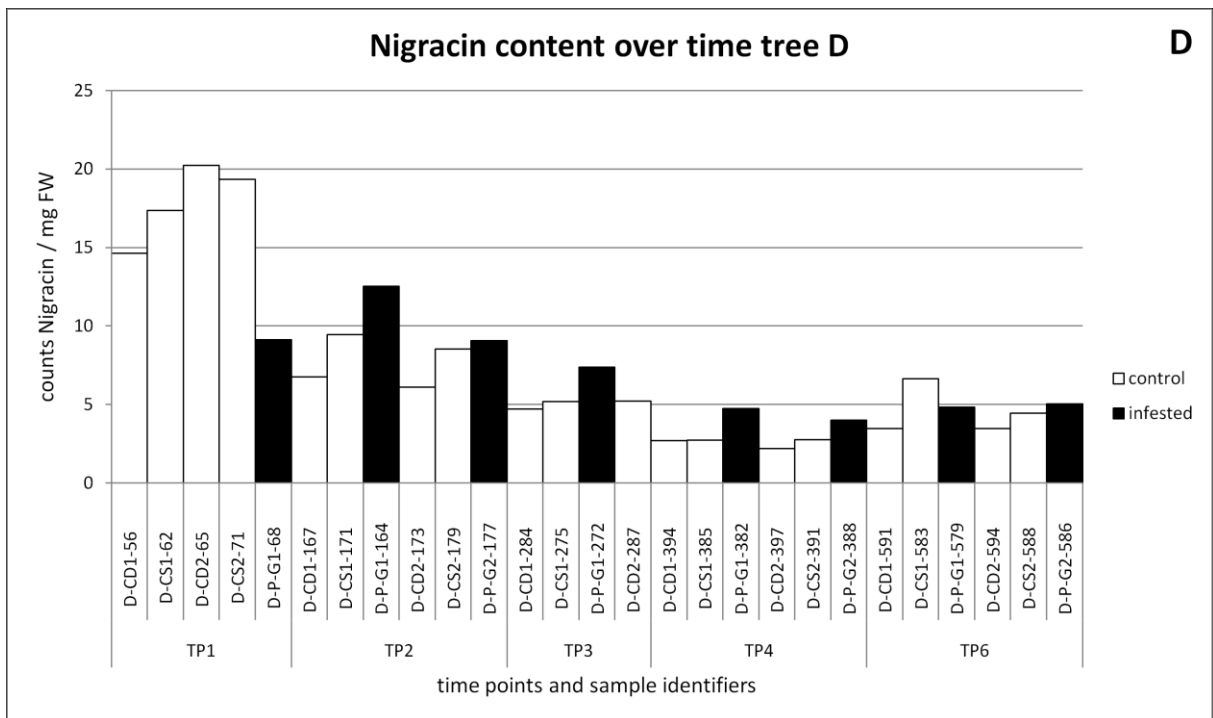
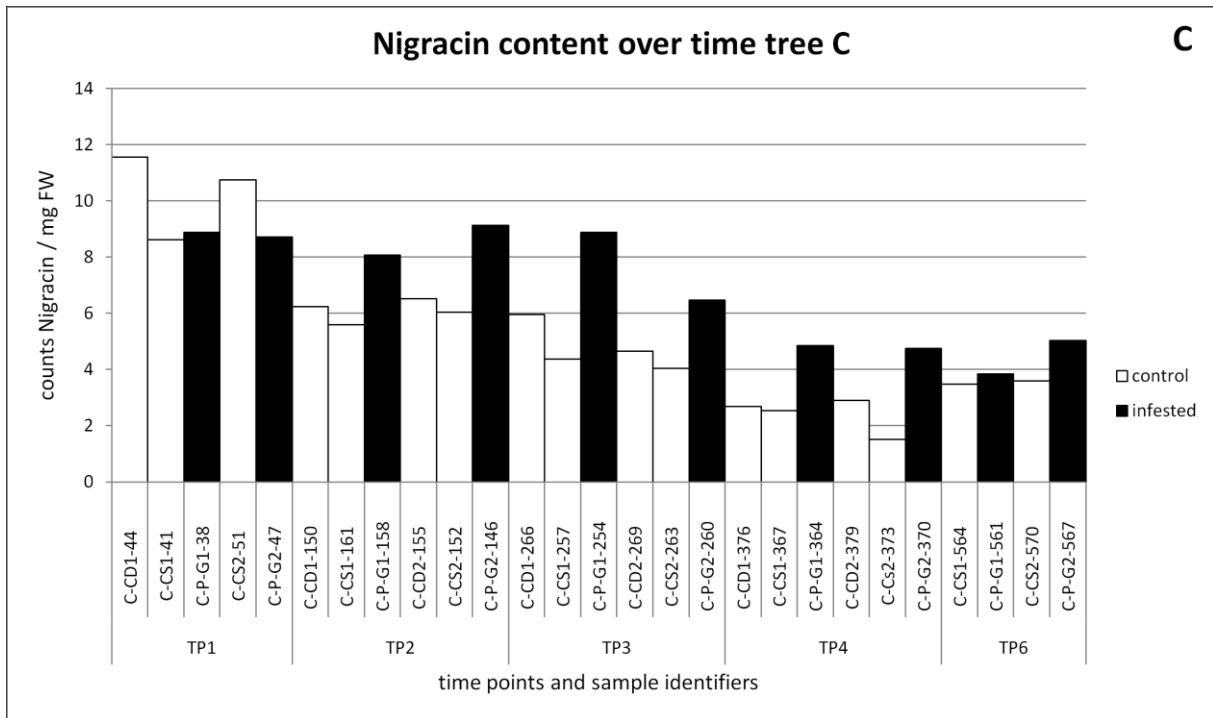


Figure VII.2.2.3.1: continued on the next page, see legend at the end of this figure

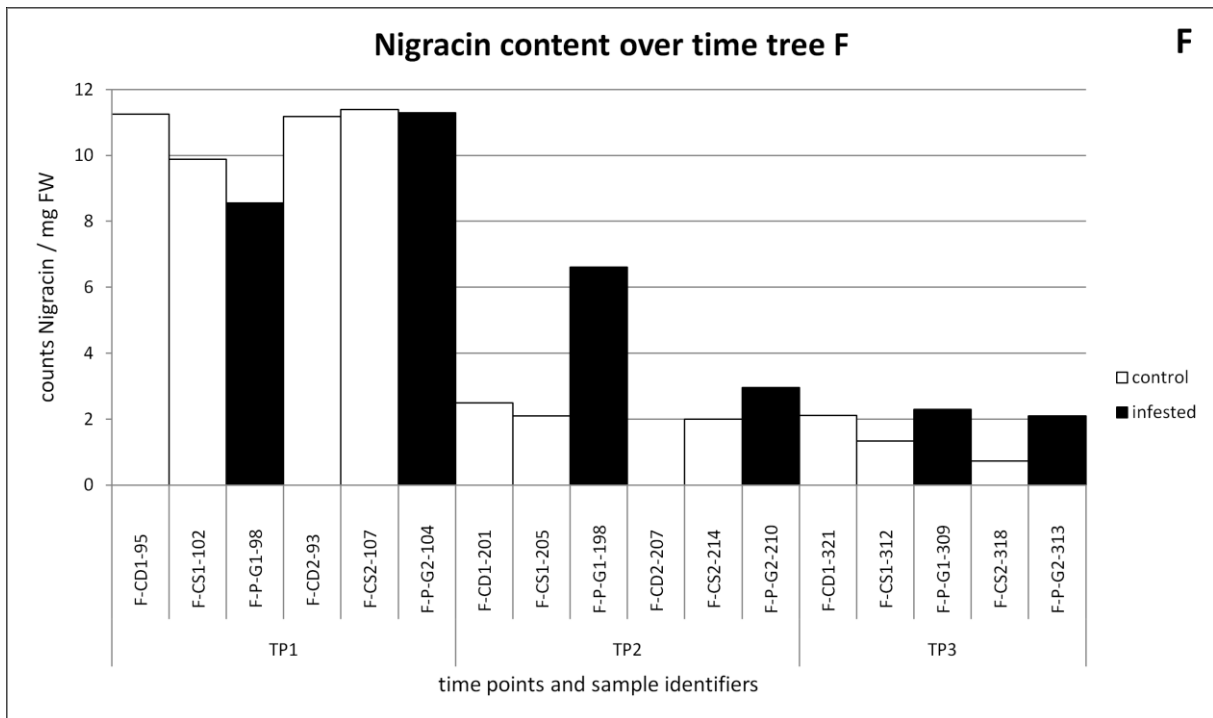
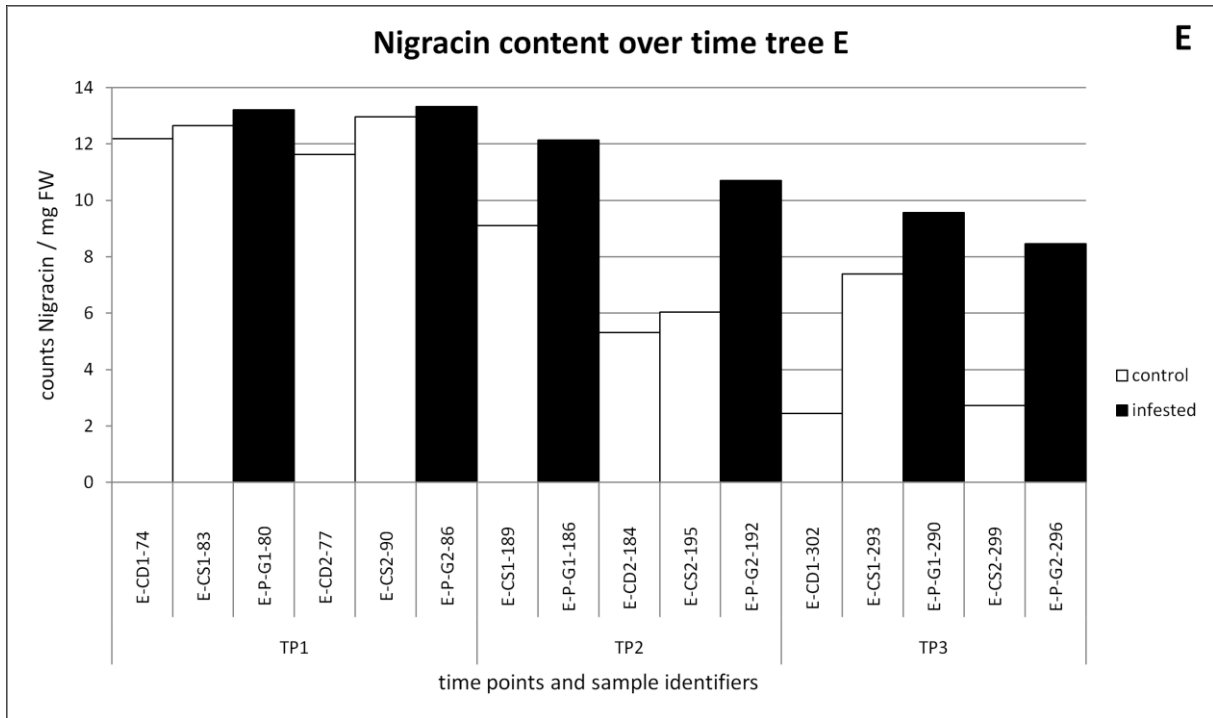


Figure VII.2.2.2.1: The six panels A-F depicted above show the levels of Nigracin in infested (black bars) and non-infested (empty bars) leaf blades of individual trees A-F (from top to bottom). Abbreviations: CS = control same, CD = control different and P-G = Pemphigus gall. All data obtained from CD leaves was not considered in the main body of the work and positions 1 and 2 were pooled in the main work as no statistical differences were observed.

VII.2.2.4 HOMALUSIDE D

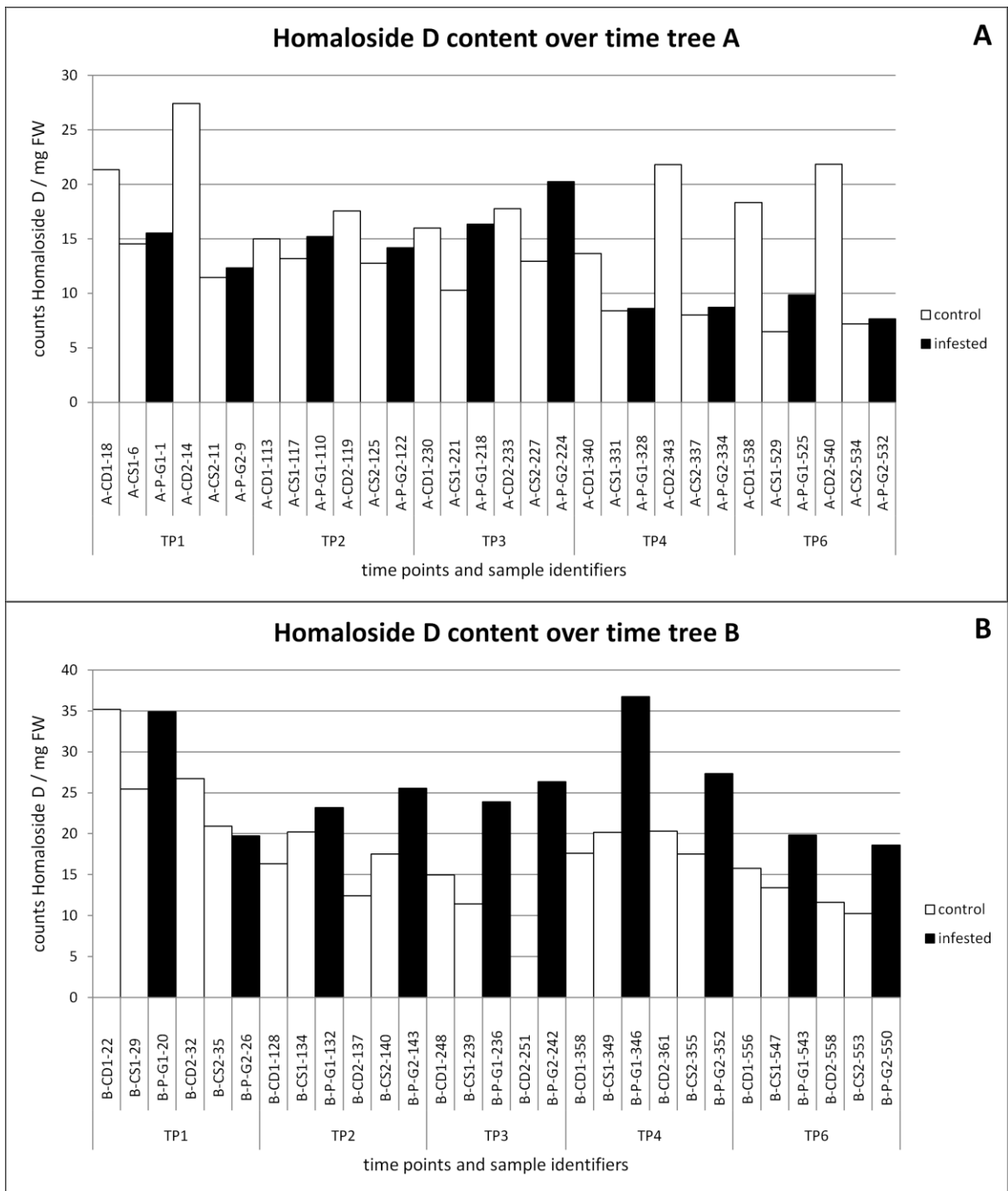


Figure VII.2.2.4.1: continued on the next page, see legend at the end of this figure

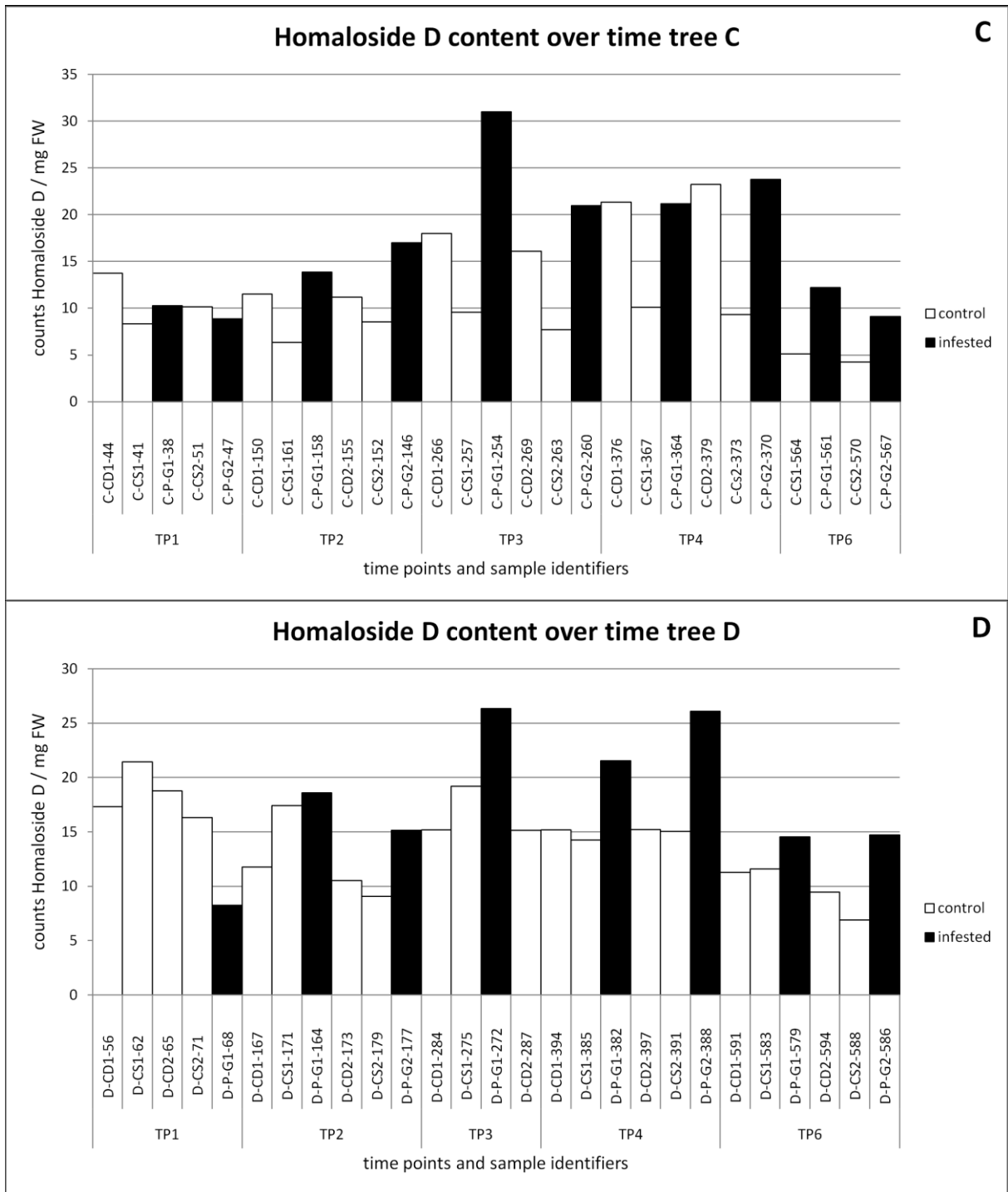


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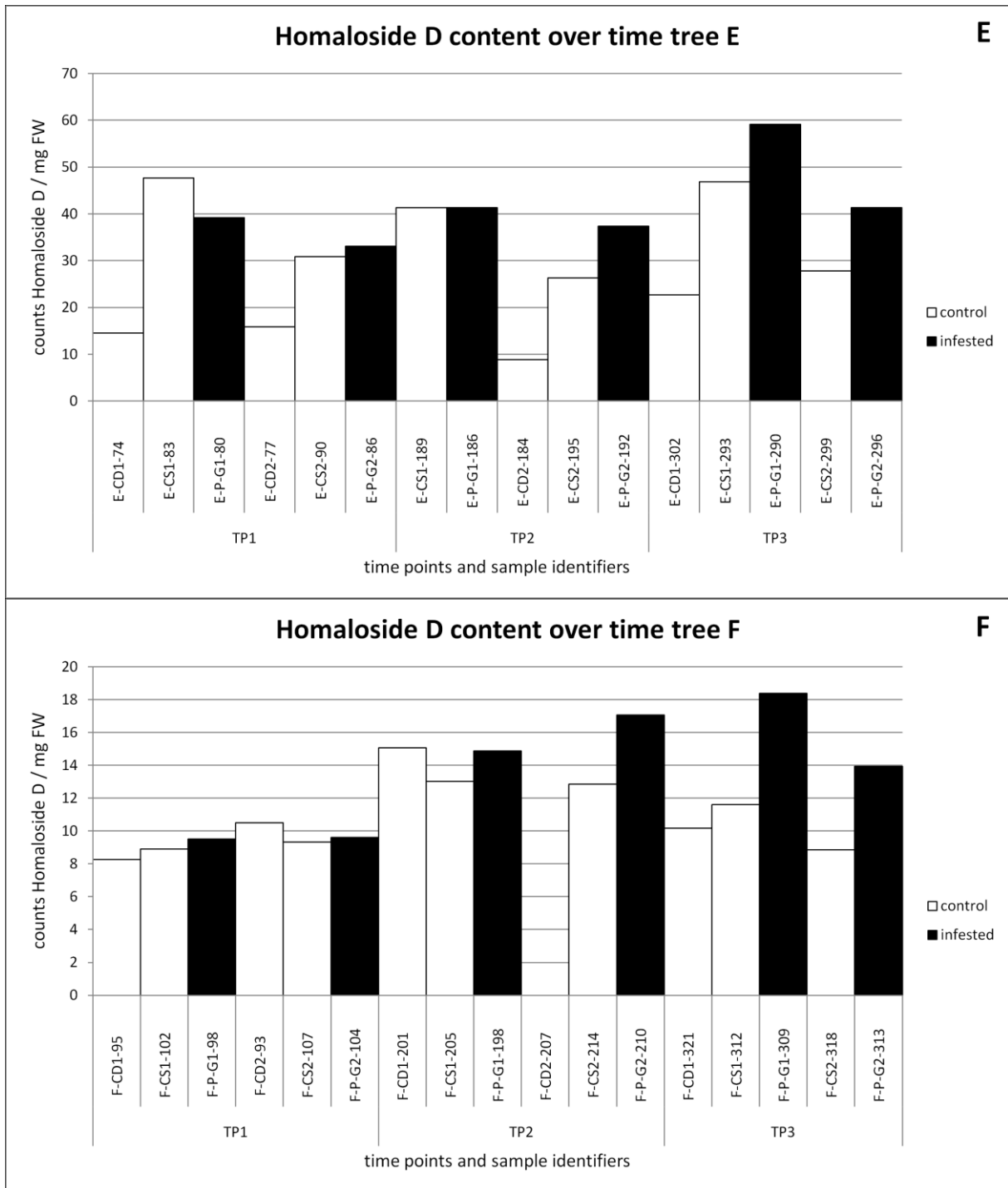


Figure VII.2.2.4.1: In this figure, the levels of Homalosite D in leaf blades of individual trees are shown. From top to bottom are panels A-F, which represent levels of Homalosite D in infested (black bars) and non-infested (empty bars) leaf blades in trees A-F. Abbreviations: CS = control same, CD = control different and P-G = Pemphigus gall. All data obtained from CD leaves was not considered in the main body of the work and positions 1 and 2 were pooled in the main work as no statistical differences were observed.

VII.2.2.5 TREMULACIN

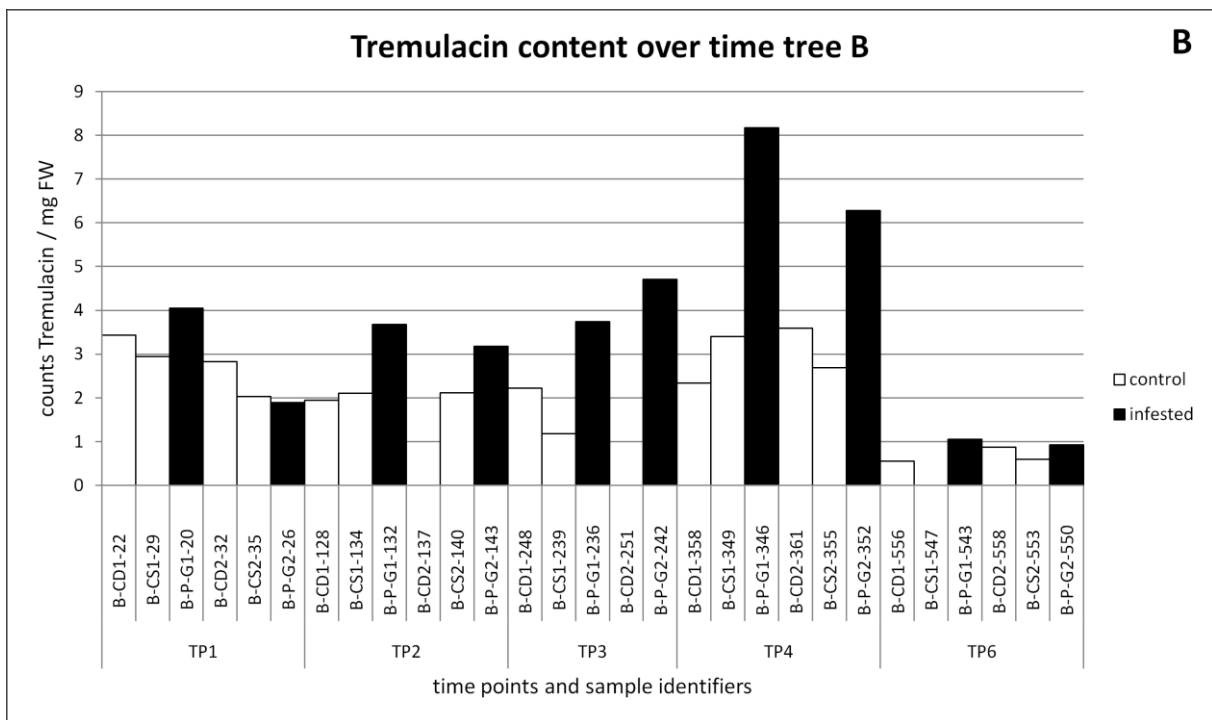
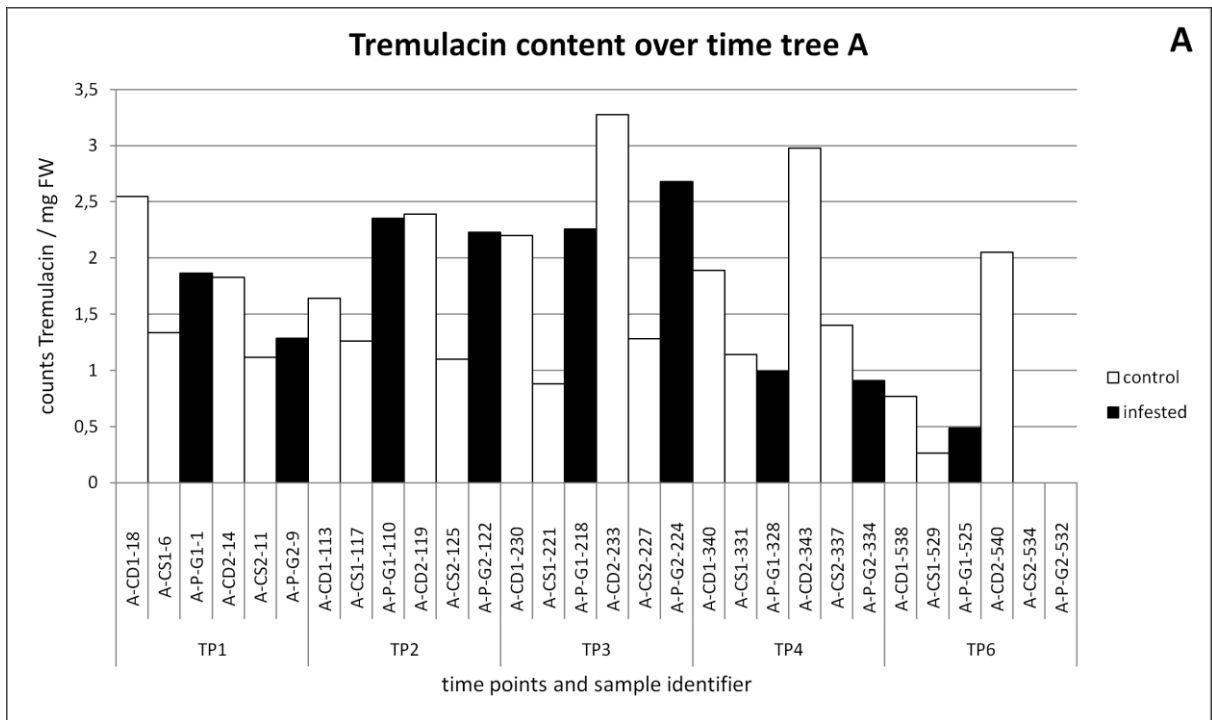


Figure VII.2.2.5.1: continued on the next page, see legend at the end of this figure

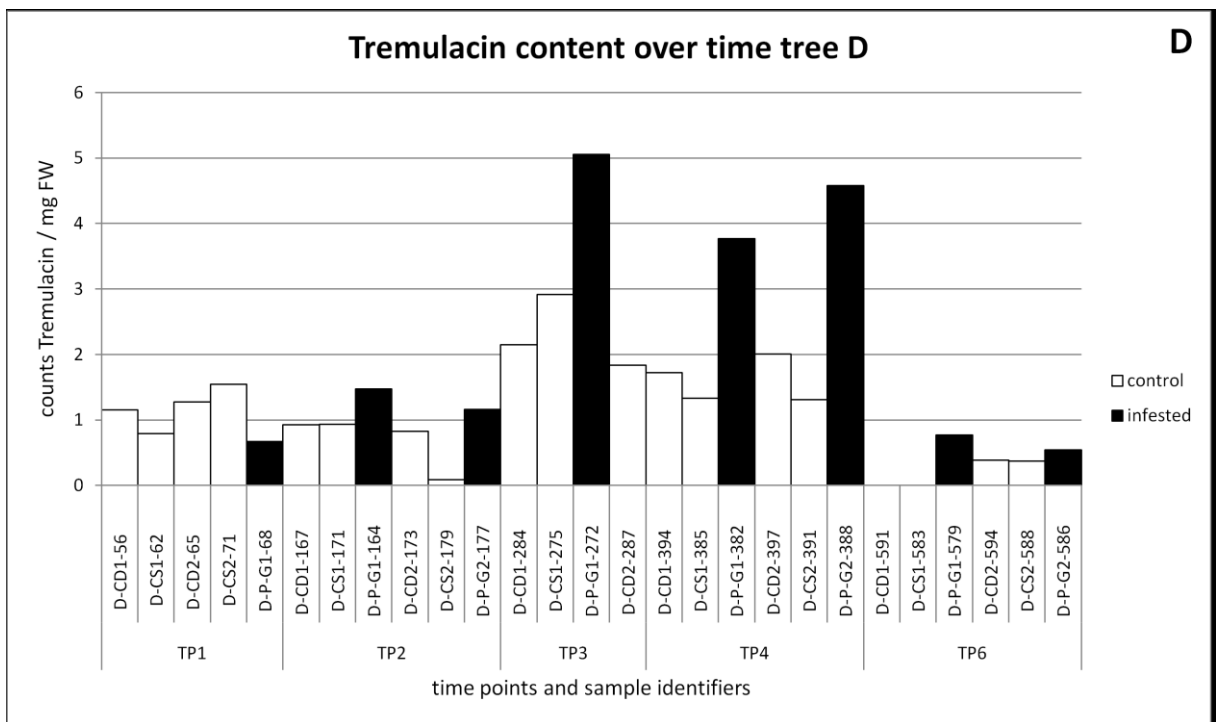
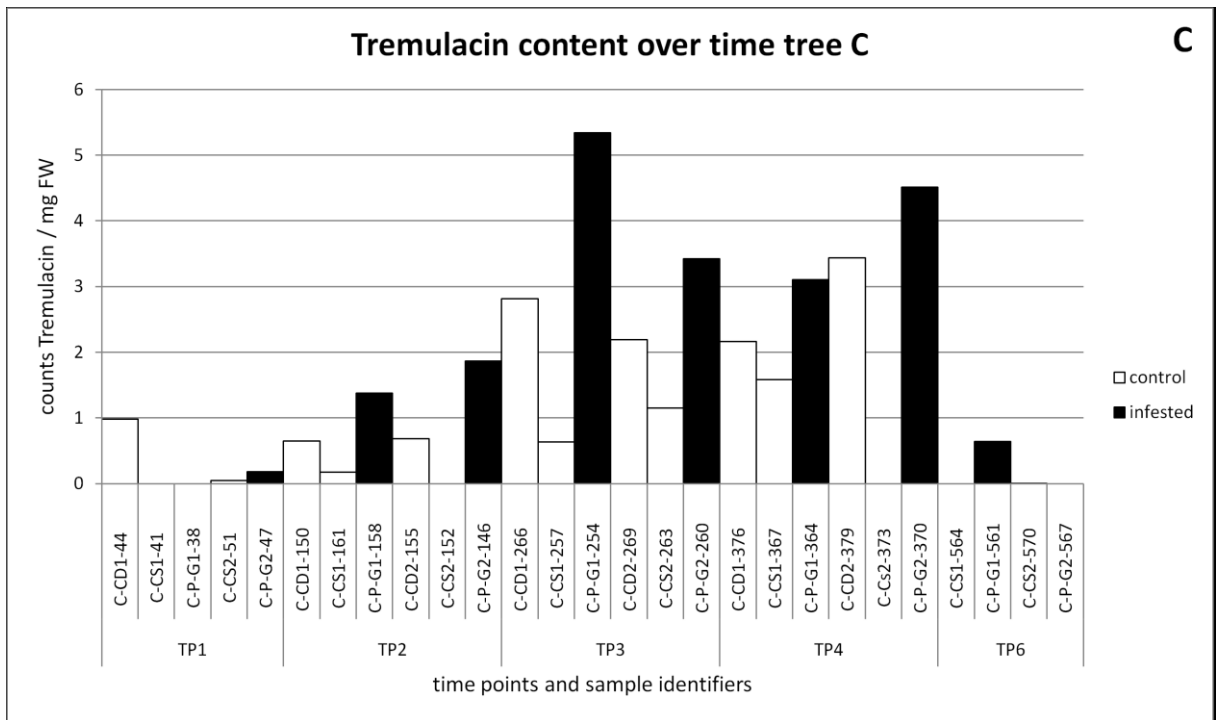


Figure VII.2.2.5.1: continued on the next page, see legend at the end of this figure

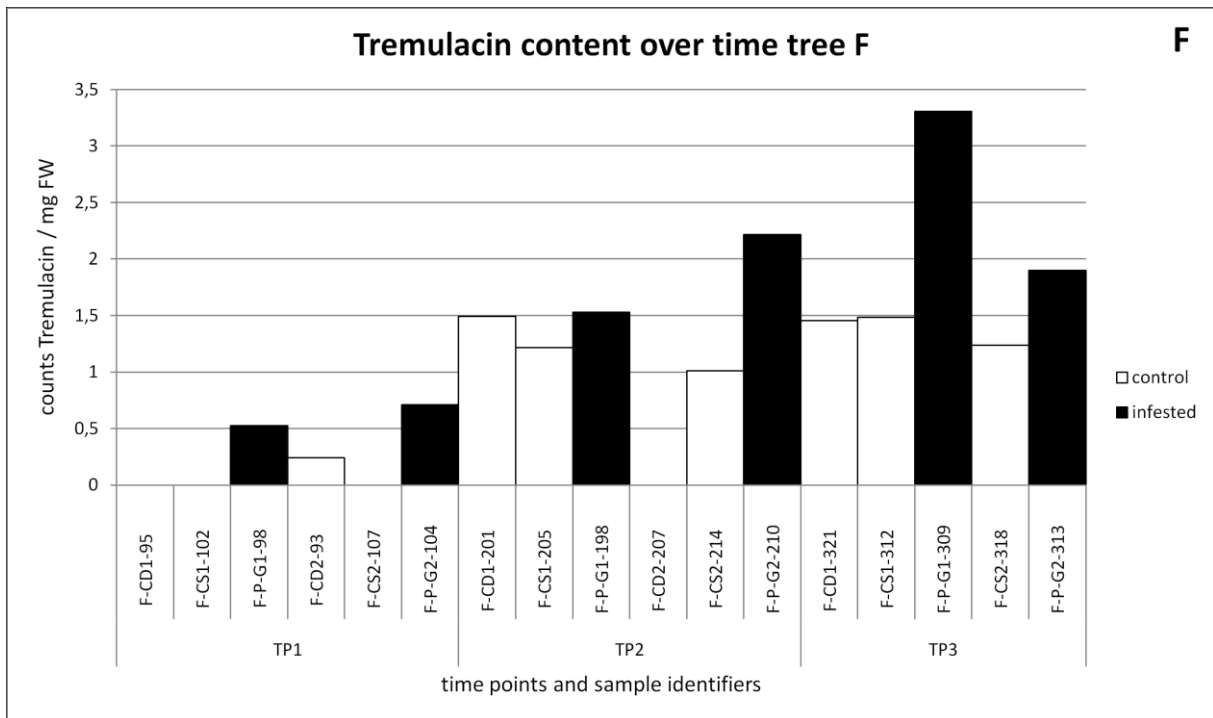
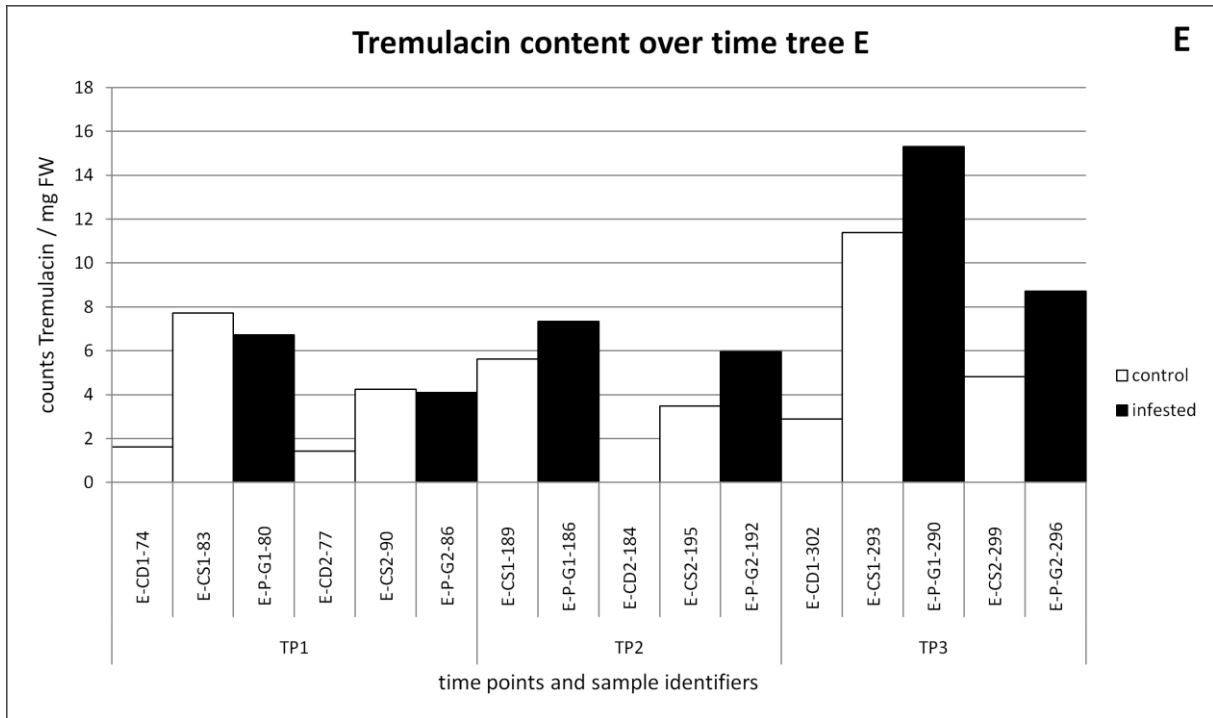


Figure VII.2.2.5.1: This figure shows the levels of Tremulacin in infested (black bars) and non-infested (empty bars) leaf blades of individual trees. Panels A-F denote to trees A-F. Abbreviations: CS = control same, CD = control different and P-G = Pemphigus gall. All data obtained from CD leaves was not considered in the main body of the work and positions 1 and 2 were pooled in the main work as no statistical differences were observed.