CHAPTER 4

RESULTS

The layout of this chapter is divided into the three different tree species examined in this thesis. The diversity in phonologies of these trees, the unpredictable occurrence of their affiliated insect pest and the variety of parameters analyzed, constructed these divisions to be individually shaped. In all results displayed, the observed effects of NeemAzal-T/S characterize the focus of considerations.

4.1 Results on Oak

Due to the removal of Oak processionary moth (OPM) nests at Schwieberdinger Street in the first week of June, data collection at this research site came to an abrupt end. Until the time of nest removal, three applications with the biological insecticide NeemAzal-T/S had been conducted on April 20th, May 5th and May 18th. On May 25th the first and only leaf collection on Oak trees were taken.

Responsible authorities for public green in Stuttgart (Gartenbauamt Stuttgart) decided to remove the OPM nests out of concern for human health. Due to this event, data on Oak is limited and may not show complete pest-insecticide succession.

4.1.1 Results from visual observations on Oak trees

OPM behavior and nest development were observed on five occasions (April 20th, May 5th, 13th, 18th and 25th) (see Table 4.1). This table also shows summarized results from these occasions on Oak, considering OPM processing, OPM nest presence and OPM nest total volume.

Table 4.1: OPM occurrence with mean average and standard deviation

Date	Option	OPM processing	Number of OPM processing	Number of OPM nests	Total OPM nest volume
April 20	Control		0	0	0
April 20	Treated		0	0	0
May 5	Control		0	0	0
May 5	Treated	X	510	0	0
May 13	Control	X	630	12	5898 cm^3
May 13	Treated	X	400	5	2010 cm^3
May 18	Control		0	20	17825 cm^3
May 18	Treated	X	49	10	9325 cm^3
May 25	Control	X	30	17	17075 cm^3
May 25	Treated		0	9	7083 cm^3

		OPM processing	OPM nests	OPM nest volume
Mean average	total	161,9	7,3	5921,6
	control	132	9,8	8159,6
	treated	191,8	4,8	3683,6
Standard deviation	total	249,04	7,50	6948,89
	control	278,69	9,39	8820,11
	treated	244,22	4,76	4281,36

Most of the OPM larvae processing, which describes the style of locomotion they are known for, happened on the observation days May 5th and 13th. Finding only a few OPM larvae processing on the later dates gives reason for the conclusion that they had reached the phase of retreating into the nests and becoming more sessile.

This is also indicated by the highest amounts of OPM nests found on the observation days of May 18th (control 20 nests and treated 10 nests) and May 25th

(control 17 nests and treated 9 nests). For this analysis, only nests with sedentary larvae were considered, leaving aside not free-ranging or processing larvae. On May 5th, remarkably on treated trees, the first OPM larvae were observed.

Remarkable as well is that on the observation days that followed (May 13th, 18th and 25th) the control always showed higher amounts of OPM presence. During the three observation days, the ratio of control to treated nest volume stayed in a similar range with an average ratio of 2,4:1.

Table 4.2 indicates the significant differences in OPM nest volume between the control and the treated Oak trees on the mentioned observation days.

Table 4.2: OPM nest volume and relations on different observation days

Date	Option	Total OPM nest volume	Relation of control to treated nest volume
May 13	Control	5898 cm^3	2,934
May 13	Treated	2010 cm^3	1
May 18	Control	17825 cm^3	1,912
May 18	Treated	9325 cm^3	1
May 25	Control	17075 cm^3	2,411
May 25	Treated	7083 cm^3	1

Culmination of OPM nest volume happened on May 18th for the control and a few days later for the treated trees. The nest volume ratio stayed similar from the first day of observation, but did decrease some over time. The treated trees had already received two applications of NeemAzal-T/S on May 13th. This was also the case for May 18th.

Until May 25th, the treated trees were receiving three applications of NeemAzal-T/S. Since there were no obvious reasons for the OPM preferably

infesting the control trees rather than the treated ones, the conclusion may be that for counteracting the tendency of relative increase of OPM infestation also on the treated trees, it seems that the NeemAzal-T/S application intervals should have been shorter than two weeks for effective OPM pest control.

So far, only the total nest volume had been under observation for analysis, whereas the single nest volume of treated trees compared to the control on the different observation days is of interest as well. The development of free-ranging larvae, nest volume and number of nests per tree is shown in Table 4.3.

Table 4.3: OPM nest volume per tree and observation day

		May 5			May 13			May 18			May 25		
		Free-range	Nest	Number	Free-range	Nest	Number	Free-range	Nest	Number	Free-range	Nest	Number
Tree #	Option	larvae	volume	of nests	larvae	volume	of nests	larvae	volume	of nests	larvae	volume	of nests
56	treated												
55	treated	*180*		2		1000 cm³	1	*29*	1250 cm³	2		1250 cm³	1
54	treated												
52	control				*130*		1		1875 cm³	1		1875 cm³	1
51	control					900 cm³	3		2150 cm³	4	*30*	2700 cm³	4
50	control				*100*	500 cm³	2		2250 cm³	2		2250 cm³	2
48	treated				*200*	750 cm³	2	*20*	4275 cm³	5		3583 cm³	6
47	treated	*330*		4		260 cm³	3		1200 cm³	3		750 cm³	1
46	treated								2000 cm³	1		1500 cm³	1
45	treated				*200*		1		600 cm³	1			
44	control					2148 cm³	4		3225 cm³	4		4000 cm³	3
43	control				*300*	1500 cm³	2		6875 cm³	6		5000 cm³	5
42	control				*100*	100 cm³	3						
41	control					750 cm³	1		1450 cm³	3		1250 cm³	3

April 20th is not considered in this table, since no free-ranging larvae or OPM nests were observed on this day.

The OPM larvae may appear in two different ways on a tree, sedentary in the nest (expressed in nest volume in cm³) or free-ranging (presented in cursive written larvae numbers) whereas free-ranging cluster are also counted as a nest.

The mobility of the larvae further increases the difficulty of larvae and nest counts for estimation of population density.

The drastic increase in OPM presence on May 13th compared to May 5th, especially in the control trees is eminent, due to no signs of OPM presence found on the earlier observation date. Table 4.3 also shows drastic OPM increase comparing observation day May 13th with May 18th, but only moderate increase or even decrease in some cases untill May 25th.

Besides the lower OPM pest presence, expressed in total nest volume (2,4:1 ratio of treated to control trees), this analysis displays no significant differences between treated and control trees, meaning that the more detailed analysis of nest volume development did not gain further insights.

The analysis of nest volume development indicated, that the application of NeemAzal-T/S influenced nest volume and pest occurrence, but due to inconsistent trends, does not necessarily assure significant and effective control of OPM pest.

For detailed data see Appendix A 1.

The statistical one-way-ANOVA analysis yielded the following results for total OPM nest volume per tree and per treatment option on different observation days. On all observations days with OPM nest occurrence, no relationship with significant interdependence between total nest volume per tree and treatment option (treated and control) was observed.

Table 4.4 indicates the corrected correlation coefficient (r^2) and the probability of error (p) on each observation day concerning the relationship of total nest volume per tree and the treatment option.

Table 4.4: Corrected correlation coefficient and probability of error of total nest volume per tree to treatment option

Date	r^2	p
May 13	-0,0724	0,5194
May 18	-0,09831	0,6106
May 25	0,055567	0,2513

The results show a very marginal relation of corrected correlation coefficient (r^2) and a very high probability of error (p) on each of the observation days.

The same is true for single nest analysis, shown in Table 4.5, where nest volume in cm^3 per treatment option are tested and their corrected correlation coefficient (r^2) and the probability of error (p) are displayed.

Table 4.5: Corrected correlation coefficient and probability of error of single nest volume per tree to treatment option

Date	r^2	p
May 13	-0,05759	0,7247
May 18	-0,03516	0,9032
May 25	-0,02277	0,5118

The results show a very marginal relation of corrected correlation coefficient (r^2) and a very high probability of error (p) on each of the observation days. Since the amplitude of samples was mean, careful interpretation of these results is required.

Overall, the assessed data allowed the assertion of no significant and effective control of NeemAzal-T/S against OPM pests to be documented, even though nest sizes of treated trees were generally smaller.

4.1.2 Results from visual analysis on Oak leaves

Tree leaf collection on Oak happened on May 25th at randomly selected trees on two different canopy locations (center and lower). Visible leaf damage such as different feeding patterns, leaf miners and other insects were analyzed to distinguish possible differences between treated trees and untreated control.

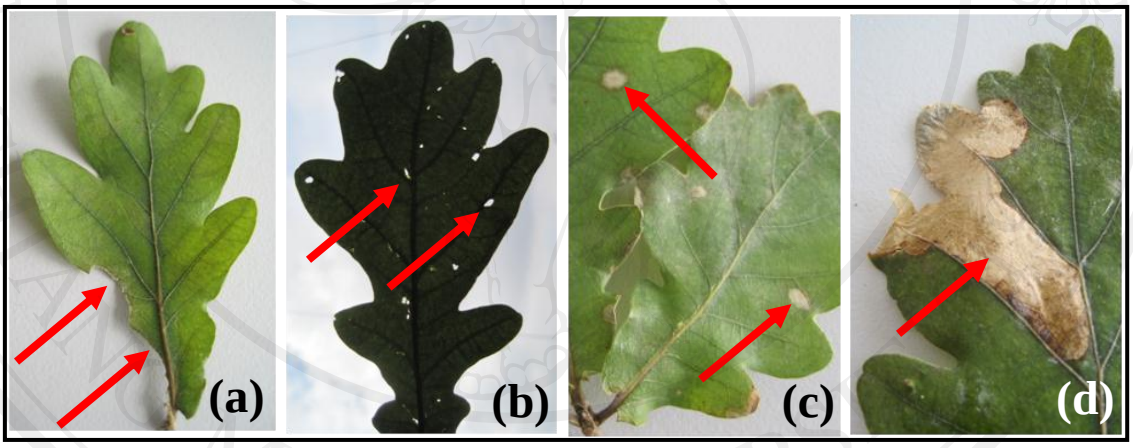


Figure 4.1: Marginal feeding (a), hole feeding (b), dead spots (c) and leaf mines (d), as different feeding patterns observed on Oak leaves

Table 4.6 summarizes and displays observed leaf damages and compared the number of undamaged leaves with total collected leaves for each option on Oak leaves on observation day May 25th.

Table 4.6: Summery of observed leaf damage on Oak on May 25th

Visual leaf assessment on Oak		Number of leaves with damage						
Date	Option	marginal feeding	hole feeding	dead spots	leaf mines	sucking damage	other insects	leafs free of damage
May, 25	treated	29	14	8	0	1	0	58 of 98
May, 25	control	28	22	0	1	8	3	57 of 107

In most cases, only a little damage on leaf surfaces was observed, in which the marginal feeding pattern was the most often recognized. The quantity of occurrences of this feeding pattern did not vary significantly between treated (29) and control trees (28). As OPM larvae are counted to be the initiators of marginal leaf feeding, and no distinct difference between treated and control trees was observed after three applications of NeemAzal-T/S, the effectiveness of spot stem application in this setting on Oak trees has to be questioned.

The total number of leaves without any leaf surface damage also does not vary significantly between treated trees and the control. Whereas for the treated trees 58 out of 98 (59,18%) leaves were undamaged, this is true as well for 57 out of 107 (53,27%) leaves of the control trees.

The other feeding patterns do not show distinct and significant changes between the treated trees and the control either.

Due to the low sample number of leaves, derived only from one leaf collection day on Oak, caused by the early removal of OPM nests, significant effects were not seen and these results may not be representative.

4.1.3 Results from HPLC-MS analysis on Oak leaves

The scan in the positive ESI-mode of the HPLC-MS analysis detected mass traces with 703,7 and 743,7 as the most intensive ones, which qualified them for determination of Azadirachtin A content. The analysis was standardized with 5mg NeemAzal-T/S and showed a distinct peak at 5,877 min retention time, indicating the trace of the active ingredient Azadirachtin A.

For each of the samples an injection volume of 5µl was used. Table 4.7 displays the Azadirachtin A peak, to qualify further detection of this active ingredient.

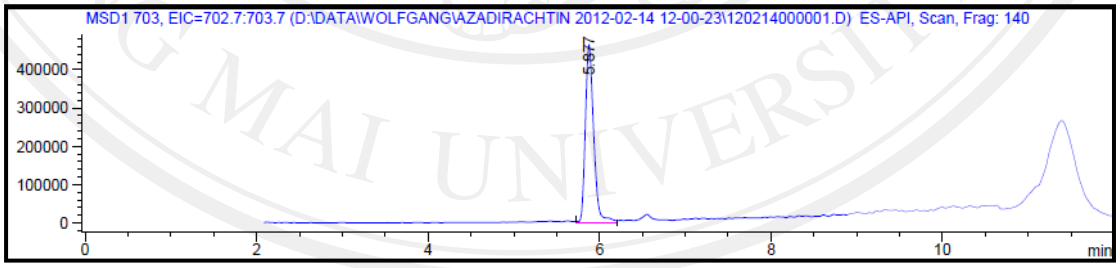


Table 4.7: Azadirachtin A standard for 5mg NeemAzal-T/S

Table 4.8 displays the printout to describe the graphical curve of Table 4.7

Table 4.8: Description of standard curve with the mass of 703

Signal 3: MSD1 703, EIC=702.7:703.7

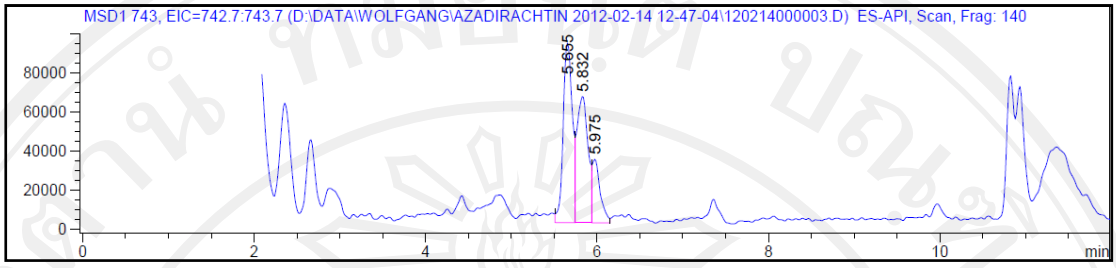
Peak #	RetTime [min]	Type	Width [min]	Area	Height	Area %
1	5.877	VV	0.1027	3.15705e6	4.67424e5	100.0000
Totals :				3.15705e6	4.67424e5	

For the qualitative analysis of HPLC-MS results, all peaks were separated from the distinct peak of the Azadirachtin probe. The exact concentration or amount of substance within the extracted sample was needed, so that by using the peak area, the assay could be analyzed.

To verifiy systemic distribution of Azadirachtin within the tree after spot stem application of NeemAzal-T/S, a well primed Oak leaf assay of 2g was analyzed with HPLC-MS. For this analysis three samples were chosen for the Oak trees, from tree 43 for the control trees and from tree 46 and 47 for the treated trees, each from the lower canopy layer.

Already the control essay with the number 43 showed at the mass of 743,7 and the retention time of 5,832 min a co elution with Azadirachtin A, caused by matrixes of other substances. Those unknown substances covered the signal of the analyzed assays as well of the treated trees, which inhibited the possibility of an assertion. Table 4.9 displays these co-eluted signals at the mass of 743,7

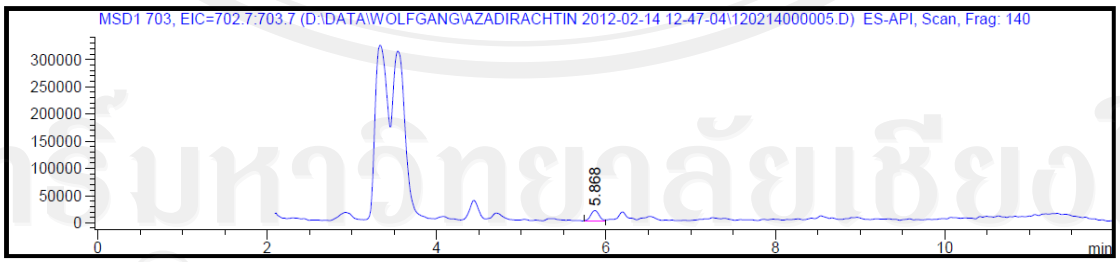
Table 4.9: Co-eluted signals of Azadirachtin analysis on Oak with the mass of 743,7



The attempt to complete an assertion with the analysis on a third mass, showed co elution as well. Merely the mass 703,7 showed a distinct signal, which had not been confirmed by one of the other masses. Therefore, following calculations are only considered as appraisal values. For definite identification and quantification a well adjusted reprocessing of assays will be necessary.

Table 4.10 displays Azadirachtin detection through HPLC-MS analysis for mass 703,7 and tree 46, were leaf samples were collected from lower tree canopy on May 25th.

Table 4.10: Azadirachtin A detection on Oak tree number 46



The peak at a retention time of 5,868 min, similar to the peak at the 5mg standard (5,877 min) indicates, that this probe contains Azadirachtin A.

Table 4.11 displays printout of mass 703,7 to describe graphical curve of Table 4.10.

Table 4.11: Description of analysis on Oak tree number 46 with the mass 703

Signal 3: MSD1 703, EIC=702.7:703.7

Peak #	RetTime [min]	Type	Width [min]	Area	Height	Area %
1	5.868	BV	0.1076	1.31932e5	1.93293e4	100.0000
Totals :				1.31932e5	1.93293e4	

Azadirachtin A concentration for tree 46 is than calculated in the following procedure.

$$\text{C (Assay 46)} = \frac{Area\ Assay}{Area\ Std} * C\ Std = \frac{1{,}31932e5}{3{,}15705e6} * \frac{5mg}{L}$$

$$\text{C (Assay 46)} = 0{,}209 \frac{mg}{L} \equiv 0{,}209\ \mu g/ml$$

2g of leaf compound dissolved in 25ml of Acetonitril makes:

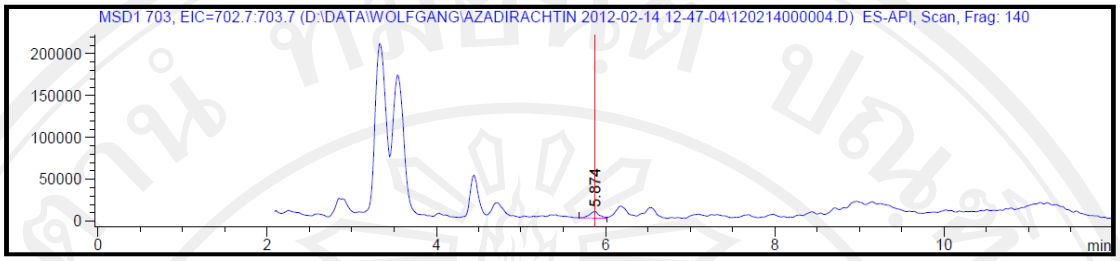
$$\text{C (Assay 46)} = \frac{0{,}209 \frac{\mu g}{ml} * 25ml}{2g} = 2{,}6125\ \mu g/g$$

$$\text{C (Assay 46)} = 2{,}6125\ mg/kg \equiv \mathbf{2612{,}5\mu g/kg}$$

An Azadirachtin A content of 2612,5µg/kg leaf mass was measured and detected on tree 46.

Table 4.12 displays Azadirachtin detection through HPLC-MS analysis for mass 703,7 and tree 47, were leaf samples were collected from lower tree canopy.

Table 4.12: Azadirachtin A detection on Oak tree number 47



The peak at retention time of 5,874 min, similar to the peak at the 5mg standard (5,877 min) indicates, that this probe contains Azadirachtin A as well.

Table 4.13 displays the printout of mass 703,7 and tree 47 to describe graphical curve above.

Table 4.13: Description of analysis on Oak tree number 47 with the mass 703

Signal 3: MSD1 703, EIC=702.7:703.7

Peak #	RetTime [min]	Type	Width [min]	Area	Height	Area %
1	5.874	VV	0.1174	6.38054e4	7960.38477	100.0000
Totals :				6.38054e4	7960.38477	

Azadirachtin A concentration for tree 47 is than calculated in the following procedure.

$$\text{C (Assay 47)} = \frac{Area\ Assay}{Area\ Std} * C\ Std = \frac{6{,}38054e4}{3{,}15705e6} * \frac{5mg}{L}$$

$$\text{C (Assay 47)} = 0{,}101 \frac{mg}{L} \equiv 0{,}101\ \mu g/ml$$

2g of leaf compound dissolved in 25ml of Acetonitril makes:

$$\text{C (Assay 47)} = \frac{0{,}101 \frac{\mu g}{ml} * 25ml}{2g} = 1{,}2625\ \mu g/g$$

$$C\ (\text{Assay } 47) = 1{,}2625\ \text{mg/kg} \equiv \mathbf{1262{,}5\mu g/kg}$$

An Azadirachtin A content of 1262,5µg/kg leaf mass was measured and detected on tree 47.

After three applications of NeemAzal-T/S (April 20th, May 5th and May 18th), very small amounts of Azadirachtin A were detected within the leaves and therefore approve systemic distribution of active compound. But as mentioned earlier are these calculations only considered as appraisals, as long as results are not verified through newly invested and well adjusted assays.

Together with the observation of generally smaller OPM nest sizes observed on the treated Oak trees, a clear indication for effects of NeemAzal-T/S application is given, even though effectiveness need to be considered as not significant in terms of real pest control.

4.2 Results on Sycamore

During the whole NeemAzal-T/S application and pest observation period from April to August, no SLB pest (*C. ciliata*) at the test location at "Schwieberdinger Street", was recorded. The Sycamore trees as potential hosts as well as the appropriate weather conditions were present without evident signs for unfavorable preconditions, but did not attract SLB at this location in 2011.

4.2.1 Results from visual observations on Sycamore trees

Even though SLB as pest did not occur, frequent field visits with visual observation on Sycamore trees and NeemAzal-T/S applications had been conducted. The whole set of six suggested applications with NeemAzal-T/S happened as well and were realized on following dates: April 20th, May 5th, May 18th, May 31st, June 15th and June 28th. Leaf collection on Sycamore happened twice on May 25th and June 22nd.

Due to the absence of SLB, there is no data for display available.

4.2.2 Results from visual analysis on Sycamore leaves

Since no SLB pest was recorded, the visual leaf analysis had been effected as well. The known feeding pattern of SLB is sucking on leaf tissue to extract plant saps, which causes a punctual yellowing of leaf surface with the consequence of possible early leaf fall. The fact, that these feeding patterns, evoked by sucking insects were documented, if at all, only in very small amounts, does verify the assumption of general SLB absence.

Sycamore tree leaf collection happened twice on May 25th (center and lower canopy layer) and June 22nd (top, center and lower canopy layer). In most cases, only

exiguous damage on leaves were recorded, whereas total leaf damage may be expected finally in cases of infestation with the pathogen *Apiognomonia venata*, appearing as leaf vein damage in early stages. Next to leaf mines, damages from *A. venata* were amongst the most frequently observed. This is true for the observation day May 25th as well as June 22nd.

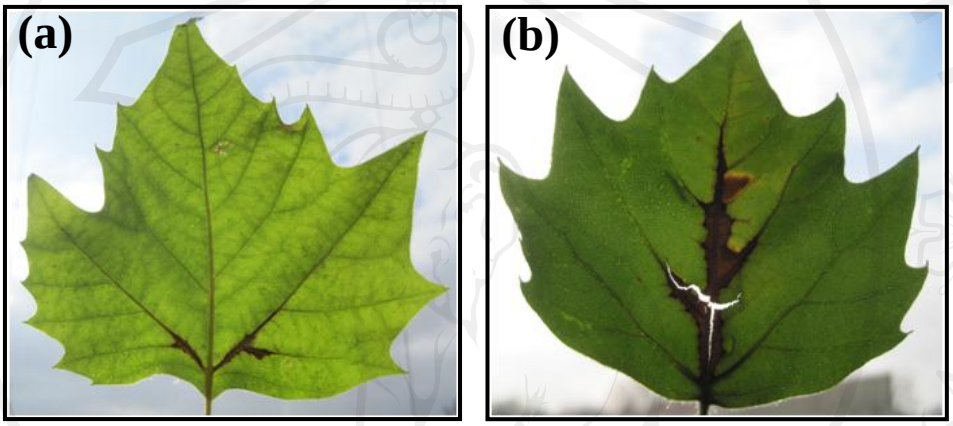


Figure 4.2: *A. venata* on secondary veins (a) and on primary vein

In later stages of infestation, the whole leaf surface wilts and turns brown.

For May 25th, of the analyzed leaf samples of treated trees, 52 out of 79 leaves were undamaged, making 27 (34,12%) being damaged, with a total of 9 (33,33%) evoked from *A. venata*. Also for May 25th, analyzing the leaf samples of the control, 37 out of 88 leaves were undamaged, making 51 (57,95 %) being damaged, with a total of 7 (13,73%) evoked from *A. venata*.

Other leaf damages frequently observed on Sycamore were leaf mines, hole feeding and diverse leaf spots. Leaf mines caused by *Phyllonorycter platani*, are also observed quite commonly. Considering the treated leaf samples on May 25th, out of the 27 damaged leaves, 5 (18,52%) showed leaf mines. In contrast showed 29

(56,86%) leaves out of the 51 damaged leaves from the control leaf mines on the same date.

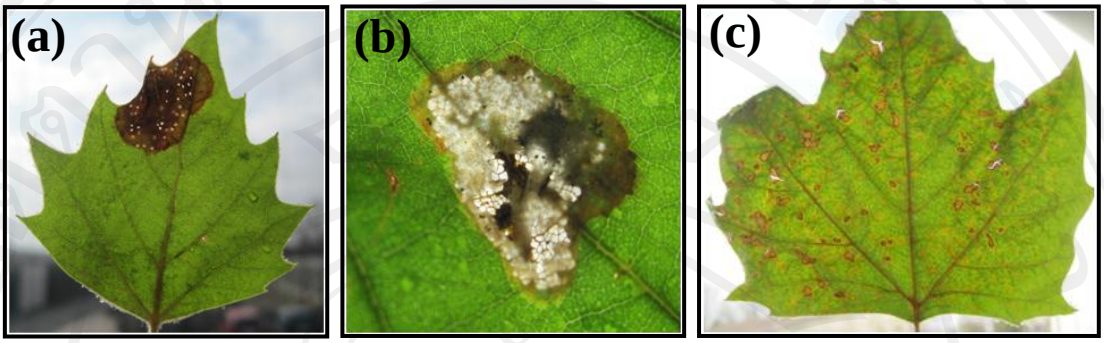


Figure 4.3: Leaf mines (a, b) and leaf spots (c) on Sycamore leaves

Table 4.14 summarizes and displays all recorded leaf damage of leaf samples collected on May 25th. Next to leaf vein damage caused by *A. venata* and leaf mines caused by *P. platani*, marginal feeding, hole feeding, leaf spots, sucking damage and other insects were under observation.

Table 4.14: Summery of observed leaf damage on Sycamore on May 25th

Visual leaf assessment on Sycamore		Number of leaves with damage							
Date	Option	marginal feeding	hole feeding	leaf spots	leaf mines	sucking damage	other insects	leaf vein fungus	leafs free of damage
May 25	treated	1	5	7	5	0	0	9	52 of 79
May 25	control	4	5	2	29	0	0	7	37 of 88

Till the date of May 25th, applications with NeemAzal-T/S had happen three times. Considering the effects of these applications, only the leaf mines seem to be responsive and vary in number from 5 of the treated trees to 29 of the control trees. Other leaf damages, such as hole feeding and leaf spots appeared only in exiguous amounts or in marginal proportions of leaf surface. Other insects or sucking damage evoked from SLB were not even documented.

For June 22nd, of the analyzed leaf samples of treated trees, 49 out of 103 leaves were undamaged, making 54 (52,43%) being damaged, with a total of 14 (25,93%) evoked from *A. venata*.

Also for June 22nd, analyzing the leaf samples of the control, 29 out of 102 leaves were undamaged, making 73 (71,57 %) being damaged, with a total of 20 (27,40%) induced from *A. venata*.

Considering the treated leaf samples, out of the 54 damaged leaves, 21 (38,89%) showed leaf mines. In contrast showed 46 (63,01%) leaves out of the 73 damaged leaves from the control leaf mines on June 22nd. Table 4.15 displays all recorded leaf damage of leaf samples collected on June 22nd. Next to leaf vein damage caused by *A. venata* and leaf mines by *P. platani*, marginal feeding, hole feeding, leaf spots, sucking damage and other insects were under observation.

Table 4.15: Summery of leaf damage on Sycamore on June 22nd

Visual leaf assessment on Sycamore		Number of leaves with damage							
Date	Option	marginal feeding	hole feeding	leaf spots	leaf mines	sucking damage	other insects	leaf vein fungus	leafs free of damage
June 22	treated	7	7	13	21	1	0	14	49 of 103
June 22	control	5	12	4	46	0	0	20	29 of 102

Till the date of June 22nd, applications with NeemAzal-T/S had happen five times. Considering the effects of these applications, as it has been observed on May 25th, the number of leaf mines seems to be responsive and decrease in treated samples compared to the control (21 on treated leaves to 46 in the control).

Other leaf damages, such as hole feeding and leaf spots appeared only in exiguous amounts or in marginal proportions of leaf surface. Other insect damage was not even documented, whereas one case of sucking damage had been recorded, which may indicate at least little SLB presence. Table 4.16 displays the percentage of damaged leaves being affected either by *A. venata* or *P. platani*, counting the total number of damaged leaves as 100%.

Table 4.16: Percentages of *A. venata* and *P. platani* on Sycamore

		leaf vein damage (*A. venata*)	leaf mines (*P. platani*)	total number of damaged leaves
May 25	treated	33,33%	18,52%	27
May 25	control	13,73%	56,86%	51
June 22	treated	25,93%	38,89%	54
June 22	control	27,40%	63,01%	73

Considering the summarized results of leaf collection days May 25th and June 22nd concerning *A. venata* clearly indicates, that *A. venata* as a plant pathogen is not effected by NeemAzal-T/S, if applied as done in this research setting.

Looking at the same results for *P. platani*, a positive trend concerning effects of NeemAzal-T/S are documented, even though the relation of infected leaves with leaf mines from *P. platani* decreased some on June 22nd compared to May 25th. This may be interpreted as indication for too long intervals between the different application occasions. (see results on Oak)

4.2.3 Results from HPLC-MS analysis on Sycamore leaves

The same procedure of standardization for analysis as it had been for the Oak trees happened for Sycamore. The assay of 5mg NeemAzal-T/S showed at the mass of 703,7 a distinct peak at 5,877 min retention time, indicating the trace of Azadirachtin A.

To ascertain systemic distribution of Azadirachtin within the tree after spot stem application of NeemAzal-T/S, a well primed Sycamore leaf assay of 2g were analyzed with HPLC-MS. For this analysis three samples were chosen for the

Sycamore trees, whereas sample number 98 represents the control trees and sample numbers 90 and 109 the treated trees, each from lower canopy layer.

No Azadirachtin A was detected in the tree sample 98, since it represents the control with no NeemAzal-T/S application. But looking at the scrutinized extracts of Sycamore leaf samples of the control tree 98, an interference of the leaf matrixes in the form of co elution of substances had been recognized at the same retention time as Azadirachtin A. Also for the treated trees, no clear accordance of retention time of the NeemAzal-T/S standard to analyzed assays of selected Sycamore trees were documented, neither on the sample of tree number 90 nor at the sample of tree 109.

On none of the three mass traces used for identification and quantification, later conducted analyses were able to actually quantify masses of Azadirachtin A. The systemic distribution of NeemAzal-T/S has therefore not been observed in those samples of the Sycamore trees.

4.3 Results on Horse Chestnut

Data collection and analysis of the Horse chestnut trees, which were location wise apart from Oak and Sycamore, was characterized by a mass occurrence of HCL (*C. ohridella*). Population density of HCL, contrary to insect pests on Oak and Sycamore, was monitored and displayed through distributed pheromone traps.

The whole set of six suggested applications with NeemAzal-T/S happened on Horse chestnut and were conducted on following dates: May 18th, May 31rd, June

15th, June 28th, July 12th and July 27th. Leaf collection happened on three occasions and was realized on May 25th, June 22nd and July 19th.

4.3.1 Results from visual observations on Horse chestnut trees

Due to favorable weather conditions, HCL population developed well and therefore infested observed Horse chestnut trees intensely. Beginning of July onwards, the visually observed infestation of leaves with HCL reached an estimated rate of 15% of total leaf number. This assessment contains data for the treated trees as well as the control. During progression of larvae development, the mine sizes increased, whereas the rate of infested leaves stagnated at around 15%.

The challenge of distinguishing leaf rate infestation and rate of leaf surface coverage through HCL mines, analyzed by visual observation, describes the crucial weighting of considerations for this assessment.

The obvious and visually observed change from healthy looking trees with green leaves and small amounts and sizes of leaf mines from HCL changed within the course of observation (May to August) into severely stressed looking trees with wilted leaves and growing numbers and sizes of leaf mines.

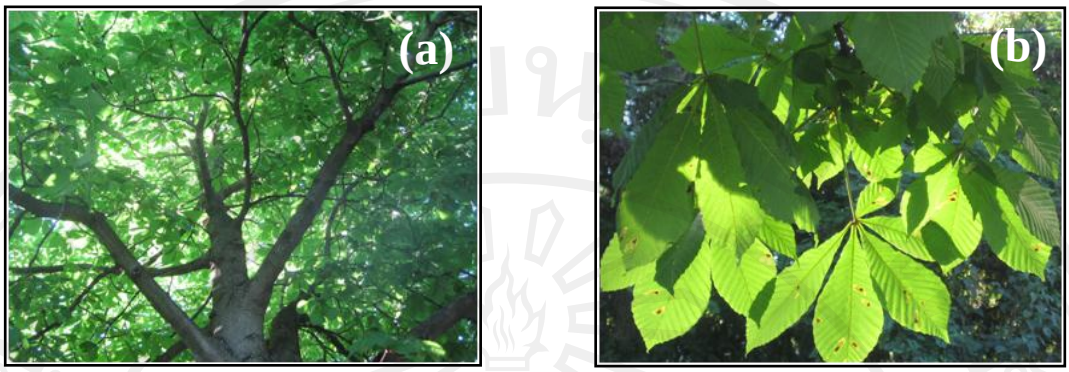


Figure 4.4: Healthy Horse chestnut tree (a) and leaves with little signs of HCL infestation (b), in May

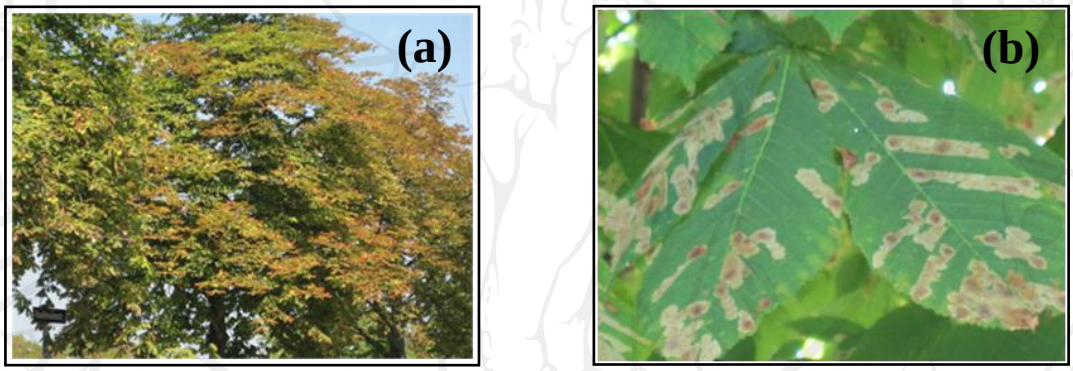


Figure 4.5: Stressed Horse chestnut tree (a) and severe HCL leaf infestation (b), in July. Consequences of this increased HCL pest infestation was early leaf fall, which was observed already in late August.

Towards the end of July, the rate of visually observed infestation of leaves with HCL increased to above 20% of total leaf number. This incident corresponds well with the second peak of HCL moth flying, which indicated the appearance of the second HCL generation, also monitored through the pheromone traps. (see Tab. 4.17)

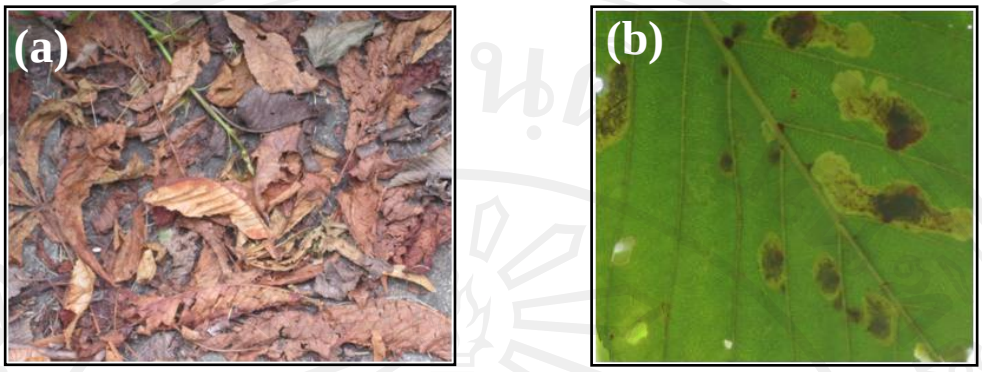


Figure 4.6: Early leaf fall (a) documented in August and mines of I. and II. Generation of HCL (b) on same leaf

Leaf roll patterns of Horse chestnut tree leaves were first observed at July 26th. Supposably evoked by the growing leaf mines covering up to estimated 40% of leaf surface at this point and the related degradation of the nutrient transport system within the leaf causing wilting phenomena, the leaf ends started to roll. This as well was observed at treated and control trees.

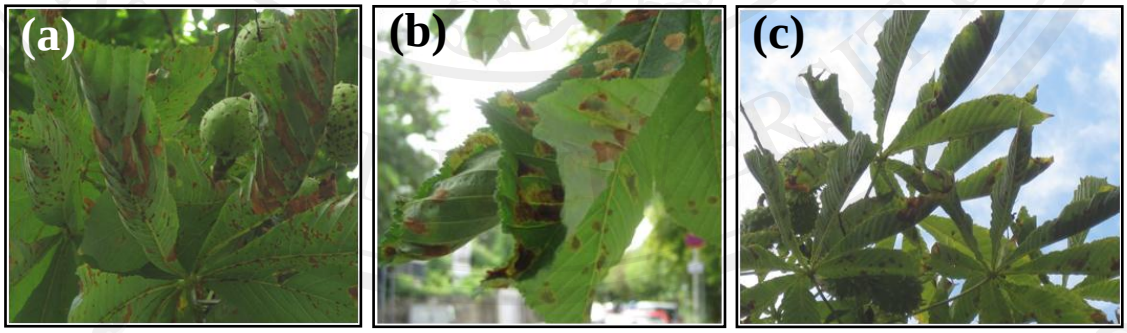


Figure 4.7: Different cases and stages of leaf roll (a-c)

Another phenomenon observed was the marginal leaf wilting, which sometimes came with a yellowing of leaf surface, separating the wilted leaf part from the green and healthy looking one. There are two possible causes for this wilting, which are the fungus *Guignardia aesculi*, also called Guignardia leaf blotch, and the

consequence of de-icing salt, a man-made problem along roadsides, appearing as leaf-edge wilt.

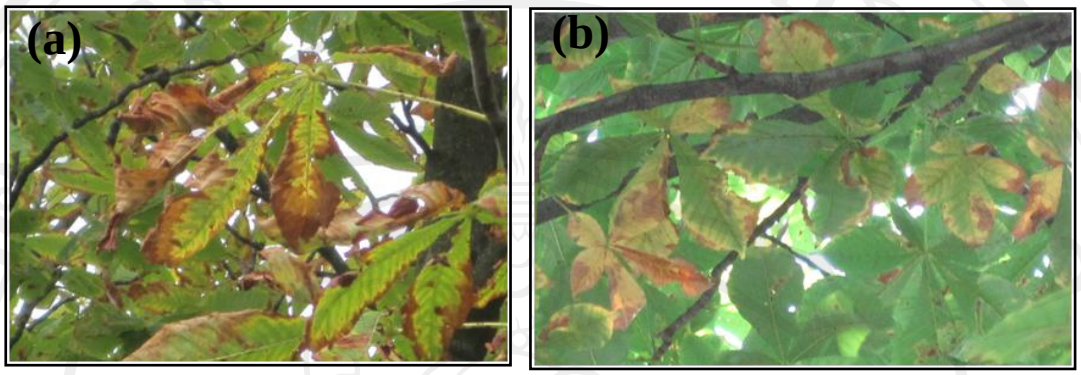


Figure 4.8: Different cases of leaf wilting, supposably caused by *G. aesculi* (a, b).

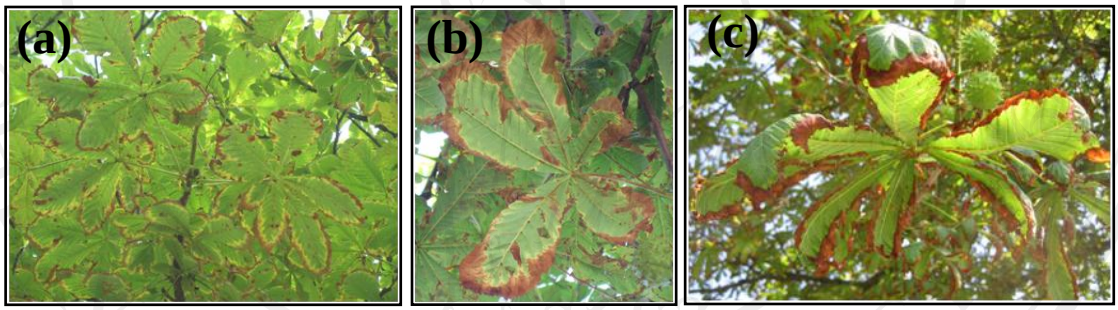


Figure 4.9: Different cases of leaf-edge wilting (a-c), which are supposably caused by de-icing salt.

Another finding of the field visits and visual observations of Horse chestnut trees was, observed on July 19th, the appearance of leaf holes and damaged leaves, more commonly in the outer canopy sphere, evoked by a summer storm with a hail incident.

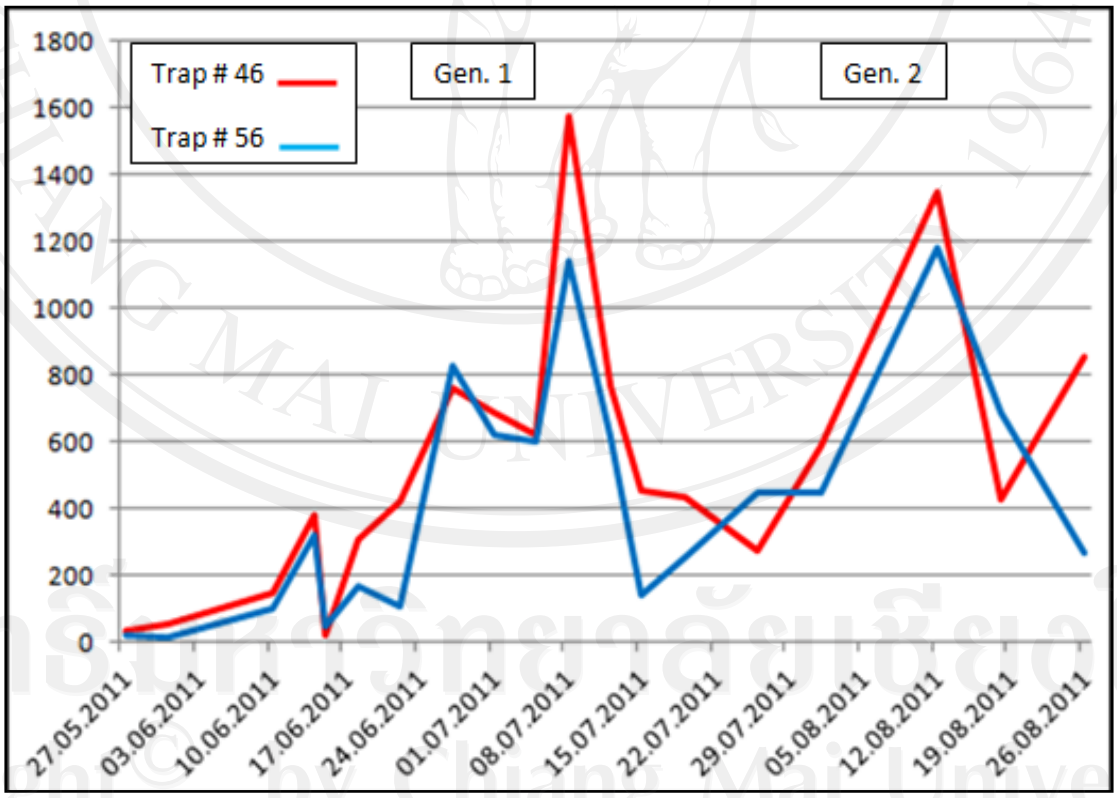
During the three month between May 27th and August 26th, the pheromone baited delta traps placed into the lower canopy layer of tree number 46 and 56, had been visited and monitored 19 times. The HCL moths stuck to the lure varied in

number between 19 at the lowest and 1576 moth at the highest. The HCL moth counts correlated quite well between the two different traps.

As described earlier, the monitoring of the HCL moth well displayed the occurrence of the different moth generations, in which the first generation peaked around July 8th and the second one around August 12th.

Table 4.17 displays the occurrence of HCL moths in the two pheromone traps at their date of appearance.

Table 4.17: Occurrence of HCL monitored through delta traps placed in tree 46 and 56. For more detailed data on HCL moth monitored in the traps see Appendix A3.



Summarizing the visual observations of the field visits on Horse chestnut trees, no distinct effects of NeemAzal-T/S application were displayed, since high

numbers of HCL infestation as well as the other leaf damages observed, were found on the control and also on the treated Horse chestnut trees in comparable numbers.

4.3.2 Results from visual analysis on Horse chestnut leaves

This analysis happened in a laboratory setting with visual support of a binocular and expanded over 144 leaves examined, 1605 leaf mines measured and related to their corresponding larvae, of which the head capsule was measured as well. This leaves had been collected on three occasions (May 25th, June 22nd and July 19th) and from up to three different canopy layers (top, center and lower).

Table 4.18a, b summarizes the condensed average values of following parameters, distinguished between the control (yellow) and the treated (green) treatment options: Average counts of mines, avg. single mine area, avg. leaf mine area and avg. diameter of larvae head capsule, separately for each leaf collection day and the affected tree numbers.

Table 4.18a: Average leaf mines of HCL, mine area and larvae head capsule of the control trees

Control	Tree #	Avg counts of mines	Avg Mine area in mm²	Avg leaf Mine area in mm²	Avg head capsule in mm
May 25	63, 70, 73	7,78	28,43	233,05	0,43
June 22	70, 71, 72	3,33	293,20	660,33	0,53
July 19	62, 70, 72	22,56	37,94	928,52	0,29

Table 4.18b: Average leaf mines of HCL, mine area and larvae head capsule of treated trees

Treated	Tree #	Avg counts of mines	Avg Mine area in mm²	Avg leaf Mine area in mm²	Avg head capsule in mm
May 25	65, 66, 69	9,78	28,78	307,95	0,34
June 22	65, 67, 68	3,92	238,18	701,78	0,52
July 19	64, 66, 69	17,59	38,56	683,52	0,28

The results show, that the average number of mines per leaf, the average mine area as well as the average sum of mine area per leaf was lower in the control as in the treated trees for the dates in May and June. But surprisingly did the average diameter of larvae head capsule not follow this trend, but instead showed higher values in the control as in the treated trees for the same dates in May and June.

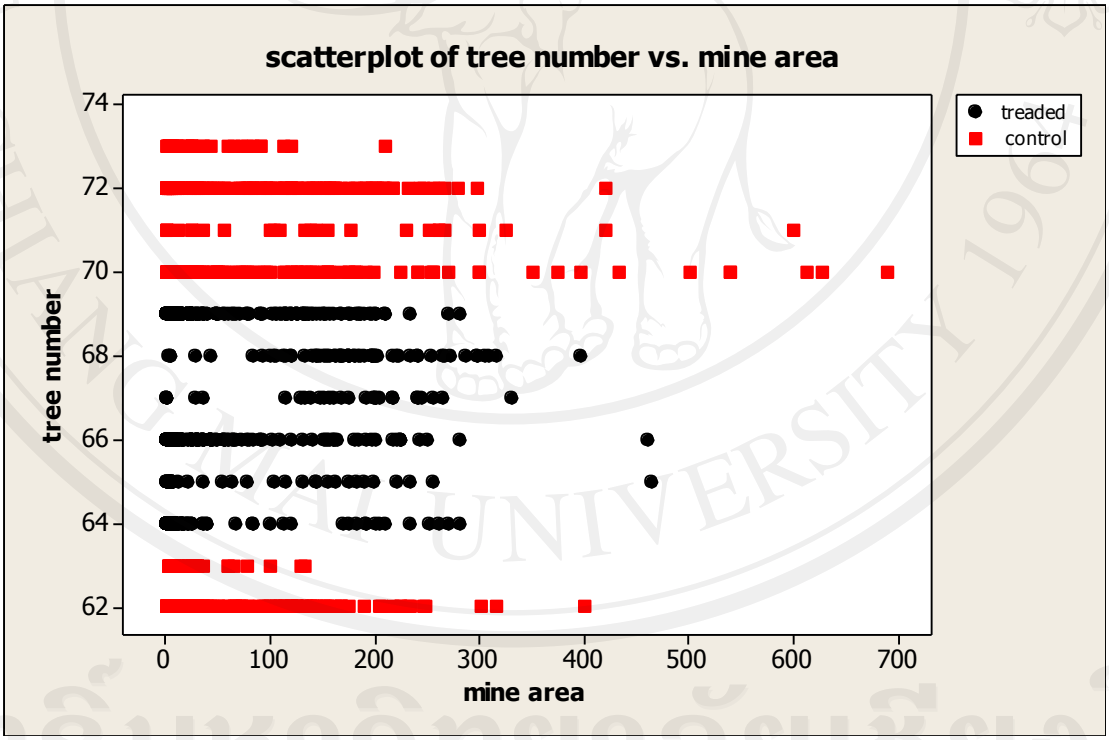
For the leaf collection date in July, this trend is inverted, since now all values, except for the average mine area, are higher in the control trees than they are in the treated ones. Also the average diameter of head capsule of larvae stays smaller in the treated option. If interpreted with the effects of NeemAzal-T/S, these results on Horse chestnut would show a late effect of this active compound, since there have been five applications, before treated values reached lower levels as the control trees did.

The escalated increase of average counts of leaf mines on July 19th is induced by the appearance of the second generation of HCL. This becomes obvious as well in comparing the average leaf mine areas, which decreased dramatically on July 19th for both treatment options, displaying the newly hatched HCL larvae starting to feed on plant tissue.

But do these differences not show trends towards significant effects of pest control of NeemAzal-T/S; neither in average number of mines per leaf, average mine area, average sum of mine area per leaf nor in the average diameter of larvae head capsule.

A general overview of the distribution of leaf mine area on Horse chestnut trees (treated trees displayed in black, control trees displayed in red) is given in following scatterplot (Tab. 4.19).

Table 4.19: Overview of total mine area per tree and treatment option



The above scatterplot displays, that most of the mines of the control as well as the treated trees, cover an area from 1 to around 300 mm². Even though the biggest mines were documented at the control trees with the number 70 and 71 (500 to nearly 700 mm²), the tree with the smallest mines (tree number 63) is of the control as well.

This brief analysis already indicates, that distinct and significant differences of treated trees compared to control trees concerning their leaf mine area, may not be documented in findings, since not displayed in the above scatterplot.

The detailed display of leaf mines per tree of different leaf collection days and treatment options is presented in Table 4.20.

Table 4.20: Leaf mines per tree, collection day and treatment option

Total number of leaf mines per tree and leaf collection day								
May 25			June 22			July 19		
Tree #	Treatment option	Number of leaf mines	Tree #	Treatment option	Number of leaf mines	Tree #	Treatment option	Number of leaf mines
63	control	36	70	control	21	62	control	272
70	control	59	71	control	22	70	control	123
73	control	45	72	control	47	72	control	214
	Total	**140**		**Total**	**90**		**Total**	**609**
65	treated	22	65	treated	16	64	treated	172
66	treated	72	67	treated	25	66	treated	137
69	treated	82	68	treated	65	69	treated	175
	Total	**176**		**Total**	**106**		**Total**	**484**

The above Table 4.20 visualizes clearly the higher numbers of leaf mines found at the treated trees of leaf collection day May 25th and June 22nd. Only at the last leaf collection day July 19th, documentation of leaf mine numbers of the treated option showed lower numbers (484), than the control (609). Looking at the general trend of the single trees, no significant difference appears distinctive.

For the same leaf collection dates in May, June and July, results of total values of mine number and mine area had been summarized as well, and displayed for their different treatment options.

These total values had then been scrutinized in more detail and the larvae found in the mines divided into all observed forms. These conditions and forms of larvae observed had been larva, pupa, parasitized larvae or pupae, larva or pupa not found in mine, larva or pupa not to measure or larva or pupa hatched and therefore not found in mine. In addition, total beings of first as well as the second generation larvae have been displayed.

Table 4.21a, b show these summarized total values of these parameters for the different leaf collection dates of the control as well as for the treated trees.

Table 4.21a: Total mine area and observed larvae forms of control trees (a) of HCL

Control	tree #	total mines	total mine area per collecting day in mm²	total beings	larva	no larva found	pupa	parasitic larva/pupa	not to measure	old mine la. hatched	Gen I.	Gen II.
May 25	63, 70, 73	140	4195	45	45	0	0	0	0	0	45	0
	total of 18 leaves		Prozent %	32,14	32,14	0	0	0	0	0	32,14	0
June 22	70, 71, 72	90	17829	90	19	5	18	2	0	47	82	3
	total of 27 leaves		Prozent %	100,00	21,11	5,56	20,00	2,22	0,00	52,22	91,11	3,33
July 19	62, 70, 72	609	25070	609	414	78	1	0	11	105	137	383
	total of 27 leaves		Prozent %	100,00	67,98	12,81	0,16	0,00	1,81	17,24	22,50	62,89

Table 4.21b: Total mine area and observed larvae forms of treated trees (b) of HCL

Treated	tree #	total mines	total mine area per collecting day in mm²	total beings	larva	no larva found	pupa	parasitic larva/pupa	not to measure	old mine la. hatched	Gen I.	Gen II.
May 25	65, 66, 69	176	5543	141	141	0	0	0	0	0	141	0
	total of 18 leaves		Prozent %	80,11	80,11	0	0	0	0	0	80	0
June 22	65, 67, 68	106	18948	106	27	8	35	3	0	33	93	5
	total of 27 leaves		Prozent %	100,00	25,47	7,55	33,02	2,83	0,00	31,13	89,62	4,72
July 19	64, 66, 69	484	18455	484	351	42	4	0	16	71	92	334
	total of 27 leaves		Prozent %	100,00	72,52	8,68	0,83	0,00	3,31	14,67	19,01	69,01

As it has been for the average results, the results for total values as well show the same tendency of higher numbers of total mines and total mine areas of the treated

trees compared to the control for the leaf collection days of May 25th and June 22nd, but showed inverted results of these parameters on July 19th.

As the treated trees had 125,71% of total number of mines compared to the control for May 25th, this value changed into 117,78% for June 22nd and 79,47% for July 19th. The same is true for the total mine area.

As the total mine area of treated trees was 132,13% compared to the control on May 25th, this value changed into 106,28% for June 22nd and 73,61% for July 19th. To what extend these results of reduced mine numbers and mine area on the last collection date are influenced and effected by the active ingredients of NeemAzal-T/S, needs to be investigated in further detail.

A further investigation of this table is the detailed analysis of all observed forms of larvae on all of the three observation days. The significant differences between total observed beings and amount of leaf mines detected on May 25th, is explained by the fact, that not all larvae found were recorded at that time.

Further development of analytical methodology allowed all larvae to be measured and to be allocated to its original mine at the later dates of June 22nd and July 19th. The table also indicates, as described earlier, the development of the second generation of HCL larvae.

In the control as well as in the treated trees, did the second generation occur slightly on collection day of June 22nd (control 3,33%, treated 4,72%) and increased intensely till July 19th, to form approximately two thirds of all mines by then (control 62,89%, treated 69,01%).

Looking at the different stages of larvae found in the mines, no significant differences in larvae mortality are observed between the treatment options.

The correlation of the total mine number and the total mine area is well displayed in these findings, whereas the increase in mine area due to the feeding activity of growing larvae needs to be put into consideration as well.

This is clearly shown, for example, in the comparison of number of leaf mines and area of leaf mines of the leaf collection days June 22nd and July 19th on the treated option. June 22nd displays 106 mines with a total area of 18948 mm², whereas July 19th displays 484 mines with a total area of 18455 mm².

The 378 fewer leaf mines of June 22nd were compensated in terms of total leaf mine area due to larger sizes of single mines, caused by the older larvae of the first HCL generation (June 22nd displays 89,62% of I. generation and 4,72% of II. Generation HCL larvae). This relation of HCL generations is inverted on July 19th, were 19,01% of I. generation and 69,01% of II. Generation HCL larvae appeared.

Interestingly, only on June 22nd, the period of high numbers of older larvae being present, were parasites and deformations on larvae observed (treated 2,83%, control 2,22%).

This verifies the findings of KAETHNER (1990), who described a higher mortality and deformation of larvae in their last instars and during pupal phase, after having accumulated Neem compounds through feeding activity during all larva stages.

In the following section, figures of those parasitic organisms, pathogens and other insects observed on Horse chestnut leaves and HCL are presented.

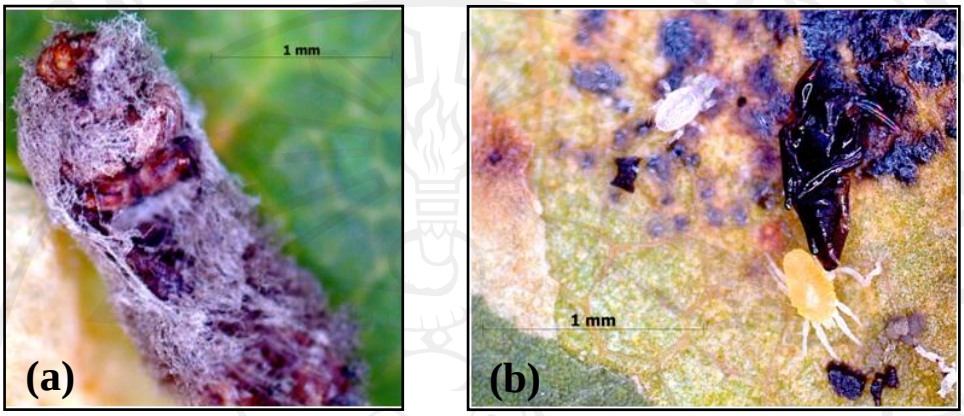


Figure 4.10: Fungi on HCL pupa (a) and predacious mites on HCL pupa (b)

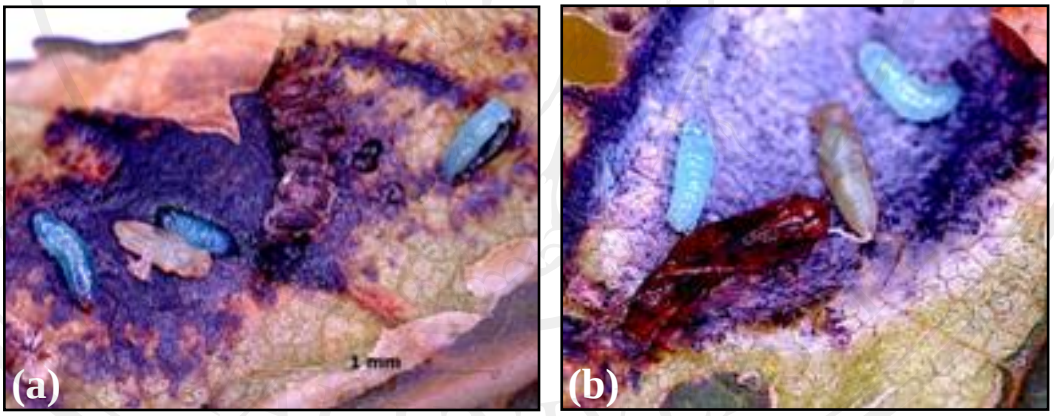


Figure 4.11: Larvae and nymph on HCL larva (a) and larvae and nymph on HCL pupa (b)

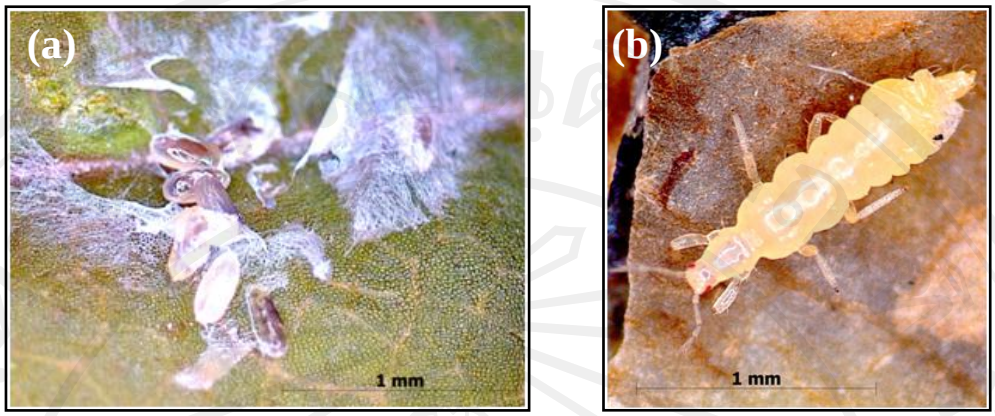


Figure 4.12: Larvae on Horse chestnut leaf (a) and thrips on HCL leaf mine (b)

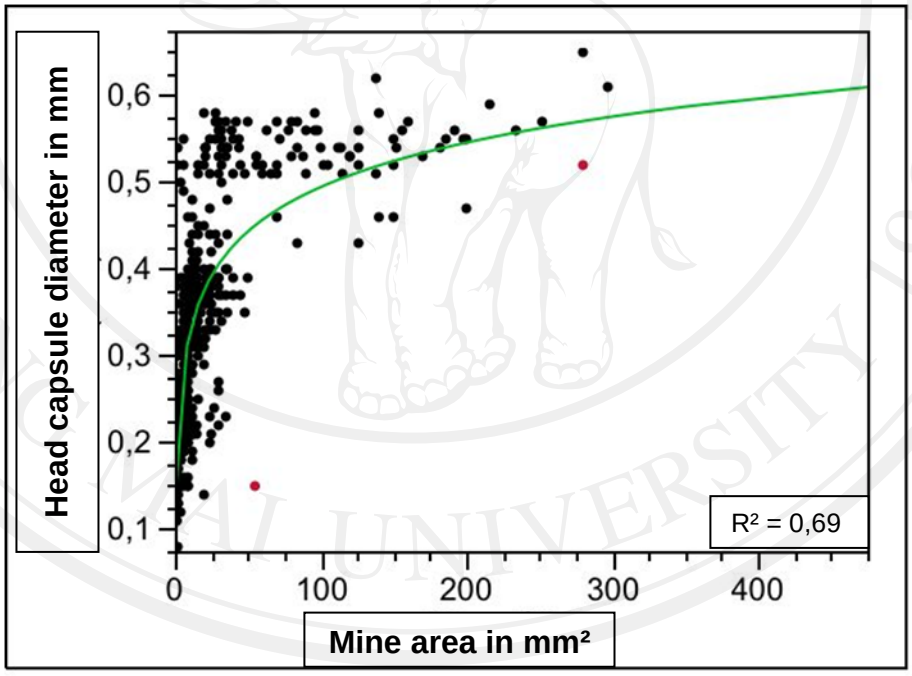
This analysis of the different HCL generations as well as the detailed analysis of the larvae forms found, did not show significant differences between the control and the treated trees, and therefore does not show effectiveness of NeemAzal-T/S applications.

These findings were verified through the statistical analysis conducted with JMP® 7.0.2 software. The general tests of effects for the mine area as the command variable resulted in statistically significant dependencies for the tree number, the date, canopy places and treatment option, but not for the number of mines. The later scrutinizing of dependencies between mine area and treatment options, did not verify significant relations. Since the trees as such are value-free, this general test already hints toward the assumption, that the tree specific phenology and physiology seems to be more influential on HCL pest occurrence, than the treatment option itself.

The results of mine area analysis showed significant dependencies on the date and canopy place, which goes well with the general circumstance of low canopy places being infested first by HCL moths. This as well was reflected in the analysis

on leaf mine area of different canopy locations at different collection dates, since simultaneous with larval development and time progression, bigger HCL mine areas were observed. The general tests of effects for the diameter of head capsule of HCL larvae as the command variable resulted in statistically significant dependencies with the mine area, but not for the canopy places, the date and the treatment options. The following scatterplot (Tab. 4.22) displays the relation of head capsule diameter to mine area.

Table 4.22: Scatterplot of relation of mine area to larvae head capsule



Scatterplot (Tab. 4.22) shows transformed adaptation of log with the formulation: Headcapsule diameter (mm) = 0,1539764 + 0,0739602 * Log (Mine area). The probability of dependency was given for p > |t| with < 0,0001, which is well displayed in this graph. Further scrutinizing of relation of larvae head capsule and mine area divided into the different canopy layers did not verify this finding. The

statistical one-way ANOVA analysis of larva head capsule for the different treatment options and canopy places showed only on May 25th and July 19th at the lower canopy place a significant trend, which in the overall consideration did not qualify.

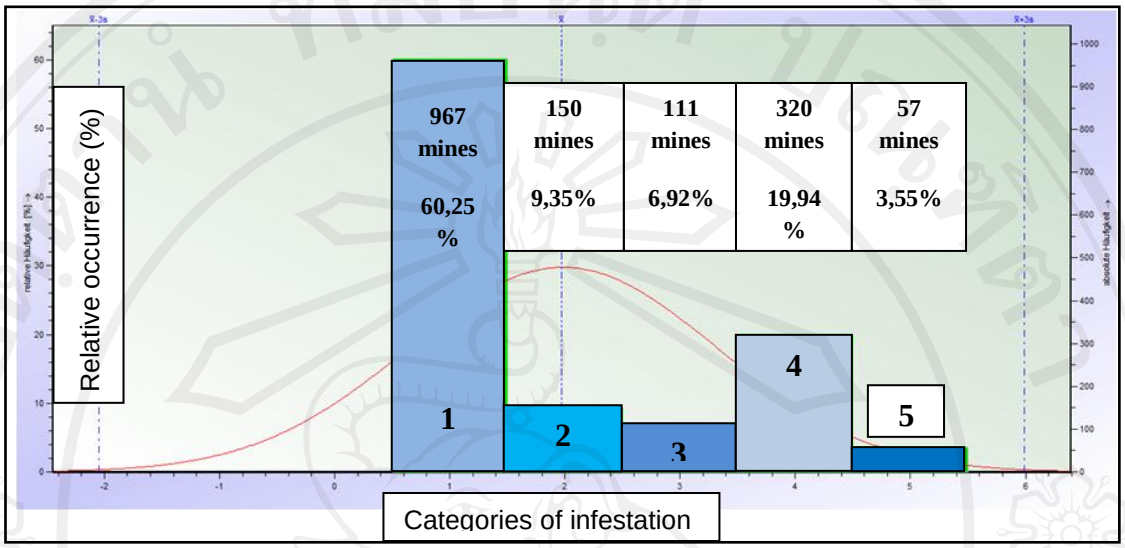
To classify the amount of infestation, the mine areas had been divided into five different categories of infestation. The following table (Tab. 4.23) displays the parameters for these five categories of infestation.

Table 4.23: Parameters of categories of infestation

Category	Mine area in mm²
1	1 to 24
2	25 to 49
3	50 to 99
4	100 to 249
5	250 and bigger

Each leaf mine detected was then allocated to one of these five categories of infestation. A general overview of the proportional distribution of the five categories is given in the following table. Relative, as well as total number of leaf mines per category of infestation, are displayed in the table (Tab. 4.24).

Table 4.24: Distribution of categories of infestation with total and relative numbers



Out of the five categories of infestation is it category 1 which is represented quite dominantly with 967 leaf mines making 60,25% of total occurrence. Second in terms of leaf mine number is category 4 with 320 mines and a share of 19,94%. This graph shows proportional distribution of leaf mines covering all three leaf collection days. The following tables (Tab. 4.25a and 4.25b) display the summarized numbers of mines and their percentage to total mine number, separated into each leaf collection day and treatment option.

Table 4.25a: Infestation categories on different collection days of the control trees

Control	tree #	total mines	Categories of Infestation 1	2	3	4	5
May 25	63, 70, 73	140	81	32	19	8	0
	total of 18 leaves	%	57,86	22,86	13,57	5,71	0,00
June 22	70, 71, 72	90	4	3	11	49	23
	total of 27 leaves	%	4,444	3,333	12,22	54,44	25,56
July 19	62, 70, 72	609	413	49	41	97	9
	total of 27 leaves	%	67,82	8,046	6,732	15,93	1,478

Table 4.25b: Infestation categories on different collection days of the treated trees

Treated	tree #	total mines	Categories of Infestation 1	2	3	4	5
May 25	65, 66, 69	176	108	34	19	15	0
	total of 18 leaves	%	61,36	19,32	10,8	8,523	0
June 22	65, 67, 68	106	3	4	4	79	16
	total of 27 leaves	%	2,83	3,774	3,774	74,53	15,09
July 19	64, 66, 69	484	358	28	17	72	9
	total of 27 leaves	%	73,97	5,785	3,512	14,88	1,86

Analyzing the amounts of infestation levels and their percentage share, an overall similar distribution between the control trees and the treated trees becomes evident. In both treatment options are the percentages high in the low infestation categories (categories 1 and 2) on May 25th, are as well low in the low infestations categories on June 22nd and peak in category 4 (control 54,44%, treated 74,53%) and are high again in the low infestation categories (category 1) (control 67,82%, treated 73,97%) at the last leaf collection date of July 19th.

A positive trend regarding effects of NeemAzal-T/S is only found at the last collection day (total number of mines from treated trees below the ones of the control for the first time), but there an equal distribution between the treatment options is ascertained as well. The high percentage of leaf mines of infestation category 1 and 2 on collection day May 25th indicates the early stages of the first generation HCL larvae. For both treatment options the highest amounts of leaf mines are found in category 4 and 5 on collection day June 22nd, displaying the late stages of the first HCL generation. Observing high amounts of leaf mines in infestation category 1 on July 19th again, is a clear indication for the arrival of the second HCL generation.

Since the overall assessment of the share of infestation categories did not show a clear trend towards reduced numbers in the treated option, again no indication for effectiveness of NeemAzal-T/S can be found.

Another investigation dealt with the total leaf area of Horse chestnut leaves, to relate them to their HCL total mine area. Table 4.26 displays total leaf area, total mine area and mine area percentage of leaf area for each leaf collection date, treatment option, tree number and canopy place.

Table 4.26: Ratio of leaf mine area to total leaf area for different collection days

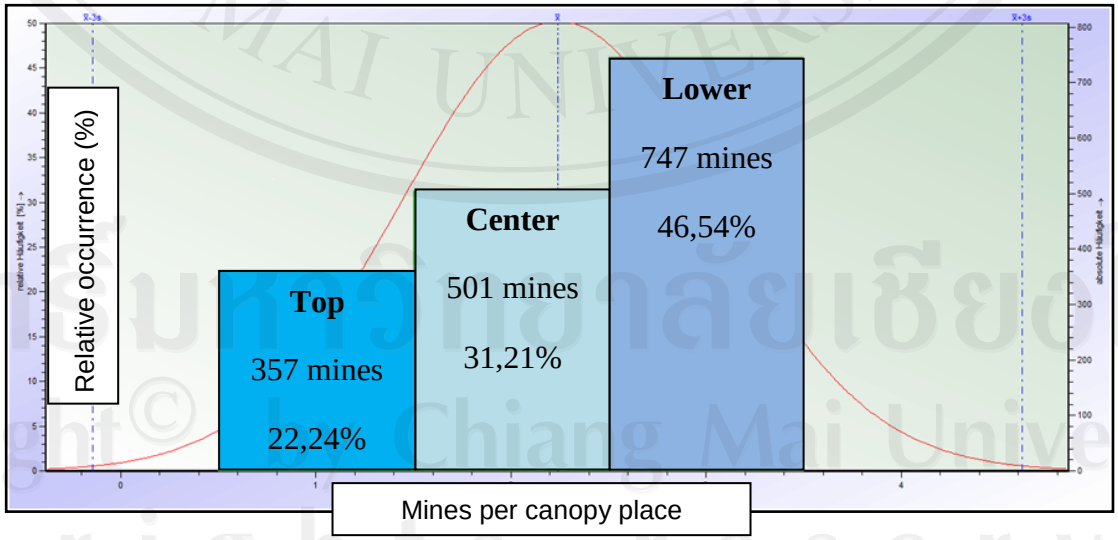
Date	Option	Tree#	Canopy place	Leaf area in mm²	Mine area in mm²	% Mine area of Leaf area
25. Mai	C	70	C	180388,50	1366	0,757
25. Mai	C	70	L	150263,50	371	0,247
25. Mai	T	66	C	97617,05	198	0,203
25. Mai	T	66	L	128091,50	1924	1,502
22. Jun	C	71	T	63154,05	1218	1,929
22. Jun	C	71	C	63527,60	910	1,432
22. Jun	C	71	L	72661,50	1995	2,746
22. Jun	T	68	T	61358,60	1161	1,892
22. Jun	T	68	C	76409,05	3268	4,277
22. Jun	T	68	L	98629,25	7064	7,162
19. Jul	C	72	T	83952,35	1324	1,577
19. Jul	C	72	C	71926,45	1589	2,209
19. Jul	C	72	L	121126,60	4248	3,507
19. Jul	T	69	T	58297,90	1626	2,789
19. Jul	T	69	C	45006,75	3393	7,539
19. Jul	T	69	L	74288,25	3047	4,102

On none of the leaf collection dates, the percentage of leaf mine area to total leaf area showed effects evocated of NeemAzal-T/S treatments.

In some cases the leaf mine percentage was even higher at the treated trees, as it had been detected for the control. On each observation day, the highest value of leaf mine percentage detected, was documented on a treated tree. Except for the control tree on May 25th, always the lower canopy layer showed higher amounts of leaf mine coverage. This goes well with the general observation of development of HCL infestation, where the lower canopy places in a tree are infested first, before infestation moves upwards. These results as well give reason for the conclusion, that the systemic distribution of active ingredient did not happen, if at all, not to all parts of the canopy.

For scrutinizing these results in more detail, the distribution of leaf mines per canopy place is now under observation. Following table (Tab. 4.27) displays the percentage share and total number of leaf mines per canopy place, summarized for all three leaf collection dates.

Table 4.27: Distribution of leaf mines at different canopy places



The generally observed beginning of HCL pest infestation in the lower canopy before moving upwards, is clearly indicated in this table, since the percentage share is highest in the lower canopy (46,54%), followed by the center (31,21%) and the top canopy place with 22, 24%.

To split up and assess these values in greater detail, the following table (Tab. 4.28) displays the total number of leaf mines, separated into the treatment options, canopy places, separately for each day of leaf collection.

Table 4.28: Leaf mines per canopy place, treatment option and collection day

Total number of mines per canopy place					
Option	**Canopy place**	**May 25**	**June 22**	**July 19**	**Total**
Control	top		31	141	172
Control	center	74	24	175	273
Control	lower	66	35	293	394
Total		**140**	**90**	**609**	**839**
Treated	top		16	169	185
Treated	center	42	28	158	228
Treated	lower	134	62	157	353
Total		**176**	**106**	**484**	**766**
Overall		**316**	**196**	**1093**	**1605**

Here quite apparent again, as mentioned before, that only on the 3rd and last leaf collection day of July 19th, the total number of leaf mines was lower in the treated option (484) as it had been observed in the control (609). On the two collection days before, the treated leaves showed higher numbers of leaf mines.

If this trend of reduced numbers in the treated option is resulting out of the applications of NeemAzal-T/S, which had been five at this point, then this is a retarded effect. Then the application intervals should have been chosen more

frequently, to increase NeemAzal-T/S effects. The escalated increase of leaf mines on July 19th, as mentioned before, did indicate the occurrence of second HCL generation.

Not always for the single leaf collection days, but in the summarized view of consideration of all collection days, the assertion of finding highest amounts of leaf mines in the lower canopy, followed by the center and smallest numbers in the top layer, is then correct.

But as well as it has been for the above observations and their conclusive results, did NeemAzal-T/S again not show significant effects, evaluated on the comparison of leaf mines and their distribution within the different canopy layers.

4.3.3 Results from HPLC-MS analysis on Horse chestnut leaves

The standardization of analysis on Horse chestnut happened with 10mg NeemAzal-T/S and showed at the mass of 703,7 a distinct peak at 3,817 min retention time, indicating the traces of the active ingredient Azadirachtin A. On each of the samples an injection volume of 5µl was used.

Table 4.29 displays the Azadirachtin A peak at 3,817 min retention time, to qualify further detection of this active ingredient.

Table 4.29: Azadirachtin A standard for 10mg NeemAzal-T/S

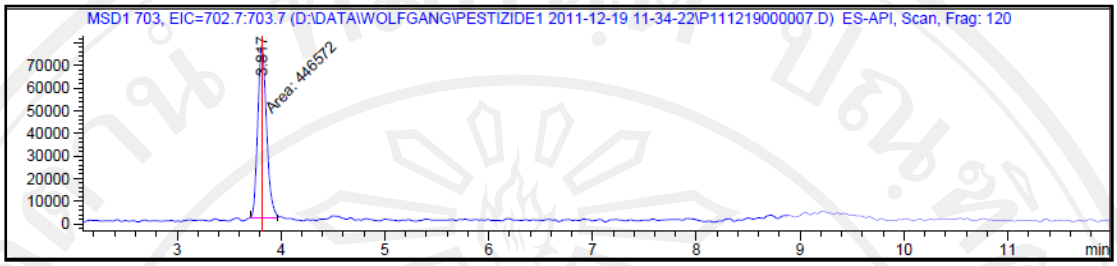


Table 4.30 displays the printout for the mass of 703,7, describing graphical curve above with signal number, retention time, signal type, width, area and height.

Table 4.30: Description of standard curve with the mass of 703

Signal 3: MSD1 703, EIC=702.7:703.7

Peak #	RetTime [min]	Type	Width [min]	Area	Height	Area %
1	3.817	MM	0.0962	4.46572e5	7.73764e4	100.0000
Totals :				4.46572e5	7.73764e4	

For the qualitative analysis of HPLC-MS results, all received peaks need to be separated from the distinct peak of the Azadirachtin sample. The exact concentration or amount of substance within the assay is needed, so that by using the peak area, the sample can be analyzed. To ascertain systemic distribution of Azadirachtin within the tree after spot stem application of NeemAzal-T/S, a well primed Horse chestnut leaf assay of 2g were analyzed with HPLC-MS. For this analysis four samples were chosen for the Horse chestnut trees, two from tree 72 for the control and two as well for tree 69 for the treated option, each from top and lower canopy layer.

As expected was no Azadirachtin A detected in the tree sample 72, since it represents the control with no NeemAzal-T/S applications. But also in the treated Horse chestnut tree (69), no traces of Azadirachtin A were detected. No interference of leaf matrixes in the form of co elution of substances were observed during Horse chestnut analysis. But the assertion of no detected Azadirachtin A was caused by the missing accordance of retention times with the standard assay. This missing accordance of retention times was documented on the treated samples of tree number 69 as well as the samples of tree 72.

On none of the four mass traces used for identification and quantification, later conducted analyses were able to actually quantify masses of Azadirachtin A. The systemic distribution of NeemAzal-T/S has therefore not been observed in those samples of the Horse chestnut trees.

4.4 Possible repellent and / or phytotoxic effects of NeemAzal-T/S

In 1956, DETHIER defined a repellent effect as an oriented movement away from the stimulus source. Parts of the Neem tree, especially the seed kernels, but to some extend also the leaves, have been known to contain compounds, that are repellent to insects, and therefore protect Neem foliage against insect feeding.

The repellent action of Neem may result from the presence of volatile sulfur-containing compounds (BALANDRIN *et al.*, 1988). The effect of repellency has a positive correlation with the concentration of active ingredients, observed on Neem

seed kernels (NSKs) (2,5%, 5% and 10% NSKs) (SCHAUER and SCHMUTTERER, 1981).

Due to the method used in this research of Neem compound applied onto the tree trunk with systemic distribution within the tree, only low amounts of active ingredients (e.g. Azadirachtin A) were documented in leaf tissue. This may indicate reasons for the fact, that during the course of this research, no repellent effects had been observed.

As it has been documented on various occasions, do high concentrations of Neem compounds as well have phytotoxic effects (SCHMUTTERER, 1995, SAXENA and KHAN, 1984, 1985). Oil-containing Neem products do partially destroy the outer waxy layer of plant leaves, if compound is applied in higher concentrations. This may cause a higher transpiration of plants to a large extent (FREISEWINKEL, 1989).

To reduce or fully avoid phytotoxic effects of oily Neem compounds, a concentration below 2,5% is recommended. Since the concentration used in this research was 20%, phytotoxic effects on waxy leaf tissue of plants should even be expected.

Phytotoxic effects observed in this research occurred either due to spray mist of NeemAzal-T/S reaching the lower canopy layer of trees and covering their leaves or due to young stem sprouts at a low height above ground, which were covered with spray mist during the application process. Phytotoxic effects were observed on Sycamore trees (*Platanus x hispanica*) on various occations and are displayed in the following.

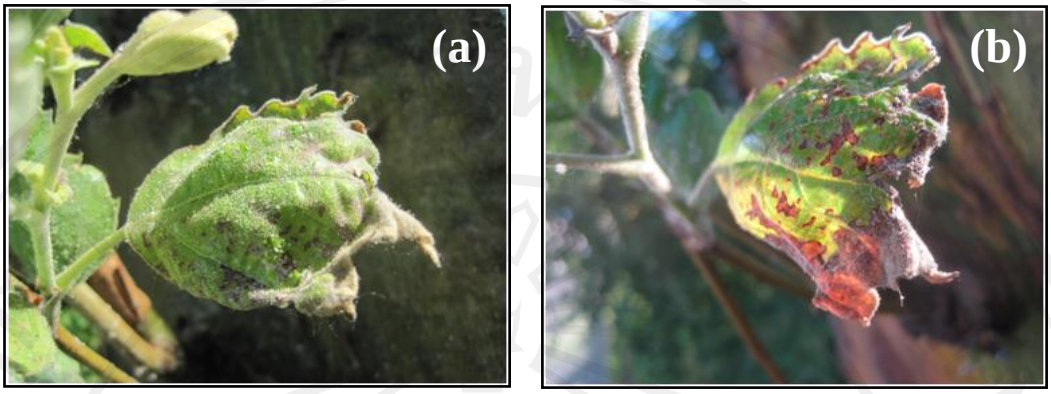


Figure 4.13: Sycamore leaf effected from spray mist of NeemAzal-T/S on May 17th (a), and same leaf on June 27th (b). Both figures derive from tree number 92 and display the progression of wilting as a result of phytotoxic effect.

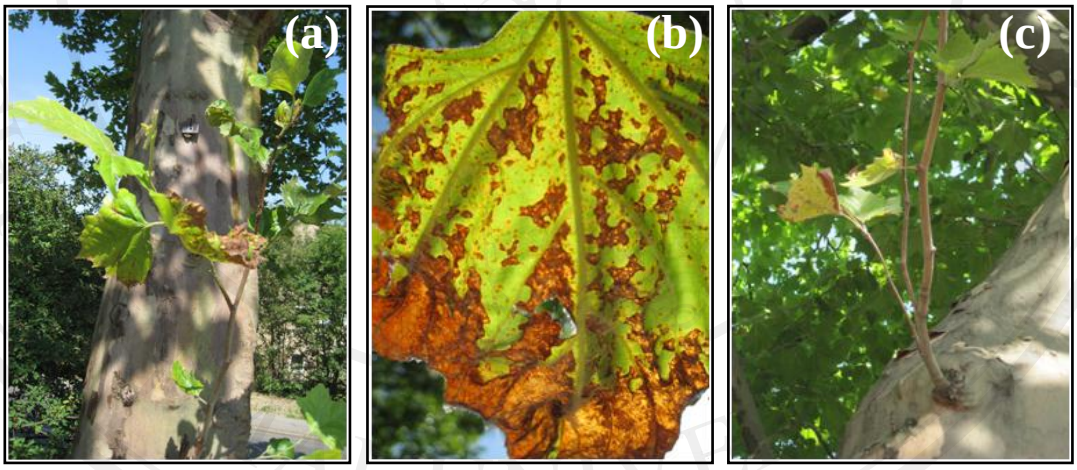


Figure 4.14: Progression of phytotoxic effects observed on leaves of the tree number 92 on July 12th (a), August 1st (b) and August 24th (c). Even though the leaves show severe damage to plant tissue, no total die-back of leaves had been observed on Sycamore leaves.

Next to the Sycamore tree number 108, a small *Acer campertre* L. trees was located. With the frequency of Neem compound application, the *A. campestre*

received parts of the spray mist destined for the Sycamore tree. Effects of the spray mist on *A. campestre* are displayed in following figures (4.15a-c).

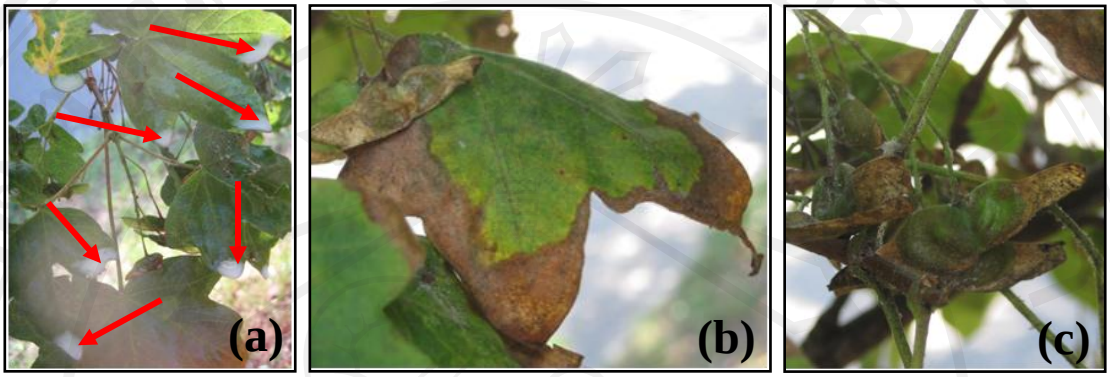


Figure 4.15: Accumulated spray mixture on leaf edges of *A. campestre* on June 27th (a), leaf edge wilting caused by spray mixture on August 1st (b) and wilting effects observed on seed husks at same date (c)

Phytotoxic effects have been observed on Horse chestnut trees as well.

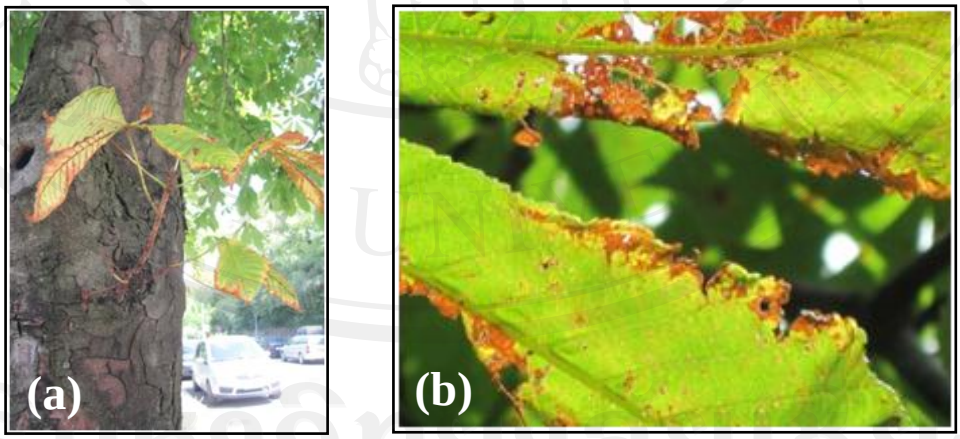


Figure 4.16: Horse chestnut leaves damaged by spray mist of active compound applied to the tree trunk (a) and partly decay of Horse chestnut leaf after contact with NeemAzal-T/S (b)

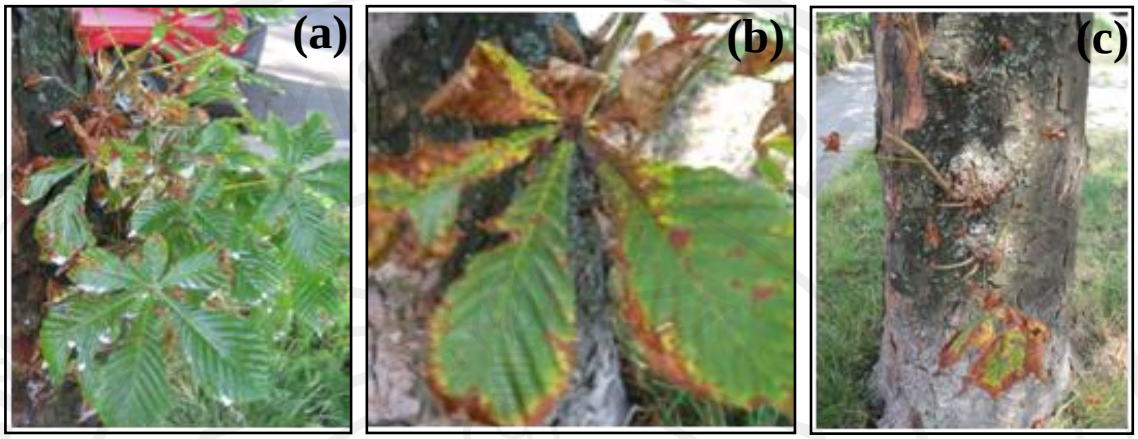


Figure 4.17: Accumulated spray mist on Horse chestnut leaves of the lower tree trunk on June 27th (a), wilting of leaves on July 8 (b) and died, as well as partly fallen off leaves on August 1st (c)

All three figures show the same spot of tree trunk number 65, and display gradually decay of leaves after NeemAzal-T/S application, and therefore document phytotoxicity.

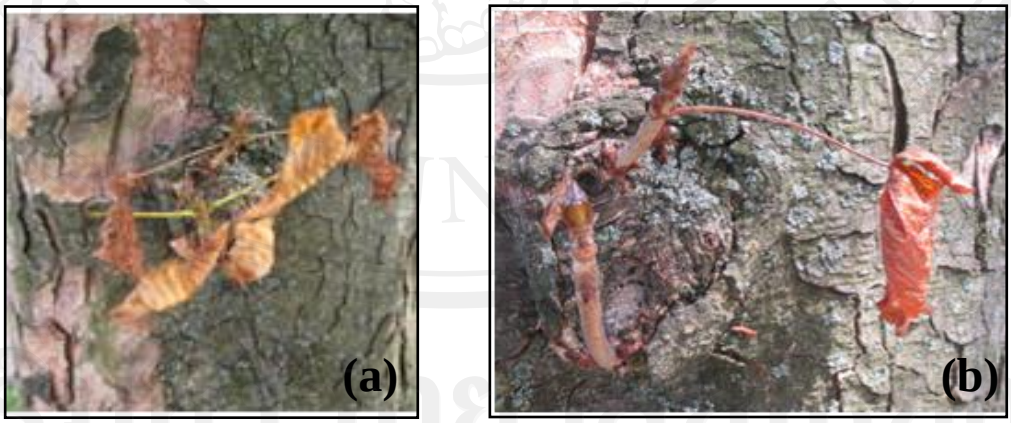


Figure 4.18: Sequence of leaf wilting on Horse chestnut due to the impact of NeemAzal-T/S at the same spot of tree number 65 at two different occasions At the right figure, a re-sprouting of buds is visible, indication that NeemAzal-T/S does not harm their development permanently.

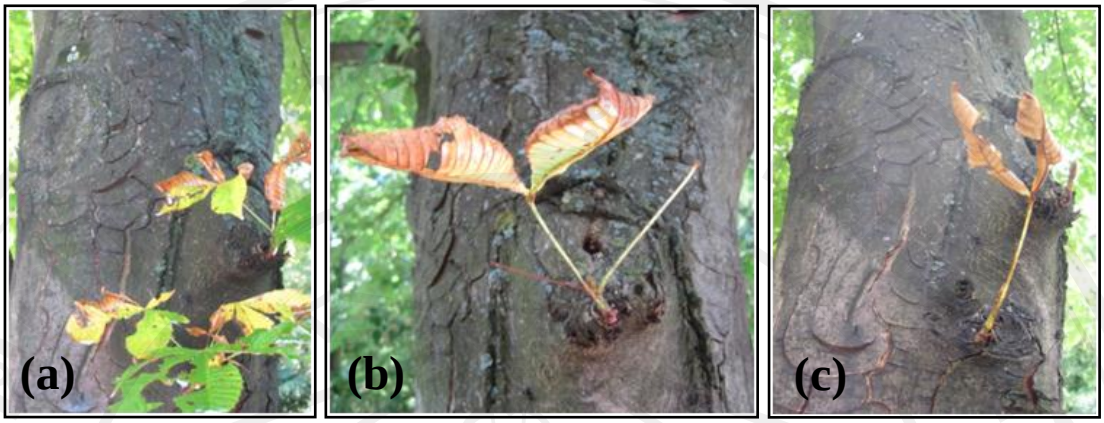


Figure 4.19: Sequence of leaf decay on Horse chestnut tree number 68 on June 27th (a), on July 8th (b) and on July 26th (c), at the same tree trunk location.

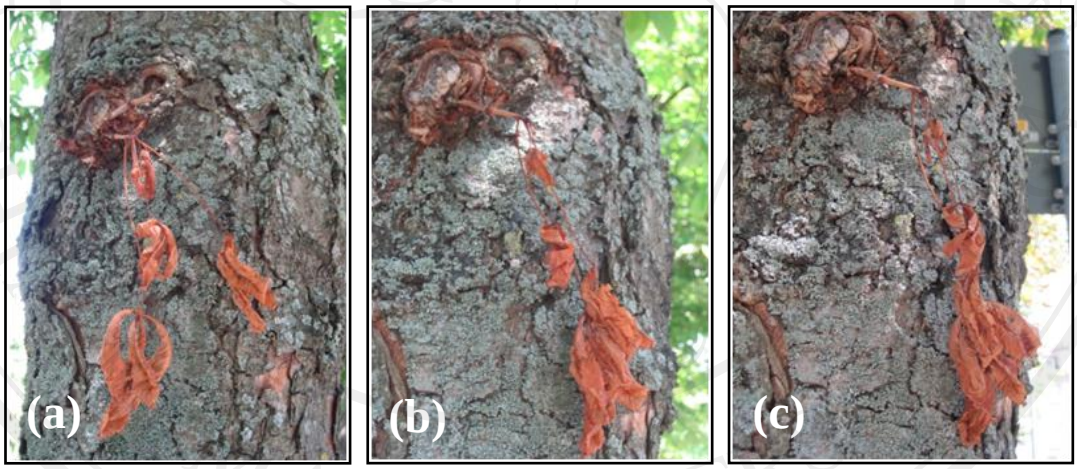


Figure 4.20: Sequence of irreversible damage after application of lethal concentration to leaves of tree 70 on July 8th (a), on August 1st (b) and on August 24th (c)

Horse chestnuts are considered having relatively fast decaying leaves. The consequence is a faster degradation of leaf surface after Neem compound application.

Comparing Horse chestnut leaves with the ones of *A. campestre*, a significant difference in cuticula thickness becomes evident. Horse chestnut leaves with a thin

cuticula layer, show phytotoxic effects of NeemAzal-T/S application faster than *A. campestre* does. Since Azadirachtin A is sensible to photodegradation, the assessment of thickness of cuticula influencing the degradation after substance application is evident, since *A. campestre* leaves were shaded and therefore exposed to phytotoxic effects for a longer time period, but did resist these effects better than Horse chestnut leaves did.

Phytotoxic effects were as well observed on *Hedera helix* L.

Figure 4.21:

H. helix leaves on Horse chestnut tree 66 June 27th, after three NeemAzal-T/S applications

(a, b)

Figure 4.21:

H. helix leaves on Horse chestnut tree 66. July 26th, after five NeemAzal-T/S applications

(c, d)

Fig. 4.21e, f

H. helix leaves on Horse chestnut tree 66.

August 24th, after six NeemAzal-T/S applications

Figure 4.21a-f: Sequence of *H. helix* leaves effected by NeemAzal-T/S at center and lower tree trunk on three different occasions on the Horse chestnut tree 66. Even though *H. helix* leaves are characterized by a slow natural decay and a thick cuticula layer, phytotoxic effects are evident.

H. helix leaves showed signs of yellowing after three applications on June 27th (Fig. 4.21a, b), as well on July 26th (Fig. 4.21c, d) after five applications. Comparing *H. helix* of the last observation day on August 24th (Fig 4.21e, f) with the two earlier occasions, an absence of yellow leaves becomes evident. The shedding of those leaves created obvious interspaces, which were consequences of phytotoxic effects as well, but did not decrease vitality to such an extent, that plant life would have been endangered.

Summarizing these results of phytotoxic effects of NeemAzal-T/S on the observed plant tissues, distinct differences on the plant species, mainly influenced by the thickness of cuticula, were evident. The high concentration of active compound (20%) needs to be considered in this assessment as well.

Except for the Horse chestnut, phytotoxic effects of NeemAzal-T/S were not permanently harmful to plant tissue. In cases where Neem compound killed the leaves eventually, new sprouts regenerated and assured continuous plant life.