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The effect of a short period of elevated temperature on the silique formation in *Arabidopsis thaliana*

Matthijs van der Houwen
900202369040

Supervisor: Johanna Molenaar
Examinator: Dick Vreugdenhil
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Preface

This is a Master thesis report of an experiment about silique formation in response to a short elevated temperature done in the Radix Clima at Wageningen University. The experiment was not possible without my supervisor Johanna Molenaar and Dick Vreugdenhil, who guided me the whole project and were always available for questions. Finally I would like to thank the chair group for this opportunity.

Matthijs van der Houwen

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Abstract

With this study we aim to identify processes in the development of siliques which are sensitive to a short period of heat stress. We have used different natural accessions of the model species *Arabidopsis thaliana*. With this set we tried to cover the spectrum of sensitivity present within the species. The plants were grown in a climate cell and approximately one week after the start of the flowering a heat shock of 35°C for 13.5 hours was given. Along the inflorescence flowers of different developmental stages were present, which gave us the opportunity to identify the sensitive and resistant developmental stages. For 5 days the pistil and stamen length, pollen release and pollen germination rate were measured for the flowers that opened that day. Also the silique length of the full-grown siliques was measured. Four accessions showed disturbance in their silique formation and for two of them the pollen germination rate was significant lower for treated plants (31.8% and 71.9% against 43.8% and 83.4%). This lower germination rate could influence the fertilization and seed formation and in this way result in shorter siliques. For one of this four affected accessions the pollen release rate was lower for treatment plants on day 4, 5 and 6 after the treatment. This low pollen release could be due to aberrant pollen development which makes normal silique formation impossible. The flowers on day 4, 5 and 6 were in floral stage 9 when they received the treatment, probably this stage is more sensitive to the heat treatment than the other stages tested. We never observed a consistent shortening of the anthers (evaluated by taking the difference between the length of the stamen and the pistil) if we compared the treated plants with the controls.

Unfortunately, eleven accessions could not be incorporated in the analysis due to low uniformity observed within the accessions. We observed large variation in flowering time and plant biomass. The bad plant performance is probably caused by non-optimal growing conditions (i.e. high air velocity) in the climate cell. This stress probably made the plants less vulnerable to the heat treatment.

1. Introduction

1.1 Background

Introduction

A plant can suffer from a lot of different stresses; biotic and abiotic stress. Biotic stress is defined as a form of stress which influences the growth of plants in a negative way and is caused by a living creature. For example, damage caused by fungi or insects that consume parts of the plant. Abiotic stress is defined as a form of stress which is caused by the environment such as drought, flood, high radiation and also high temperature.

In the summer the temperature in a greenhouse can be high, growers try to manage the temperature with screening, spraying the roof with water and whitening the windows. Still the temperature can exceed the optimal growth range. A higher temperature within the optimal growth range of a crop will cause a faster development. When the temperature is out of the optimal growth range, plant processes are not optimal anymore and the growth is influenced in a negative way. The response of a plant to high temperatures is not for all organs the same. Especially the reproductive parts of the plant are more vulnerable to stress. Hazra and Ansary (2008) and Saeed *et al* (2007) noticed that the yield of a tomato crop decreased after exposure to high temperatures. In case of Hazra and Ansary (2008) it decreased for 16 genotypes with an average of 70% at day/night temperatures of 34.5/19.2°C (optimal 18-28 C).

Fruit set in tomato

Yield in tomatoes is dependent on a lot of factors but it is highly influenced by the percentage of fruit set. Researchers determine the percentage of fruit set by scoring flower counts on all inflorescences present and fruit produced until at least six inflorescences had set fruit. In a research of Abdul-Baki and Stommel (1995) a fruit is considered set when its diameter reaches ≥0.5 cm.

Exposure to a high temperature influences the fruit set in a negative way. For example in a research of Abdul-Baki *et al* (1995) the percentage of fruit set significantly decreased with tomato crops grown by 35/23°C (day/night) compared with the control treatment of 27/23°C. The development of a flower is influenced by the high temperature and some processes in the development of the flower are disrupted. The main reason or exact process for poor fruit set is not known but there are probably several processes involved. Fruit set occurs in this way, the stigma of a tomato flower is pollinated and after germination of the pollen a stigma tube grows to the ovule and after fertilization a fruit will develop, in other words a fruit will set (Figure 1).

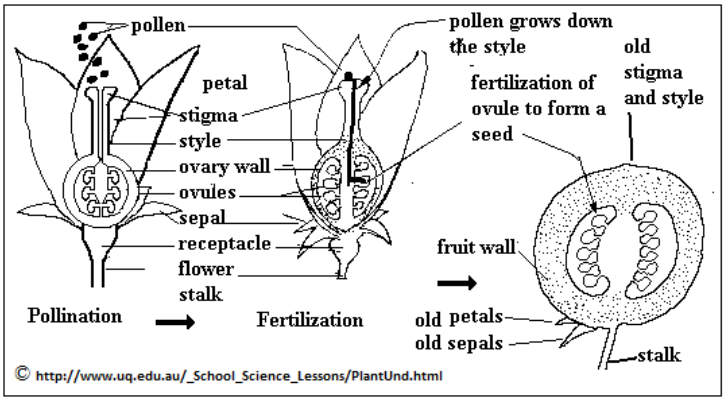

Figure 1. Schematic overview of pollination and fertilization in tomato flowers.

In a research of Hazra and Ansary (2008) it is found that pollen viability and germination emerged as the major limiting factors for fruit set under chronic high temperature stress. This seems logical because when the pollen are not viable there will be no fertilization and no fruit development. The underlying reason for the lack of viable pollen is probably carbohydrate stress. It works in this way, under control conditions (28/22°C) starch is accumulated in the pollen grains, where it reached a maximum value 3 days before anthesis; it then diminished towards anthesis, because starch is broken down to soluble sugars. During anther development, the concentration of soluble sugars gradually increased in the anther walls and in the pollen grains, reaching a maximum at anthesis. The reason for a decrease in pollen viability of tomatoes is probably that the heat stress prevents the build-up of starch which

may be associated with lower levels of soluble sugars derived from the hydrolysed starch in mature pollen grains (Pressman, Peet et al. 2002). Next to reduced pollen germination as a reason for reduced fruit set, Sato *et al* (2002) found that pollen release and disturbed microsporogenesis also plays a role. Microscopic investigation of anthers in plants grown continuously at high temperature indicated disruption of development in the pollen, endothecium, epidermis and stomium (Sato, Peet et al. 2002). Stomium is cell tissue that is important at the dehiscence of the anther, a disruption in the development of the stomium could explain a poor pollen release.
Another reason for a poor fruit set can be poor stigma tube elongation. When the stigma tube does not elongate, the pollen are not able to reach the ovule and pollination will not occur. Dane *et al* (1991) found that there is a negative correlation between stigma tube elongation and tomato fruit yield in a crop under high temperature stress. In conclusion, Saeed *et al.* (2007) thinks that the main reasons for poor fruit set at high temperature in tomato are stigma tube elongation, poor pollen germination, poor pollen tube growth and carbohydrate stress.

Fruit set in Arabidopsis

The fruit set in Arabidopsis plants is also vulnerable to high temperature stress. When the stamen in the flower of an Arabidopsis plant is pollinated a reproductive organ is formed. The reproductive organ in Arabidopsis is called a silique, which is a sort of pod with small seeds in it. When the plants are exposed to a temperature of 35°C for a time (in our case 13.5 hours) the development of siliques is disrupted due to the high temperature stress (Figure 2). In Arabidopsis plants the response to a high temperature is comparable with the reaction of tomatoes because also the fruit set is disrupted. Kim *et al.* (2001) found that when they applied a heat shock of 42°C for 4 hours to Arabidopsis during flower development, floral organs such as sepals, petals, stamens, and carpels developed normally. The disruption in silique development is probably due to poor fruit set. In tomatoes pollen viability and germination emerged as the major limiting factors for fruit set under chronic high temperature stress (Hazra and Ansary, 2008). In Arabidopsis the disruption in silique development is caused by poor pollen development. According to Sakata *et al.* (2010) is male sterility in Arabidopsis caused by abortion of pollen development due to a lower endogenous auxin biosynthesis. A lot of flowers are formed by an Arabidopsis plant and due to a short term heat shock only flowers in a certain developmental stage are affected. Warner *et al.* (2005) found that in different accessions also other stages are sensitive to the heat treatment (Ranging from floral stage 9-12). The effect on these different developmental stages can be investigated to find the cause of disruption in silique development. Kim *et al.* (2001) did this research for Columbia-wildtype plants and found that floral stage 9 primordia (during floral stage 9, pollen mother cells are separated from tapetum tissues and meiosis occurs) failed to produce any pollen grains. Morphological analyses suggested that heat shock causes a failure of separation of pollen mother cells followed by microspore differentiation and/or inhibition of male meiotic processes. Heat shock also caused sterility in floral stage 12 flowers (during this period of development, desiccation of pollen grains and anther dehiscence occurs) but the sterility was due to the failure of pollen release from the pollen sacs (Kim *et al.*, 2001). In a research of Sakata *et al.* (2010) heat shock injury was observed in recombinant Arabidopsis grown at 33 °C for >7 days, with plants forming short stamens and rarely producing any pollen because of premature abortion of microsporogenesis.

Figure 2. A) Arabidopsis plant that received a heat treatment for 13.5 hours. B) Control Arabidopsis plant.

1.2 Objectives and Hypothesis

The aim of this study is to find which developmental/physiological processes are involved in the disruption of siliques after a short period of elevated temperature in *Arabidopsis thaliana*. We expect to find that the pollen in sensitive developmental stages are not viable, or that fertilization is not possible due to a bad pollen release or delayed anther development leading to a mismatch in timing of pollen release and stigma development.

2. Material and Methods

2.1 Cultivation conditions

In this experiment the *Arabidopsis thaliana* plants were grown in a climate cell (C3 Radix Klima, Wageningen University and Research Centre), with controlled conditions. In total 380 plants of 19 accessions were placed in the climate cell. The plants were placed in plastic trays on a table with elevated borders. In the climate cell the temperature was kept constant at 20°C ±1°C during light hours and 18°C ±1°C during dark hours. A humidity level of 70% ±3% is obtained and the CO_2 level is around 700 ppm. The plants were watered every 2 or 3 day by hand with a watering can. The plants were lighted by fluorescent lamps (125 µmol/m^2/s), from 5:00 AM till 9:00 PM (16 hours light/8 hours dark). This light is gradually switched on and off within half an hour (Figure 3).

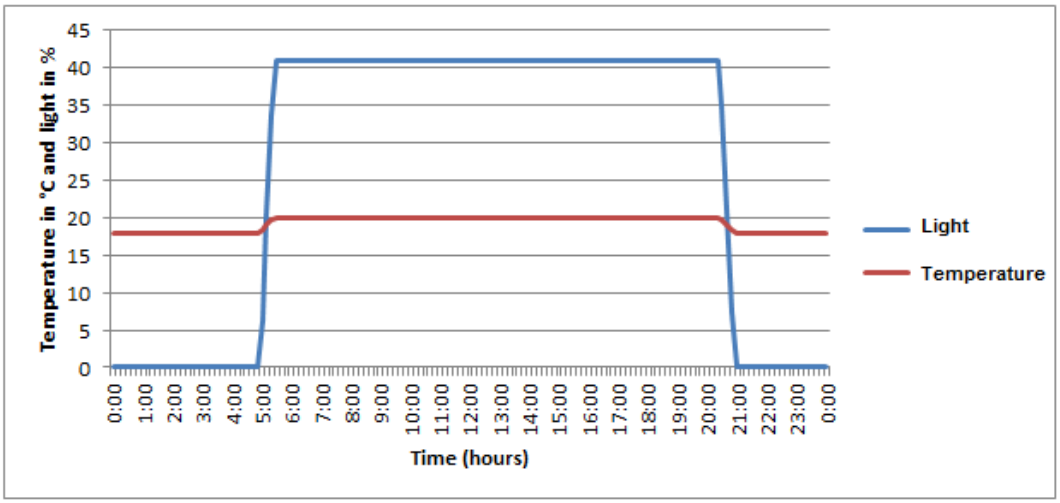

Figure 3. Time schedule for light (%) and temperature (°C) in the climate cell (C3).

2.2 Experimental setup

Our experiment is based on an experiment done by Johanna Molenaar, in this previous experiment 360 accessions were grown (HapMap population), out of this population 27 accessions were selected based on their genetic variation. Out of the 27 accessions, 19 accessions were selected by phenotype. This selection was based on their reaction to the heat shock, 9 were selected because the silique formation was not disturbed and another 6 were selected because the silique formation was heavily disturbed. These 19 accessions were grown again, around 40 seeds were sown on a filter paper in a petri dish, the filter paper was watered with demi water. After sowing the petri dishes were placed in a cooling cell at a temperature of 4°C for four days to stimulate germination. After four days the seedlings were put in a growth chamber for two days at 20°C. After a total of six days the 20 most developed seedlings were picked with a brush and individually planted in a wet rockwool cube and placed in the climate cell (growing conditions described in chapter 2.1).

There are 20 plants per accession, 6 were kept at control conditions and 9 received the treatment, the other 5 were used as back up plants. All accessions are numbered and the plants of the accession are labelled from A to T. Most of the times A, B, C, D and E are control labels, the others are used for treatment plants. The plants were placed in plastic trays, 5 accessions per tray in vertical rows.

The flowering time per accession was scored and approximately one week after the most plants of an accession were flowering, the plants were collected and transported to a Weisscabinet on Wednesday. In the Weisscabinet the plants received a heat treatment. First the plants can acclimatise for a day at 20°C and then on Thursday at 2.00 AM the temperature gradually increases within 1.5 hour to 35°C. This temperature is contained for 13.5 hours, after that the temperature gradually decreases again and the plants can acclimatise tot 20°C again (Table 1).

Table 1. Time table of the heat treatment for plants in the Weisscabinet.

Date	Time	Humidity	Temperature	Light
				125 umol/m2/s
Wednesday	2.00	70%	20°C	0% start increasing
	2.30			100%
	17.30			100% start decreasing
	18.00			0%
Thurday	2.00	70%	20°C start increase	0% start increasing
	2.30			100%
	4.00	74%	35°C	
	17.30	74%	35°C start decrease	100% start decreasing
	18.00			0%
	19.30	70%	20°C	
Friday	2.00	70%		0% start increasing
	2.30			100%
	17.30			100% start decreasing
	18.00			0%

There was only one small problem during the experiment. There was a difference in climate conditions for the Weisscabinet and the climate cell (C3). In the Weisscabinet, where the plants received the heat shock, the light was gradually switched on at 2:30 AM and gradually switched off at 5:30 PM. In the climate cell (C3) the light was gradually switched on at 5:30 AM and gradually switched off at 20:30 PM. In the Weisscabinet on the day that the plants received the heat shock, the temperature gradually increased from 20°C to 35°C, starting at 2:30 AM. While during normal conditions and for the control plants the temperature increased from 18°C to 20°C at 5:30 AM (Figure 4).

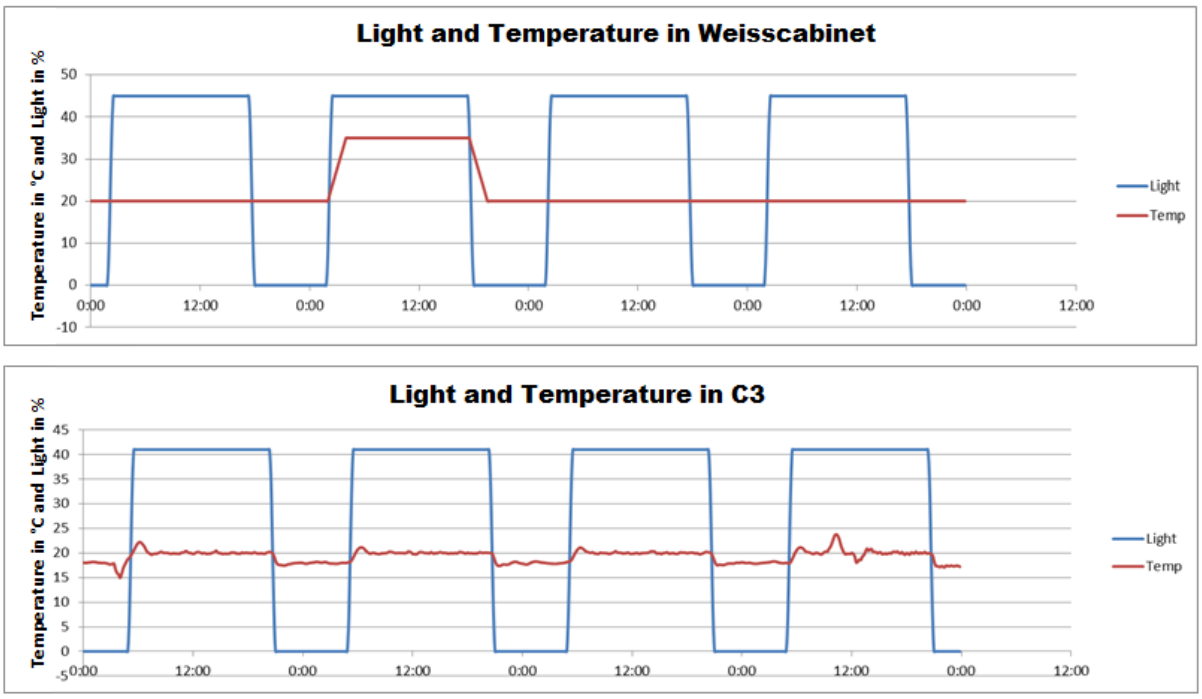

Figure 4 . Schedule for light and temperature in the climate cell (C3) and the Weisscabinet.

2.3 Measurements

The flowering time per accession is checked every day except for Saturday and Sunday. Approximately 7-10 days after flowering the accessions are selected for the heat treatment and a little thread was tied on the stem of the flower that will open the next day, this is a flower of the main branch. The flower with a thread on the stem was used as a reference flower also known as flower 0. The treatment plants received a heat shock on Thursday. Measurements started on Monday after the heat shock. The following measurements were done for 5 days:

- Pistil and stamen length (2 control, 3 treatment)
- Pollen germination (2 control, 3 treatment)
- Silique length (2 control, 3 treatment)

To measure the pistil and stamen length, every day all open flowers were picked from the main stem from control and treatment plants. Two petals were removed with a tweezers and the flowers were glued on a microscope slide with nail polisher in the right order of flowering (left to right, latest to earliest flowering). Then a photo was made with a microscope-camera and with Image J the length of the pistil and stamens was calculated (Figure 5).

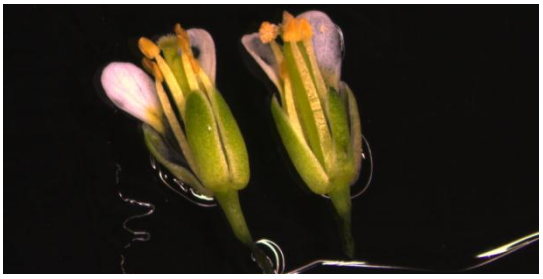

Figure 5. Arabidopsis flower on a microscope slide.

The pollen germination rate for treatment and control plants was checked every day for all open flowers on the main stem. The flowers were picked with a tweezers and dipped in a droplet of germination solution (18% Sucrose, 0.01% Boric acid, 1mM $CaCl_2$, 1mM Ca $(NO_3)_2$ and 1mM $MgSo_4$) on a microscope slide and hung upside down during the night (Appendix 7.1). In this way the pollen were able to germinate. The next morning the germination rate was checked by counting 30 pollen under the microscope. When a pollen tube was visible the pollen was counted as germinated, when it was not visible is was recognised as not germinated, in this way the rate of germination was calculated (Figure 6). It was also noted when there were no pollen visible in the solution.

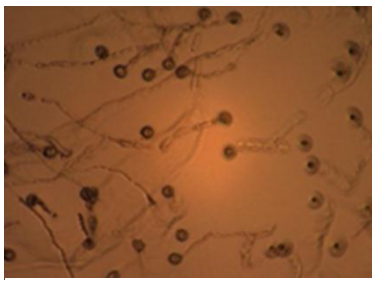

Figure 6. Germinated pollen on a microscope slide.

The silique length was measured per plant with an electronic caliper in millimetres, starting at the stem of the plant to the top. The flower with the threat was set as flower 0, in this way the effect of the heat shock was visible.

Table 2. Overview of accessions, abbreviation and origin for the non-disturbed, medium and disturbed accessions (disturbed in the experiment of Johanna Molenaar), which were used in this experiment.

Non-disturbed			**Medium**		
Accession	Abbreviation	Origin	Accession	Abbreviation	Origin
77	Nw-0	Germany	33	Di-1	France
111	Sp-0	Germany	165	Col-0	Columbia
117	Tsu-0	Japan	168	Cvi-0	Cape Verde Islands
146	Bay-0	Germany	279	Sha	Tajikistan
152	Bor-4	Czech Republic	**Disturbed**		
185	Ga-0	Germany	3	Alst-1	United Kingdom
228	Lp2-2	Czech Republic	103	RRS-7	USA
243	Mrk-0	Germany	157	Bur-0	Ireland
263	Petergof	Russia	172	DraIV1-5	Czech Republic
			181	Fei-0	Sweden
			270	Rennes-1	France

3. Results

3.1 Overview

The silique length was measured for 19 accessions, for 11 accessions the results were, for various reasons, not used in the analysis (Appendix 7.2.1). For 4 accessions (33, 146, 181 and 117) the silique formation was disturbed, which means that the treatment plants showed a decrease in silique length after applying the heat treatment (Table 3). For 4 accessions (185, 263, 270 and 279) the silique length was not disturbed, which means that the treatment plants do not show a decrease in silique length compared with the control plants (Table 3). For the accessions with disturbed and non-disturbed silique formation also the difference in pistil-stamen length, pollen release rate and the pollen germination rate is shown (Table 3).

Table 3. Overview of results for silique length, pistil-stamen length, pollen release rate and pollen germination rate for 9 accessions. + = difference in treatment and control, - = no difference in treatment and control.

Accession	33 (Di-1)	146 (Bay-0)	181 (Fei-0)	117 (Tsu-0)
Silique length	disturbed	disturbed	disturbed	disturbed
Pistil-stamen length	+	-	-	-
Pollen release rate	-	+	+	-
Pollen germination	-	-	-	-

Accession	185 (Ga-0)	263 (Petergof)	270 (Rennes-1)	279 (Sha)
Silique length	non disturbed	non disturbed	non disturbed	non disturbed
Pistil-stamen length	+	-	-	-
Pollen release rate	+	-	-	-
Pollen germination	-	-	-	-

3.2 Silique length

Accession 33 (Di-1) had a disturbed silique length, after receiving the heat shock, starting at silique -6 till silique 19. The siliques (silique -6 till 19) of treatment plants were on average 9.13 mm and the siliques of the control plants were 14.78 mm, this is a difference of 5.65 mm. The silique length from silique -20 till silique -6 was higher for the treatment plants compared with the control plants (Figure 7). The silique length of accession 146 (Bay-0) was influenced by the heat shock, the siliques -5 till 18 of the treatment plants were smaller than the control plants. The average silique length (silique -5 till 18) was 6.46 mm for the treatment plants and 13.12 mm for the control plants, a difference of 6.66 mm. For accession 181 (Fei-0) the silique length is disturbed after applying the heat treatment (Figure 7). From silique -14 till -5 the length is equal to the control, but from silique -5 till 30 the silique length is smaller than the control plants. The average silique length (silique -5 till 30) is 8.34 mm for the treatment plants and 12.87 mm for the control plants, a difference of 4.52 mm. Starting at silique 30 the silique length increased and at silique 36 the length of the siliques of the treatment plants are larger than the control plants. Accession 117 (Tsu-0) also had a disturbed silique length after the heat shock, the siliques 8 till 12 of the treatment plants were smaller than the control plants. The average length of the siliques 8 till 12 was 11.53 mm for the treatment plants and 16.86 mm for the control plants, a difference of 5.33 mm. The other siliques did not differ a lot in length, only after silique 29 the siliques of the control plants became smaller than the treatment plants (Figure 6).

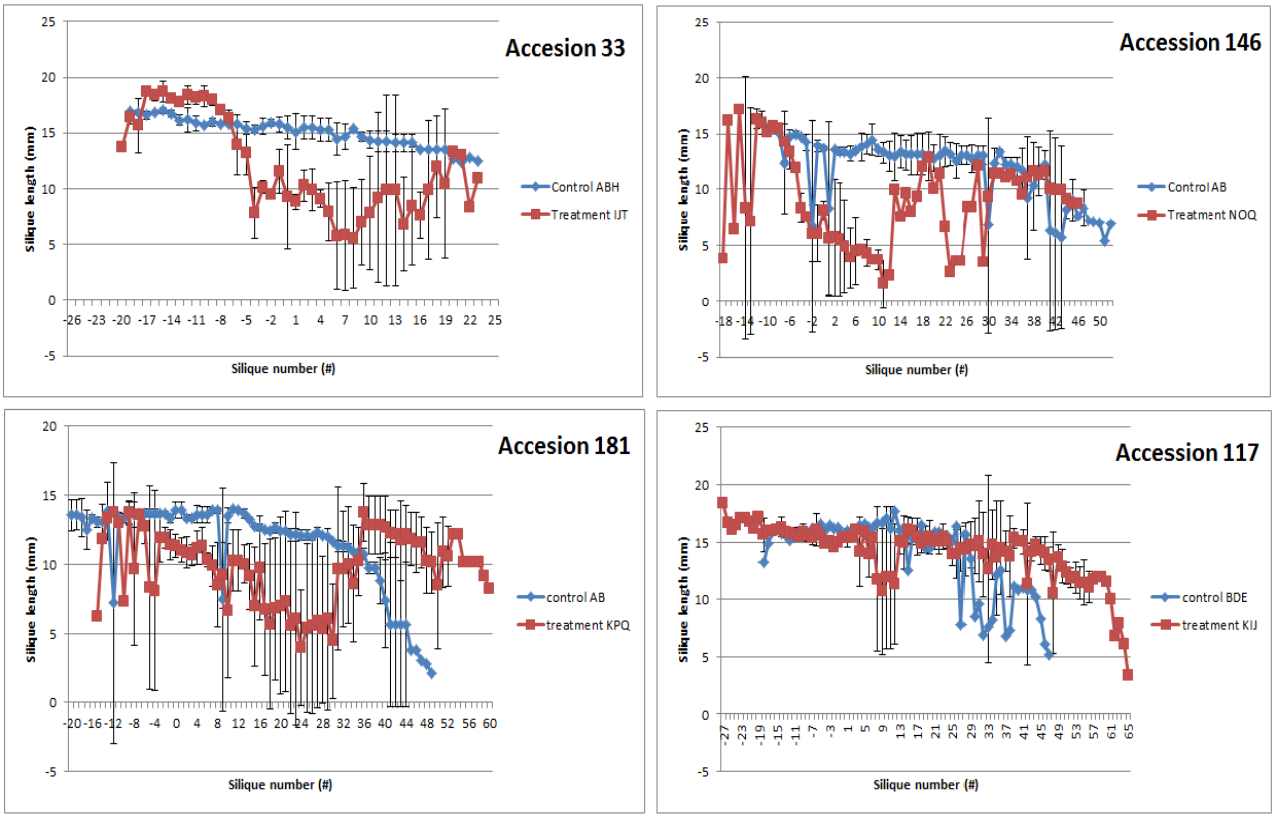

Figure 7. The silique length in mm per silique on the main stem for accession 181 (Fei-0), accession 33 (Di-1), accession 146 (Bay-0) and accession 117 (Tsu-0).

In short or in conclusion: Accession 33, 146, 117 and 181 show a disturbed silique length (Figure 7 and Appendix 7.2.2). The silique length is for most siliques in the range of -10 till 10 significant lower than the control plants (Table 4). There is no significant difference between the silique length of the control and treatment plants for the accessions with a non-disturbed silique formation (185, 263, 279 and 270).

Table 4. The P-values of the difference in silique length (from silique -10 till 10) between the treatment and control plants of accession 33, 146, 181 and 117.

Silique number	Accession 33	Accession 146	Accession 181	Accession 117
-10	0.16	-	-	0.45
-9	0.05	-	0.98	0.70
-8	0.07	-	0.49	0.78
-7	0.51	0.67	0.70	0.46
-6	0.36	0.37	0.50	0.96
-5	0.20	0.01	0.33	0.12
-4	0.03	0.09	0.31	0.14
-3	0.02	0.04	0.23	0.32
-2	0.00	0.94	0.05	0.01
-1	0.05	0.13	0.08	0.04
0	0.14	0.06	0.03	0.03
1	0.09	0.72	0.02	0.34
2	0.02	0.28	0.05	0.16
3	0.02	0.27	0.02	0.81
4	0.03	0.21	0.06	0.31
5	0.03	0.11	0.08	0.34

6	0.08	0.15	0.02	0.36
7	0.08	0.05	0.04	0.06
8	0.06	0.02	0.09	0.31
9	0.07	0.05	0.81	0.20
10	0.15	0.01	0.13	0.29

3.3 Pistil-stamen length

The pistil and stamen length was measured for all accessions. The difference between these two lengths (pistil-stamen) was calculated for every flower and these values were averaged for control and treatment plants. The accessions 146, 181 and 117 with a disturbed silique length did not show a significant difference in pistil-stamen length between the control and treatment plants (for 181 and 117 were data not shown). For accession 146 the average pistil-stamen length was 0.27 mm for the control plants and 0.59 mm for the treatment plants, not a significant difference. Only the first 5 measured pistil-stamen lengths had a bigger difference, silique 9 till 13 had an average pistil-stamen length of -0.01 mm for the control plants and 0.65 mm for the treatment plants (Figure 8). For the siliques 9 till 13 the pistil extents the stamen for the treatment plants. Only for accession 33 (disturbed silique length) the average difference in pistil-stamen length for control plants was -1.06 mm and for treatment plants -0.38 mm (Figure 8). The stamen were significantly bigger for the treatment plants compared with the control plants.

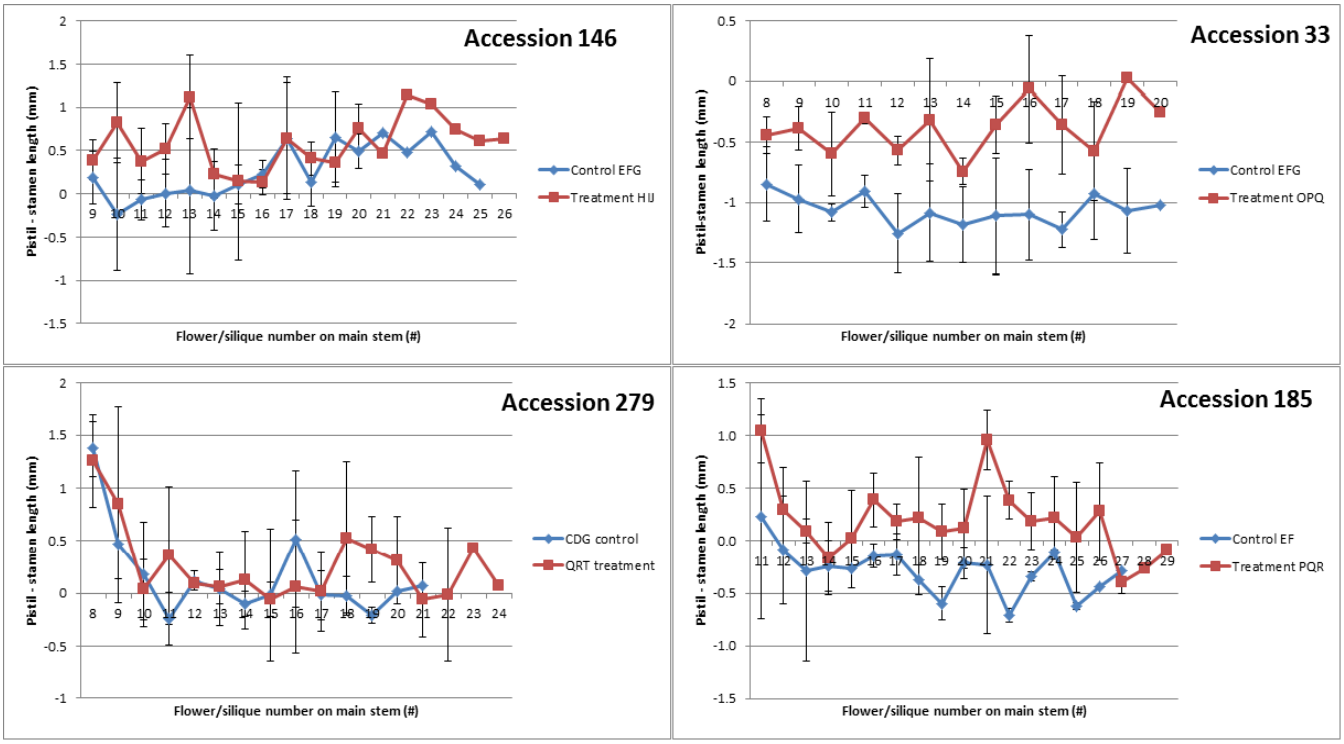

Figure 8. The pistil-stamen length in mm per silique/flower on the main stem for accession 146, 33, 279 and 185.

For accession 279 with a non-disturbed silique length did the pistil-stamen length not significantly differ (Figure 8). The average pistil-stamen length for the control plants of accession 185 was -0.285 mm and the average pistil-stamen length for the treatment plants was 0.188 mm. The stamen of the treatment plants was for most of the flowers on the main stem significantly bigger than the control plants (Figure 8). The other accessions with a non-disturbed silique length (263 and 270) did not show a significant difference in pistil-stamen length.

3.4 Pollen germination rate

The pollen germination rate was measured on Monday and the following four days, after the heat treatment on Thursday.

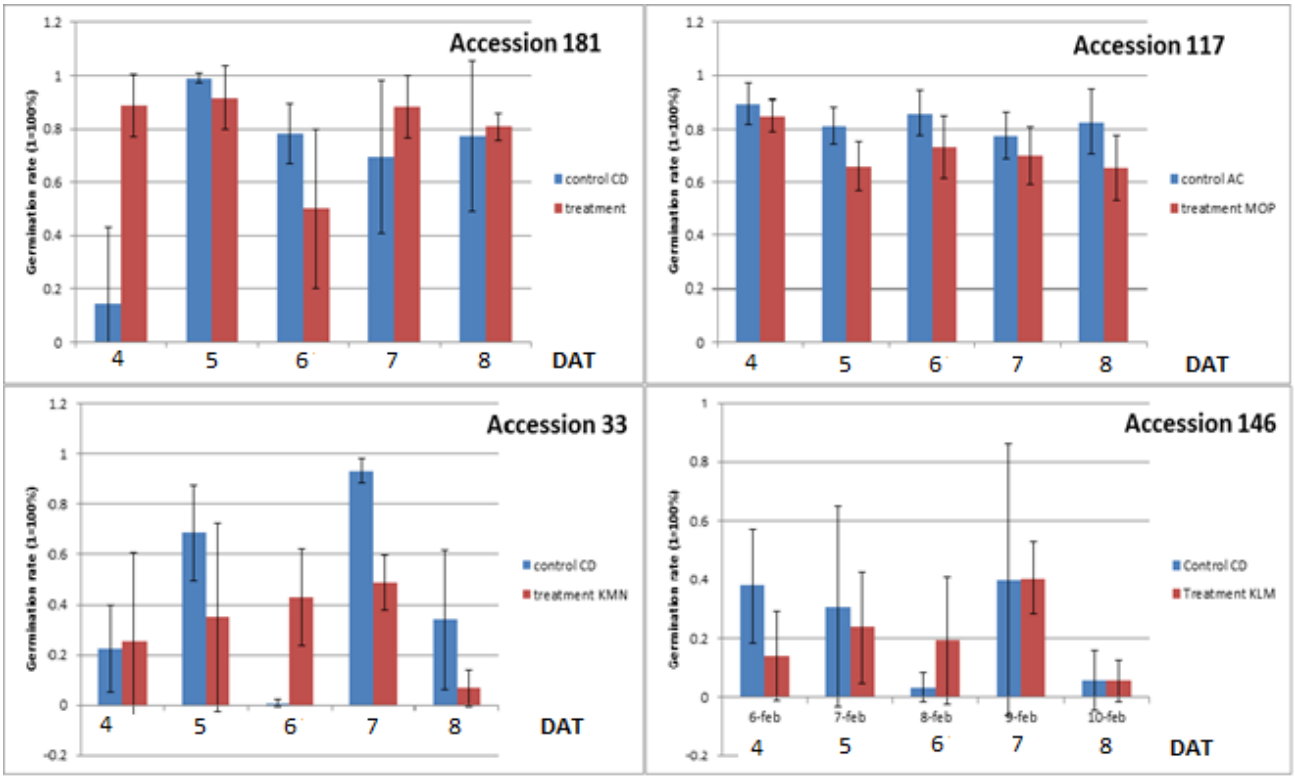

Figure 9. The germination rate for control and treatment plants in five days (DAT = Days After Treatment) for accession 181, 117, 33 and 146.

For all the accessions the germination rate was measured. The accessions with a non-disturbed silique length (185, 263, 270 and 279) did not have a significant difference in pollen germination rate between the control and the treatment plants. The differences in control and treatment plants for the average pollen germination rate of the non-disturbed accessions are between 1.7% and 8.9% (Table 5).

For the accessions with a disturbed silique length (33, 117, 146 and 181) some interesting observations were made. On the day 4 (13th of February) the germination rate of the control pollen of accession 181 was significantly lower than the treatment pollen but for the other days there was no significant difference between the control and treatment plants. Accession 117 had a constant germination rate for the control and treatment plants, the average germination rate for the control plants was 83% and for the treatment plants 72%, this difference was not significant (Table 5). Accession 33 had a low germination rate for the control and treatment plants. On day 6 (Wednesday 8th of February) the germination rate was significant lower for the control plants, and on day 7 (Thursday 9th of February) the germination rate was significant lower for the treatment plants. On the other days, not a significant difference was found. Accession 146 had a low germination rate for control and treatment plants. There was no significant difference in the control and treatment plants for the five different days (Figure 9).

Table 5. Average pollen germination rate (%) for control and treatment plants of 8 accessions.

Accession	Control germ. rate (%)	Std. dev.	Treatment germ. rate (%)	Std. dev.
33	43.8	0.14	31.8	0.22
146	23.5	0.23	20.7	0.15

181	67.6	0.20	79.9	0.14
117	83.4	0.09	71.9	0.10
185	41.8	0.23	43.5	0.15
263	54.9	0.18	46.0	0.14
270	33.6	0.19	35.3	0.14
279	11.2	0.09	9.8	0.11

3.5 Pollen release rate

For accession 181 the pollen release rate of the control plants was 1 for all five days, this means that every day all picked flowers released pollen, most of these pollen germinated. The treatment plants had a lower pollen release rate for 14, 15 and 16 February (Figure 10). On the 14th of February only 40% of the flowers released pollen, for the other days this is respectively 89% and 33%.

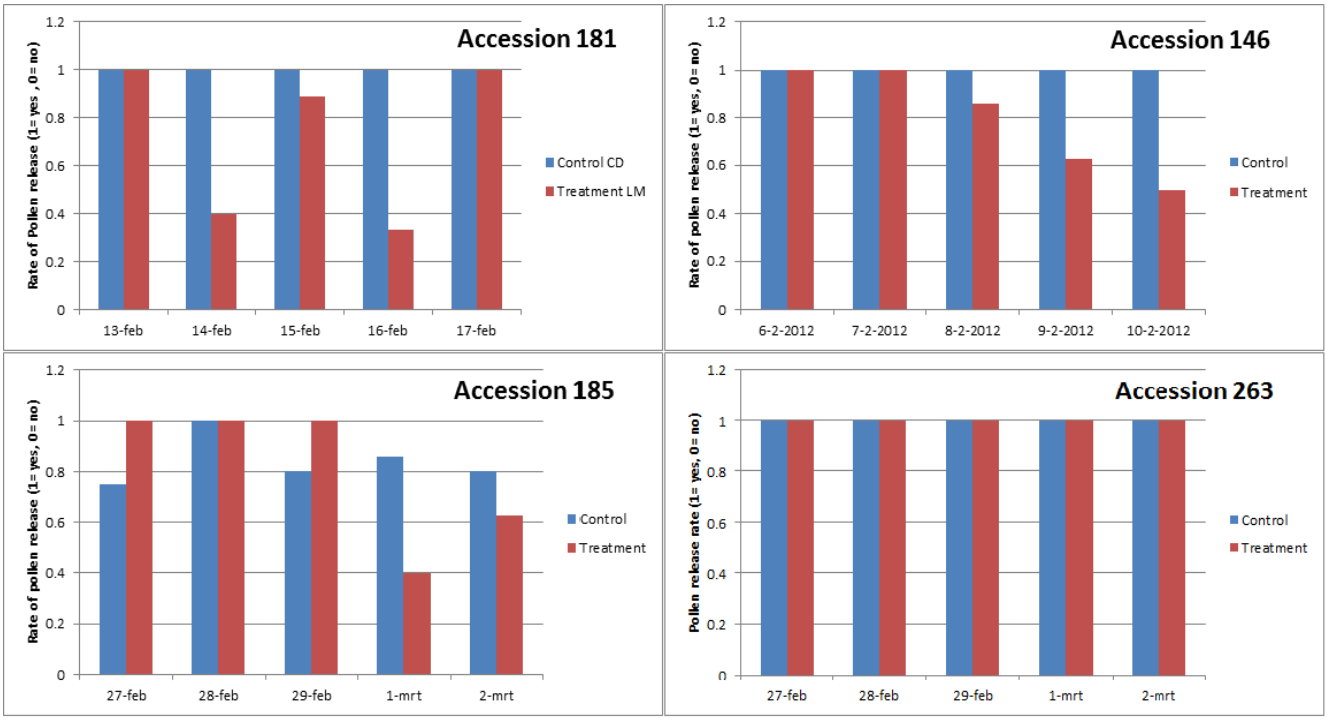

Figure 10. The pollen release rate for 5 days of accession 181, 146, 185 and 263.

The control plants of accession 146 did release pollen every day, but for the treatment plants the pollen release rate started to decrease on the 8th of February. The pollen release rate of the treatment plants of accession 146 was respectively 86%, 63% and 50% for 8th, 9th and 10th of February (Figure 10). For the other accessions with a disturbed silique length, 33 and 117, there was no difference between the control and treatment plants in the pollen release rate. For accession 263, 270 and 279 with a non-disturbed silique length, there was also not much difference in the pollen release rate between control and treatment plants. For accession 185 of the group with a non-disturbed silique length the pollen release rate did differ much, for the control plants the rates were 75%, 100%, 80%, 86% and 80% for the days 27th, 28th, 29th of February and the 1st and 2nd of March. For the treatment plants this rates were 100%, 100%, 100%, 40% and 63% (Figure 10).

4. Discussion

The aim of this experiment is to find which developmental processes are involved in the disturbance of siliques formation due to a short period of elevated temperature. In this experiment 19 accessions were grown, from 11 accessions the results were not incorporated in the analysis mostly due to a shortage of well grown plants. For four accessions (33, 117, 146 and 181) the silique length was disturbed and for four accessions (185, 263, 270, 279) the silique length was not disturbed. There could be various reasons for a disturbed silique length, bad pollen germination, no pollen release for the pollen sacs, low pollen viability or the pistil could extent the stamen.

4.1 Pollen germination

A reason for the disturbed silique formation in the accessions 33, 117, 146 and 181 could be due to aberrant pollen development. Kim *et al.* (2001) applied a heat shock of 42°C for four hours and they did not detect normal pollen grains in flowers that were in floral stage 9 at the moment that the plants received the heat shock. Approximately 65% of the treated flowers showed huge clamps instead of pollen grains inside the pollen sacs and 35% of flowers showed irregular shaped pollen-like structures. In our research the average germination rate of 5 days for accession 33 was 43.8% for control plants and 31.8% for treatment plants (Table 5). Also for accession 117 the average pollen germination rate is 83.4% for the control plants and 71.9% for the treatment plants. This lower germination rate for treatment plants could indicate unviable pollen and in this way influence the fertilization and cause the malformation of siliques. The flowers were in floral stage 7, 8 and 9 when the heat treatment was applied.

In accession 146 the average pollen germination rate is 23.5% for the control plants and 20.7% for the treatment plants, both germination rates are low but there is no significant difference between the two (Table 5).

For the accessions 33, 117 and 146 the germination rates were almost constant and not a special developmental stage was different. For accession 181 the average pollen germination rate was 67.7% for the control plants and 79.9% for the treatment plants, in this case the average germination rate was high for the treatment plants compared with other accessions (Figure 9). The low average germination rate for the control plants is due to a germination rate of 14.4% on day 4 (13th of February). The high germination rate for the treatment plants indicates a good pollen viability, only on day 6 after treatment (the 15th of February) the germination rate was 50%. According to Smyth *et al.* (1990) it will take 144 hours (6 days) from floral stage 9 to reach floral stage 13, the stage when the flower opens and the pollen are released. The flowers on day 6 after treatment were in floral stage 9 when they received the heat shock, in a research of Kim *et al.* (2001) the pollen of flowers in floral stage 9 were malformed due to the heat shock leading to male sterility. In our research the plants receive a lower heat shock temperature of 35°C, probably in some flowers the pollen formation was influenced which lead to a 50% germination rate. A reason for the heat treatment caused abortion of pollen could be due to a lower biosynthesis of endogenous auxin in developing anthers (Sakata *et al,* 2010).

In our experiment we only compare germination rates within accessions because there is a high variation in the different accessions. For some accessions the germination rate is low for the control plants, this low germination rate is probably caused by the temperature during the germination. In a report of Boavida *et al.* (2007) the optimal germination temperature was 22°C (germination 80%, below or above caused a germination drop of 30%. This shows the temperature sensitivity for in vitro pollen germination for *Arabidopsis*.

4.2 Pollen release

In a research of Kim *et al.* (2001) the heat shock induced sterility in flowers is also due to a failure of pollen release from pollen sacs in certain floral developmental stages. In our research the pollen release rate for accession 33 and 117 did not significantly differ between the control and the treatment plants. So probably another mechanism should cause the observed reduction in silique length. In accession 146 the pollen release rate is 100% for the control plants and also for the treatment plants in the first two days. The release rate for treatment plants starts to decrease in the last 3 days: 86%, 63% and 50% (Figure 10). It is possible that due to the lower release rate less fertilization occurred, this could be a reason for the disturbed silique length.
For accession 146 the pollen release rate starts to decrease in the last days, in a research of Kim *et al.* (2001) the flowers that had a failure in pollen release from the pollen sacs, were in floral stage 12 the stage that the flowers almost open. This could not be the reason because the flowers were on that day of the heat shock still in floral stage 8/9. Also in accession 181 the pollen release rate of the control plants is 100% every day, for the treatment plants this rate is 100%, 40%, 89%, 33% and 100% (Figure 10). In a research of Kim *et al.* (2001) flowers at floral stage 13 during the heat shock, had a failure in anther dehiscence and pollen release. Probably this is not the case in our research because the flowers on 12, 13 and 14 February, which corresponds to 40%, 89% and 33% pollen release rate, were in floral stage 9 at the moment when the heat shock was applied.
The different temperature or duration of the heat shock, compared with Kim *et al.* (2001), probably influenced the formation of pollen of flowers in floral stage 8/9 because significant less pollen were released. The low pollen release rate probably influenced the fertilization and is in this way the cause for a disruption in the silique formation.

4.3 Stamen/pistil elongation

For accession 33 the silique length is disturbed, from silique -4 till silique 5. The siliques are significantly shorter for the treated plants (Table 4). In a report of Dane *et al.* (1991) a negative correlation between stigma tube elongation and tomato fruit yield was showed. In a report of Sakata *et al.* (2010) the heat shock effect was observed in Arabidopsis plants grown at 33 °C for >7 days, these plants formed short stamens and rarely produced any pollen. Our heat treatment is shorter, so probably less severe but still the pistil and stamen development could be influenced. The pistil could extent the stamen and in this way pollination is not possible. In our research the average pistil-stamen length for accession 33 for the control plants was -1.06 mm and for treatment plants -0.38 mm. The difference in pistil and stamen length was smaller for the treatment plants than the control plants, this means that the stamen are closer to the pistil (Figure 8). When the pistil-stamen length is negative, the stamen are larger than the pistil and pollination is possible, so probably the silique disruption observed is not caused by the differences observed in stamen and pistil development.
For accession 117 and 181 the silique length was also disturbed, but there was not a significant difference for the pistil-stamen length between the control and treatment plants of these two accessions (Table 4).
For accession 146 the silique length was disturbed, the siliques -5, -3, 8 and 10 were significant smaller than the siliques of the control plants (Table 4). Also most of the other siliques were smaller than the control plants (Figure 8). The average pistil-stamen length was 0.27 mm for the control plants and 0.59 mm for the treatment plants, not a significant difference, but the first 5 measured pistil-stamen lengths had a bigger difference. Silique 9 till 13 had an average pistil-stamen length of -0.01 mm for the control plants and 0.65 mm for the treatment plants. The positive value indicates that the pistil extents the stamen and probably this difference had an influence on the pollination and development of these siliques.
For the non-disturbed accessions the pistil-stamen length did also not differ for the control and the treatment plants. Only for accession 185 the average pistil-stamen length for the control plants was -0.285 mm and the average pistil-stamen length for the treatment plants was 0.188 mm (Figure 8). This significant difference probably did not have influence on the silique formation. Probably in some

accessions the heat shock influences the elongation of the stamen and pistil but for most accessions the difference in pistil-stamen length did not cause the disturbed silique length.

4.4 Climate conditions

In our research 4 out of 19 accessions had a clearly disturbed silique formation, 11 accessions were not incorporated in the results because of various reasons (Appendix 7.2.1). Most of the times the plants did not develop normally and a high variation in plant length was noticed. Also the flowering times did differ within accessions. Accession 165 (Colombia) was for example not incorporated because only 1 treatment plants was available for the germination and pistil-stamen length measurements, also only 10 out of 20 plants were flowering. This low uniformity within the accessions indicates that the growing conditions are not optimal and that the plants are experiencing stress.

In a research of Johanna Molenaar (*personal communication*) the same experiment was conducted and also the same accessions were used. The experiment of Johanna Molenaar took place in another climate cell. For accession 117 the difference in silique length is clearly visible (Figure 11).

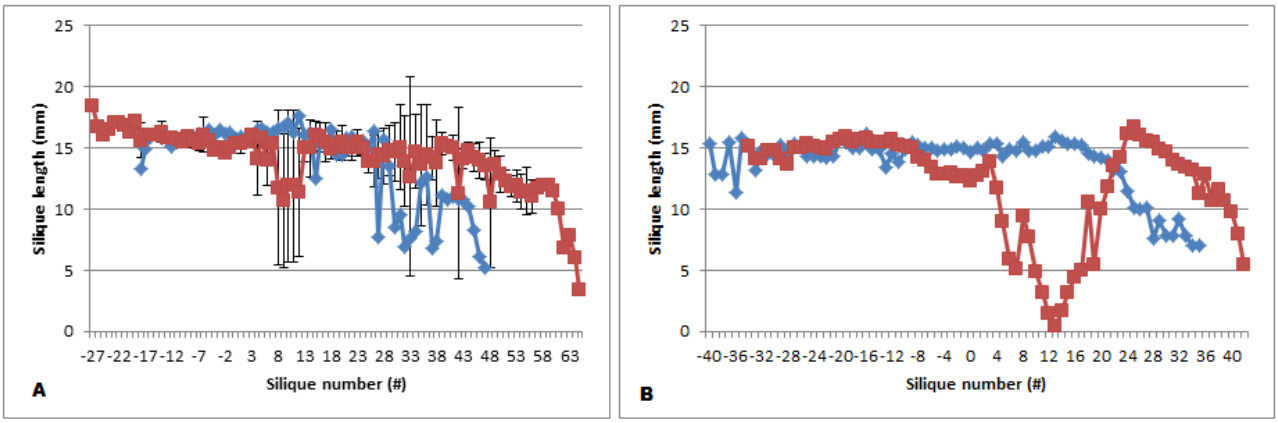

Figure 11. The difference in response to a heat shock on silique length between A. accession 117 in our experiment and B. accession 117 in the experiment of Johanna Molenaar (Personal communication, 2012).

For accession 117:A (our experiment) the average silique length for silique 8 till 12 is 11.53 mm for the treatment plants and 16.86 mm for the control plants, a difference of 5.33 mm. The average silique length of accession 117:B (experiment of Johanna) for silique 4 till 21 was 6.16 mm for the treatment plants and 14.96 mm for the control plants, a difference of 8.80 mm (Figure 11). The effect of the heat shock is heavier for the plants of the research of Johanna, because the difference in length is bigger and the number of siliques affected is larger.

The plants of Johanna Molenaar did receive a vernalization treatment after the seedlings had grown for a week. This vernalization treatment contributed to a high uniformity within the accessions. A reason for the low uniformity in our accessions is due to the non-optimal growing conditions and the lack of a vernalization treatment. The plants of Johanna were grown in a different climate cell, probably the growing conditions in this climate cell were more optimal for the plant development. In the climate cell of our experiment the ventilation system circulated air at a high velocity. According to Bossdorf *et al.* (2009) did high wind speed significantly affect plant growth and phenology. Due to this mechanical stress maybe the plants were less vulnerable to the heat shock. Probably also the different light schedule of the Weisscabinet had an influence on the effect of the heat shock.

5. Conclusion and recommendations

5.1 Conclusion

In our experiment 4 accessions (33, 117, 146 and 181) did have a disrupted silique formation. In accession 33 and 117 the silique length was disturbed and the average germination rate of the pollen of these accessions was lower for treatment plants compared with the control plants, respectively 31.8% and 71.9% against 43.8% and 83.4%. The lower pollen germination rate for the treatment plants probably influenced the fertilization and the formation and development of siliques.
Another influencing factor for the disturbed silique formation is a low pollen release. In accession 181 the release rate of the pollen of the treatment plants is constant only on day 4, 5 and 6 after the treatment the pollen release rate is 40%, 89% and 33% instead of 100%. The flowers had a significant lower pollen release rate compared with the control plants, probably the pollen were not developed or malformed which prevents release from the pollen sac. The flowers on day 4, 5 and 6 after treatment of accession 181 were on the day of the treatment in floral stage 9, in this stage the pollen mother cells are separated from tapetum tissues and meiosis occurs. Probably this developmental stage is sensitive for the elevated temperature.
In most accessions the pistil-stamen length was not significant different for the treatment plants compared with control plants, only for accession 33 a significant difference was observed but this value was negative which indicates stamen extending the pistil. Probably this did not influence the silique formation.
Four accessions (185, 263, 270 and 270) did have a non-disrupted silique formation. However we found a significant difference for stamen-pistil length and pollen release rate (Figure 8 and 10). We could not explain from our data why these observed differences did not result in shorter siliques. Compared with the research of Johanna the effect of the heat shock is less severe in our treatment, because only 4 accessions had a disturbance in silique formation. The plants in our experiment were less sensitive to the heat shock, this lower sensitivity is probably caused by the non-optimal growing conditions, which also resulted in a low uniformity within the accessions.
Plants which received a heat shock had a disruption in the silique formation, this is due to a decreased pollen germination and pollen release rate. The flowers are sensitive for this heat treatment in floral stage 9 when the flowers are developing and the meiosis and pollen mother cell separation is taking place.

5.2 Recommendations

In this research the most important thing was to find the developmental processes that are involved in the disruption of siliques, therefore it is important that the moment the flowers are affected is easy noticeable. It is important that the plants are uniform and that the reactions to the heat shock within accessions does not differ too much. For further research I would use a vernalization treatment to guarantee a higher uniformity. Also the growing conditions should be optimal and the plants should not experience stress. Less accessions and more plants per accession is desirable, because in that way the research will be more significant. When the pistil-stamen measurements are skipped more time can be used for other measurements.
To really find out which developmental processes are involved it is maybe an possibility to use a scanning electron microscopy device to find out how the pollen and flowers develop after the treatment.

6. Literature References

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7. Appendices

7.1 Pollen Germination Solution

Solid Pollen Germination Medium (PGM):
(Zhengbio Yang laboratory, University of California, Riverside)

18% Sucrose
0.01% Boric acid
1mM $CaCl_2$
1mM $Ca(NO_3)_2$
1mM $MgSo_4$
0.5% Noble agar (Difco)

pH the medium to 7.0. pH is important to get good pollen tube growth. Dissolve sucrose first, then add other ingredients. Finally add agar, place medium in a hot water bath and let the contents boil until agar completely dissolves (3-4 minutes). There is no need to autoclave the medium. Pour medium onto petri dishes (Fisher Brand small mini dishes,35 X 10 mm, catlog number 08-757-11YZ) or slides (in this case add medium to the slides by Pasteur pipettes). Store plates at 4^0 C. I have used plates for up to 6 weeks without any problem.

Liquid medium for pollen growth *in vitro*: (reference: Hicks et al, 2004; Plant Phys. 134: 1227-1239)
1. Prepare liquid medium as per receipe above (of course, omit the agar and related steps).
2. Place 30ul of liquid pollen growth medium on a glass slide (we use slides from eriesciences, www.eriesci.com; catlog #:10-175A-Black)
3. Touch pollen-containing anthers on the liquid meniscus.
4. Rapidly flip the slide upside down (do not worry, liquid won't fall; do not do the inverting process slowly, instead just flip the slide!) and let the slide remain in a wet chamber (tip box with wet kimwipes) for 6hrs-overnight. The drop should be
small enough that surface tension keeps it hanging there and it doesn't fall off,
yet not too small.
5. To have the slide in the inverted position, have them stand in between two raised surfaces, so that there is airspace below the liquid medium.
6. When done growing, reinvert the slide, add staining solution (optional), and place a cover slip and observe under microscope.

7.2.1 Silique length not incorporated

Accession 243 was not incorporated because there was only 1 treatment plant, and this treatment plant showed a strange growth pattern. Accession 165 was not incorporated because there were not enough treatment plants for the other experiments. For Accession 152 the control plants showed a strange growth pattern. For accession 77 the control plants did not grow very well and the silique length of the control plants is a lot smaller than the treatment plants. The control plants of accession 228 are not stable and show a very variable growth pattern. For accession 172 the variance in silique length for the treatment plants is too big to see the effect of the heat shock. For accession 111 the control plants show a strange growth pattern. For accession 3 the variance in silique length for treatment plants is too big. For accession 157 there is also a big variance in the silique length of the treatment plants and it is not visible when the heat shock affected the plants. For accession 103 there were no results for the germination rates of the pollen, this is why the result of the siliques are not used.

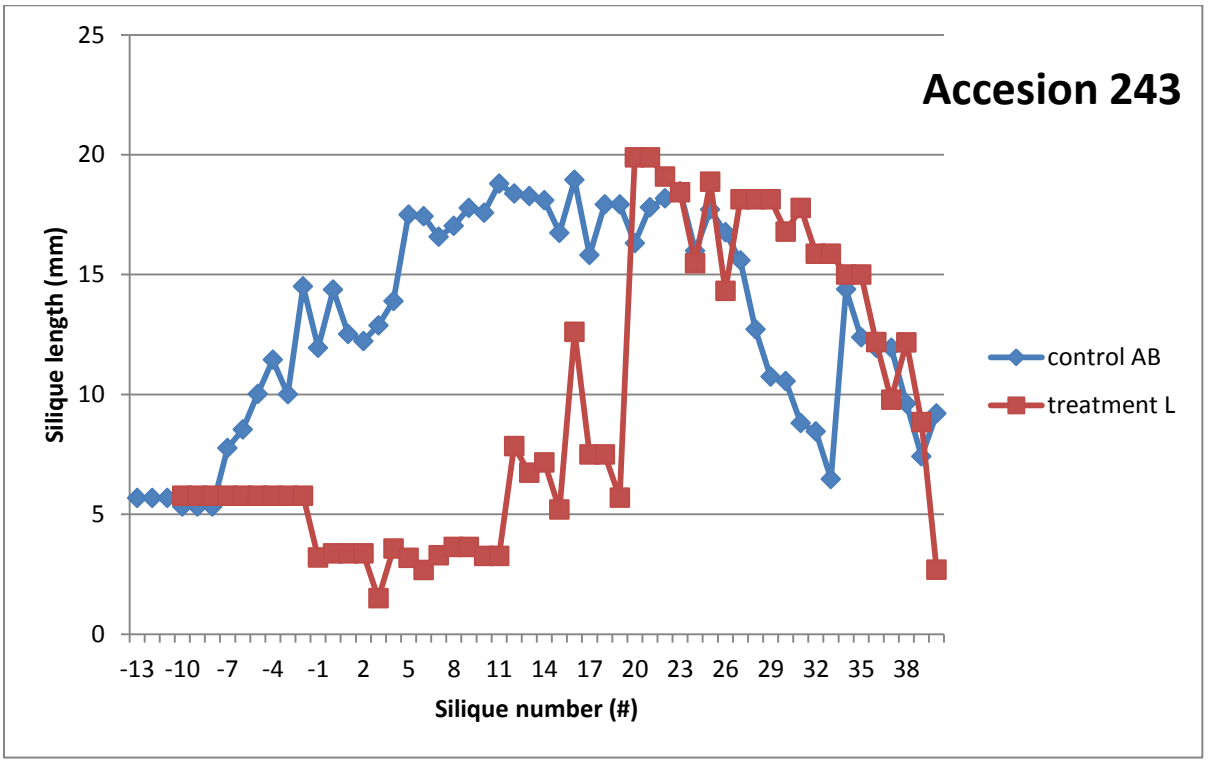


Figure . Silique length in mm per silique on the main stem for accession 243.

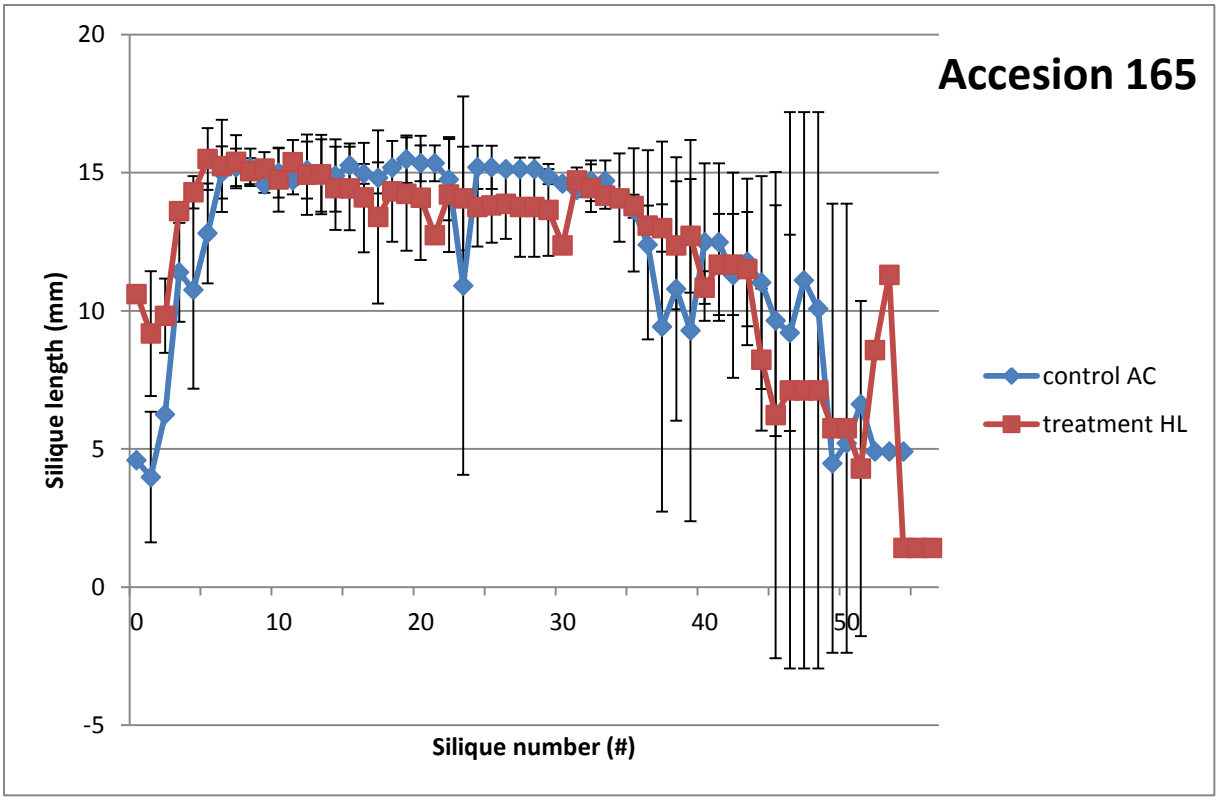


Figure . Silique length in mm per silique on the main stem for accession 165.

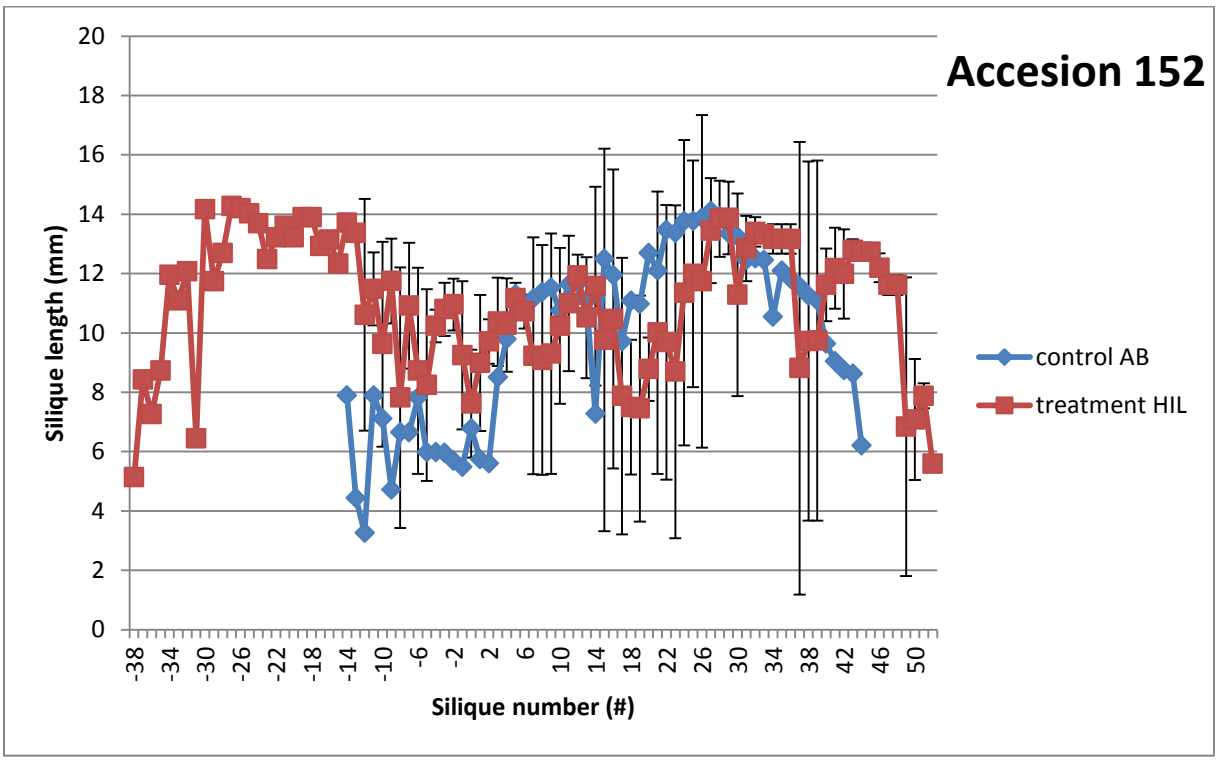


Figure . Silique length in mm per silique on the main stem for accession 152.

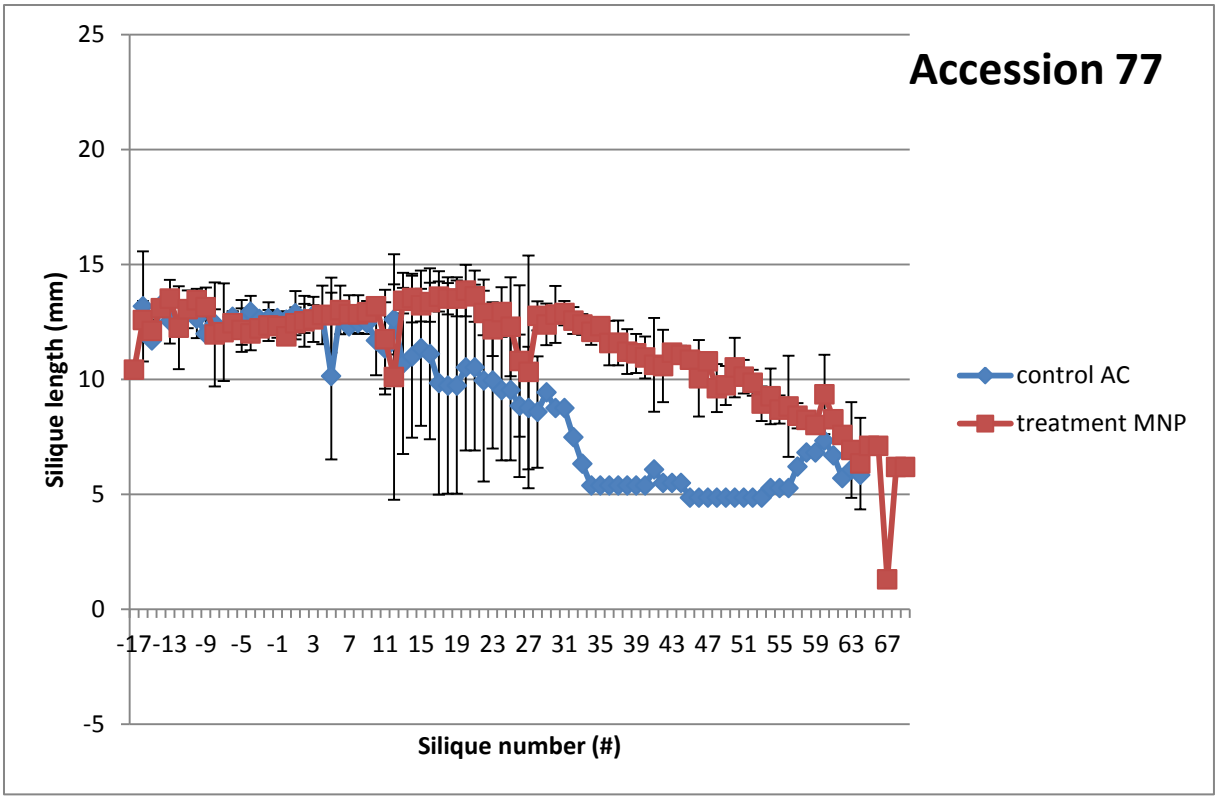


Figure . Silique length in mm per silique on the main stem for accession 77.

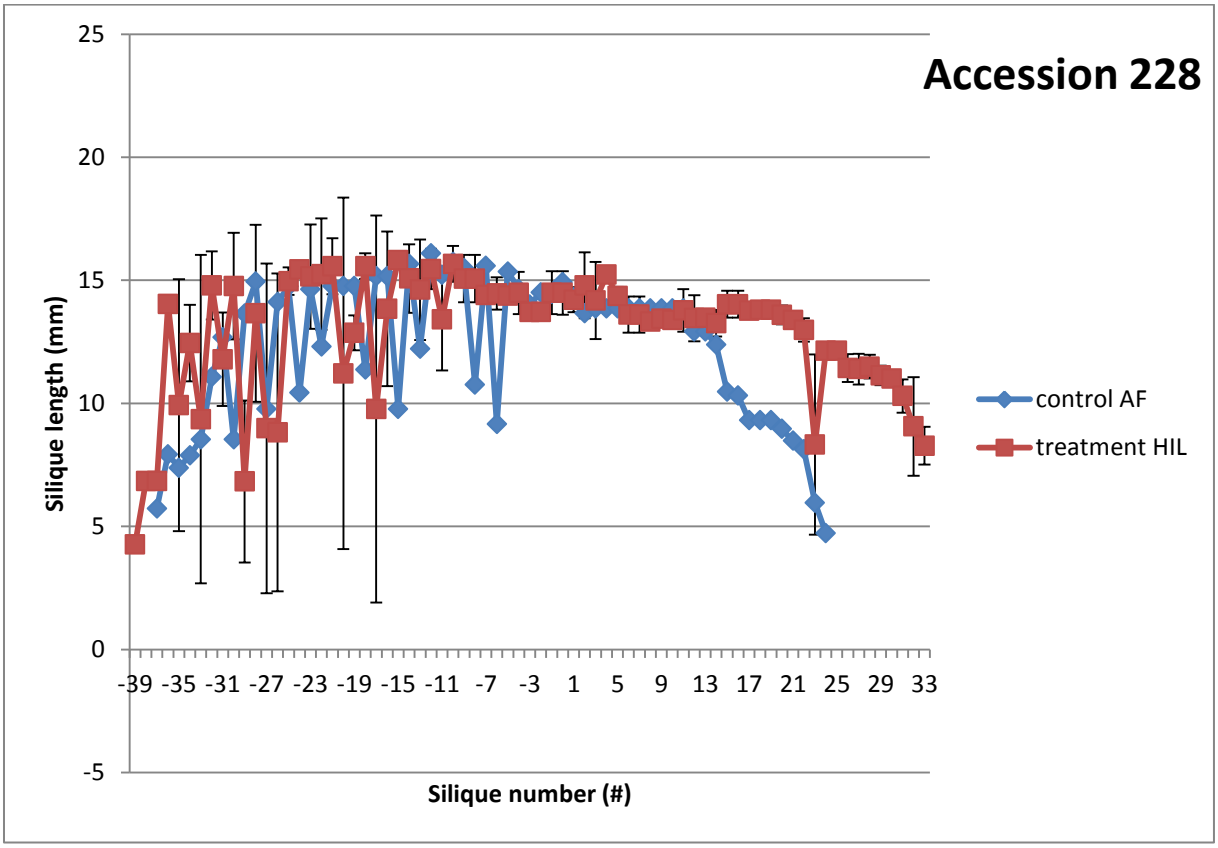


Figure . Silique length in mm per silique on the main stem for accession 228.

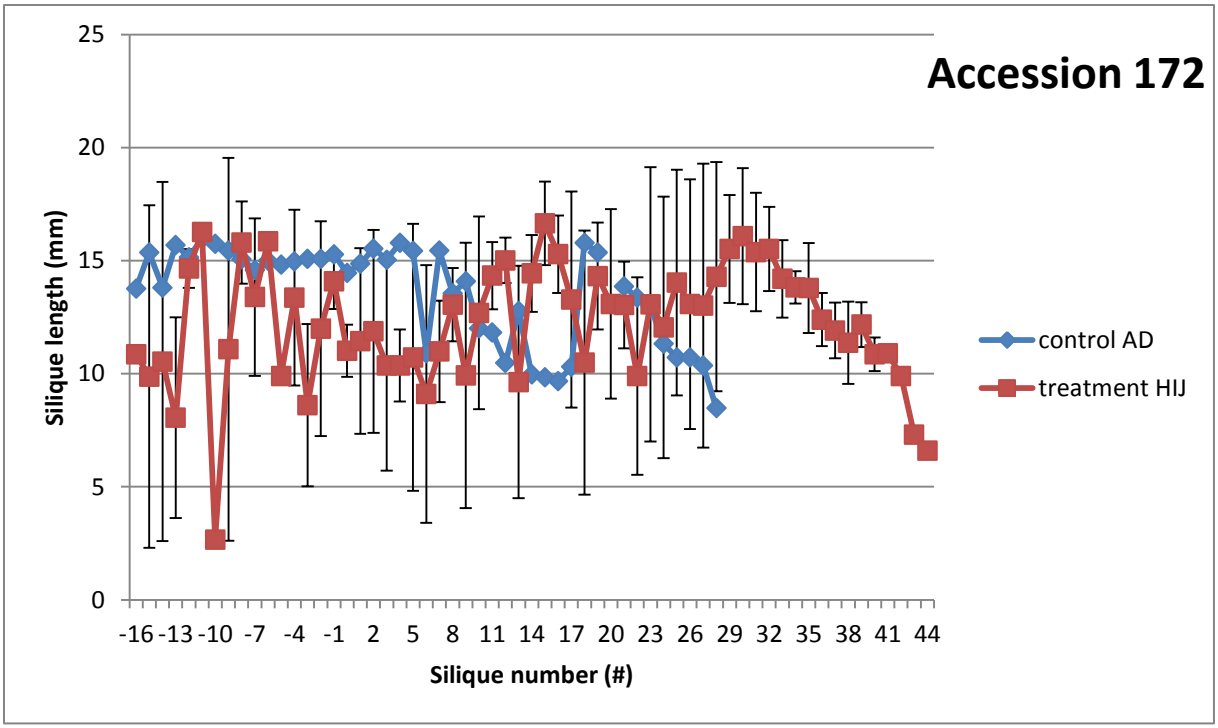


Figure . Silique length in mm per silique on the main stem for accession 172.

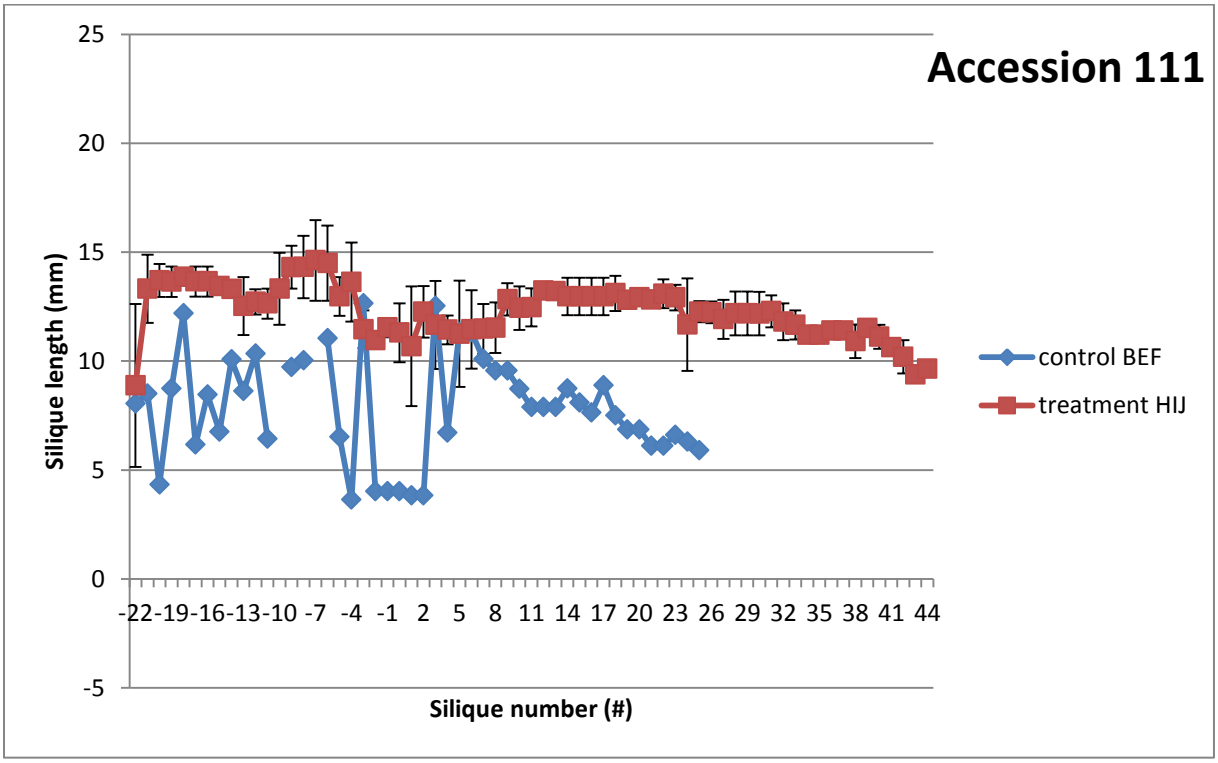


Figure . Silique length in mm per silique on the main stem for accession 111.

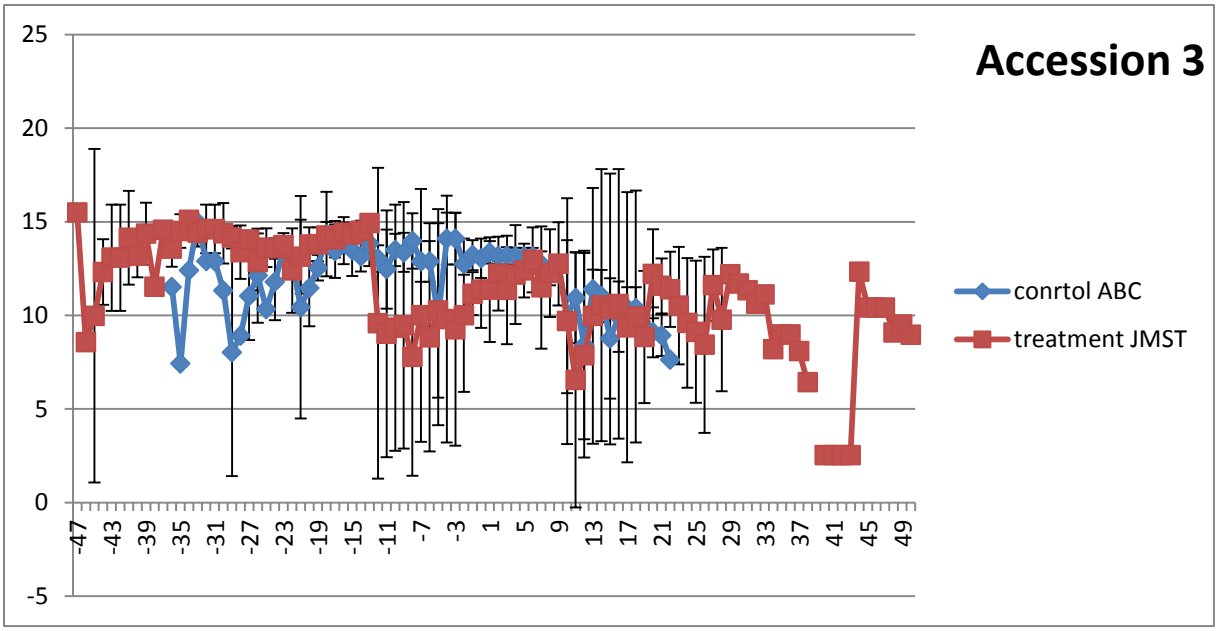
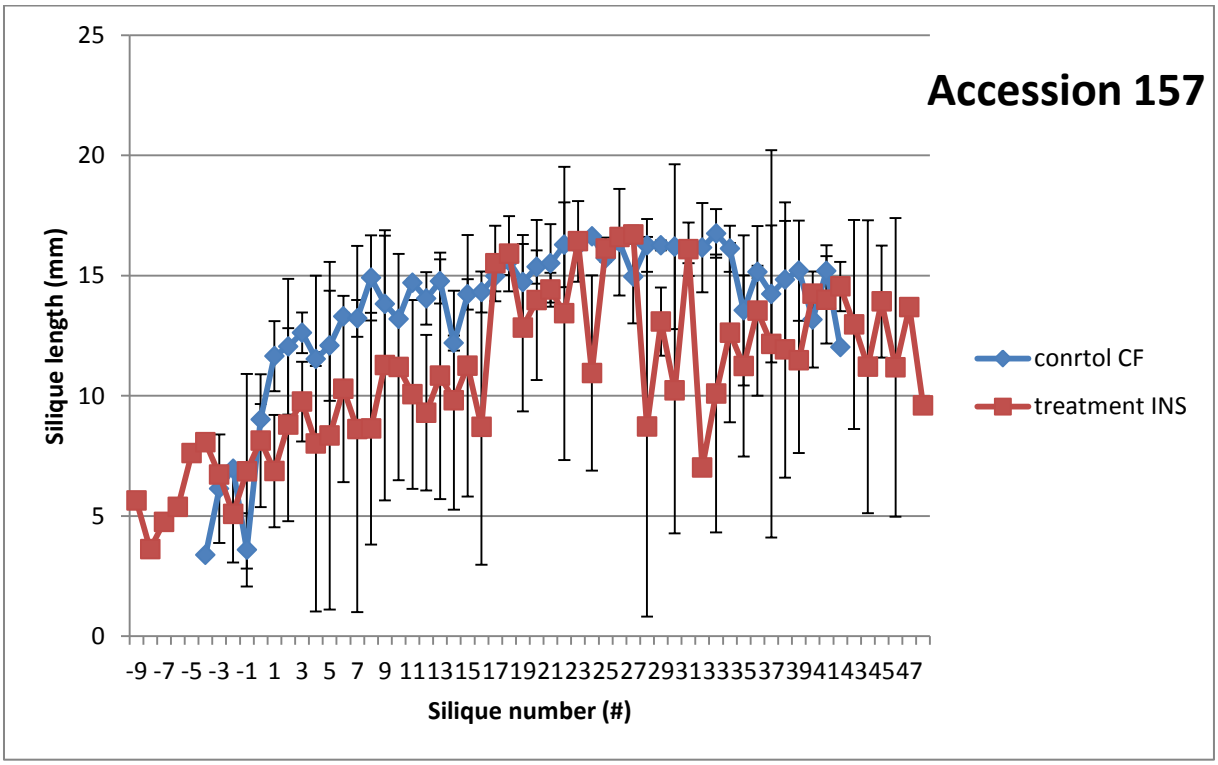


Figure . Silique length in mm per silique on the main stem for accession 3.



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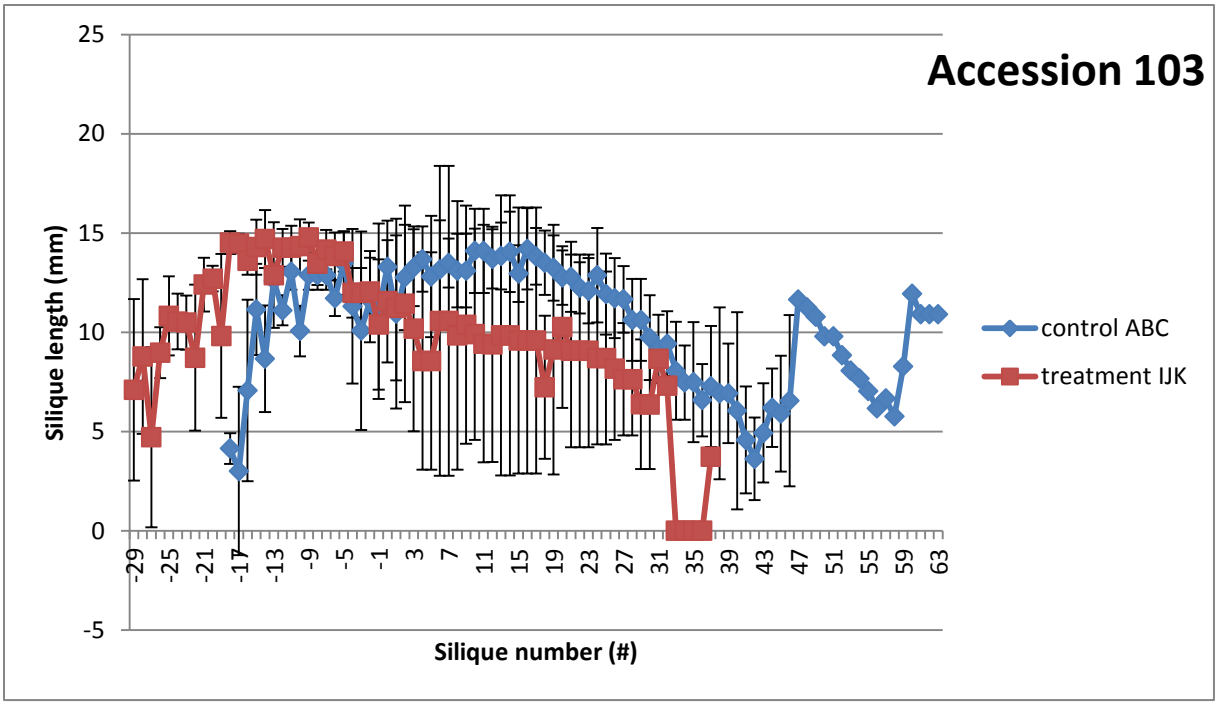

Figure . Silique length in mm per silique on the main stem for accession 103.

7.2.2 Silique length disturbed

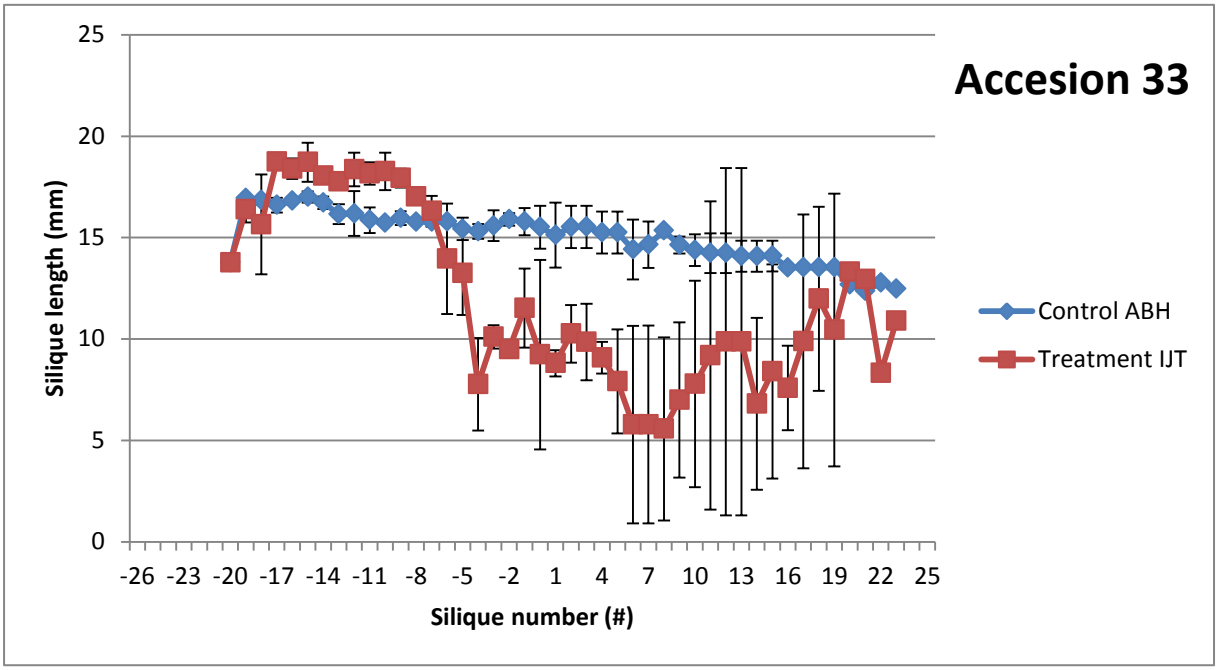

Figure . Silique length in mm per silique on the main stem for accession 33.

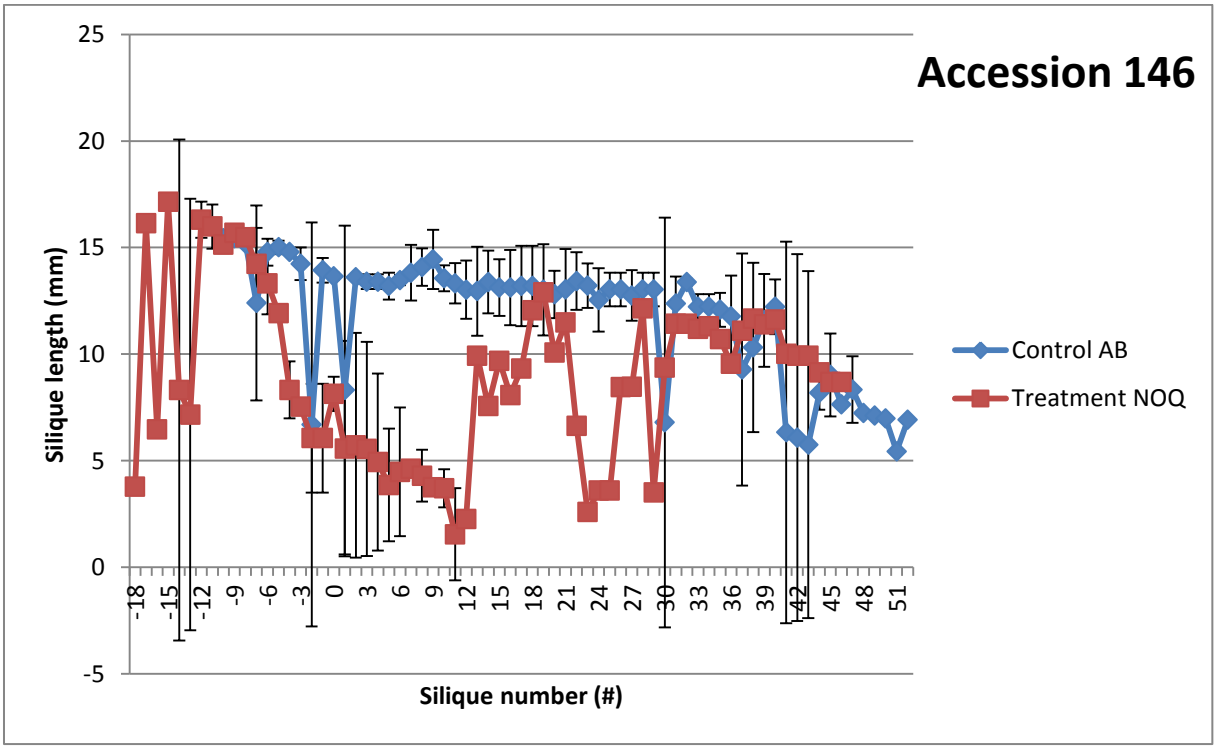

Figure . Silique length in mm per silique on the main stem for accession 146.

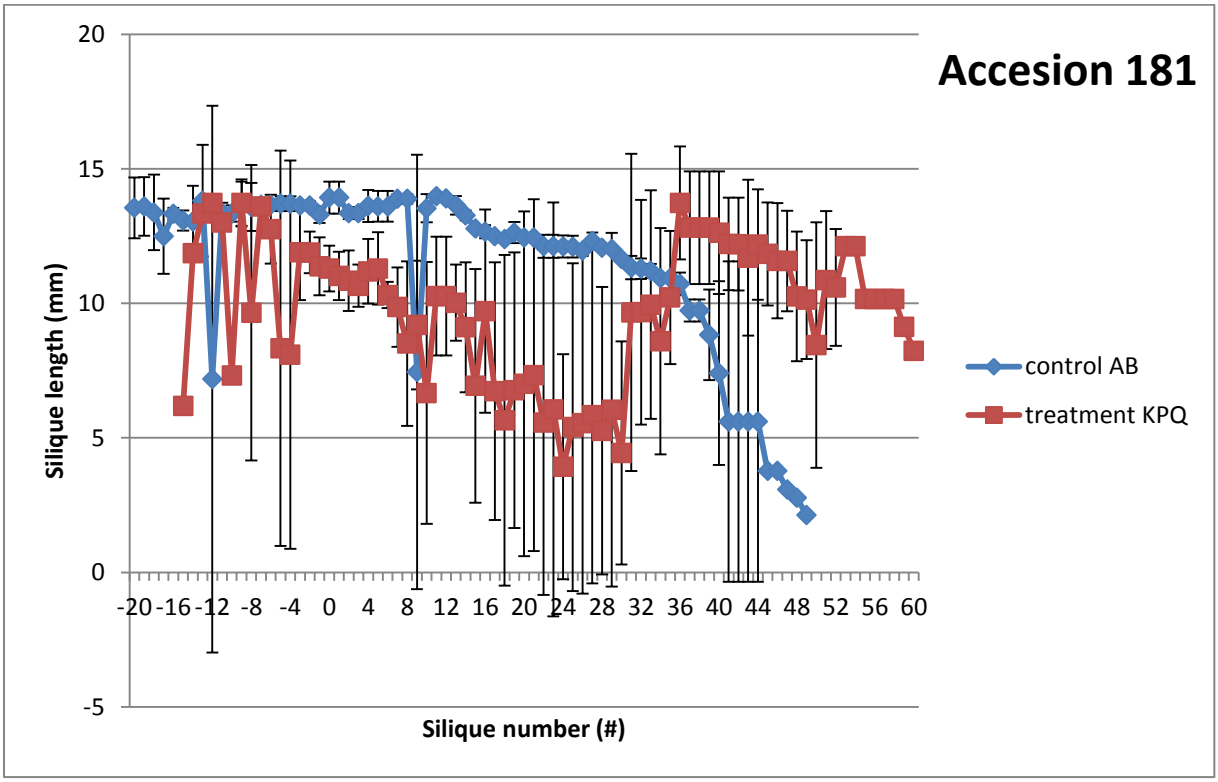

Figure . Silique length in mm per silique on the main stem for accession 181.

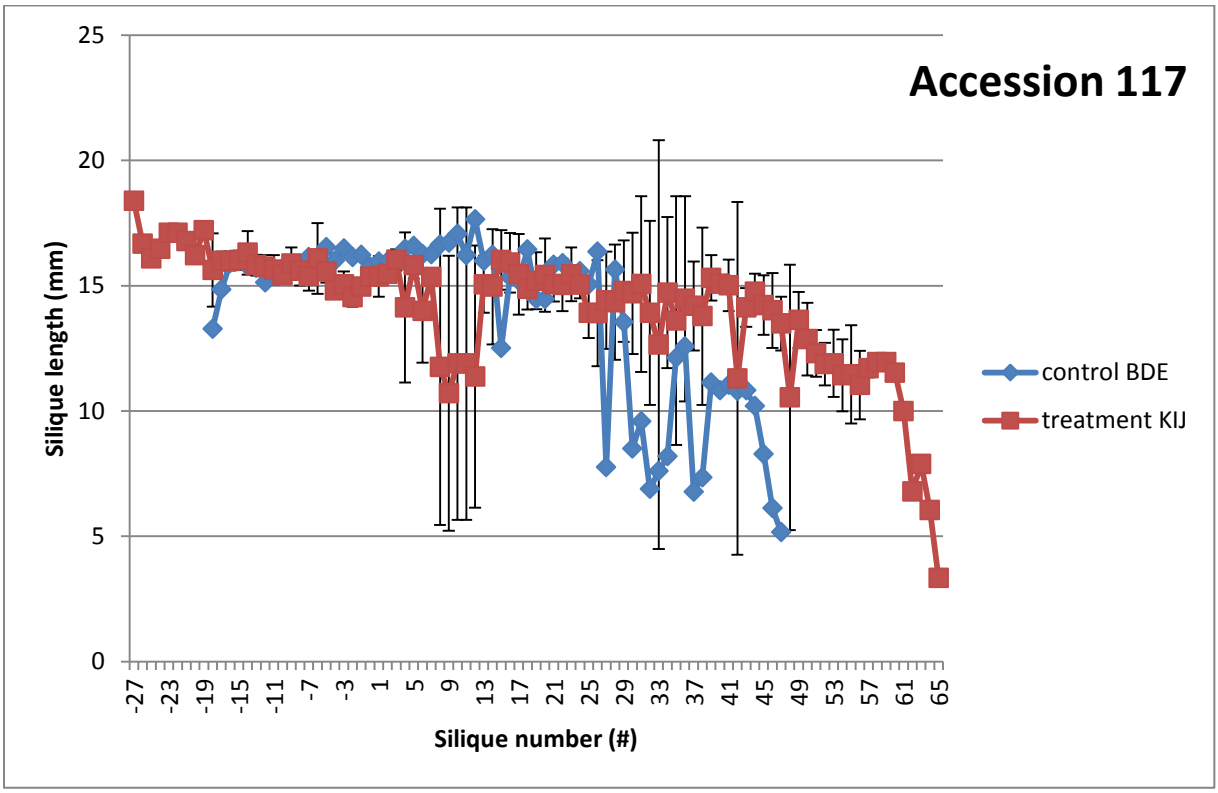

Figure . Silique length in mm per silique on the main stem for accession 117.

7.2.3 Silique length non-disturbed

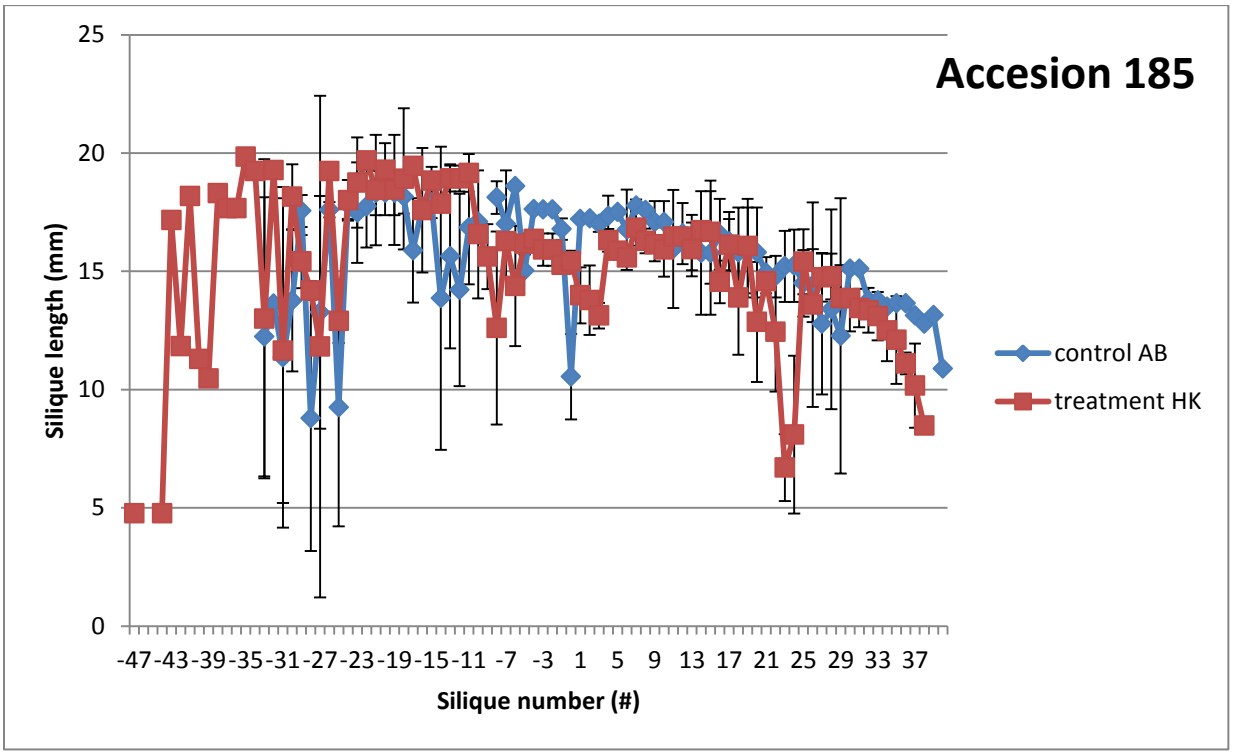

Figure . Silique length in mm per silique on main stem for accession 185.

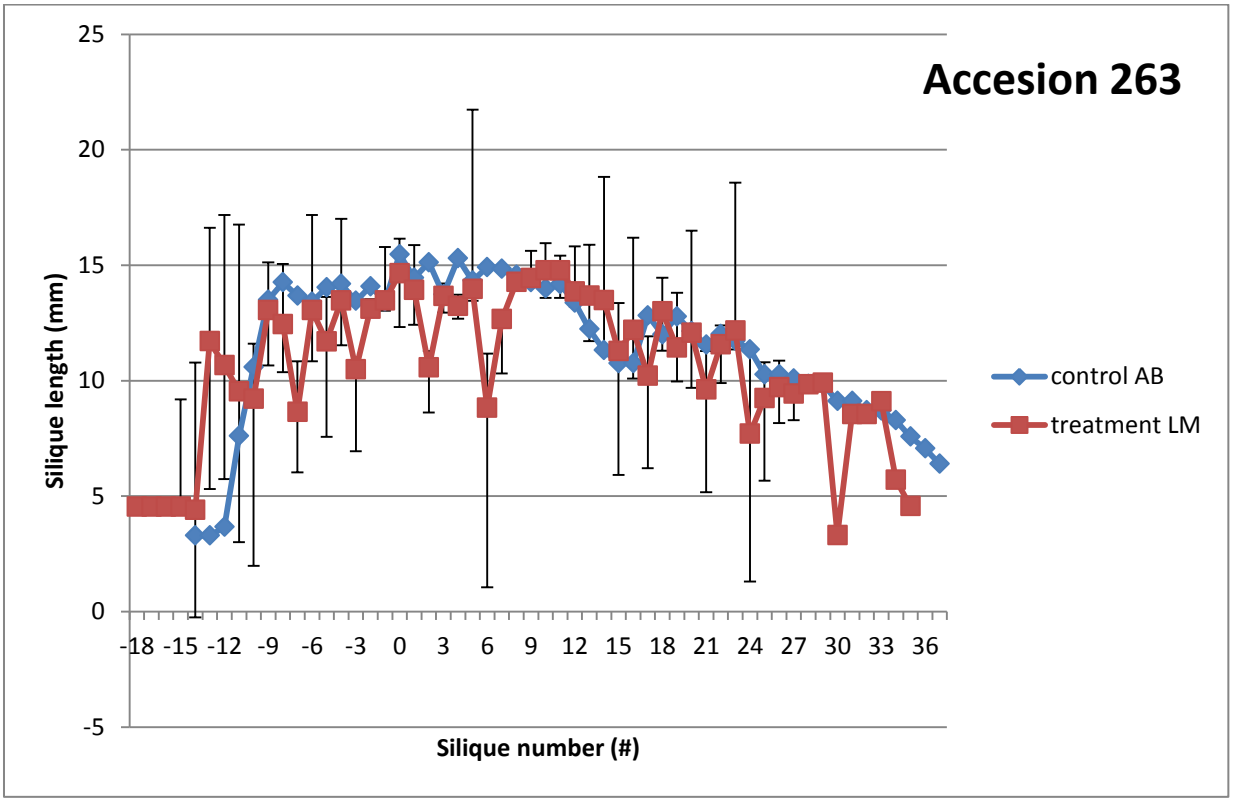

Figure . Silique length in mm per silique on main stem for accession 263.

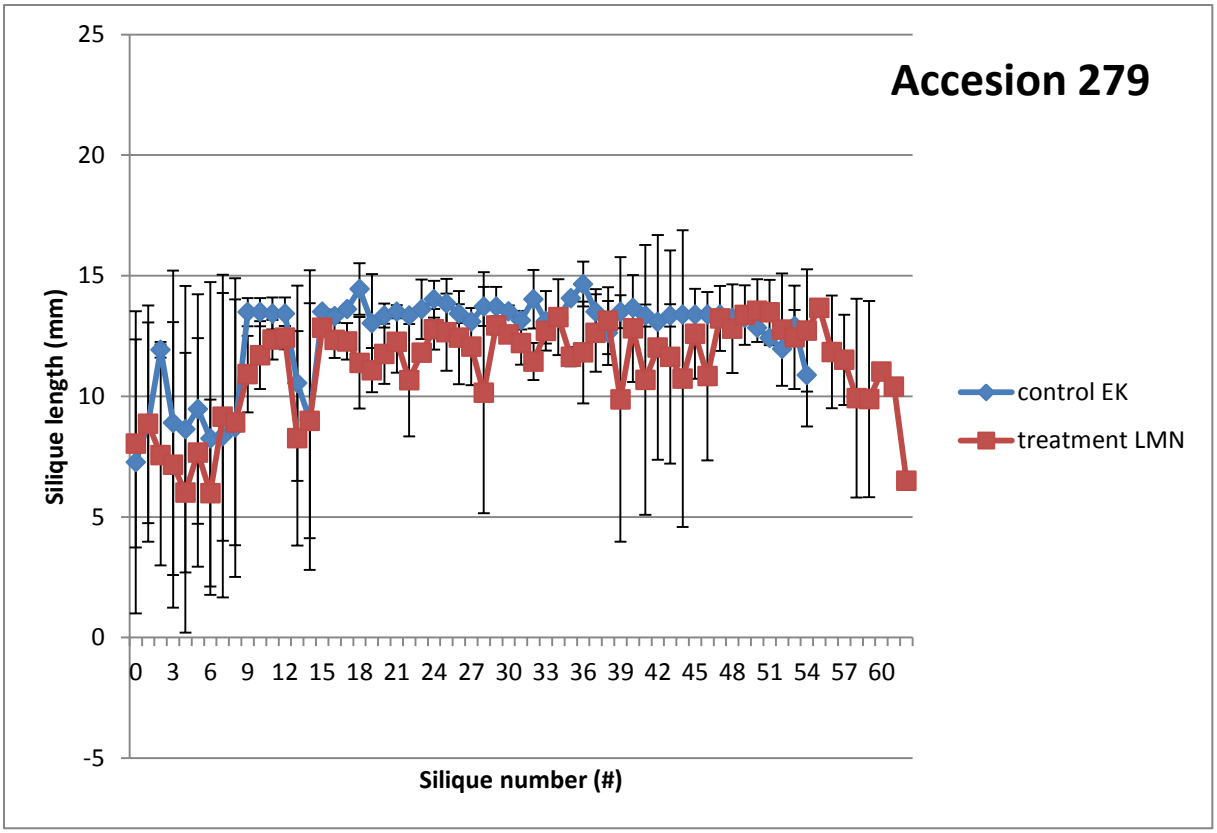

Figure . Silique length in mm per silique on the main stem for accession 279.

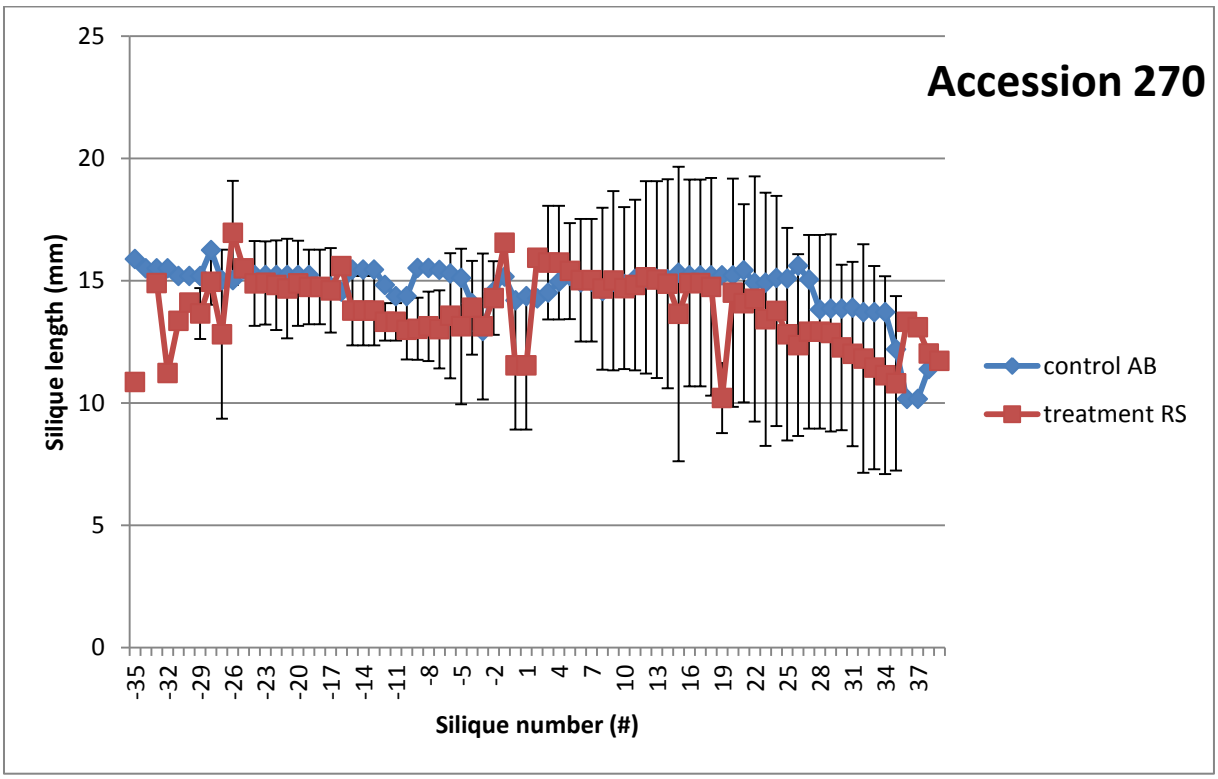

Figure . Silique length in mm per silique on the main stem for accession 270.