Defense of Garlic *(Allium Sativum L.)* Against Herbivory and Heavy Metals with Insights on the Sulfur Metabolism

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Abstract

Garlic, grown in a controlled environment, was exposed to either herbivory, copper excess or both stresses. Chlorophyll content in the leaves was measured using a photospectrometry method, FRAP assay (Ferric reducing antioxidant power) was carried out and allicin content was determined using HPLC. Cayman glutathione assay kit was also used to determine glutathione content in the leaves, but molecules from our matrix inhibited the kit from working properly. The experiment should verify that the plant's allicin metabolic pathway is affected by different biotic and abiotic stresses, and that these stresses also affect chlorophyll synthesis which was verified by trend. Fungi infestation and other physical illnesses indicated that the plant was affected by the simulated stresses. Whereas allicin content in the leaves remained unaffected by copper. One could estimate, that the plant used an alternative pathway to synthesise the essential defense molecule.

A. INTRODUCTION

For visual explanation see figure 1 in the annex.

In nature, plants have to deal with biotic stressors and whether they can defend themselves or not, became a crucially important and selective element [1]. Additionally there are also abiotic stressors that can harm plants and one of the major challenges for plants growing on places with former industry is dealing with pollution of the soil, such as heavy metals. To know how they defend themselves is of importance when considering e.g. recultivation aspects and a detailed understanding of how plants manage to grow on such heavily contaminated grounds, is paramount. These heavy metals, especially iron and copper, have been frequently shown to be major soil contaminants in industrialized areas [2]. These pollutants force plants to adapt their metabolism in order to survive. One of these adaptions concerns the sulfur metabolism which is known to play a major role in plant defense [3]. In addition, we know that this sulfur metabolism is severely affected by copper excess, as thiolate groups of the

molecules, resulting from the sulfur metabolism such as glutathione (GSH) or cysteine (Cys), often tend to chelate copper in order to prevent heavy metal damage. Furthermore, many phytochelatins are synthesised from cysteine and glutathione. The role of these phytochelatins is to bind copper and evacuate it to the vacuole [4, 5]. Altogether, heavy metals and especially copper are known to decrease sulfur metabolism [6].

On the other hand it is known that biotic stresses, such as herbivory, that is, the attack of an animal or pathogen, may cause the plant to increase the amount of its secondary metabolites, such as allicin, this is the case for garlic. It was proved in previous investigations [7] that both, simulated herbivory and copper excess, compromise the growth of garlic plants and resulted in strongly decreased growth of the garlic leaves for copper excess, whereas herbivory, increased growth of the plant. Allicin produces the characteristic, deterrent smell of garlic, and it is originally meant to act as a bactericide and fungicide with the function of chasing away potential herbivores [8]. Its flavour precursor alliin is synthesized from glutathione and Serine which will bind together with one molecule of cysteine. In fact, Allicin is produced upon rupture of the plant tissue, when alliin, stored in vesicles in the vacuole, enters in contact with the enzyme alliinase, stored in the cytoplasm [9, 10]. All facts considered, herbivory, contrary to heavy metal pollution, is meant to increase the activity of sulfur metabolism. Thus, it would be interesting to observe the plants' reaction if both stresses were combined because herbivory increases the activity of sulfur metabolism, whereas heavy metals tend to decrease its productivity.

Based on our previous work [7], we believe that copper excess will induce oxidative stress by denaturation of several important enzyme systems in the plant, including those required for the production and stability of chlorophyll, resulting in the formation of copper chlorophyllin. This will be measured by a decrease in chlorophyll content in the leaves. The derivation of chlorophyll supposedly results in the production of active oxygen species [11], which have to be removed by the plants' antioxidant defense system. Among this antioxidant defence system, glutathione is of great importance due to its high concentration in the cytosol. Thus, active oxygen species such as singlet oxygen are scavenged by two molecules of glutathione (GSH) to its oxidized form (GSSG); an increased presence of glutathione can thus be measured by an increased reducing capacity of the extract.

Furthermore a first line defense system consists of chelating copper with thiolate groups and by this way, it can evacuate the copper in excess to the vacuoles. Also, two single molecules of GSH can form a complex with copper to avoid further interferences. Subsequently, GSH and cysteine combine to form phytochelatins which bind copper to finally dispose of the heavy metal and cope with them properly. This is the reason, why the plant will invest more glutathione to its abiotic defense responses, than to the production of allicin, which would be measured by a decrease in the amount of allicin per gram of dry weight. So this results in a decreased defense against the biotic stressors, such as herbivores and fungus.

The usual manner for garlic plants to defend themselves against herbivores, bacteria and fungi, is to increase the production of alliin. To achieve higher alliin concentrations, the plant has to produce more cysteine and GSH which will result in an increase of the general sulfur metabolism. Supplementary, cysteine will also be invested into more growth to compensate the loss from the herbivory.

When both stressors are combined, the plant should basically show the same symptoms as the plants treated with only copper, but being exposed also to herbivory, the additional stimulation may help the plant to protect itself better from copper, as the increased productivity, due to herbivory will be

compensated by the slowdown of sulfur metabolism due to copper excess.

The objective of this project is to analyse the activity of the sulfur metabolism in plants that are exposed to these two different stressors. Garlic was used because of its high dry weight sulfur content, reaching up to 1 % [12]. As it is also well known that copper heavily affects the sulfur metabolism [6]; we added a supplementary of 10 mM of copper sulfate ($CuSO_4$) as heavy metal stress [13]. Even though copper is an essential trace element, in excess it induces the formation of active oxygen species, just as other heavy metals do. For this purpose, garlic plants were cultivated in a hydroponic system in order to be able to have full control of nutriment and heavy metal supply. As the concentration of allicin could also be affected by temperature and light conditions, [14] plants were placed in light and temperature controlled incubators. For our analysis we attempted to measure total chlorophyll, allicin and glutathione content as primary endpoints. For chlorophyll quantification, the absorbance of an extract was measured. Allicin content was determined by a HPLC (High Pressure Liquid Chromatography) analysis and for glutathione quantification we used an assay kit employing DTNB (5,5'-dithio-bis(2-nitrobenzoic acid), Elman's Reagent) for quantification. However, substances from our matrix interfered with the DTNB and enzymes involved in the assay kit, which inhibited colour development and so quantification by this way became impossible. We switched methods and used the FRAP Assay (Ferric reducing antioxidant power assay) to be able to determine the plant's general antioxidant defense system, in order to obtain an indication for the amount of oxidized glutathione and another phenolic compounds present in the sample.

B. MATERIAL AND METHODS

1. Chemicals and reagents

Unless otherwise mentioned, all chemicals were purchased from Sigma (St. Louis, MS, USA), and 18 MΩ water was used for all experiments.

2. Plant Culture

Garlic tubers, bought at a local store were placed in a box filled with about 1cm of tap water in a place illuminated by natural light. After 2 weeks of germination and growth, seedlings were placed in previously prepared boxes. Four boxes were prepared in the following manner: 15 holes were cut into the cover plate in order to fix isolating tubes with a diameter of 5cm. Plants were kept in place with cling film and elastic bands on the bottom of the isolating tubes. To each of the four boxes, 15L of Hoagland solution [15] were added. (Figure 2)

The nutrient solution was permanently supplied with ambient air by aquarium air pumps (Wave, Mouse, 2,5 L/min). In order to concentrate pressure onto the tip, we mounted the plastic ends of Eppendorf pipettes (Eppendorf Research T.I.P.S. 1mL) onto the tip of each tube [7]. Plants were placed in incubators (Forma Scientific Diurnal Growth Chamber), with a day temperature of 20°C and 16°C at night with a day/night cycle of 12 hours.

The experiments were carried out with plants under 4 different treatments: a control group with no added stressors, a herbivory group, where 5 cm of the tallest leaf were cut, a copper group, where we added 10 mM $CuSO_4$ and a combination of copper sulfate and herbivory. With each box containing 15 plants and 6 plants for the first day, which were the same for each treatment, 65 plants were used.

Samples were taken at day 0, 2, 7 and 12 of the experiment. At each measurement day, 5 replica of each condition were withdrawn. On day 0, only 5 plants were withdrawn as it was

supposed that all plants were at the same development stage.

3. Sample Preparation

Plant leaves were cut into big pieces (ca. 10 cm long) and bulbs and leaves of each plant were put into separate 50 mL Falcon tubes (Figure 2). Immediately, samples were shock frozen with liquid nitrogen and transferred into a lyophilisator (Christ Alpha 2-4 LSC). Plant material remained there for about 2 days to ensure absolute dryness of the samples. Afterwards, the dry plant leaves were crushed with a mortar and a pestle in to a homogeneous powder that was mixed and stored at -80°C until analyses.

4. Chlorophyll

A modified extraction method developed by Wellburn in 1994 [16] was adapted. To ensure stability, samples were stored at a cold, dry and dark place.

About 50 mg of the powder was transferred to a 15 mL Falcon-tube. 10 mL of a cold aqueous 80% acetone solution was added and the mixture was vortexed and sonicated afterwards for about 5 min. The samples were centrifuged then for 15 min at 5.000 g and 4 °C and the so obtained supernatant was transferred to a 1 mL spectrometry cuvette. After a complete absorbance scan for the visible light spectrum which confirms specific absorbance wavelengths, absorbance was measured for chlorophyll [a] at 663 nm and for chlorophyll [b] at 646 nm. Photospectrometer Spectro DU 800 Transport was used for analysis. The concentrations were calculated as follows, with 80% acetone solution used as a blank control.

- [Chlorophyll a] (mg/g)

$$= \frac{(12{,}21 \text{ x A663} - 2{,}81 \text{ x A646}) \frac{mg}{L} * 0{,}01\text{L}}{used\ dry\ weight\ of\ the\ plant\ in\ gramm}$$

- [Chlorophyll b] (mg/g)

$$= \frac{(20{,}13 \text{ x A646} - 5{,}03 \text{ x A663}) \frac{mg}{L} * 0{,}01\text{L}}{used\ dry\ weight\ of\ the\ plant\ in\ gramm}$$

- [Total Chlorophyll](mg/g)

$$= \frac{(7{,}18 \text{ x A663} + 17{,}32 \text{ x A646}) \frac{mg}{L} * 0{,}01\text{L}}{used\ dry\ weight\ of\ the\ plant\ in\ gramm}$$

A = Absorbance with the specific wavelength

5. Glutathione analysis

We used the Cayman glutathione assay kit (Item No. 703002) [17] and the extraction method for homogenated organ tissue, described in the accompanying manual.

To ensure that total glutathione was liberated from the vegetal cells, samples were ultrasonicated for 10 min and in order to avoid any proteins to interfere within the assay kit, samples were deproteinated according to the given protocol. For sample analysis, kinetic method was used.

6. Allicin analysis

a) Allicin extraction

A modified extraction and quantification method was adapted according to the Pharmacopeia Europea 2008 [18], but we used ethanol instead of methanol, because best stability and solubility was earlier observed in this solvent [19]. Blania and Spangenberg et al. verified this extraction method and also observed best HPTLC (High performance thin layer chromatography) results with this method [20].

About 0.08 g of the freeze dried plant sample was transferred into a 15 mL Falcon tube with 2 mL milli-Q water at room temperature and remained there for 15 min. The samples were sonicated in ice water for 20min and centrifuged then at 5.000g for 15 min at 4°C. 1 mL of supernatant was removed and mixed with 1.5 ml of a solution composed to 60% of ethanol and 40% of a 1% formic acid aqueous solution. Once more, samples were centrifuged at 5.000g for 5 min and 4°C and 1 mL of supernatant was removed and kept for HPLC analysis at -80°C.

b) HPLC Parameters

We used a Dionex UV 254 nm HPLC. The column (Column Grace Smart RP 18, 250 x 4.6 mm, 3 µm) was kept at 30°C. This elevated fluidity of the solvent to a flow rate of 0.7 ml/ min. For the solvent, we used a gradient, meaning that proportions of polar (water) and non-polar solvent (methanol) were changed during analysis in order to enhance peak separation. The broken line on the chromatogram shows variation in proportion of methanol and water over time (Figure 3). As allicin is relatively unstable and only available in small quantities, we additionally used an internal standard for quantification with a known response factor to allicin. Knowing the proportions (areas) in which they eluted, and having both detected at the same absorption wavelength (254 nm), we could calculate he exact amount of allicin in each sample.

7. FRAP-Test

a) Extraction

The extraction was carried out according to Bouayed et al. (2011) [21]

50 mg of crushed and dried material was mixed with 2.5 mL of 80 % aqueous methanol in a 15 mL falcon tube. The solution was then vortexed and sonicated for 20 min. Thereafter it was put in a centrifuge at 4000 g for 5 min. 1.5 mL of supernatant was transferred into a new 15 mL falcon tube. Then, 3 mL of 100 % methanol were added to the original falcon tube, vortexed and also sonicated for 20 min, as well as centrifuged at 4000 g for 5 min. Of the so obtained supernatant, 3 mL were transferred to the new falcon tube. In order to fully extract the antioxidant compounds, one can repeat the extraction with 100 % methanol several times, in our case the centrifuged samples showed a colourless resident, so we felt comfortable that all compounds were fully extracted. Thus, we did not re-extract with 100 % methanol.

A speed vac (Centrivap Lavonco) was used to evaporate under vacuum the methanol out of the samples. Then water was refilled up to 2 mL and 2 mL methanol were added.

For storage, the tubes were layern with argon and sealed with parafilm. Samples were kept at -80°C until analysis.

b) FRAP Assay

Frap Assay was carried out according to Benzie and Strain (1996) [22].

FRAP reagent was freshly prepared on a daily basis mixing in proportions of 10:1:1 respectively the three constituents acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) in 10 mM HCl and 20 mM $FeCl_3$. The FRAP reagent was then heated to 37°C prior to use. Standard curve was obtained using 100-2000 µM $FeSO_4$ (n=7). As a blank, FRAP reagent was mixed with 50 µL of a 50% aqueous methanol solution. In a photospectrometry cuvette, 100 µL of the sample extract were mixed with 1.5 mL FRAP reagent. Left shaking for 4 min, the absorbance of the mixture was measured at 593 nm against the blank solution. Photospectrometer Spectro DU 800 Transport was used for analysis.

8. Statistical Section and Data Interpretation

Statistical evaluation was carried out with SPSS version 19.0 (IBM, Chicago, IL). All values are expressed as mean plus/minus standard deviation, unless otherwise stated. Normality of data was verified with normality plots, equality of variance by boxplots. Following these assumption testings, several linear mixed models were developed, each with the two fixed factors "day of analysis (0, 2, 7, and 12)" and “treatment (control, copper, herbivory, copper + herbivory)”, and with the dependent (observed) variables allicin, FRAP values, chlorophyll, and ratio of leaf over tuber, respectively. Following significant Fisher F-tests, post-hoc tests (Bonferroni) were performed. Given that a significant interaction of "day" and "treatment" were found, the

models were run again, keeping "day" constant, in order to study the effect of the treatment at each day of analysis. P-values below 0.05 (2-sided) were considered as statistically significant.

C. RESULTS AND DISCUSSION

1. Chlorophyll

The following results were obtained for total chlorophyll content:

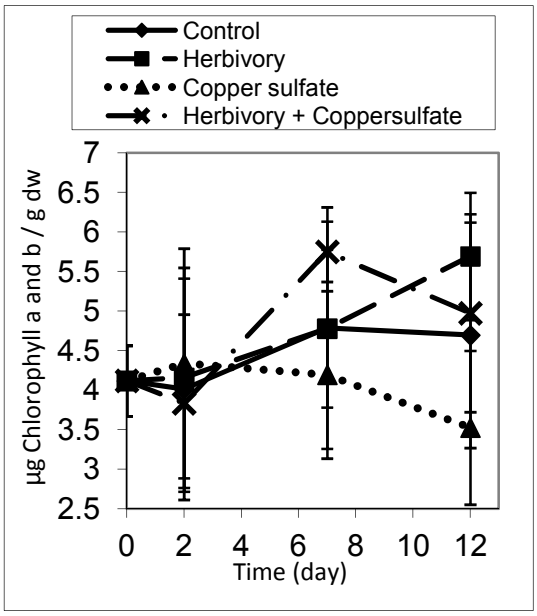

Figure 5: Total chlorophyll content in the leaves in function of the experiment duration. Error bars represent standard deviation (n= 0.5)

Chlorophyll content of the control plants, expressed per gram dry weight, remained fairly constant over the 12 days. The content of the herbivory treated plants goes slightly up compared to the other conditions, albeit this did not reach statistical significant difference. Plants treated with copper sulfate had the lowest value of all conditions and a clear decreasing tendency was observable. Plants treated with both stressors showed a fast increase in chlorophyll content until day 7 where it started to decrease and reached a level similar to the control plants at day 12.

These results confirm our hypothesis, meaning that due to herbivory exposure the activity of the general metabolism of the plant increases and by elevating the amount of chlorophyll in its leaves the plant can take up more energy from the sunlight, thus counterbalancing negative effects of herbivory. Because of copper excess, chlorophyll is derived to chlorophyllin. In fact, copper ion may replace magnesium ion in the centre of normal chlorophyll and, due to saponification, chlorophyll loses the methyl and cyclophytol moieties of the ester. In addition acid functions are neutralised to form chlorophyllin [23]. Furthermore, we could hypothesize that copper, as a two plus charged ion, could undergo dipolar interactions with proteins in the cell. This also affects enzymes involved in chlorophyll synthesis which would also explain the decrease, meaning a certain amount of chlorophyll is naturally destroyed by sunlight and enzymes reproducing chlorophyll cannot compensate the loss anymore because they are harmed by copper.

When both stressors are combined, the beneficial effects of herbivory on chlorophyll content are dominating. To deal with both stressors, the plant elevates its amount of chlorophyll in the leaves so that there is more energy available to synthesise essential defence molecules and compensate negative effects from herbivory. Even though copper stress affects chlorophyll content the plant is able to compensate the loss and increase the amount of chlorophyll in its leaves.

The absolute chlorophyll content we could find was consistent with the values found by Bideshki et al. [14]. Values reported were at 4.7 mg chlorophyll per gram dry weight, which is perfectly coincident with our results. Only total chlorophyll content is being discussed, because diagrams for Chlorophyll a and b complement one another.

2. Glutathione

For the Glutathion assay, there was normal colour development for the standard curve and

a good calibration curve, which clearly indicated that the assay was working properly. But there was no colour development for any of the samples. This is probably due to other substances from our matrix interfering with the assay kit. Other papers also gave clear evidence that phenols priorily react with DTNB (5,5'-Dithio-bis(2-nitrobenzoic acid) or Ellman's Reagent) [24]. Hence, there was no more DTNB for thiol identification. Furthermore, it is known that phenols inhibit the enzyme glutathione reductase [25] involved in the assay, catalysing the reaction with TNB (1,3,5-trinitrobenzene).

3. FRAP Assay

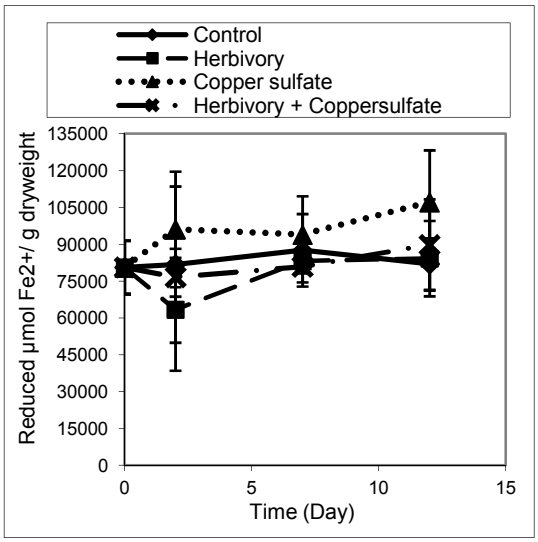

Figure 6: Ferric reducing antioxidant power of the tissue samples. Error bars representing SD of a mean of 5 replicas.

The reducing capacity of the control plants, plants treated with herbivory and plants treated with herbivory and copper remained relatively constant over the whole experiment. Only plants treated with copper sulphate had a tendency for a change (not significant), showing an increase over the days of experiment.

In fact these results are difficult to interpret, even though they show some tendencies. The FRAP test gives us the reducing capacity (Fe3+/ Fe2+) of the sample, but as we extracted many different antioxidants from our matrix, we can barely draw a conclusion on the amount of oxidized GSH in the sample, albeit GSH may be one of the major compounds implicated in the plants antioxidant defense system [5]. In addition, we cannot give any information about the redox potential of the molecules we are analysing. So we also cannot say which of the molecules reduced iron and which didn't. Overall, the FRAP assay kit only gives us an idea about the total free radicals scavenged by these molecules, meaning that we can only observe a slight increasing trend here for copper stress being linked with the loss of chlorophyll over the days in the sense that due to chlorophyll derivation, reactive oxygen species are formed and these induce oxidative stress. Copper, similar as to iron, could result in increased formation of free radicals, via the Haber-Weiss reaction, and this deploys an increase of free radicals in the garlic plants. At the same time, many of the free radical scavenging molecules are reduced over time by enzymatic reactions, which then again makes interpretation all but impossible.

We could find no other sources confirming our results for reducing capacity except Benkeblia et al. [26] measuring antioxidant capacity of garlic bulbs, which again is not comparable to our results.

4. Allicin

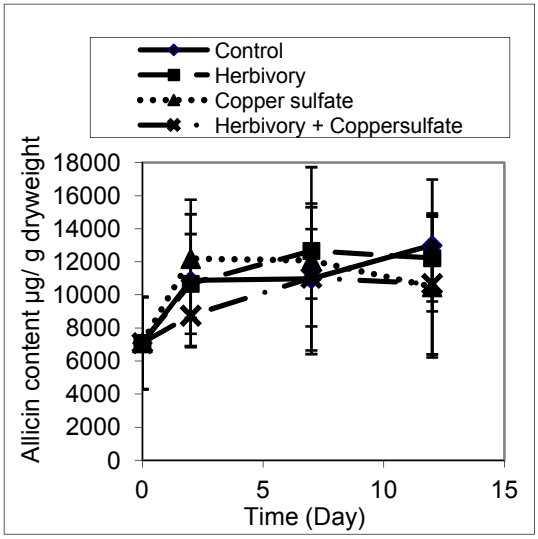

Figure 7: Allicin Content in the Plant leaves.

The response factor, calculated for Allicin, was 0.0525, 0.0521 and 0.0494 for concentrations of 50, 100 and 150 µg/ml respectively which confirms the representativeness of our standard curve and that the HPLC method works as expected. However, the results show a high standard deviation and do not fulfil a bell-shaped distribution curve, for reasons remaining speculative, such as a remaining activity of the enzyme alliinase and/or instability of allicin. Even if samples were treated with greatest care, exactly the same working conditions for allinase in each of the samples cannot be guaranteed.

Our results for the allicin test show little to no difference in the final concentrations between the 4 treatments and neither herbivory, nor copper sulfate affected significantly the final allicin content in the leaves.

The reasons for these observations remain unclear. It is possible that the stressors were not strong enough to induce clear changes of the Sulfur metabolism. Another explanation for these results could be that in various stress situations, the plant can compensate the copper based stress by changing its metabolic pathway or activity for the same final chemical product (allicin), thus, even if some pre-metabolic substances are affected, the plant has other means of maintaining allicin concentrations, which appear to be important enough to be maintained at a constant level.

In fact, there are two different pathways to produce alliin, the precursor to Allicin. One uses the antioxidant glutathione (GSH) and an alyll group from an unknown source in the plant [9], the other also needs this alyll group, but instead of GSH it uses the amino acid serine.

As it is a well-known fact that glutathione content is affected by copper excess, due to its antioxidant [27, 28] and detoxicative [29] properties, we can assume that glutathione is used to protect the plant and so is no longer available for allicin production, even though we were not able to measure the GSH content of each plant in our case. The fact, that heavy metal stress affects GSH level in the plant and that GSH is used to synthesize phytochelatins in response to heavy metal stress, is also verified by Zhang et al. [30] and Knecht et al. [31]. These factors show that Glutathion is here rather used for detoxification, than for the production of allicin. That is why the only possible way for the plant to synthetize enough allicin under heavy metal stress and guarantee best defence properties, has to be the serine metabolic pathway. Future studies in this domain are necessary in order to clearly reveal the pathways involved in allicin formation as it is yet unknown in which situations the plant tends to use the second instead of the first pathway. Whereas it is yet known that standard pathway is up from GSH and allyl group. These results are of great importance considering the determination in which situation, which pathway is used.

The results we found out for allicin content in garlic were also found by Bideshki et al. [25] in the sense that they found similar absolute allicin concentrations as we did for our control plants.

Effect of drought stress, also known to be at the source of ROS, on allicin content in garlic bulbs was also analysed by Bideshki et al. Values of 2685 µg per gram dry weight in the bulb where reported at field crop. But these results are not comparable to ours as content in bulb and leaves vary. Whereas it was also reported in the same paper, that stresses did not affect allicin content.

5. General Condition of the plants

a) Percentage of plants infested by fungi on the last day

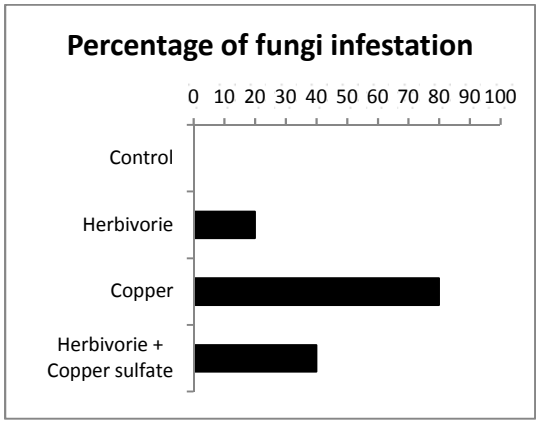

Figure 8: Percentage of Fungi infestation on the plants

As all plants had the same growing conditions, one can assume, that they were also exposed to the same grade of fungus infestation. Plants treated with copper, had a higher proportion of infested specimens compared to the ones treated only with herbivory and control showing none. Plants treated with herbivory and copper stress seem to have kept better defence abilities than the ones treated only with copper (Figure 4).

Fungi affection and final allicin content in the bulb are clearly linked because allicin acts as a bactericide and microbial for the plant. And as the allicin content decreases, the ability to defend itself against fungus would simultaneously decrease. But again, herbivory seemed to have a beneficial effect on the plant´s Sulphur metabolism linked defence abilities contrary to copper.

Very interesting is that we found out that allicin content in the leaves remained relatively constant, independent on the stressor speculatively due to changes in the metabolic pathway. We can only assume that perhaps repartition of allicin in the whole plant changed, as we measured only allicin content in the leaves and fungus was exclusively observed at the neat between the roots and the bulb. Meaning that alliin production was disarranged from the bulb to the leaves. Alternatively, the fact that despite the merely slight differences of allicin following herbivory and copper exposure suggest that other plant compounds were also important in the defence against fungi.

b) Fraction of the bulb on the total mass

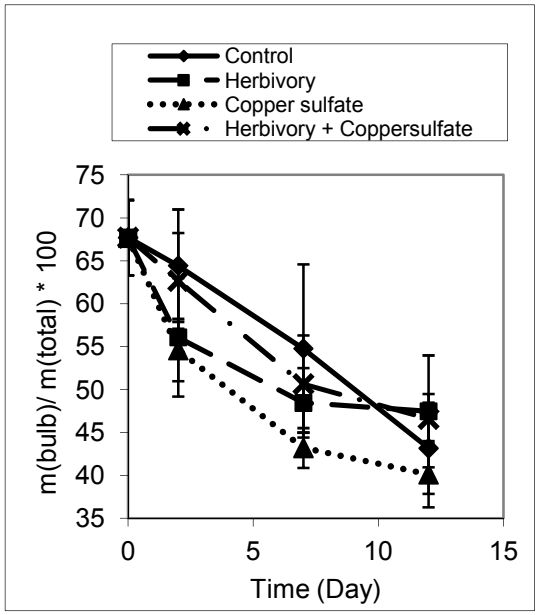

Figure 9: Ratio of tuber weight over total weight of the plants.

The values all decrease in function of time. We could notice that the copper treated plants had a stronger decreasing trend and had a lower fraction of the bulb on the total mass in comparison to the other treatments.

It is a normal growing process that the plant transfers matter from the storage organ, bulb, to the leaves in order to grow. However, the copper treated plants may had to consume their reserves to be able to defend themselves against heavy metal exposure and had to mobilize more rapidly these reserves compared to other plants.

At the same time, we could speculate that amongst others, alliin and its flavour precursors are dislocated from the bulb to the leaves which results in a reduced defense capacity of the bulb against fungus which we could observe on the last day.

Whatever is the case, further analysis concerning the measurement of allicin in the bulb is paramount.

D. CONCLUSION AND OUTLOOK

The work consisted of measuring chlorophyll content, allicin content and the ferric reducing antioxidant capacity of garlic leaves, with plants submitted to four different treatments: control, herbivory (cut 5cm of the tallest leaf), copper (10 µM) and herbivory with additional copper stress. Tuber ratios of the individual plants and abnormalities, such as fungi infestation were observed and statistically documented.

Chlorophyll content in the leaves was measured, because our hypothesis was that copper converts chlorophyll to chlorophyllin. This was verified by trend as the total amount of chlorophyll shows a decreasing tendency over the days of the experiment. For the FRAP assay, one cannot draw a lucid conclusion on the amount of reduced or oxidized glutathione, as the FRAP Assay is determining total antioxidant reducing capacity of the tissue samples. For the allicin test, no treatment affected significantly allicin production. This is hypothetically due to the possibility of the plant to use alternative metabolic pathways to synthetize allicin, as it is an essential biochemical defense molecule. Due to this change in pathway, more glutathione can be used for detoxification and scavenging of free radicals. However, we still confirm that the plant was affected and damaged by our treatments, as the plants treated with copper and a combination of copper and herbivory, clearly showed higher fungi infestation to none for control plants and the ones treated with herbivory. This might be due to a dislocation of allicin and its flavour precursors from the bulb to the leaves. The shift aims to have more allicin in the leaves of the plant, to chase away potential herbivores and guarantee defence against these even if the plant is harmed e.g. by copper excess.

In conclusion, one can say that beneficial effects of herbivory compensate the stress induced by copper excess, being the case for chlorophyll and fungi infestation. But at the same time, one can see that plants have a complex defense system against the different stressors. Even though a combination of the two stressors resulted in theory in a lack of glutathione in the plant, which would cause great harm to the whole plant, it is not the case. The plant can flexibly react on the combination of the stresses by changing pathway or disarrange allicin repartition, so that essential defence mechanisms are still guaranteed (Figure 11). It is really astonishing how plants developed such a complex and self-regulating defense system against the different biotic and abiotic stressors. These results show that plants are much more complex and well advanced creatures as one might think. Over time, only these plants that could deal with a variety of different stressors could survive and by the molecular changes in metabolism and pathway plants managed to do this feat.

In future studies, adaptation of experiment parameters and further tests have become paramount and in order to minimize the standard deviation and to get clear statistically valuable results, one may increase the experiment's duration and/ or the inflicted stress, or would have to switch to a much higher number of plants in order to decrease standard deviation.

RNA analysis could give us final certitude about the used pathway for alliin production as well as other metabolic adaptations. In addition, comparison of the pathway for allicin in the leaves and in the bulb as well as absolute allicin concentration in both tissues would offer us valuable clues to allicin production at this development stage.

Overall, one could observe effects that suggest a beneficial effect of herbivory treatment on the plant´s sulfur metabolism, whereas copper treatment appeared to inhibit its activity. Unfortunately results only showed tendencies

(as defined for p-values between 0.05 and 0.1). This is likely due to the plant having alternative ways synthesizing the products needed and/or because the stress was not intense enough and the days of experiment too short, and/or the number of plants too low. These preliminary results obtained in this investigation suggest that future studies in this research domain are warranted.

E. ACKNOWLEGEMENTS

Special thanks go to our tutors, Torsten Bohn, Boris Untereiner and Hanna Deußer, for their expert help and patience. Furthermore we'd like to thank Anouk Kaulmann for helping us with the FRAP Assay and handing us a detailed method on FRAP extraction and assay protocol. We also thank Marc Behr for explaining us the chlorophyll measurement. Thanks go to Montano Garces Mauricio for helping us to analyse the results from the Glutathione Assay. Then we would like to thank Fred Fack and Monica Dieterle from "Centre de Recherche Publique de la Santé - Luxembourg" for their background knowledge and motivation. We also want to thank Marc Charpentier for his reviewing and last but not least, very special thanks go to Paul Kieffer because he marked the origin of our garlic experiences and made the pathway for this paper.

Thank you, without all of you this would not have become possible for us.

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Hoagland solution consisted of (quotation from the original document [8]):

"Micropropagated plants at the same developmental stage […] were transferred to modified Hoagland solution containing:

1.25 mM KNO_3,
1.25 mM $Ca(NO_3)_2 \cdot 4H_2O$,
0.5 mM $MgSO_4 \cdot 7H_2O$,
0.25 mM $KH_2(PO_4)$,
11.6 µM H_3BO_3,
4.6 µM $MnCl_2 \cdot 4H_2O$,
0.19 µM $ZnSO_4 \cdot 7H_2O$,
0.12 µM $Na_2MoO_4 \cdot 2H_2O$,
0.08 µM $CuSO_4 \cdot 5H_2O$ and 10 µM Fe supplied as Fe(III)-EDTA or Fe(III)-citrate"

We used Fe(III)-citrate as iron source

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Programs used :

- for molecule drawing:

MarvinView 5.9.3 (20.04.2012) Sun Microsystems Inc. Java 1.6.0_29 [x86 Windows Vista 6.0] Copyright © 1998-2012 ChemAxon Ltd.

- for diagram:

Adobe Photoshop CS5 extended ver. 12.1x64 [Windows 7 64bit home premium] Copyright © 1990-2011 Adobe Systems incorporated. all rights reserved

- for statistical analysis:

SPSS version 19.0x96 (20.08.2011) [x86 Windows Vista 6.0] Copyright © 1990-2011 IBM Cooperation, Chicago, Illinois

F. ANNEXIS

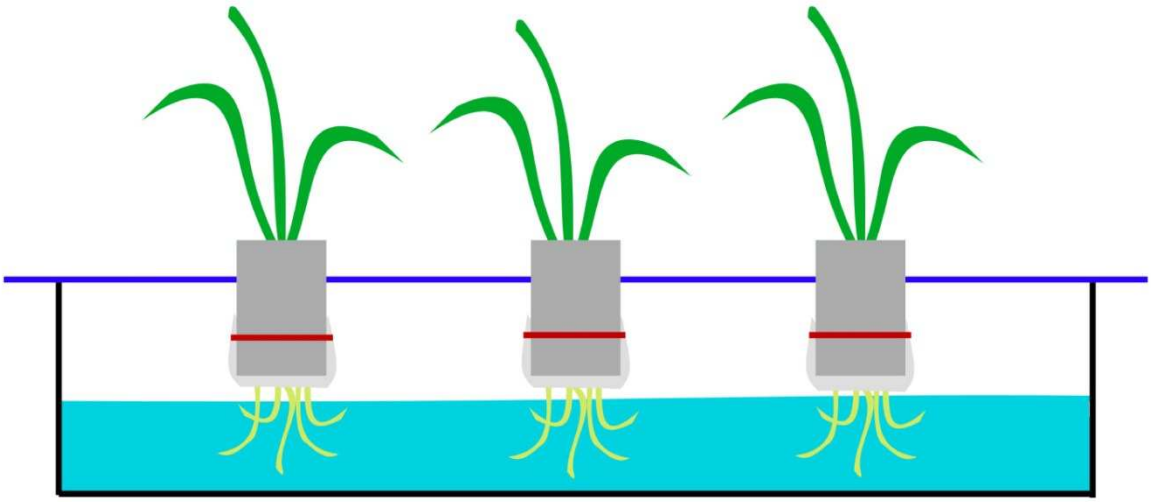


Figure 1: This is a schematic representation of the hydroponic system used for the experiment. It consists of a blue cover plate and a black painted box, both inhibiting photosynthesis and formation of chlorophyll in the leaves. Each plant is hold in 5cm of an isolating tube fixed with cling film and an elastic band, so that the plant is fixed and the roots are in the nutriment solution. The solution is aired in permanence to avoid any reactions varying concentration in the solution.

Figure 2: If necessary, leaves and bulbs, were cut in as big pieces as possible and immediately frozen. After that samples were freeze dried and stored at -80°C before analysis.

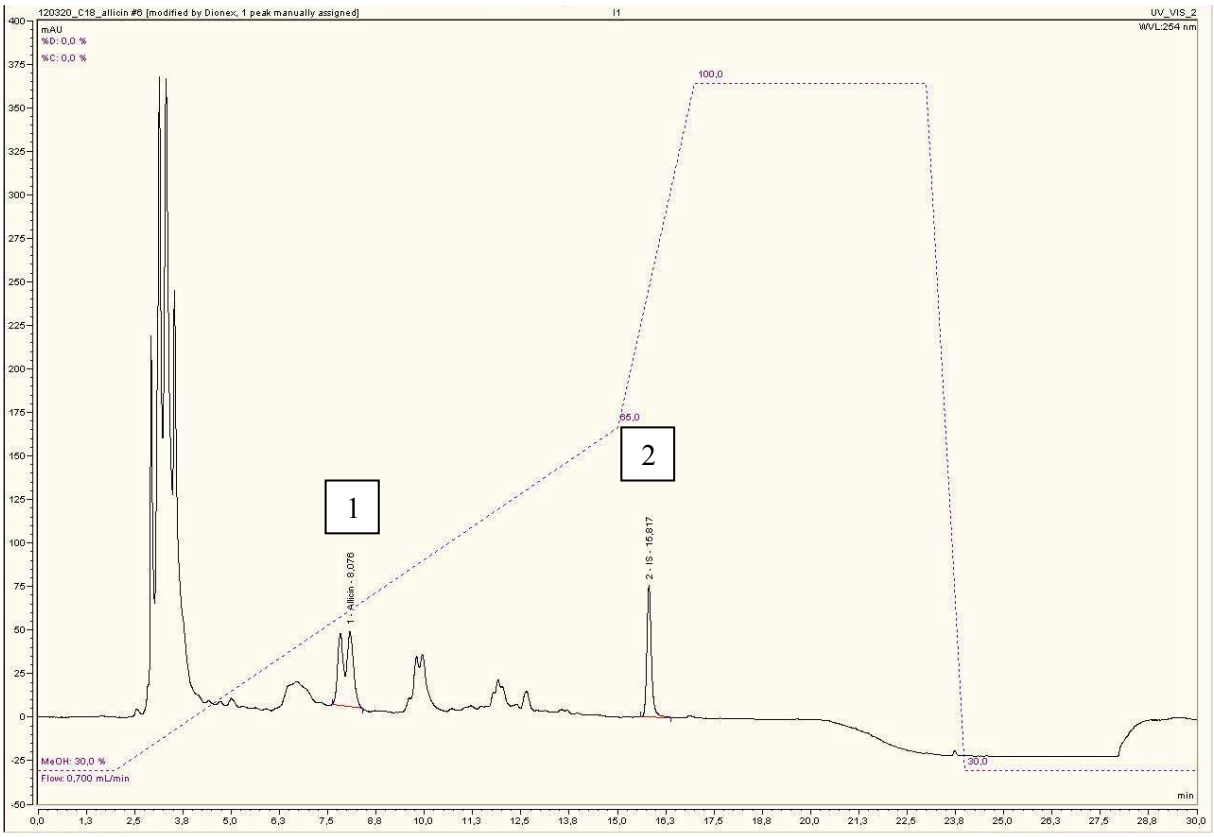


Figure 3: This is a typical HPLC diagram obtained from samples. The dotted line on the diagram shows concentration of water and methanol. Gradient starts with 30% methanol, for 2 minutes. Then, there is a linear increase of the concentration to 65% at 15min. At this time lap allicin peak (1) can be observed and the internal standard (2) also migrates. Thereafter, concentration goes up to 100% at 17.5min where it remains for exactly 5 minutes to decrease to 30% methanol at minute 23 of analysis.

Figure 4: On the last day, fungus infestation could be observed at the bulbs of 80% of copper contaminated plants.

The following results were obtained for total chlorophyll content:

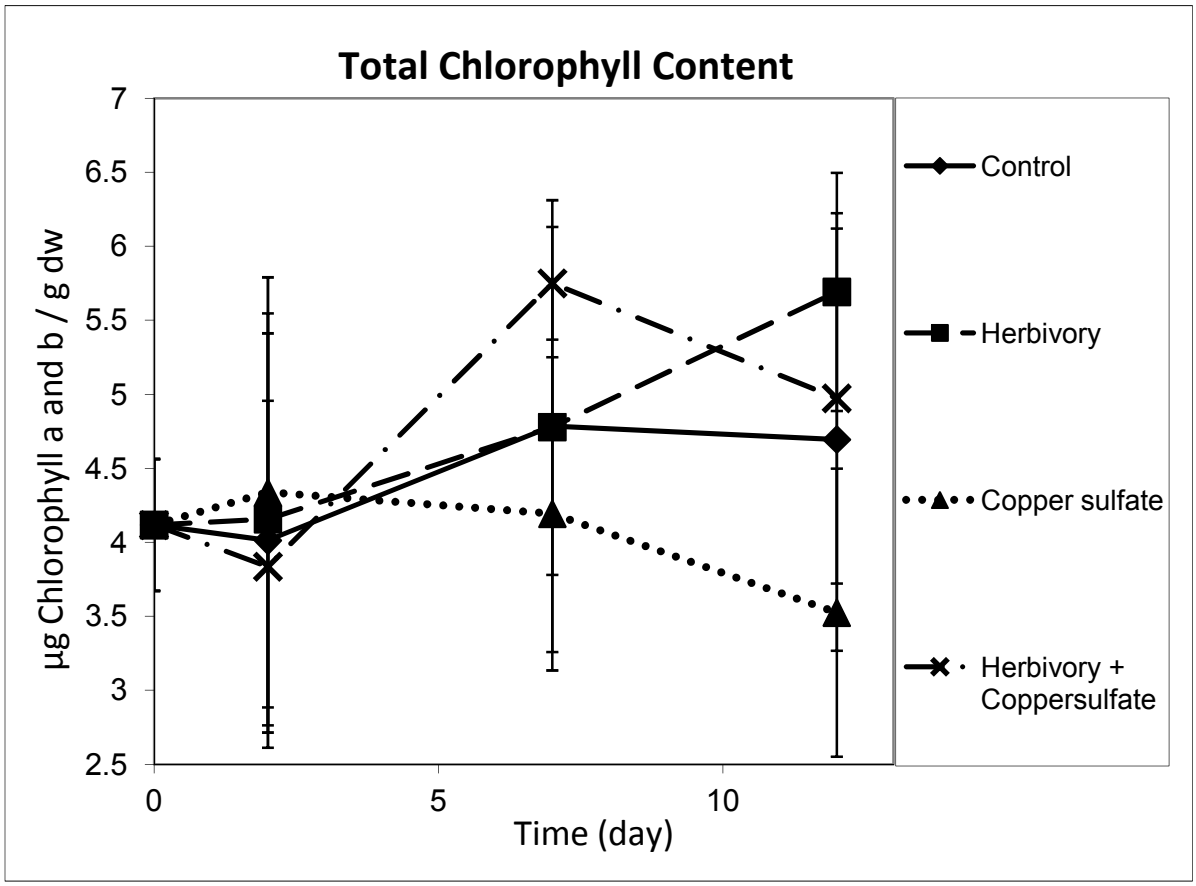


Figure 5: Total chlorophyll content in the leaves in function of the experiment duration. Error bars represent standard deviation (n= 0.5)

The following results were obtained for “Ferric Reducing Antioxidant Power” of the samples:

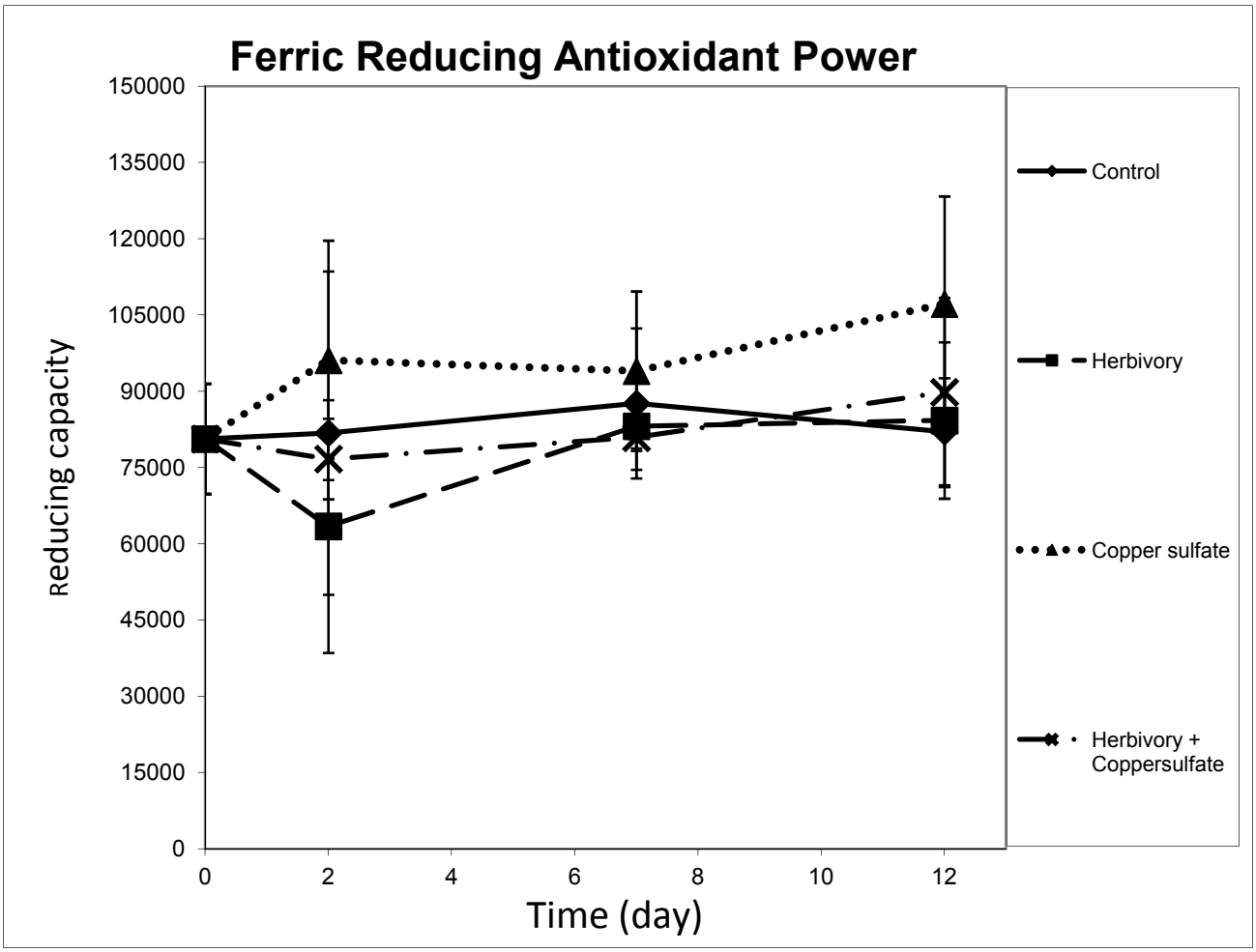


Figure 6: Ferric reducing antioxidant power of the tissue samples. Error bars representing SD of a mean of 5 replica.

The following results were obtained for allicin content in the leaves over the days of experiment :

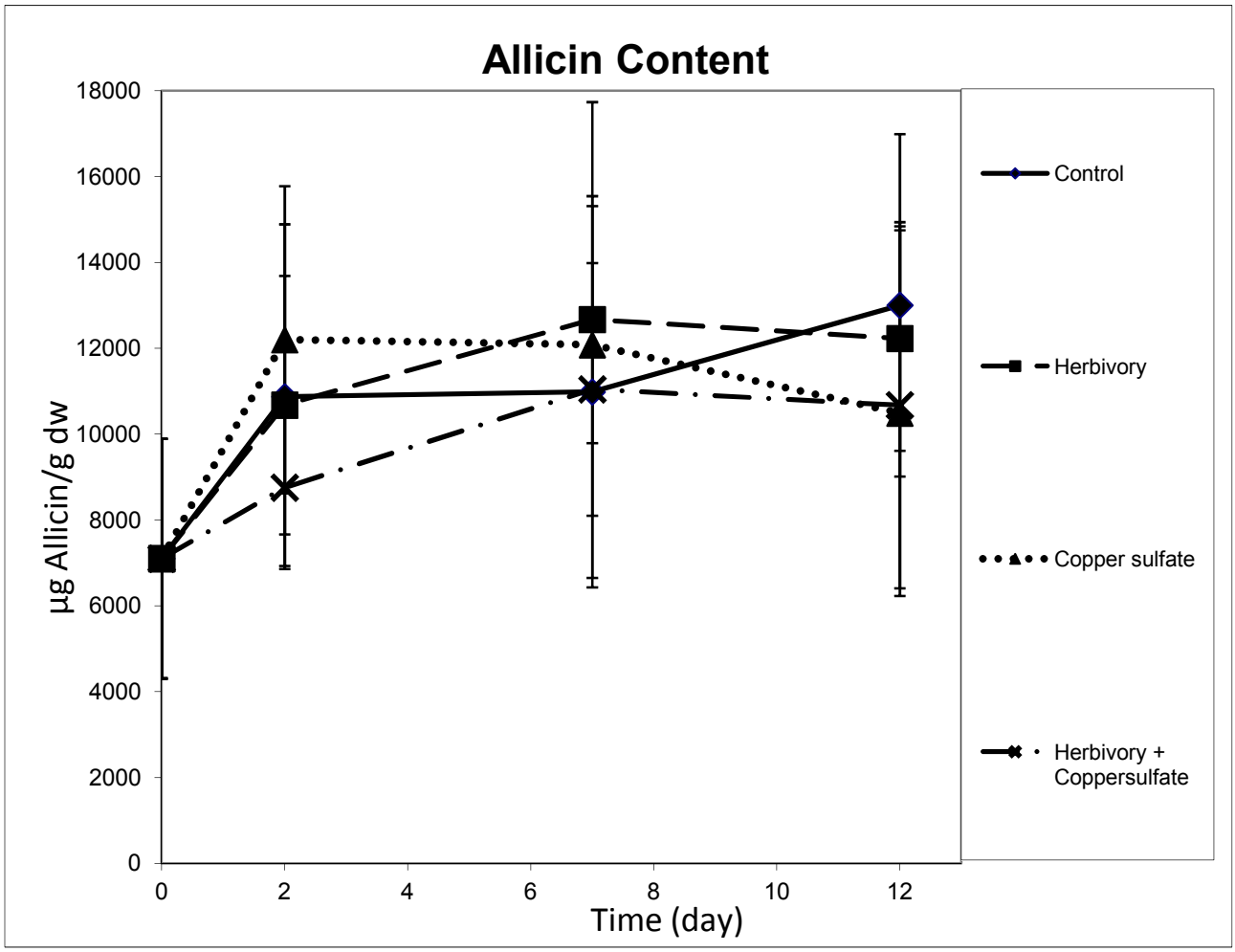


Figure 7: Allicin content in the garlic leaves in µg Allicin per gram dry weight

The following proportions of plants were infested by fungus the last day (12) :

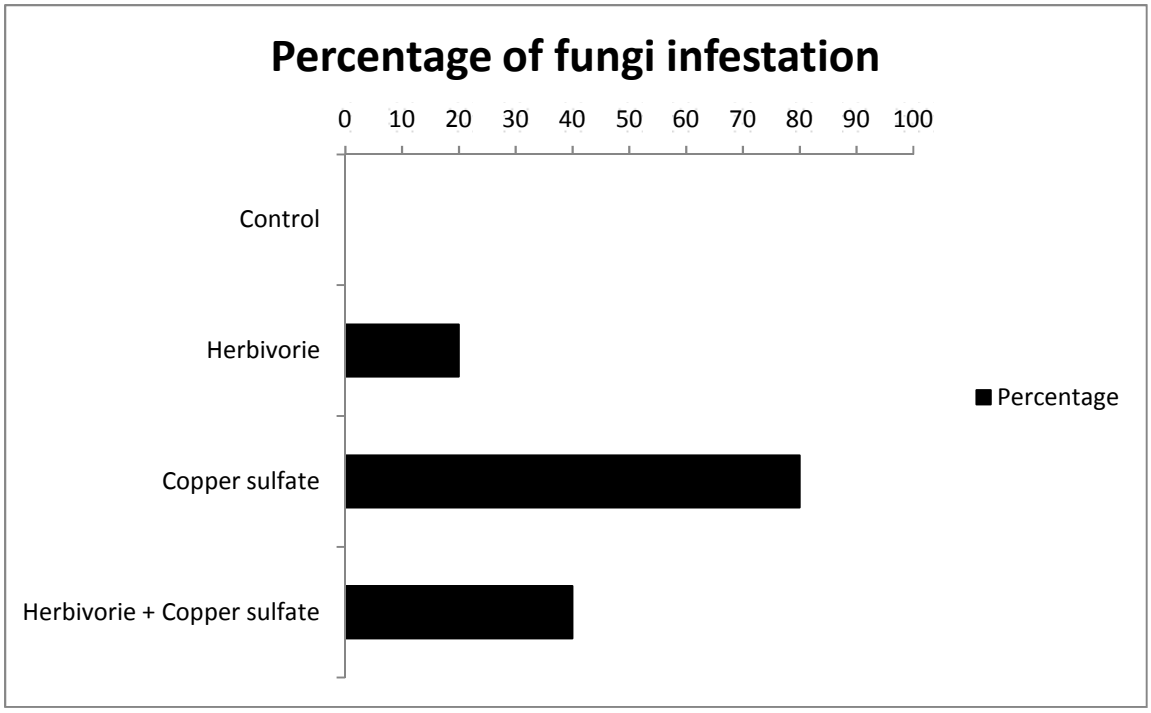


Table 8: Percentage of Fungi infestation on the plants

These results were obtained for the proportion of the bulb on total dry weight:

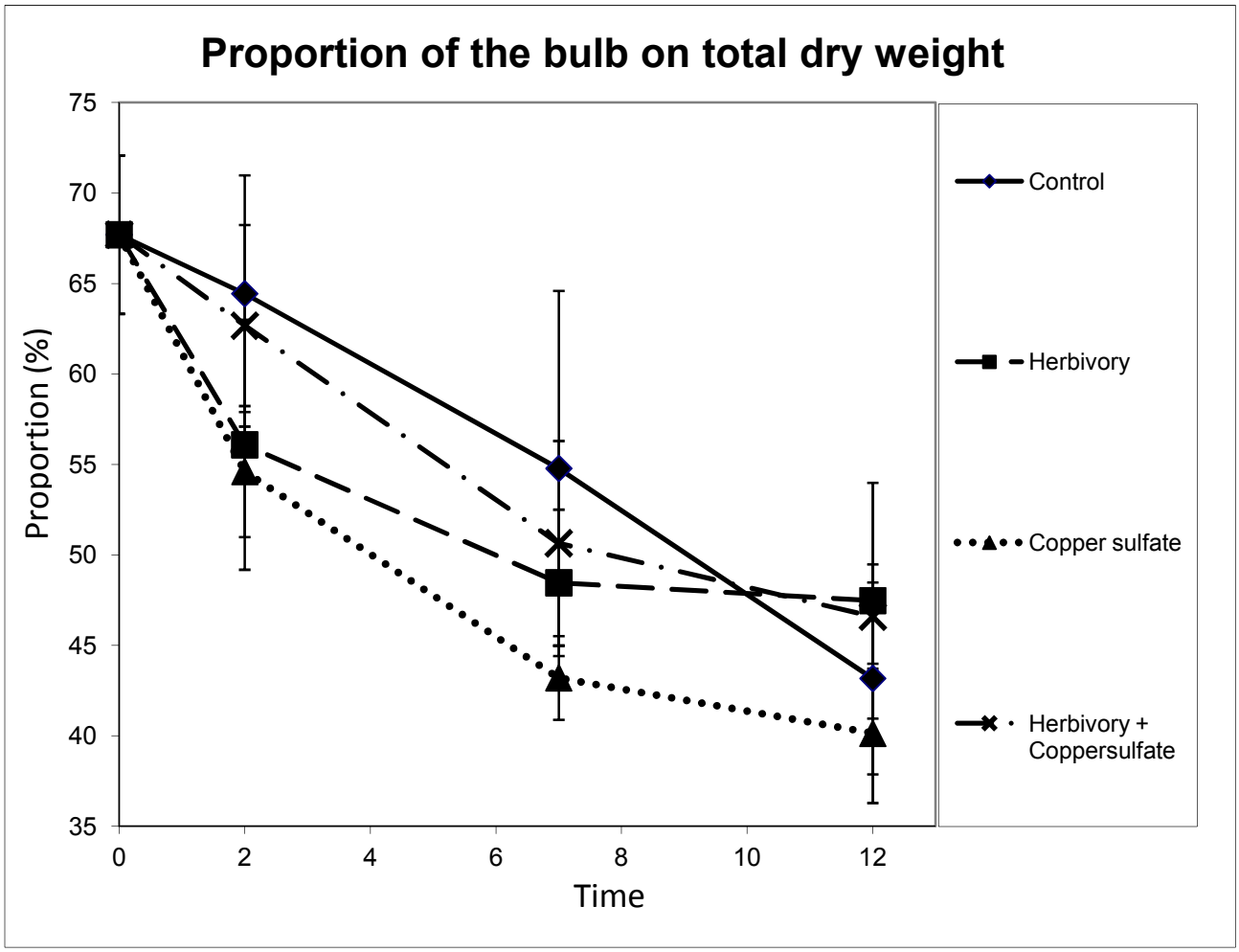


Table 9: Diagram showing the proportion of tuber weight over total weight of the plants over time.

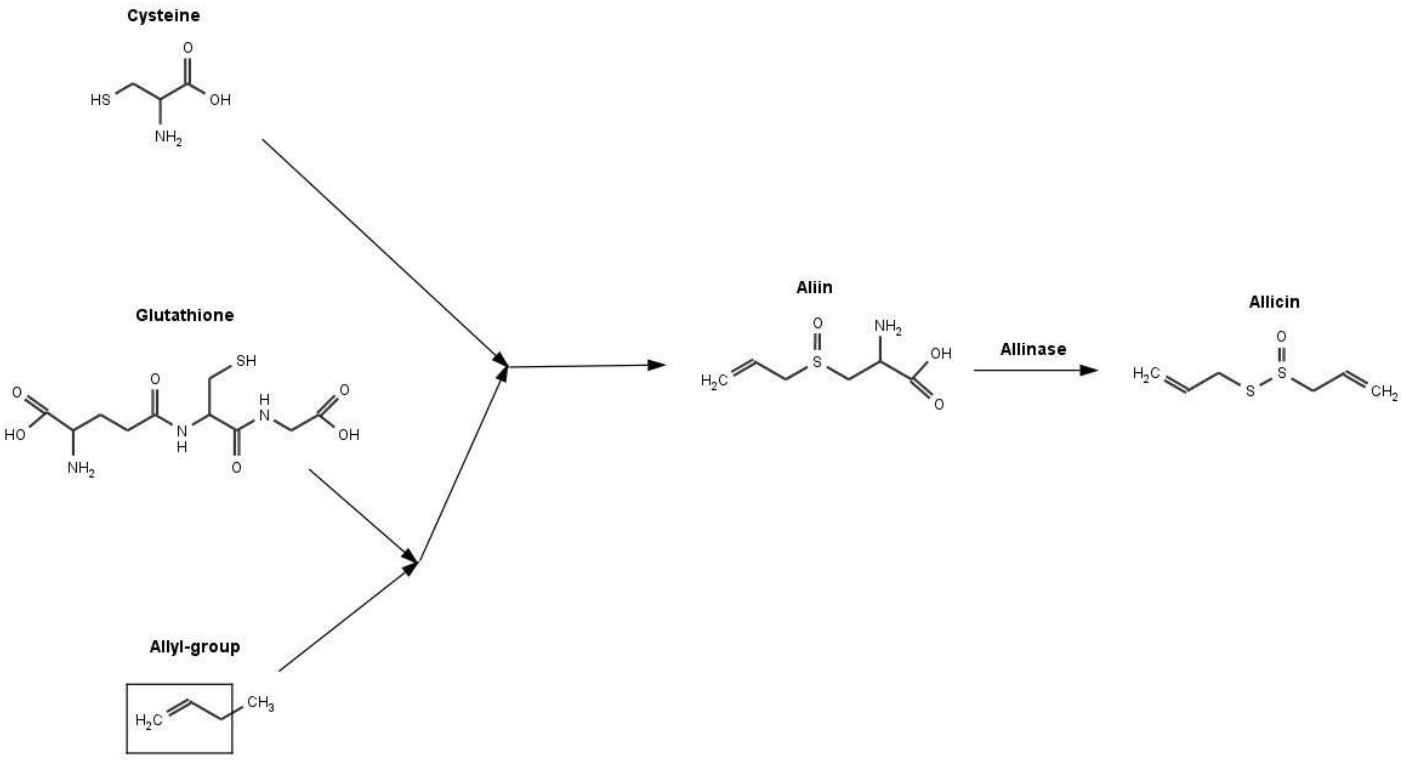
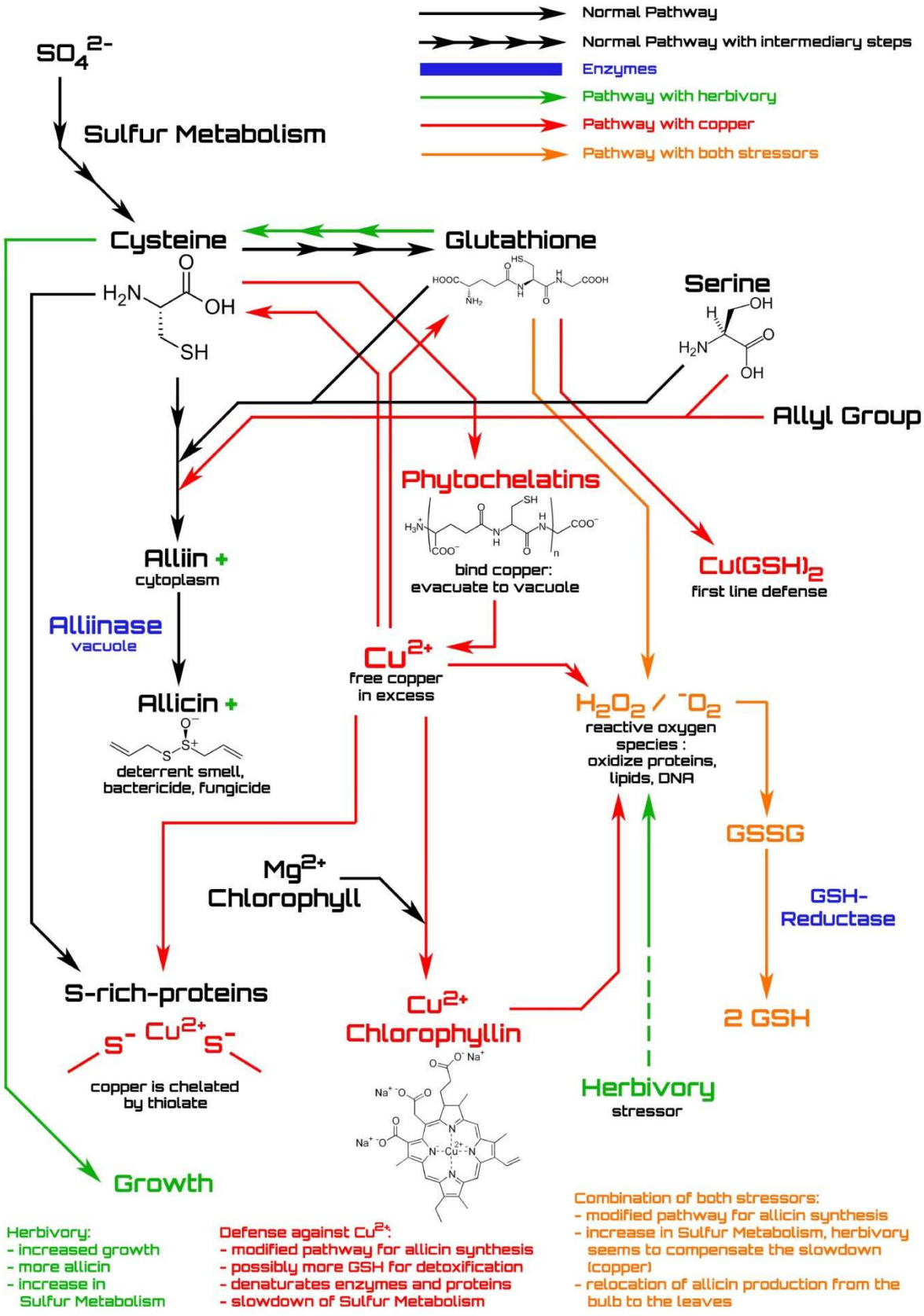


Figure 10 : Scheme showing a brief out cut of the normal pathway leading to the production of allicin in the plant. Glutathione and an allyl group (black box) from an unknown source bind together with cysteine to form alliin. When alliin, stored in vesicles in the cytosol, comes together with Alliinase, from the vacuole, allicin is produced. Allicin acts as a fungicide and bactericide and its deterrent smell chases away herbivores.

Figure 11: The following diagram shows the adaptions of the Sulfur metabolism on copper excess and the consequences. The diagram is easy to read, when first looking at the black arrows, then at the red arrows and the green, and again look at it more globally.


Normal Pathway
Normal Pathway with intermediary steps
Enzymes
Pathway with herbivory
Pathway with copper
Pathway with both stressors
SO_4^{2-}
Sulfur Metabolism
Cysteine
Glutathione
Serine
Allyl Group
Phytochelatins
bind copper:
evacuate to vacuole
$Cu(GSH)_2$
first line defense
Alliin +
cytoplasm
Alliinase
vacuole
Allicin +
deterrent smell,
bactericide, fungicide
Cu^{2+}
free copper
in excess
H_2O_2 / $^{-}O_2$
reactive oxygen
species :
oxidize proteins,
lipids, DNA
GSSG
GSH-
Reductase
2 GSH
Mg^{2+}
Chlorophyll
S-rich-proteins
$S^- Cu^{2+} S^-$
copper is chelated
by thiolate
Cu^{2+}
Chlorophyllin
Herbivory
stressor
Growth
Herbivory:
- increased growth
- more allicin
- increase in
Sulfur Metabolism
Defense against Cu^{2+}:
- modified pathway for allicin synthesis
- possibly more GSH for detoxification
- denaturates enzymes and proteins
- slowdown of Sulfur Metabolism
Combination of both stressors:
- modified pathway for allicin synthesis
- increase in Sulfur Metabolism, herbivory
seems to compensate the slowdown
(copper)
- relocation of allicin production from the
bulb to the leaves