

Supplementary materials for the work:

The use of image analysis in improving knowledge and skills on the example of teaching the biology of algae and fungi

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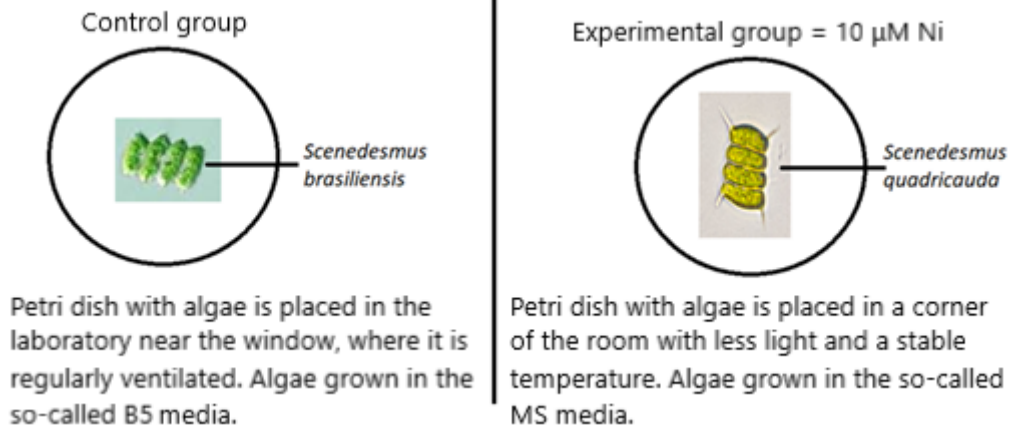
<https://orcid.org/0000-0002-5701-0598>

Test used in this work

Gender: male / female

Identification code:

1. Thomas tried to determine the effect of Ni on the content of ROS (reactive oxygen species) in the algae *Scenedesmus quadricauda*. He proposed the following experiment:



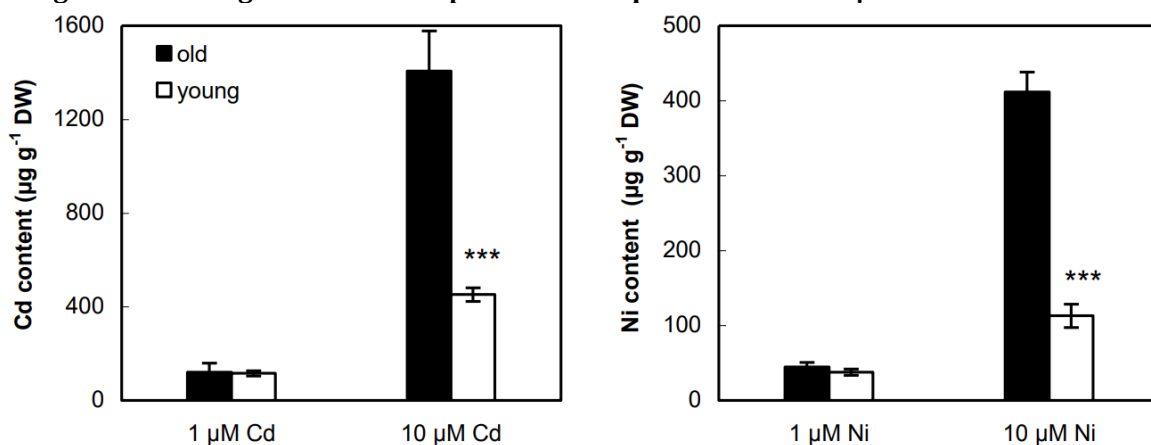
Various nutrition media have various mineral compositions. After 2 days, the content of ROS in the experimental group was higher than in the control group, and therefore Thomas formulated the following conclusion: „Nickel induces a higher production of ROS and has a negative effect on *Scenedesmus quadricauda*.“

- Could Thomas validly formulate this conclusion based on his experiment? Justify your answer.
- What is oxidative stress?
- Do you think that ROS occur only in algae and plants or also in other organisms? Justify your answer.
- Why hydrogen peroxide molecule occurs in cells?

2. Ascorbic acid (vitamin C) is a metabolite synthesized mainly by plants and is important in regulating the constant level of ROS. The ROS are formed in larger quantities in plants under unfavorable conditions (e. g. higher cadmium/Cd concentrations). Plants therefore need to reduce the ROS content in their body. Simone wants to carry out an experiment in which she will investigate the effect of lycorine (inhibitor of vitamin C synthesis, note: inhibit means to block) on the content of ROS in the alga *Scenedesmus quadricauda* exposed to Cd.

- Help Simone make a hypothesis about the amount of ROS in the algae exposed to lycorine + cadmium compared to cadmium alone:
- Can you give examples of plants/fruits that contain a lot of vitamin C?
- Why is it important to get enough vitamin C through food?

3. Dominika found a scientific article with graphs showing the content of Cd and Ni in old and young cultures of alga *Scenedesmus quadricauda* exposed to 1 and 10 μM Cd or Ni.



- Did the algae contain more Cd or Ni?
- Did the age of the algal culture affect the uptake of Cd and Ni, and if so, how?
- Does metal absorption by algae depend on the concentration that was applied?
- Describe what you know about cadmium and nickel.
- What is the effect of Cd or Ni on the organisms?

4. Sebastian found a scientific article with table presenting the effect of the fungal pathogen *Verticillium dahliae* (Vd), salicylic acid (SA) and mycorrhizal fungus (AMF) on shoot length (SL) of *Capsicum annum* seedlings. Help Sebastian formulate the conclusions from the table.

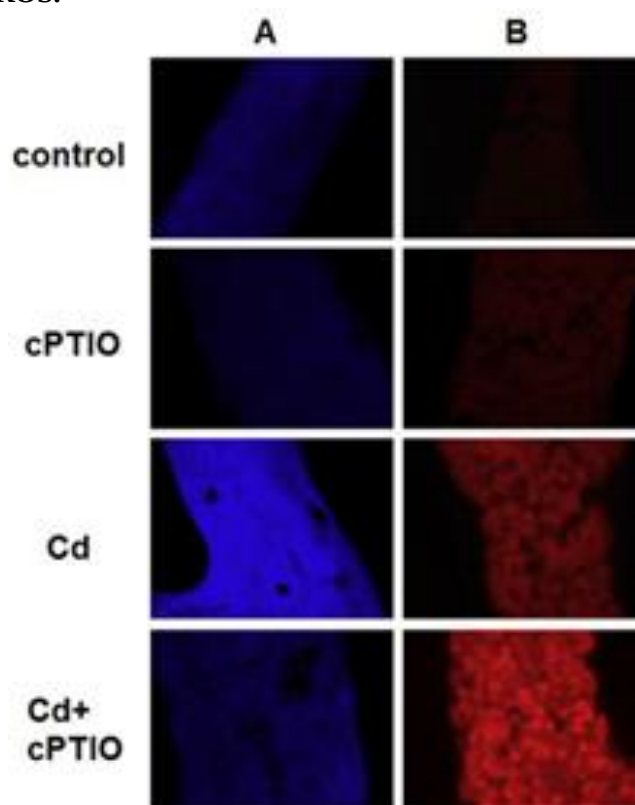
Treatments	SL (cm)
Control	37.91bc
Vd	30.00e
AMF	41.76a
SA	38.31b
AMF+SA	40.76a
AMF+Vd	35.58 cd
SA+Vd	33.79d
AMF+SA+Vd	33.62d

- What was the effect of *Verticillium dahliae* on shoot length?

b. What was the effect of salicylic acid and mycorrhizal fungi on the shoot length of *Capsicum annum* infected with the pathogen *Verticillium dahliae*?

c. Do you know what effect acetylsalicylic acid has on the human body?

5. Daniela found a scientific article with microscopic photos of a lichen showing the impact of cadmium (Cd) and nitric oxide inhibitor (cPTIO) on nitric oxide content (column A) and on ROS content (column B). Nitric oxide (NO) is a molecule that usually has a positive effect on organisms and protects them from ROS. The ROS are generated as a normal product of cellular metabolism, but various factors can lead to their excessive production. These microscopic photographs (always a similar part of the lichen) were observed under a fluorescence microscope. With the help of fluorescence microscopy, it is possible to identify where and in what concentration (more intense red or blue color = higher quantity of the given substance) specific substances (in this case, nitric oxide and ROS) are found in the tissue. Help Daniela formulate conclusions about the relationship between Cd, NO and ROS.



a. What is the effect of Cd on ROS and NO content?

b. What is the effect of cPTIO on ROS and NO content during co-application of Cd?

c. Does nitric oxide have a positive effect on Cd-induced changes? Justify your answer.

d. Do you know any effects of nitric oxide on the human body?

e. Nitric oxide is a substance that usually has a positive impact on organisms. Do you know how this or other oxides of nitrogen affect the environment?

f. What are lichens?

g. What is the importance of lichens in nature and for people?

6. Mark carried out an experiment and wrote the results in a table. Help him to construct a graph showing the dependence of the phenolic content on the harvest day of the alga *Ulva lactuca*. Create a graph based on the data in the table (use a ruler):

harvest day	phenolic content
1.	80 mg of phenols per gram of dry matter
5.	50 mg of phenols per gram of dry matter
7.	30 mg of phenols per gram of dry matter
10.	20 mg of phenols per gram of dry matter

a. Graph drawing:

b. Phenols are a group of plant substances that can make up to 45% of the organic matter in plants. Do you know the effect of phenols on humans?

c. If you know, give examples of foods that contain a lot of phenols.

Table S1. Examples of answers in the test mentioned above. Tasks are marked as science process skills (grey rows) and knowledge (white rows). We assigned 1 point for a correct answer (any correct information from the given examples in the first column) and 0 points for an incorrect answer.

question no.	examples of the most common <i>correct</i> answers	examples of the most common <i>incorrect</i> answers
1. a.	no, because he worked with two species of algae, and the control/experimental groups didn't have the same experimental conditions	yes, he could because the algae changed color yes, he could because Ni has a negative effect on algae yes, he could because he carried out the experiment
1. b.	stress caused by imbalance in the formation and degradation of reactive oxygen species (ROS) or enhanced formation of ROS	an excess of oxygen that the organism cannot cope with stress caused by reactions with oxides
1. c.	ROS also occur in other organisms, because they are formed during aerobic metabolism (mentioned by some university students based on the previous education)	yes, because they are dangerous/deadly for animals yes, they are also found in plants
1. d.	because the oxygen in the cell reacts with different enzymes/processes or because it is formed from other oxygen radicals or because it is a molecule that is formed in peroxisomes (mentioned by some university students based on the previous education)	because oxygen and hydrogen are present in the cell for disinfection
2. a.	the formation of ROS will increase because lycorine will inhibit the production of vitamin C	the formation of ROS is reduced by the addition of lycorine, because it inhibits the formation of vitamin C
2.b.	<i>Citrus</i> sp. (lemon, orange), <i>Rosa canina</i> (rose hip), <i>Malpighia</i> (mentioned by some university students based on the previous education)	-
2. c.	because humans do not synthesize it and are dependent on its intake from food or improves immunity or essential in collagen synthesis (mentioned by some university students based on the previous education)	for energy because it is important
3. a.	algae contained more cadmium (Cd)	algae contained more nickel (Ni)
3. b.	yes, older culture absorbed more metals at higher dose	no yes, younger absorbed more metals
3. c.	yes, metal uptake increased with increasing concentration	no
3. d.	they are (heavy) metals or metallic elements	they are elements in the periodic table

3. e.	excess of metals is negative, they accumulate in organisms, causing disruption of metabolic functions	positive neutral
4. a.	the effect is negative, or shoots are shorter	great influence shoots are longer
4. b.	the effect is positive, or shoots are longer	shoots are shorter
4. c.	antipyretic (against fever), antiphlogistic (alleviation of inflammation) and analgesic (against pain)	negative/toxic
5. a.	stimulated the formation of ROS and NO	it became colored or it was more visible
5. b.	stimulated ROS formation but inhibited NO	almost none
5. c.	yes, because the application of cPTIO (an inhibitor of NO formation) induced increased ROS production = NO may protect against ROS	no
5. d.	blood flow regulation, signaling molecule	a waste product of metabolism that a person exhales negative, breathing problems
5. e.	causes acid rain, damages the ozone layer, contribute to global warming	improves plant growth positive
5. f.	symbiotic organisms consisting of a fungus and algae/cyanobacteria	plants that live in humid environments mosses algae that live on land
5. g.	bioindicator, production of perfumes, food for animals	water retention in the environment, for decoration
6. a.	-	sketch without marking x and y axis
6. b.	they have many health benefits for humans	negative
6. c.	green tea, dark chocolate	preservatives, food additives with E numbers, meat products

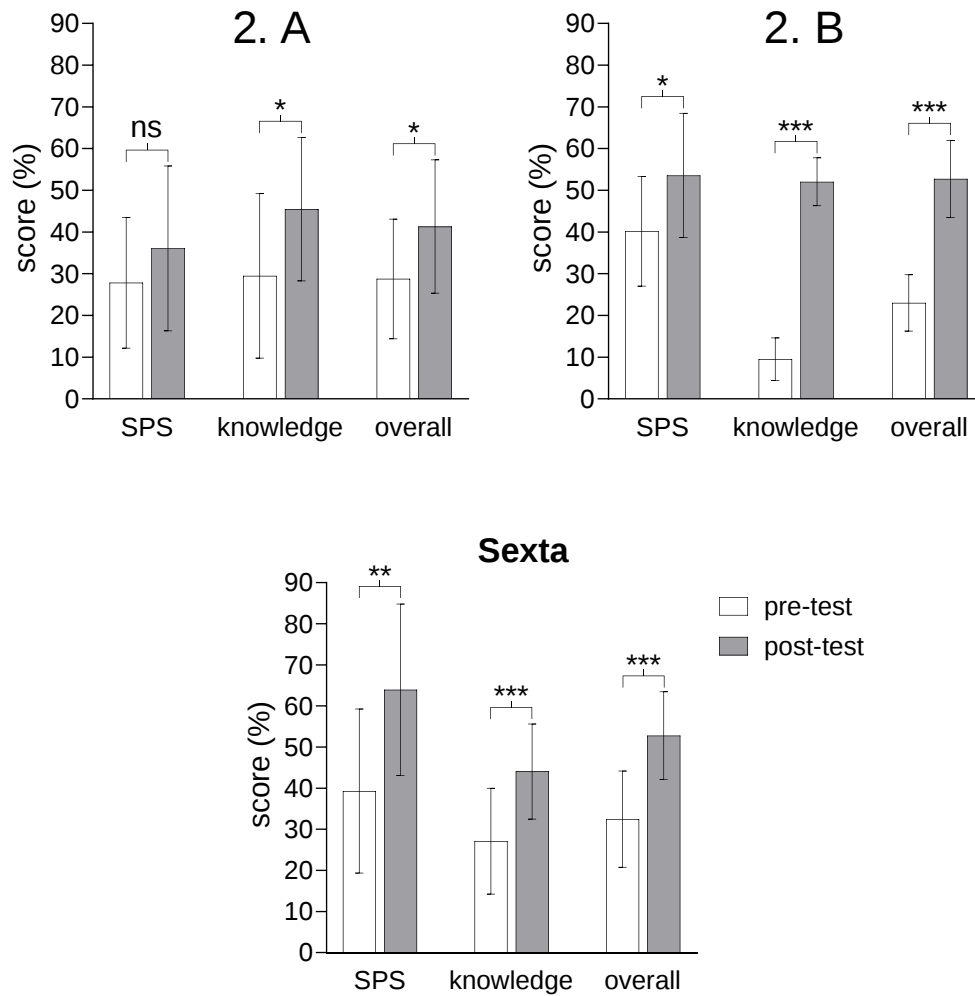


Figure S1. Comparison of pre-test and post-test results in three independent classes (belonging to “our experimental gymnasium students“ as mentioned above). The test monitored either science process skills (SPS) or knowledge (and sum of both parts is shown as columns “overall”). Data are given as a percentage of success out of a maximum score of 100% and bars represent standard deviations. Values above columns with *, ** and *** indicate significant differences at 0.05, 0.01 and 0.001 levels of the Wilcoxon signed-rank test between the pre-test and post-test (and ns means non-significant difference with $P > 0.05$). Classes 2. A and 2. B are students of 4-year gymnasium program (meaning only gymnasium education, ISCED3) while class Sexta are students of 8-year program (meaning lower secondary followed by gymnasium education, i.e. ISCED2 and 3). For comparison of the sum of 4-year program versus 8-year program see Fig. 1 in the main text.

Worksheet no. 1: Algae as food and a source of nutrients

Introduction: One of the biggest problems of the 21st century is the provision of sufficient resources for the constantly growing world population. A partial solution to this problem is the cultivation of seaweed. *Ulva lactuca*, also known as sea lettuce, is an edible green alga. This alga is used locally in soups and salads; other species of the genus are used in different parts of the world. Just as farmers try to maximize the yield and quality of the plants they grow, seaweed farmers also try to maximize the yield and quality of the algae. A Swedish team of biologists also dealt with it, trying to find out the optimal day for harvesting *Ulva fenestrata* during the year. One of their conclusions are these photos:



Figure 1

Research question: What is the effect of time on the size/area of the alga?

Photos can be objectively compared using the image analysis method. It is a method of image processing when assessing the properties and quality of an object, in which subjective judgment by a human is replaced. We will use the Fiji program to analyse the image. Note that each photo has own scale. **Why is a scale necessary for every photo?**

Instructions for measuring the size of alga:

1. Open the Fiji program on your computer.
2. In groups of four students, divide the pictures, which will be analysed by individual group members. Each member selects one picture.
Subsequently, open the file that you will analyse in the Fiji program (Fig. 2).

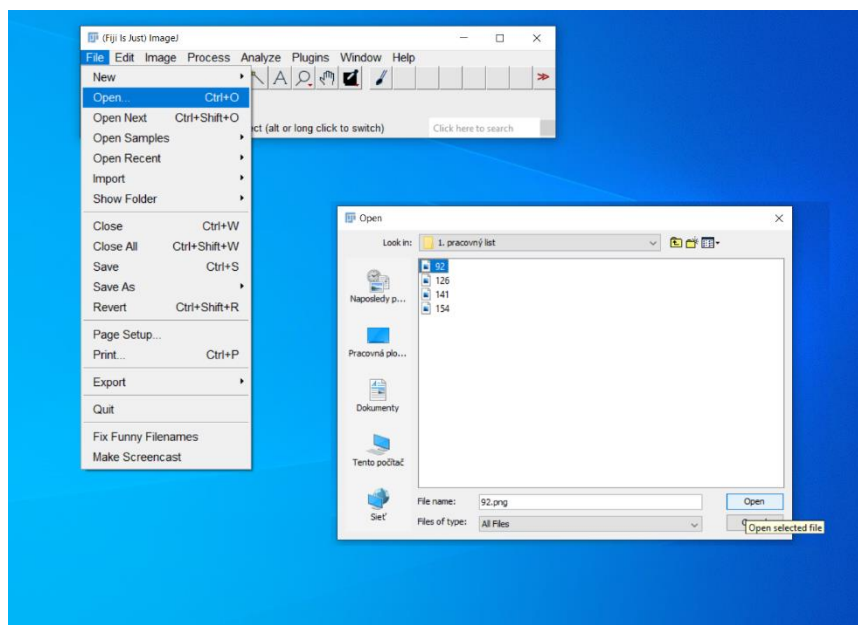


Figure 2

3. Set the scale of the photo:

- Click on the line icon (**Straight**) and mark the scale, which represents 5 cm (Fig. 3, red arrows).
- After marking the scale length, click on **Analyze** → **Set Scale**, set the scale parameters according to Fig. 3 (don't overwrite the row **Distance in pixels**). Then click on the OK button (yellow arrow).

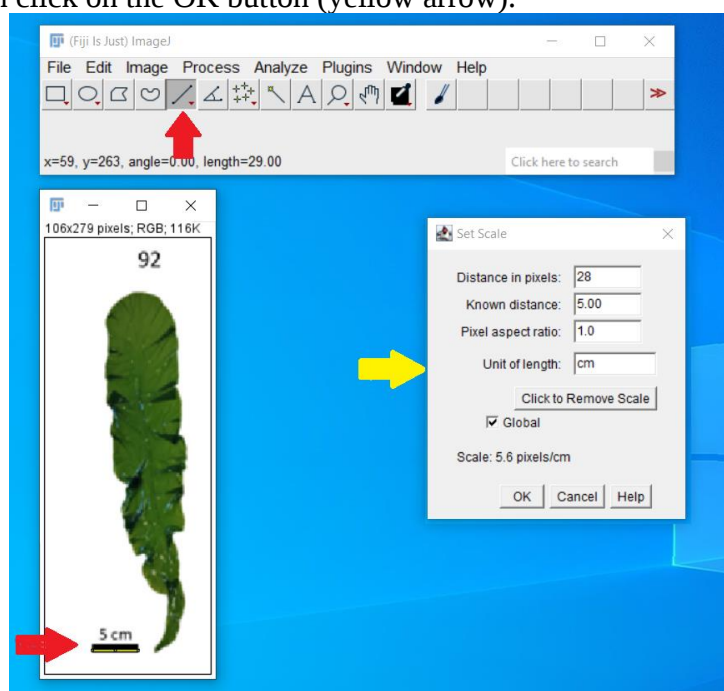


Figure 3

4. Threshold the photo: click on **Image** → **Adjust** → **Color Threshold**, set the threshold according to Figure 4 and close the given window.

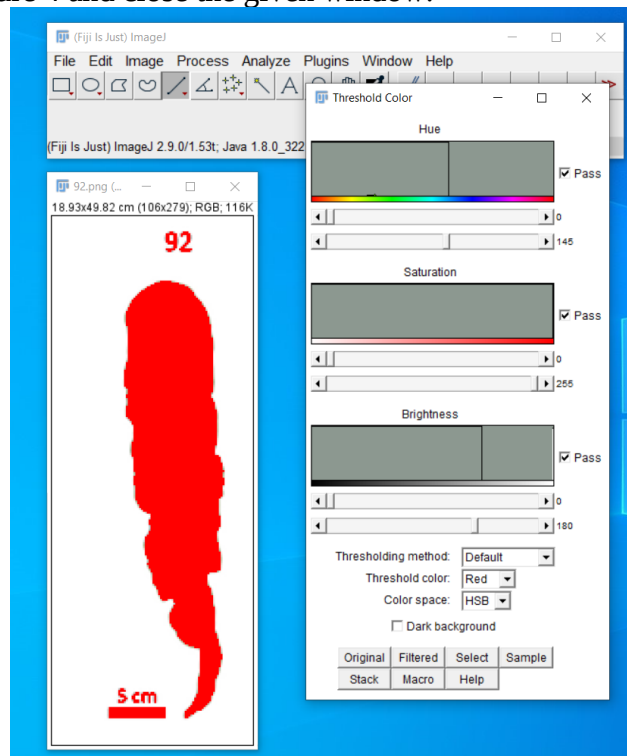


Figure 4

5. In the program, you need to set what you want to measure: click on **Analyze** → **Set Measurements**. Set the window that appears according to Fig. 5 (Area) and click **OK**.

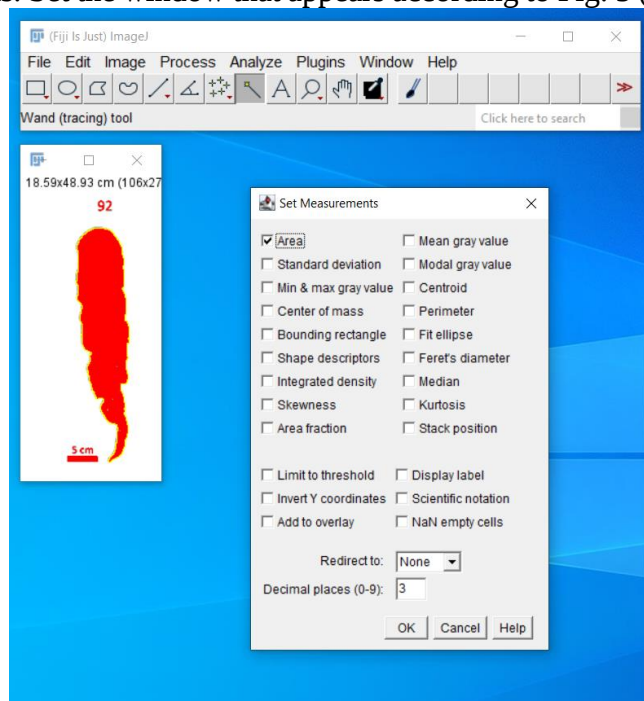


Figure 5

6. Select the object whose area you want to measure: click on **Analyze** → **Tools** → **ROI Manager**.
7. A window will appear. Click on the wand icon (**Wand tool**, Fig. 6, red arrow) and click on the object you want to measure. Then click on **Add** in the “**ROI Manager**” window (Fig. 6, yellow arrow).

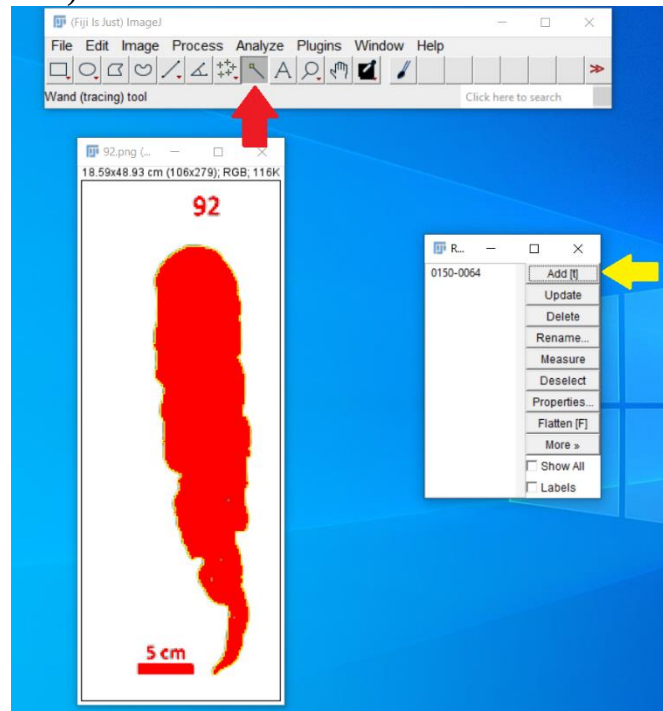


Figure 6

8. By clicking on **Measure** in the “**ROI Manager**” window, you will see a table showing the size/area data. Write your result.

From the results of all members of your group, construct a graph that will show the dependence of the size of alga on the harvesting time. Is it possible to conclude when is the best time to harvest alga from a growth point of view?

Graph:

Your conclusion:

Based on Figure 1, formulate an assumption about the change in chlorophyll content of the alga during various days of harvest:

Growth/size is not the only parameter of quality, and the chemical composition is also important. Scientists also determined other parameters (the content of proteins, fatty acids, chlorophylls, and phenols) using analytical methods.

Study these graphs and answer the questions below (DW = dry weight):

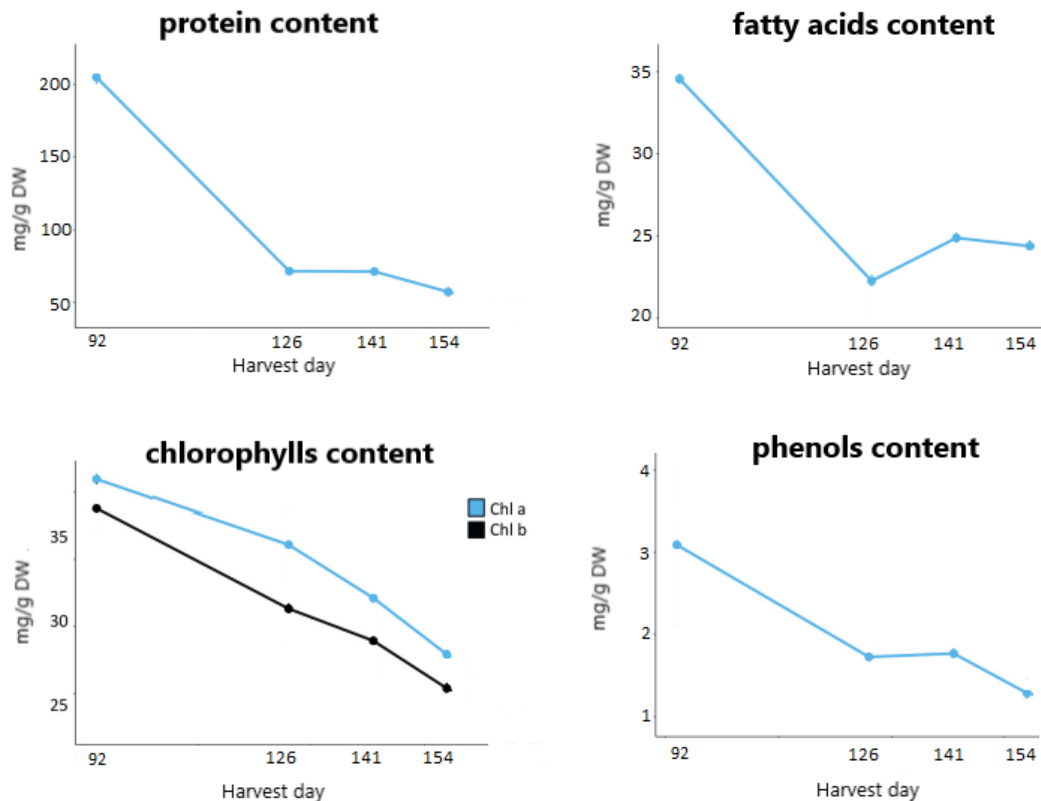


Figure 7

a) Is it possible to conclude from the graphs when is the best time to harvest algae in terms of metabolite content?

b) Is it true that the largest algae size also has the highest metabolite content?

c) Based on the metabolite content graphs on this page and the algal size graph you created on the previous page, suggest the best time to harvest algae to achieve maximum growth/area and highest metabolite content simultaneously.

Worksheet no. 2: Algae, oxidative stress and antioxidant

Introduction: *Coccomyxa* is a genus of unicellular green algae (so-called microalgae) with a small ellipsoid to spherical shape. About 40 species are known (free-living and symbiotic species in lichens). Some of the species have potential use for biofuel production. Biofuel is a substance derived from organic material that can be used as an energy source. *Coccomyxa* is often used as a model organism since its entire genome is known.

Plants, including green algae, are exposed to changing environmental conditions throughout their life. Unlike animals, they cannot migrate to another place and must cope with unfavourable conditions. In such conditions, reactive oxygen species (ROS) are excessively formed in all aerobic cells, which damage the basic components – proteins, lipids, or DNA. The ROS formation is significantly stimulated by an elevated concentration of metals. Nitric oxide (NO) is a gas that was originally discovered in animal cells. It has many functions in plants, from regulating basic growth processes to alleviating the effects of various stresses.

The results of research that investigated the formation of ROS and nitric oxide in algae exposed to mercury (Hg) and the possibilities of alleviating the negative effect of this metal are shown in Fig. 1 as microscopic photos. The researchers also investigated the effect of vitamin C (ascorbate), a molecule potentially mitigating the negative effects of the metal. Using a fluorescence microscope, it is possible to monitor the intensity of ROS (green signal) and nitric oxide (blue signal) formation (more intense green or blue color = higher quantity of the given substance).

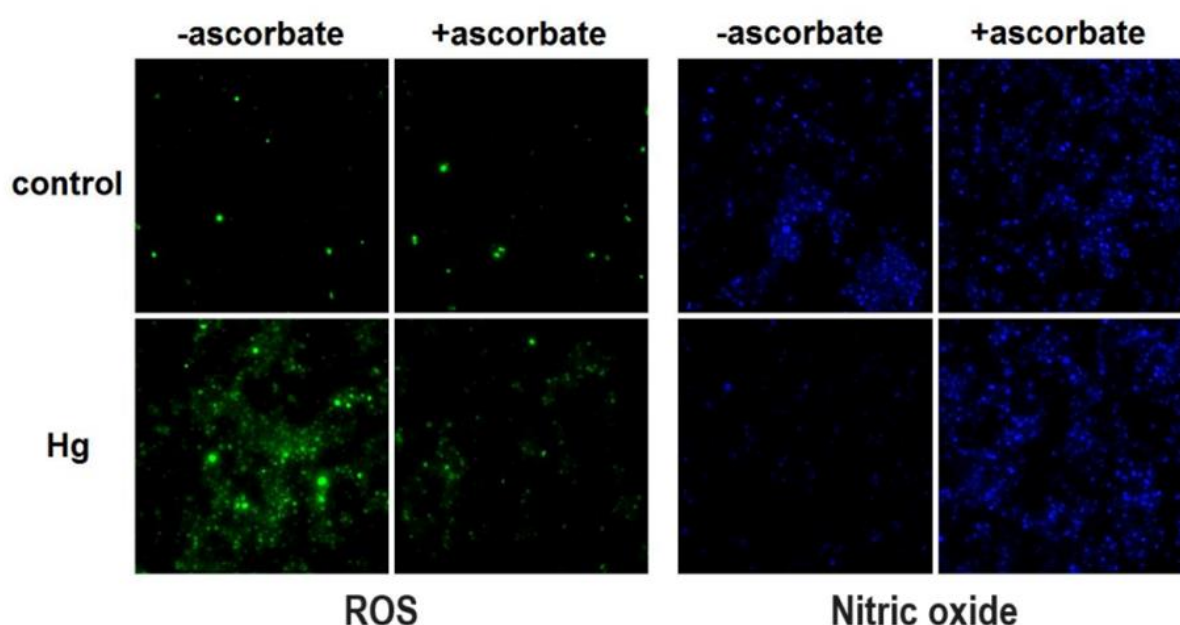


Figure 1

Research questions:

- Does the Hg induce ROS formation in algae?
- Does the content of nitric oxide change after addition of Hg?
- What is the effect of vitamin C on these changes?

Photos can be objectively compared using the image analysis method. It is a method of image processing when assessing the properties and quality of an object, in which subjective judgment by a human is replaced. We will use the Fiji program to analyse the image.

Instructions for measuring signal intensity:

1. In groups of four students, divide the pictures, which will be analysed by individual group members. Each member chooses one column (ROS without vitamin C, ROS with vitamin C, nitric oxide without vitamin C, or nitric oxide with vitamin C) with two photos for each team member (control and Hg).
2. Open the Fiji program on your computer.
3. Subsequently, open the file that you will analyse in the Fiji program (Fig. 2).

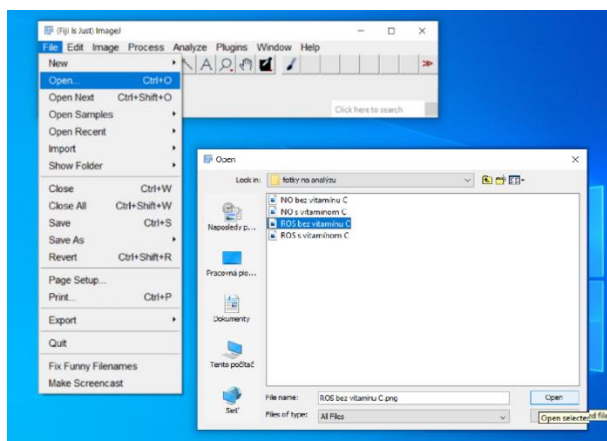


Figure 2

4. In the program, you need to set what you want to measure: click on **Analyze** → **Set Measurements**. Set the window that appears according to Fig. 3 and click **OK**.

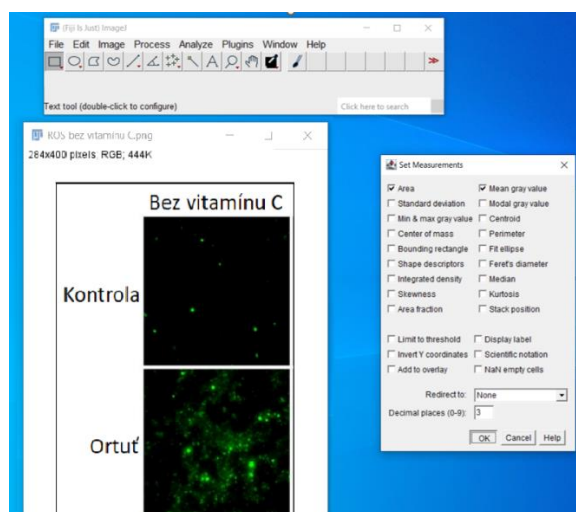


Figure 3

5. Now we will measure the signal intensity of individual microscopic photos. Using the **Rectangle** under the **File**, mark the first photo (control) you want to analyse and click **Analyze** → **Measure**. A table with the measured data will be displayed. **Area** represents the size of the area you marked and **Mean** represents the relative intensity of the signal.
6. Repeat the fifth step for the second image and save the table with results or copy it to an Excel file.
7. **From the results of all members of your group, construct two graphs showing the impact of metal Hg and vitamin C on the formation of ROS and nitric oxide.**

Graph:

Questions:

a) What was the effect of mercury on the formation of ROS and nitric oxide in the alga? Is the relationship between them positive or negative?

b) What was the effect of vitamin C on the formation of ROS and nitric oxide induced by the presence of mercury?

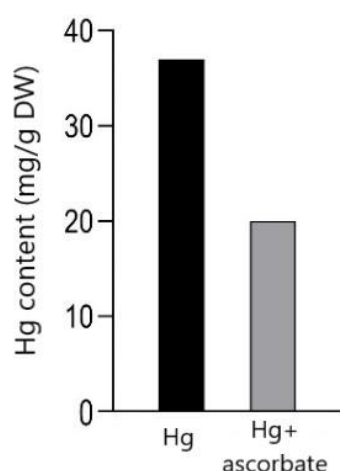
c) Did higher nitric oxide concentrations after vitamin C application contribute to better protection against ROS? Justify your answer.

d) What is the effect of nitric oxide on the human body? If you do not know the answer, use the internet.

e) Can you write the chemical formula of nitric oxide? Do you know other oxides of nitrogen? One of them is a very dangerous greenhouse gas, 1 molecule of which released into the atmosphere is equivalent to 265 molecules of released carbon dioxide. What molecule is it? If you do not know the answer, use the internet.

The graph below shows the content of Hg in algae exposed to mercury without or with the addition of vitamin C. Abbreviation DW = dry weight.

The effect of ascorbate on the Hg content in algae



f) Based on the graph, explain how vitamin C affected the mercury content in algae.

g) The graph above shows that the algae contain 37 mg of Hg in 1 g of dry weight (black column). Express the Hg content as % of dry weight:

h) Is it possible to assume that, for example, dandelions produce ROS in the presence of metals in a similar way to the algae? If yes, why do you think so?

i) Give examples of plants that contain a lot of vitamin C. What is its importance for humans?

j) Is mercury useful or harmful for humans and the environment? Give any example you know.

Worksheet no. 3: Fungal disease and Aspirin-related compound

Introduction: Plant diseases cause great economic losses to farmers worldwide. The Food and Agriculture Organization (FAO) estimates that plant pests and diseases are responsible for approximately 25% of crop losses. Plant diseases are caused mainly by viruses, bacteria and fungi. One of the fungal pests is *Glomerella cingulata* (belongs to the Ascomycota), which damages many varieties of apple trees, e.g. “Gala”, is sensitive to this fungus. On the leaves, the disease is first manifested by several small black spots which quickly increase in size, causing premature leaf fall.

The results of the research that investigated the influence of *Glomerella cingulata* on apple leaves are shown in Fig. 1. In this research, the authors applied sprays containing different concentrations of salicylic acid (SA) to the leaves and determined whether this substance slowed down the development of the fungal disease. SA is formed naturally in plants.

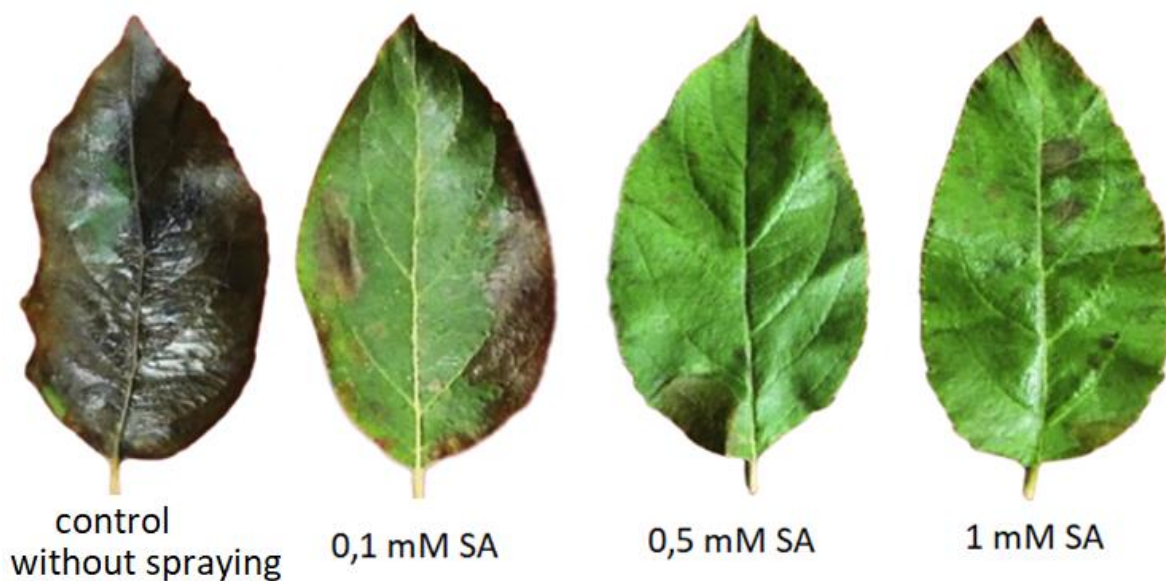


Figure 1

Research question: What is the effect of salicylic acid on the spread of the disease caused by the fungus *Glomerella cingulata*?

Photos can be objectively compared using the image analysis method. It is a method of image processing when assessing the properties and quality of an object, in which subjective judgment by a human is replaced. We will use the Fiji program to analyse the image.

Procedure for measuring leaf area and disease area:

1. Open the Fiji program on your computer.
2. In groups of four students, divide the pictures, which will be analysed by individual group members. Each member chooses one picture.
3. Open the file to be analysed in Fiji. Click **File** → **Open** and open the file.
4. Set the scale of the photo:
 - Click on the line icon (**Straight**) and mark the scale which represents 1 cm (Fig. 2, red arrows).
 - After marking the scale length, click on **Analyze** → **Set Scale**, set the scale parameters according to Fig. 2 (don't overwrite the row **Distance in pixels**). Then click on the OK button (yellow arrow).

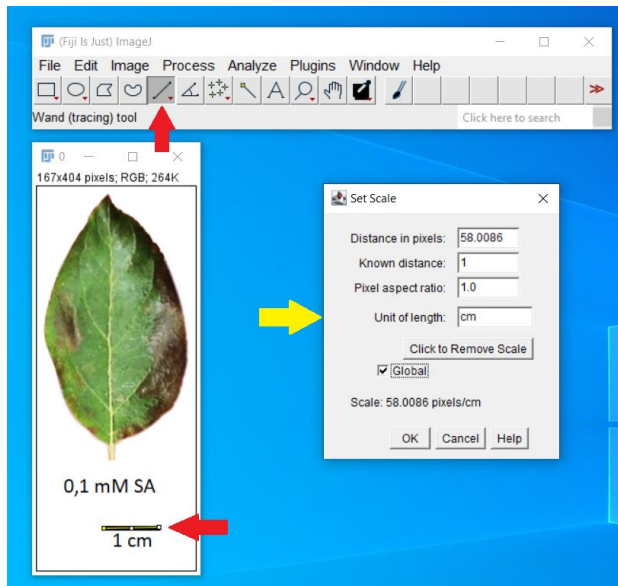


Figure 2

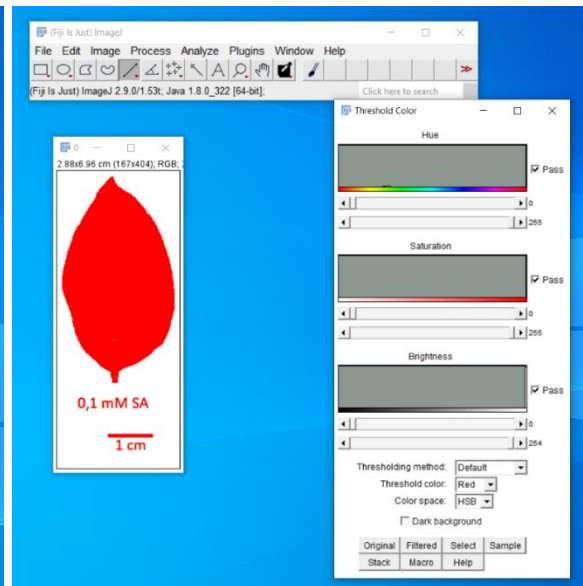


Figure 3

5. To compare the effect of salicylic acid, you need to know the area of individual leaves. Measure the area of the leaf using the already known procedure. Threshold the photo: click on **Image** → **Adjust** → **Color Threshold**, set the threshold according to Fig. 3 and close the given window.
6. In the program, you need to set what you want to measure: click on **Analyze** → **Set Measurements**. Set the window that appears according to Fig. 4 and click **OK**.

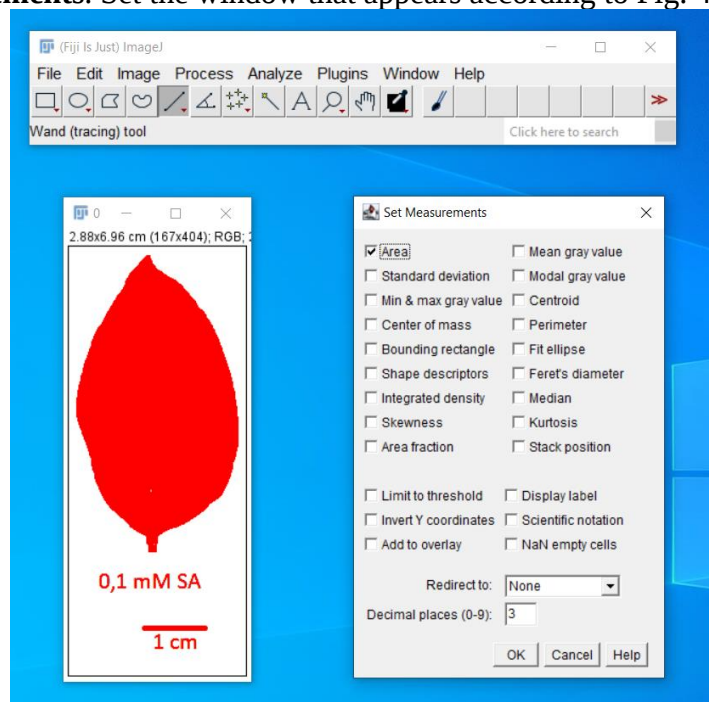


Figure 4

7. Select the object whose area you want to measure:
 - Click on **Analyze** → **Tools** → **ROI Manager**.

- A window will appear. Click on the wand icon (**Wand tool**, Fig. 5, red arrow) and click on the object you want to measure. Then click on **Add** in the “**ROI Manager**” window (Fig. 5, yellow arrow).

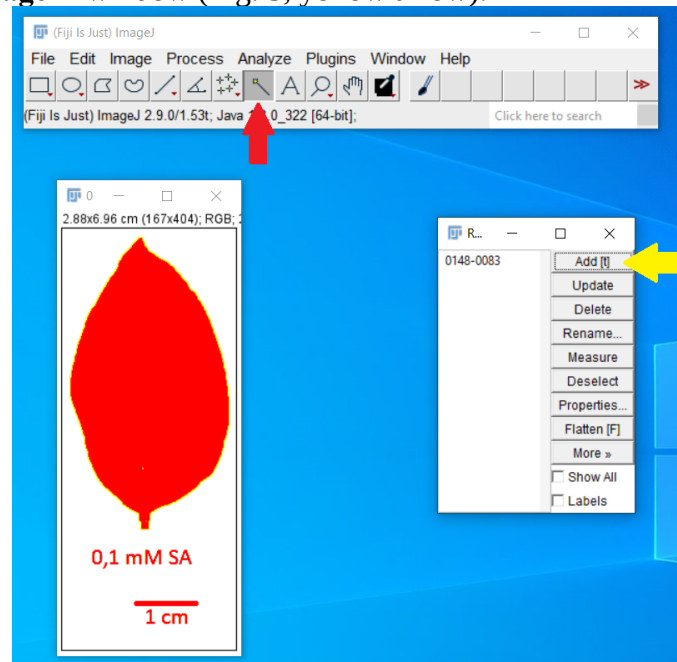


Figure 5

8. By clicking on **Measure** in the “**ROI Manager**” window, you will see a table that shows the leaf area data. Write the result.
9. Close the given photo and open the same file. Click **File** → **Open** and open the file.
10. Find out the area of the leaf that is damaged by the disease. Threshold the photo: click on **Image** → **Adjust** → **Color Threshold** and set the threshold according to Fig. 6 (don't forget to click **Dark background** and close the given window).

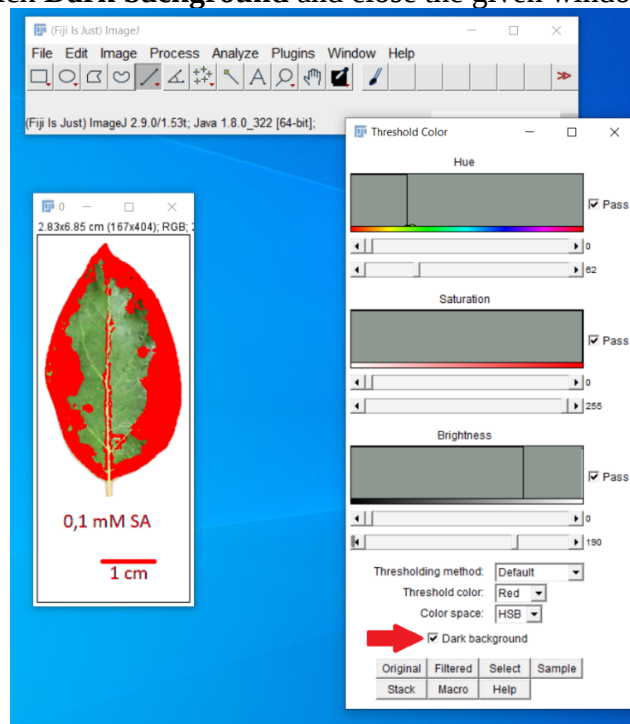


Figure 6

11. Now edit the photo to black and white: Click **Process** → **Binary** → **Make Binary**.
12. Now color the area you don't want to measure. Double-click the left mouse button on the brush (**Paintbrush tool**, red arrow, Fig. 7) and set the window according to Fig. 7 (yellow arrow), click **OK**, and color black the parts of the photo whose area you do not want to measure.

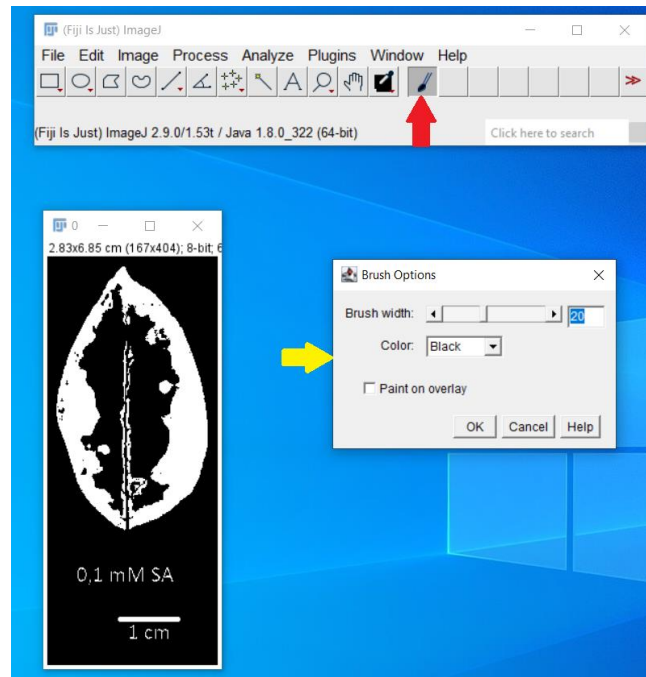


Figure 7

13. Measure the area of the disease: Click on **Analyze** → **Analyze Particles**, a window will appear that you set according to Fig. 8 (red arrow) and click **OK**. You will see the results of the leaf disease area in a table. Write this result.

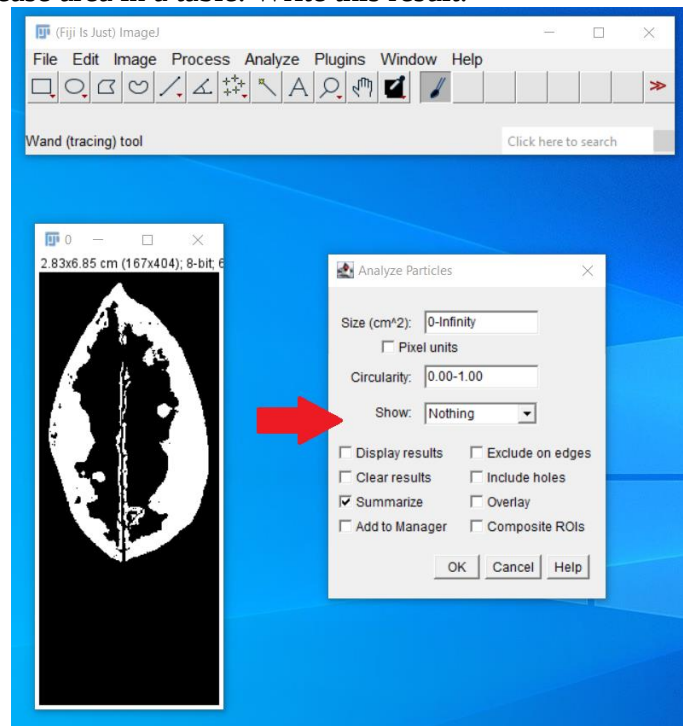


Figure 8

14. Using the results obtained from the image analysis, calculate how much % of the leaf is infected by the given fungal disease depending on the applied concentration of SA.

15. From the results of all members of your group, create a graph showing the effect of salicylic acid on the spread of disease on apple leaves.

Graph:

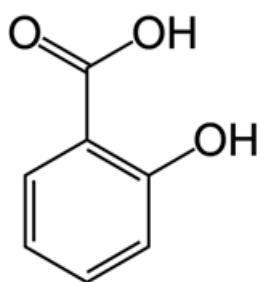
Questions:

a) Give examples of other fungal diseases on plants that commonly occur.

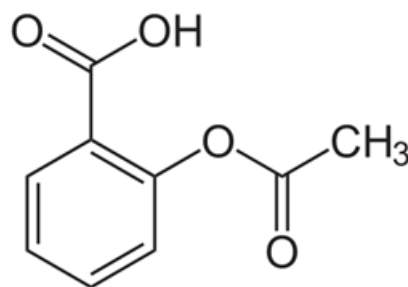
b) What was the effect of salicylic acid on the disease caused by *Glomerella cingulata*?

c) The researchers used apple trees of the same size in the experiment. They also used only one apple variety. Why are these factors important to the experiment?

d) The picture shows 2 molecules. One molecule is salicylic acid, and the other is acetylsalicylic acid. **What is the difference between these two molecules?** Highlight it:



Salicylic acid



Acetylsalicylic acid

We can say that acetylsalicylic acid is a derivative of salicylic acid. In chemistry, a derivative is a compound that is formed from a similar compound by a chemical reaction.

e) Because of what effect do people use preparations containing acetylsalicylic acid?

.....

f) Do you know the names of medications that contain acetylsalicylic acid?

.....

Salicylic acid is a substance synthesized by plants. Plants are interesting for humans due to many health-positive compounds. Many drugs/medications and their derivatives commonly used in practice are of plant origin.

g) Which medicinal plants do you use most often?