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Bakalářská práce

The influence of drought and cold-plasma treatment of seeds on the structure and function of photosynthetic apparatus of rape

Vliv sucha a ošetření semen studeným plazmatem na stavbu a funkci fotosyntetického aparátu brukve řepky olejky

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Abstract

In the period of global climate changes, the resistance of significant agricultural crops to drought has become from an economical point of view a very important topic. In order to achieve maximum productivity of agricultural crops, it is necessary to search for new technological approaches to enhance this resistance. Currently, one of the actual topics in this area is the possibility of seed treatment with cold plasma. The aim of this work is to reveal the degree of influence of cold-plasma treatment of seeds on the stress response of plants that had been grown under the influence of drought. The oilseed rape was chosen for this experiment intentionally because it remains a substantial agricultural crop with a huge variety of usage. The plants of rapeseed had been grown in fully-controlled conditions in a growth chamber. The selected photosynthetic characteristics had been checked in regular intervals and the stress response was evaluated on two variables: plants grown from seeds treated with cold plasma and plants grown from commonly used seeds. In total, three measurements of chlorophyll fluorescence and photosynthetic pigment content have been conducted. The first measurement in the beginning of development of the seedlings showed better drought resistance in non–treated seedlings but in the following second and third measurements it was vice versa: cold-plasma treated plants survived better in dry conditions. Results of the analysis of photosynthetic pigments suggest that the cold plasma treatment can obviously increase the resistance of seedlings of rape especially in conditions of long-term drought.

Key words: Cold-plasma treatment, stress response of plants, chlorophyll fluorescence, photosynthetic pigments.

Abstrakt

Odolnost významných hospodářských plodin vůči suchu je v době globálních klimatických změn z hospodářského hlediska velmi důležitým tématem. V zájmu dosažení co nejvyšší produktivity zemědělských plodin je nutné hledání nových technologických postupů tuto odolnost zvyšujících. Jedním z aktuálních témat v této oblasti je v současné době možnost ošetření semen studeným plazmatem. Cílem této bakalářské práce je zjistit, jaký vliv má ošetření semen řepky studeným plazmatem na stresovou odpověď rostliny pěstované pod vlivem sucha. Jako testovaná rostlina byla vybrána brukev řepka olejka, naše významná hospodářská plodina s mnohostranným využitím. Rostliny řepky byly pěstovány ze semen v plně kontrolovatelných podmínkách v kultivačním boxu. V pravidelných intervalech byly zjišťovány vybrané fotosyntetické charakteristiky a stresová odpověď rostlin byla posuzována u dvou variant rostlin: Rostliny vypěstované ze semen ošetřených studeným plazmatem a rostliny vypěstované z běžně používaného osiva. Celkem byla provedena tři měření fluorescence a fotosyntetických pigmentů. První měření na začátku vývoje semenáčků prokázalo lepší odolnost neošetřených rostlin vůči suchu, nicméně během dvou následujících měření bylo zjištěno příznivější přežívání plazmatem ošetřených rostlin v podmínkách sucha. Výsledky analýzy fotosyntetických pigmentů naznačují, že ošetření chladným plazmatem může zřejmě zvyšovat odolnost mladých rostlin řepky zejména v podmínkách dlouhodobějšího sucha.

Klíčová slova: Studené plazma, stresová odpověď rostlin, fluorescenční záření, fotosyntetické pigmenty.

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Prohlášení

Prohlašuji, že svoji bakalářskou práci jsem vypracoval samostatně pouze s použitím pramenů uvedených v seznamu citované literatury.

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1 Introduction

I have chosen this topic intentionally with the purpose of the future possibility to use the results not only on our family farm. The method of treating rape oilseeds with cold plasma, which is still under development, is not very known and spread to public, so I decided to be a part of this project and check out whether the influence of cold plasma treatment of rape seeds could influence the plant response to stress conditions. As the stress factor we tested the resilience against drought because according to the current climate changes, it plays an important role worldwide. In the frame of our project, the drought impact on growth has been measured and observed in several experiments, such as the germination of seeds, photosynthesis, the amount of photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids) and chlorophyll fluorescence, out of which the last two mentioned are my main field of focus. These experiments have been taken on the second leaves, which appeared to be good indicators. The plants were growing in the growth chamber. The main questions to be answered were 1: Is there any influence of cold - plasma treatment on photosynthetic apparatus of rape and 2: Is there any influence of cold plasma treatment of seeds on the rape seedlings response to limited water supplies? The collected data were interpreted in the section called Discussion later in this work.

2 Theory section

2.1 *Brassica napus L.*

2.1.1 General characteristics

In our climate zone the rape is known in the vegetative period of 300-340 days. It creates a huge taproot (with the storage function) and a large number of lateral roots. The above-ground part occurs in two stages - in the fall at the stage of leaf rosette = the vegetative phase, and in the spring in generative (elongate) phase. Stems usually reach a height of 140 to 160 cm. Six to eight branches of the first order grow in leaf axils on the stem. *Brassica napus* blooms especially in May and the whole flowering lasts about 20 to 25 days. The number of yellow coloured flowers on 1 m^2 is about 300 to 500; the average number of plant on $1m^2$ is 60. The fruit is a pods containing about 15-20 dark rounded seeds with the average weight 5 g (Vašák, 2000).

2.1.2 Terminology of Rapeseed

Brassica napus signs exotic species, initially cultivated in Asia and the Mediterranean. Canola refers to all *B. napus* varieties which are known for low levels of two toxic substances, erucic acid and glucosinates. It is grown mainly for its quality edible oils that are used in several foods such as margarines and several seed meals used as feed for animals (Vašák, 2000). According to Pots and Rakow (1999) the term represents CAN as Canadian and OLA as an Oil of Low Acids. Later in this work the terms rapeseed, oilseed rape or canola are used as synonyms for rape.

2.1.3 *Brassica napus L.* as a significant agricultural crop

Brassica napus L. var. *napus* from the family *Brassicaceae* contains 398 genera, 4765 accepted species[1] (Stevens, 2001). Oilseed rape together with other 50 species belongs to genera *Brassica* (Curran, 1962). This old cultural plant is known and described since the 13th century. This specific form of *Brassica napus L.*, nowadays the most common form, originates from two varieties: *Brassica oleaceae L*. and *Brassica rapa L.* The seeds contain approximately 47% of oil with potential for food usage (Mlíkovský and Stýblo, 2006).

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[1] Available from http://www.mobot.org/MOBOT/research/APweb

The biggest oilseed rape expansion began in Europe in 1970*s*. During this period the laboratory experiments showed, that the erucic acid which had been contained in rapeseeds had a negative influence on myocardial function of tested animals. Therefore the low-erucic acid rapeseed forms (LEAR) with a minimal level of this omega-9 fatty acid, sometimes called “00” have been discovered and since then the production is increasing (Sahasrabudhe, 1977; Bečka et al., 2007).

There are many kinds of rapeseeds. The most common and practical division is into hybrid and lineal ones. For the purposes of our experiment the lineal kind of canola was used. Sowing non-tested seeds is not recommended, because of the erucic acid which could exceed the allowed limit of 2% (Bečka et al., 2007)

According to Baranyk (2007), oilseed rape is considered to be the second most important oil crop (after soya), with the production of 46-49 million ton, out of which 15 million ton is produced and processed in EU. The development of world production of rape is shown in the figure 1.

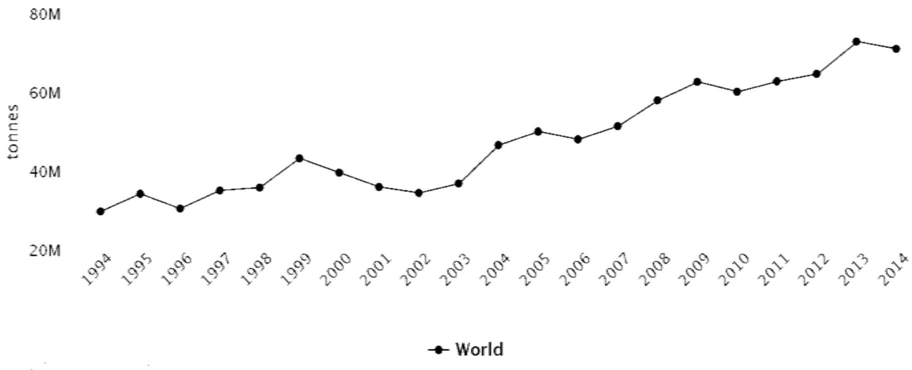

Fig. 1: Worldwide production of rape, (source: FAOSTAT: Food and agricultural organization of United Nations – statistics division)

In 2007, the areas for growing rape reached 30 million hectares, with the average production about 3t/ha. Some hybrid kinds of rape are able to produce about 5-10% more seeds - 4t/h but the chance of fungi infections increases and the ability to survive spring frosts is much lower (Bečka et al., 2007). In Czech Republic the area for growing canola was in 2006 about 250 -350 thousands hectares (Mlíkovský and Stýblo, 2006). Rape fields significantly shape the landscape in which we live (see figure 2) Czech Republic also holds the position of top 10 producers around the world. The top five states and their production are expressed in figure 3.

Fig. 2: Photography of a large rape field in the locality of Horní Třebonín (source: Drška, 2016)

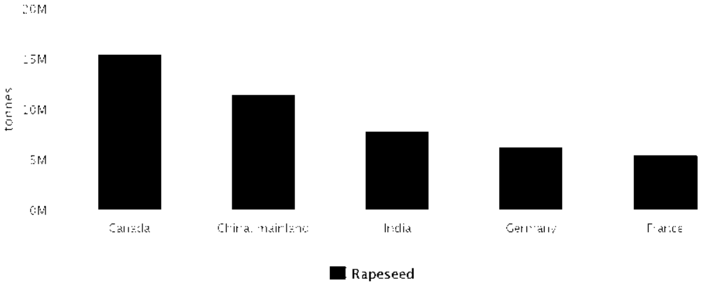

Fig. 3: The graph of top 5 world producers of rapeseed in 2014 (source: FAOSTAT)

This graph shows the world´s production of rapeseed. The biggest producer among the European Union countries is Germany.

2.1.4 The importance and utilization of oilseed rape

Canola is applied in food industry, as a feed crop, in oleo-chemistry and can also be used for energy as a renewable energy source. The advantage of rapeseed oil compared to soy oil is longer durability and the fact, that it does not contain some of the saturated fatty acids with negative effects on the level of cholesterol. Furthermore, rape is used as a feed for livestock, especially as a scrap. In comparison with the other countries, in the Czech Republic, there is a lack of awareness of the quality of rape, particularly erucic acids and glucosinolates, so this usage is less common than abroad (Baranyk et al., 2007).

In oleo-chemistry the main two chemical decomposition processes are hydrolysis or alcoholysis. As a product of these reactions there arise fatty acids, fatty acid esters and glycerol - as the common product of both reactions (Vašák, 2000;

Baranyk et al., 2007). The fatty acids have the biggest variety of usage, because they form rapeseed methyl esters, called biodiesel, which functions as a fuel in diesel engines and is often used in a mixture with rope. The biggest advantages are lower emissions and minimum content of dangerous sulphur oxides. Using rape oil as a fuel brings lots of risks and some construction mechanisms on engines are required otherwise it can cause for example poor lubrication of the engines, blocked oil filters or high viscosity in the cold winter months. The other disadvantage comprises in different approaches in gaining rape oil, so the quality can significantly differ and majority of collective farms in Czech Republic refuse to use this fuel because of the fears from damaging their machines. Rapeseed plays an important role as a grazing for animals, especially deer, then as a source of nectar for honeybees and generally growing this plant entails lots of positives. The fields are gaining more fertility, because during harvesting, only the seeds are required and the stems together with the massive roots (see figure 4) are used as organic fertilizers. Rapeseed is considered to be a great preceding crop for seed-corns such as wheat, oats, rye, barley etc. Because of the gained fertility but also because growing rape requires many chemical spraying against weeds, the rape fields are much cleaner. The next great ability of rape is to fix the sulphur, so it is possible to grow it in localities contaminated by sulphur (Vašák, 2000).

Fig. 4: Massive root of canola (source: Drška, 2016)

2.2 Definition of Plasma

The Oxford dictionary explains this term as an ionized gas composed of positive ions and free electrons in proportions resulting in more or less no overall electric charge with the typical occurrence at low pressures (for example in the upper atmosphere and in fluorescent lamps) or at very high temperatures (such as in stars and nuclear fusion reactors)[2]. This definition is also quite accepted and deeper explained in the book called Plasma Physics (Piel, 2010). It is formed by removing electrons from the electron shell or ionizing (tearing) of molecules (Mráz et al., 2013).

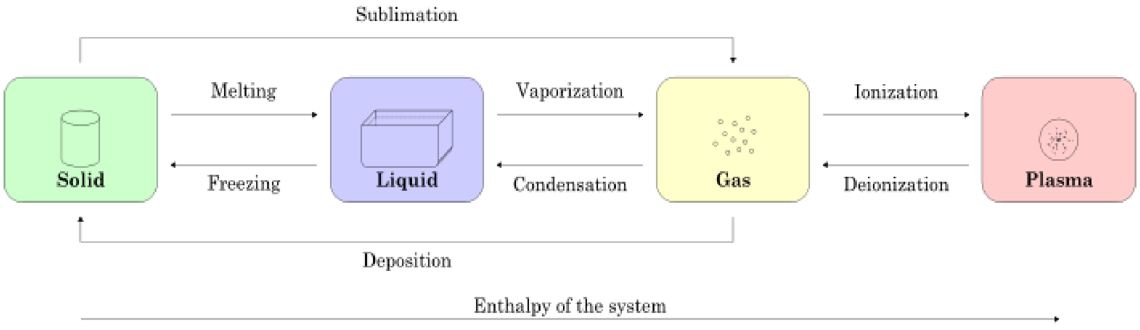

Fig. 5: The schema expressing four states of matter and the processes leading to a change of matter (source: https://commons.wikimedia.org)

Generally to call this gas plasma, two basic conditions has to be complied. The first one, **quasi-neutrality** relates to a neutral behaviour of particles despite carrying an electrical charge. The amount of positive charged particles should not differ from the amount of negative ones. The second assumption, **collective behaviour of particles**, detects a motion of particles in plasma caused by the accumulation of charged particles in one spot, which gives the start to electric fields and as a reaction the cooperative response of interactive plasma particles cause shielding (= reducing electric fields effectively) to sustain in plasma state (Piel, 2010; Maroušek, 2014; Chen, 1984; Stach, 1987). Stach (1987) also talks about plasma as a fourth state of matter (see figure 5) and the final common definition of plasma says, that it is a conductive gas containing permanently interactive particles with different energies: electrons, photons, ions (positive and negative), free radicals, atoms and molecules, out of which

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[2] Available from http://www.oxforddictionaries.com/

electrons and photons are considered light and the others as heavy particles (Chen, 1984).

Piel (2010) suggests that it is not necessary for plasmas to occur only in gaseous state, other forms like solid and liquid are also possible. This is determined by the degree of coupling between atoms. To describe this degree, the coupling parameter Γ is established. The formula is characterized as **Γ = Wpot/kBT**, as the ratio of the potential energy of nearest neighbours and the thermal energy. T expresses temperature and kB stands for Boltzmann's constant/factor. This factor regulates the relative population of different energy states. A gaseous phase is found for $\Gamma < 1$, the liquid state for $1 < 180$ and the solid phase for $\Gamma > 180$. An ideal neutral gas is determined by the density of particles *n* – number of particles per unit volume (in m^{-3}). The temperature of the gas shows the motion of particles and out of these two parameters we get the pressure *p*, counted as **p = nkBT**. The plasmas most often occur in two forms: high temperature and low temperature plasma (Piel, 2010).

2.2.1 Kinds of plasma

Plasma can be distinguished into several classes according to degree of ionisation and temperature.

According to the degree of ionisation the division into **weekly-ionized plasma** and **strongly-ionized plasma** is known, which is based on the principle of Saha equation, which is described into details in Piel´s book Plasma Physics. In the week forms the concentration of charged particles is much lower than in the neutral ones (the particles collide with gas molecules), whereas in the strong forms the concentration of charged particles dominates (the particles collide with one another). This classification also takes into account the polarizing forces during collisions of particles. In the strong forms the Coulomb forces operate between charged particles and the decrease of kinetic energy is much lower. This difference between forces influence in a significant way the characteristics of both higher mentioned forms (Martišovitš, 2004).

In the division according to a temperature we distinguish a **high-temperature**, alternatively **thermal** plasma, for which the energy of charged particles is higher than 100 eV with the temperature higher than 1 MK. This type is present in 99%, for example in the universe, thermonuclear syntheses etc. (Moreau et al., 2008). For the **low- temperature, non-thermal** plasma is valid, that the temperature of neutral gas and positive ions is comparable to a room temperature whereas the temperature of electrons can reach up to 10^5 K. This type is visible in fluorescent lamps and electric arc used in welding of metallic materials, plasma TVs etc. The thermal plasma is strongly ionized but the non-thermal is weekly ionized (Moreau et al., 2008; Martišovitš, 2004; Stach, 1987).

2.2.2 Current and possible biological usage of non-thermal plasma

Nowadays this principle is relatively new. In plant physiology cold plasma is mainly used to surface treatment of seeds. Mráz et al. (2014); Henselová (2012) studied the use of plasma as a physical tool for sterilization in the ways of protecting seeds and how it influences the germination and the first stages of growth. Henselová et al. (2012) found out the positive effect on germination and early growth of maize after treating seeds with non-thermal plasma for 60 seconds. Several researches have been done to test the effect on other significant crops. Šerá et al. (2010); Hrušková et al. (2009) studied the effect on wheat and oat, Dubinov et al. (2000) investigated the same on barley, Gavril et al. (2012), Petřík (2010) used this method on opium poppy and even some less traditional crops such as white goosefoot or tomato have been used as a subject of experiment (Šerá et al, 2008; Yin, 2005). Recently Ryplová et al. (2015) analyzed the different response of rape seedling to non-thermal plasma according to the duration of plasma treatment.

2.3 Plants under stress conditions

2.3.1 Stress in biology connotation

Stress is in a general sense defined as an overpowering pressure of some adverse force or influence[3]. The pressure can be caused only by one specific factor or more often by a combination of stressors and can be fatal for a plant. According to a Liebig´s law of the minimum the one limiting factor significantly differing from the optimum can be fatal for the plant (Townsend, 2010). In biology there appear several stressors such as soil itself – excess or lack of chemical elements, soil salts or pH in the soil. The next stressor: radiation is also very dangerous but nowadays the most discussed one is drought. Every above mentioned factor has a negative effect on the growth and because the plants do not have the ability of locomotion they defend themselves by creating some specific mechanisms that will be described in the next chapter. Generally plants appear in stress when there is an insufficient compliance with the requirements of the specific crop. The great example is inadequate water supply of plants in many parts of the world. To satisfy the market needs and retain or even increase the productivity of crops it was necessary to firstly understand better the physiology and responses to stress and secondly to develop the species of crops that will be well-adapted to the drought (Jones and Flowers, 1989). Recently, there has been made a huge progress in discovering the mechanisms which increase the tolerance of plants to abiotic stresses (Rai and Takabe, 2005).

2.3.2 Drought as an important stressor

Drought is possibly the most limiting stressor, which decreases the activity of all enzymes and causes slower growth (Bláha, 2003). The optimal water conditions for growth of rapeseed oil are in the areas with the annual precipitation between 450 to 700 mm, the height above sea level to 650 meters and annual average temperature 7 - 9 °C (Baranyk, 2007). The low precipitations are limiting mainly in the period after germination, in which the hard root is being formed and then in the period of flowering, which affects the seed production. In the case of higher rainfalls than optimal (after a longer dry period during which the soil erosion transported the

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[3] Available from http//www.oxforddictionaries.com

minerals on the surface) the risk of salinisation increases. Rapeseed gains nutrition from its root. The usual growth of root is based on several physical rules, namely on negative phototropism and positive geotropism. In areas with restricted water supply the root grows into depths to gain the needed water. In some cases the principle of geotropism completely subordinates to water resources in soil and the growth is abnormal (Pavlová, 2006). The next extreme, excessive rainfalls are also harmful, the minerals can be washed up to one area, which will then become over-salted and the other area will contain very few minerals, therefore this phenomenon is also extremely dangerous and nowadays rape is recognized as an erosion crop and cannot be grown on the slopes.

2.3.3 Water deficit: Response of plants

The plants react to water deficit by slowing down the growth and the photosynthesis. The key factor is the turgor pressure on plasmatic membrane. When the pressure declines, stomata close and the exchange of carbon dioxide is paused. The stomata are closed also due to the presence of abscissic acid (ABA), which also increases the osmotic pressure and reduces the transpiration under water shortage (Mansfield and Mc Ainsh, 1995).

Plants regulate the scale of transpiration - at first by shortening the period in which their stomata are opened and it can end in full closure of stomata, so then the plants use only cuticle transpiration (Shaw et al., 2005; Bláha et al., 2003). During drought Good and Zaplachinski (1994) found out the increased amino acids in leaves at the early stages of flowering. The most abundant was proline, which has been cumulated during water deficit as a protective mechanism in many studies (Ma et al., 2004; Norouzi et al., 2008). According to Larher et al. (2003), it does not play a primary role in osmoregulation. The osmotic adjustment can be better achieved with carbohydrates and compatible solutes whereas the proline functions in plants during recovering from stress conditions. Huang (2010) proved the increased proline concentration of wheat under drought stress after the plasma treatment. Generally the role of sugars in osmotic-protection and desiccations is more crucial, because they protect membranes and proteins from drying (Müller at al., 2011).

Bláha et al. (2003) classify between the other defence mechanisms the ability to survive the dehydration. This strategy is only effective for short period stresses. During the stressed conditions the concentration of chlorophylls is decreasing, the transport of supplies is limited and the toxic substances are cumulating. Seeds tent to be smaller and lower germination is registered (Bláha et al., 2003).

2.3.4 Climate changes and possible future predications

Tuck et al. (2006) claim that in several south European areas the aridity will be increasing and the worsen growth of bio-energy crops is predicted. As a reaction on that changes, several studies have been done with the aim to find the indices of rapeseed cultivar that will manage to grow well in these conditions (Norouzi et al., 2008; Kumar and Sing, 1998). As mentioned before the drought is an important stress factor but not the only one. In one Canadian study Qaderi et al. (2006) tested three components of global climate change on rapeseed and their separate and together influence has been measured. This study tested the increasing level of atmospheric carbon dioxide, temperature and drought. Therefore this study is so priceless, but to simulate the future development of climate changes it would be necessary to grow plants not in laboratory conditions but on fields, where the next factors (such as soil composition, radiation, winter snowfalls, frosts, fluctuation in rainfalls and temperatures, influence of micro and macro-organisms etc.) could be involved.

Houghton et al. (2001) points out that CO_2 level has been increasing in the past 150 years and same trend is expected also to the future. By the end of this century the atmospheric carbon dioxide concentration could reach 700 $\mu mol/ mol^{-1}$ and the temperature could be higher about 5, 8°C. The development of average temperatures is portrayed in the figure 6. Carbon dioxide is produced and released to the air mainly from burning coal and other fossil fuels and deforestation. Also volcanoes emit CO_2 but not as much as human activities. Volcano release between 0.2 and 0.3 billion tons every year, whereas human activities emit about 29 billion tons (Gerlach, 2011). The increase of carbon dioxide can on the other hand be useful for plants because it affects quantity and quality of crops. It enhances growth, photosynthesis, water use efficiency because of decreasing transpiration (Long et al., 2004). The next phenomena measured in Qaderi et al. (2006) study: heat and drought have only negative effects.

High temperatures increase transpiration and reduce plant biomass (Crafts, Brandner and Salvucci, 2000). Together with the drought it affects fyto-hormones during stress by increasing ABA - abscisic acids but decreasing IAA – indole-3-acietic acid. The effect of heat and drought differs at ethylene – the level is decreasing with higher temperatures and increasing with drought (Nilsen and Orcutt, 1996). ABA is decreased in conditions of drought, which helps the plants to survive but in the conditions of high temperatures the ABA is inhibited, which leads to inability to close stomata and final death of cells because of drought (Mansfield and Mc Ainsh, 1995). Out of this work we can observe that the heat is even more dangerous for plants than the drought. In addition to that, Pinheiro and Chaves (2011) discovered that seasonal timing of drought influence crops yield much more considerably then the intensity of drought. Müller et al. (2011) in their study proved the opposite. They pointed out that duration and intensity of drought are more significant than the timing of water stress. This topic therefore should be examined in future studies.

Qaderi et al. (2006) showed the shorter, thinner stems under water deficit with smaller leaves and lower biomass, which is not surprising. Davies (2004); Sharp et al. (1998) explain the shortened stem by ABA accumulation, which restricts the ethylene responsiveness and prevent plants from elongation. This Canadian study proved that drought stress increased the weight of leaves but decreased the shoot/root ratio. The more massive roots can be explained by the need of plant to search for water supply in more depths and therefore is considered as an adaptive mechanism. It is interesting to observe the chlorophyll concentration in leaves. In drought stressed ones **the level of carotenoids was increasing** not only in Qaderi et al. (2006) study but also Yin et al. (2005) proved the same. This study is extremely important because it takes notes of interactive effects of global warming phenomena and it is aware that there is a space for exploring the interactions between other environmental factors, such as light, salinity or flooding. Baranyk et al. (2007) predicts the following possible scenario of global warming. At first the fluctuation in precipitations and then the extreme weather phenomena are estimated. More useful information about global warming is available online in David Herring´s work which replies to commonly asked questions about global warming[4]. There are many approaches to this topic. Some people points out that we could observe such climatic changes when looking long back in history, anyway the trend of global warming is well-proved and it is necessary to acquaint not only the children at schools with that, and seek to change their often phlegmatic attitude that one person cannot change anything. It is necessary to convince them that we should be friendly to the environment in which we live as together with the other animal species, and think about the next generations, which will definitely need a healthy environment.

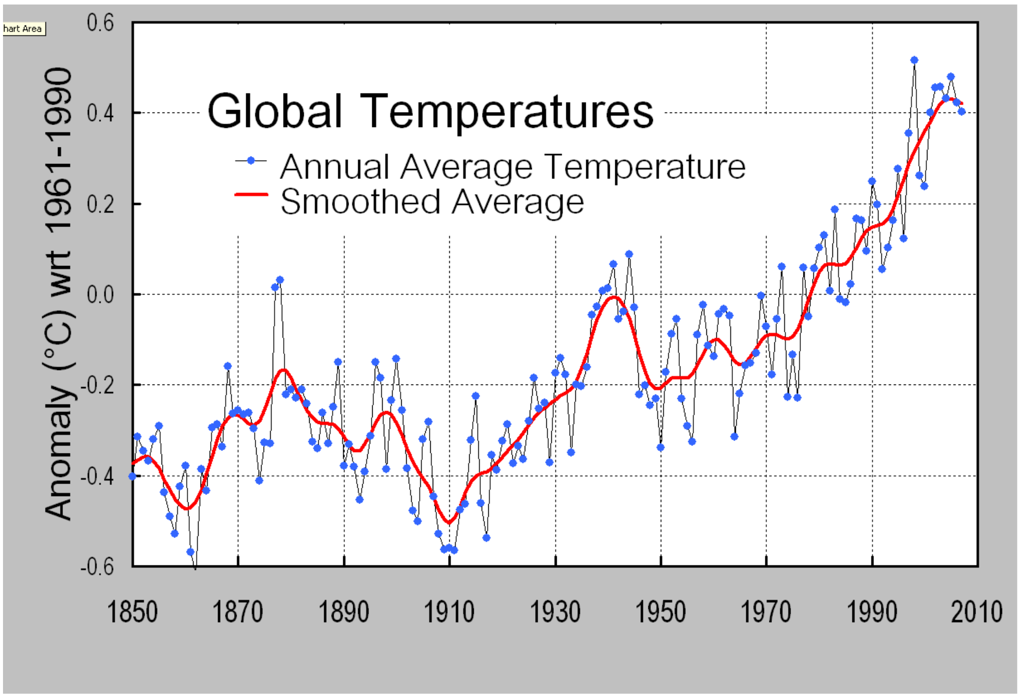

Fig. 6: The development of average temperatures since 1850s
(source: https://commons.wikimedia.org)

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[4] Available at: https://www.climate.gov/news-features/understanding-climate/global-warming-frequently-asked-questions

2.4 Fluorescence as an indicator to stress exposure

Using chlorophyll fluorescence to indicate the physiologic state of plants has lately become a reliable biological tool. The stresses of plants usually distract the function of photosynthetic apparatus but can hardly be identified when talking about short-term stresses (for few minutes, hours). The impact of long-term stresses is much more significant and it is shown by decrease of several chlorophylls in leaves. Plants can adapt to such short stresses and the measured fluorescence does not have to show them (Papageorgiou and Govindjee, 2004).

2.4.1 Principles underlying chlorophyll fluorescence

Generally, when excited molecules are transferred into the ground state the term luminescence is used. When the excitation is caused by absorption of light quanta, the term fluorescence is used. To sum it up, the basic principle of fluorescence are **the transition of the excited electron to the ground state**, wherein the excitation of electrons is caused by energy absorption of light quanta (the absorbing molecule is mostly chlorophyll a) **and subsequent emission of radiation** of a greater wavelength (still in the spectrum of visible light) and of lower energy than during the excitation state (Lakowicz and Joseph, 2013). The emission of radiation is often drawn by Jablonsky energy level diagram. The different wavelengths between absorption and emission spectra are characterized by *Stokes shift,* highlighted in figure 7 (Hlízová, 2008).

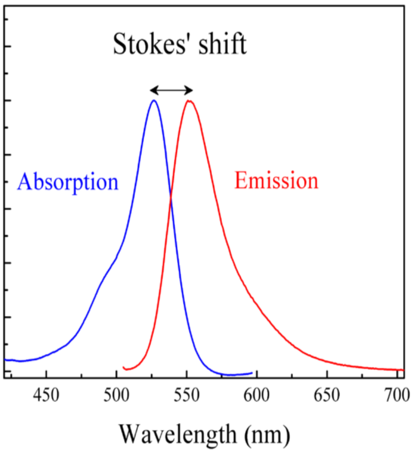


Fig. 7: Stokes´shift (source: https://commons.wikimedia.org/)

After absorption of light (photons), the electron occurs in excited state and the light energy can undergo one of the following three processes. Mostly it is used to drive photosynthesis, secondly the excess energy can be dissipated as heat or it can be re-emitted as light fluorescence: see figure 8 (Maxwell et al., 2000).

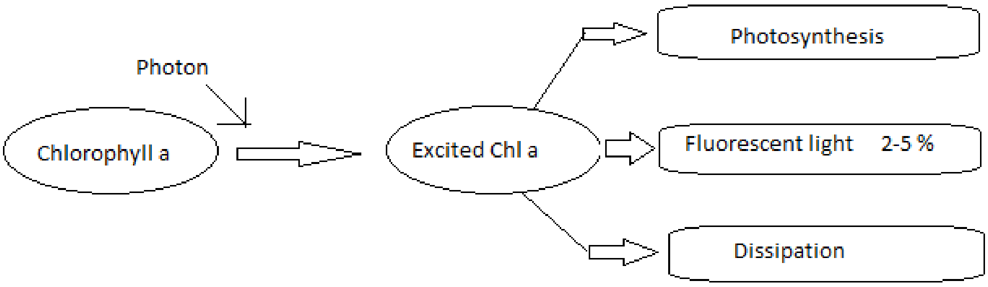
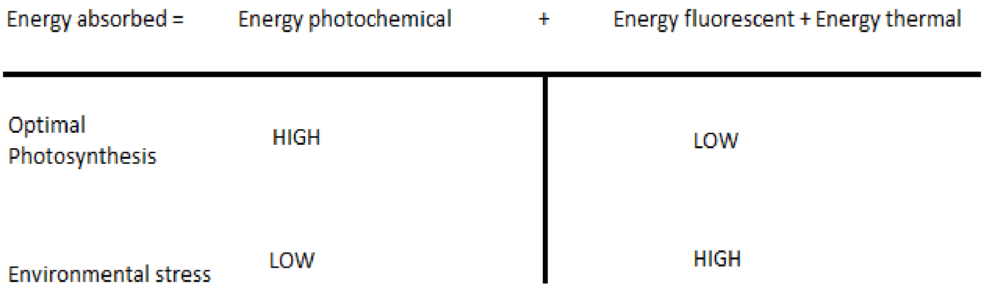


Fig. 8: The chart utilization of excited light energy (source: Drška, 2016; according to Maxwell et al., 2000)

Out of this diagram we can observe that only about 2 - 5 % of all the photon energy undergo fluorescence emission. This percentage seems to be very small but it can reliably inform us about the physiologic state of the plants due to the fact, that all three processes mentioned above in the schema occur in competition (Maxwell et al., 2000; Papageorgiou and Govindjee, 2004). If the intensity of fluorescence is low, the efficiency of photochemical reactions is high, which is illustrated in the figure 9.



Energy absorbed =	Energy photochemical +	Energy fluorescent + Energy thermal
Optimal Photosynthesis	HIGH	LOW
Environmental stress	LOW	HIGH

Fig. 9: The relationship between photosynthesis and fluorescence (source: Drška, 2016; according to Ryplová, 1997)

If the photon energy undergoes photosynthesis, it changes to the chemical energy of transporters (NADPH). Pavlová (2006) highlights the one-way energy transport from the periphery to the react centres, in which chlorophyll a is situated. The principle functions in a way that the nearer to the react centre we are, the absorbed wavelength maximum is increasing and the amount of needed energy for excitation is decreasing. Therefore on the periphery chlorophyll b molecules are present, because they have shorter absorb maxima than molecules of chlorophyll a, and for its excitation more energy is needed. From the react centre one electron is released and the sequence of redox reactions is initiated. When talking about the react centres the majority of fluorescent emission (90%) is emitted by chlorophyll a from the react centre PS II (Papageorgiou and Govindjee, 2004; Maxwell et al., 2000).

2.4.2 Kautsky effect and fluorescent induction

Chlorophyll fluorescence is not a static phenomenon but it changes with time. These changes have been firstly observed in 1930s by the legendary H. Kautsky and the term Kautsky effect has been used for that. The changes are caused by different speed of photo-chemic processes on thylakoid membrane and inside stroma. They are greatly reflected in a fluorescence induction curve, visible at Figure 10 (Papageorgiou and Govindjee, 2004; Hlízová, 2008). Many useful parameters can be read out of this curve. Unfortunately, in the literature this problematic is often hardly understandable due to the uncertain nomenclature. There have been many attempts to set one nomenclature, for example Maxwell and Jonson (2000) but still in some recent publications we can observe different signs expressing one parameter. In this work the nomenclature according to Baker is used (Baker and Rosenqvist, 2004).

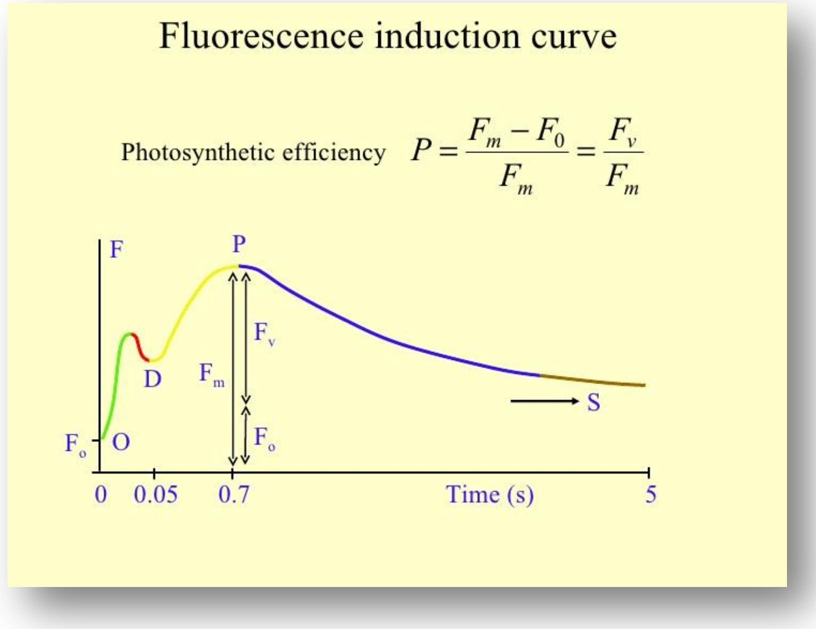


Fig. 10: Fluorescent induction curve, (source: Rubin A.)

The changes that are pictured in a graph above start after a dark adapted leaf is exposed to light. On immediate exposure the plant reacts by increasing fluorescence to a minimal level **(F_0)**. In that state the reaction centres PS II are opened - ready for photochemistry, because primary acceptor of PS II (Q_a) is fully oxidized. Then a leaf is irradiated by a short saturation pulse and the fluorescence rises firstly to the transient inflection level **F_i** (the green part of the figure 10) and then the final rising to the peak **F_p** or **F_m** occurs (the yellow part of the figure 10). In this maximum state the reaction centres are closed and Q_a is fully reduced. The term **F_v** expresses the difference between F_m and F_0. These rising changes from a minimal fluorescent to a peak are in some literature called **FAST PHASE of fluorescent induction**, because the according to the figure 10 this process is very fast. The rising fluorescent curve after short saturation pulses of light is often called OJIP (Papageorgiou and Govindjee, 2004)

- ➔ O stands for origin, which correlates with the basic value of fluorescence in dark adaptive state (F_0)
- ➔ J and I represent short steady-state flow of energy from PS II to the electro-transport chain (F_i)
- ➔ P means the peak, which shows the maximal value of fluorescence during the given irradiation (F_m, F_p)

The OJIP curve as mentioned before indicates environmental stresses out of which the temperature is the most limiting one. It decreases the oxygen production and decreases the absorption of carbon dioxide.

After reaching a peak the value decreases for a few minutes and settles to a steady state level **Fs** (the brown part of the figure 10). For this **SLOW PHASE of fluorescent induction** the permanent synthesis of ATP, $NADPH^+$ and carbon dioxide fixation is typical until it stays in balance. This phase is also called quenching - see the blue part of the figure 10 (Baker et al., 2004). This decline in fluorescence has been in history successfully used to indicate the responses to stresses (Flagela et al., 1996)

The graph also expresses the photosynthetic efficiency but for our experiment, the **fluorescent efficiency** Fv / Fm is more significant. It is given by a ratio of number of photons emitted divided by the number of photons absorbed and is called the Quantum yield (QY). When every emitted photon is also absorbed, the QY = 1, which is the maximum value. The usual values in plants not suffering from a stress lies around the value 0,8 (relative units) (*Lakowicz* and Joseph, 1999).

2.4.3 The importance of fluorescent measurement

According to Lichtenthaler (1996) the measurement of chlorophyll fluorescence provides the actual information about activity of photosynthetic apparatus during stressed conditions and nowadays this simple method increases in popularity. This sensitive indicator influences the shape of OJIP curves and out of the received parameters we can gain information about:

- qualitative and quantitative function of photosynthetic apparatus of plants
- physiological state of plants
- level of stress they are exposed
- the processes on the thylakoid membranes in both react centres
- mechanism regulating the transport through electro-transport chain

(Papageorgiou and Govindjee, 2004)

2.5 Photosynthetic pigments in leaves

Oxford dictionary describes pigments as the natural colouring matter of animal or plant tissue. It originates in Latin from the word *pigmentum, pingere*, which means to paint and is associated with the typical colouring[5]. In this work we focus on the pigments in plant tissues. Pigments are located in chloroplasts and carry the main importance during photosynthesis. During this process the light absorbance and how the light energy converts to the energy of chemical bonds is crucial (Pavlová, 2006). In this chapter the light absorbance is explained more in details. The absorbance of two main chlorophylls is portrayed in figure 11.

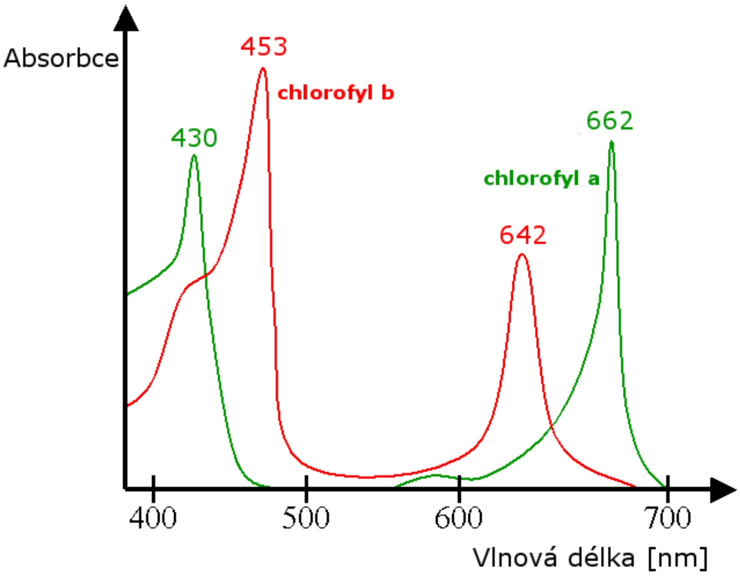


Fig. 11: The absorbing spectra of the most significant chlorophyll pigments (source: https://commons.wikimedia.org/)

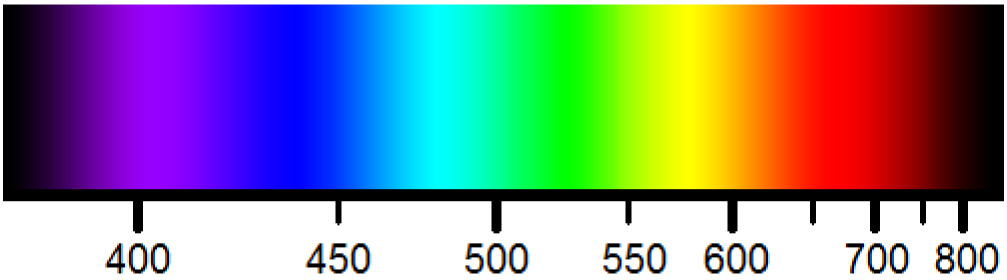


Fig. 12: Spectrum of visible light (source commons.wikimedia.org)

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[5] Available from http://www.oxforddictionaries.com/

As we can see in the figure 12 above, the visible spectrum of light ranges from red with the longest wavelength to violet with the shortest wavelength. Plants own several pigments which have absorption maxima in specific areas of the visible spectrum. They are often categorized to **chlorophyll pigments, the accessory pigments and anthocyanins** (Sims and Gamon, 2002).

- **Chlorophyll A** describes a green pigment that absorbs light for photosynthesis. We can see that its absorption maximum is in the violet/blue and then in red light part of visible spectrum but not much in the middle. It appears as "bluish green".
- **Chlorophyll B** has its absorption maxima only slightly shifted, exactly the blue and orange-red parts of the spectra. It is often called "yellowish green".

Both chlorophyll pigments are called green and the reason, why plants appear to be green is that they absorb all the other colours of visible spectra, apart from green, which is reflected so our eyes can see it.

The other produced pigments are marked as **accessory pigments**, which help to absorb more of the solar energy for photosynthesis. They can effectively absorb the parts of visible spectra that chlorophylls cannot: the green light (Šesták, 1971).

- **Carotenoids** absorb light from violet into the green part of the visible spectrum and transmit yellow shapes to our eyes. There exist over 600 described carotenoids; they are often split into two classes, *xanthophylls* (which contain oxygen) and *carotenes* (which are purely hydrocarbons, and contain no oxygen). They play an important role in the photoprotection of the photosystems. It has been shown that these pigments are crucial in protecting the photosynthesis apparatus against photo-damages, by interconversions within the xanthophyll molecules (Young et al., 1997). We are able to observe them well in autumn, when chlorophylls are degrading faster than carotenoids and leaves do not stay coloured green (Bartlez and Scolnik, 1995).

The third group of pigments consists of the **anthocyanins**. They differ from chlorophylls and carotenoids in the fact that they do not attend in photosynthesis. The colours may appear red, purple, or blue which is visible mainly during the phases of flowering and in the early stages of growth. For our purposes these pigments have not been detected (Gould, 2004).

- **The ratio Chl. a/b** is an indicator of the functional pigment equipment and light adaptation of the photosynthetic apparatus (Lichtenthaler et al., 1981). Chl. b is found only in the pigment antenna system, differing from the Chl. a, which is present in the reaction centres of photosystems I, II and also in the pigment antenna. The shaded leaves display Chl. a/b ratio lower than the sun-exposed plants (Lichtenthaler et al., 1982). Chlorophyll *a*/*b* has generally been shown to decline during senescence (Adams et al., 1990).The decreasing values of the Chl. a/b ratio may be interpreted as an enlargement of the antenna system of PS (Lichtenthaler and Buschmann, 2001). If the ratio decreases, the bigger value is in denominator. Because we evaluate chl. a/b it means that when the ratio declines more of chlorophyll b is present and this pigment occurs exclusively in antenna, therefore we can talk about enlargement of antenna system.
- **The ratio of Chl. (a + b) to total carotenoids** is an indicator of the viridity. The ratio (a+b)/(x+c) normally occurs between 4.2 with the maximum around 7.0 (Lichtenthaler and Buschmann, 2001). Lower values for the ratio are explained by senescence, stress or damage to the plant and the photosynthetic apparatus, which is expressed by a faster breakdown of chlorophylls than carotenoids (Sims and Gamon, 2002). Leaves tent to change colours to yellow shades and exhibit values decrease to 3.5, or even lower: 2.5 to 3.0. The ratio is generally higher in C4 plants and in sun leaves (Lichtenthaler and Buschmann, 2001).

The detection and evaluation of photosynthetic pigments, and consequently their relationships, are important indicators of senescence (Brown et al., 1991). Chlorophyll loss correlates with the effect of environmental stresses and the variation in total chlorophyll/carotenoids ratio may be a good indicator of stress in plants (Hendry and Price, 1993).

3 Methods

3.1 Establishment of the experiment

The experiment has been started on 19th of September, 2015. The seeds of rape have been sown into flower pots - 5 seeds into one pot, and then carefully put into the wet soil. Five pots have been put on a plate and all 12 plates (300 seeds) have been situated into a growth chamber which simulated conditions, in which the rape on the fields usually germinates.

3.1.1 Plant material

As a plant material, the variety called **Cortez** was chosen. It belongs to a "double zero" erucic acid and low glucosinolate plants. Macháčková et al. (2014) highlight the excellent overwintering ability of this variety, which has been proved in 2012, when the temperatures fell far below freezing up to -24°C repeatedly and the snow cover was minimal. This good health conditions together with excellent resistance to lodging determines an abundant harvest. Cortez is definitely one of the most productive varieties specially bred for Central European conditions. The plant material has been provided to our experiment by the company Osiva Boršov. The commercially prepared soil, called Garden Forestina, also pictured in the figure 13, from Střelská Hoštice was used.

Fig. 13: Used soil
(source: Drška, 2015)

3.1.1.1 The procedure of cold-plasma treatment

The sample of rape seeds, with the weight of 150 g, was treated by low-temperature plasma, generated by microwave plasma source at reduced pressure - of 100 Pa in the vacuum chamber. As the working gas the air with the flow rate of 100 sccm (standard cubic centimetre) was used. The seed treatment lasted four minutes. During the exposure to plasma the seeds were stirred in a mixer device with a constant speed of 60 rpm (revolutions per minute) to provide the uniform treatment to the whole surface of seeds.

3.1.2 Sowing seeds of rape into flower pots

The flower pots with the dimensions 11 x 11 x 12 cm were filled with soil about 1 cm below the rim and the soil was lightly beaten. Then using a glass rod, five holes have been excavated into 1 cm depth and the seeds were gradually situated into these holes. The distribution of seeds in a flower pot is outlined in the figure 14 below. The seeds were covered and 5 flower pots have been situated onto a plate. We got 300 seeds out of which half of them were treated with cold plasma and the second half was non-treated. Both variants were halved to normally watered plants and dry plants.

Figure 15 shows the schematic distribution of flower pots in a growth chamber. As a result, four groups have been established. The abbreviations given in brackets are used later in this work.

non-treated wet (Non-t + w; non-t, w)
non-treated dry (Non-t +d; non-t, d)
treated wet (t + w; t, w)
treated dry (t + d; t, d)

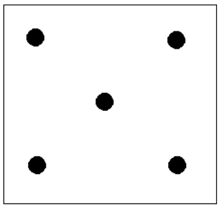

Fig. 14: The schema showing the distribution of five seeds in a flower pot (source: Drška, 2016)

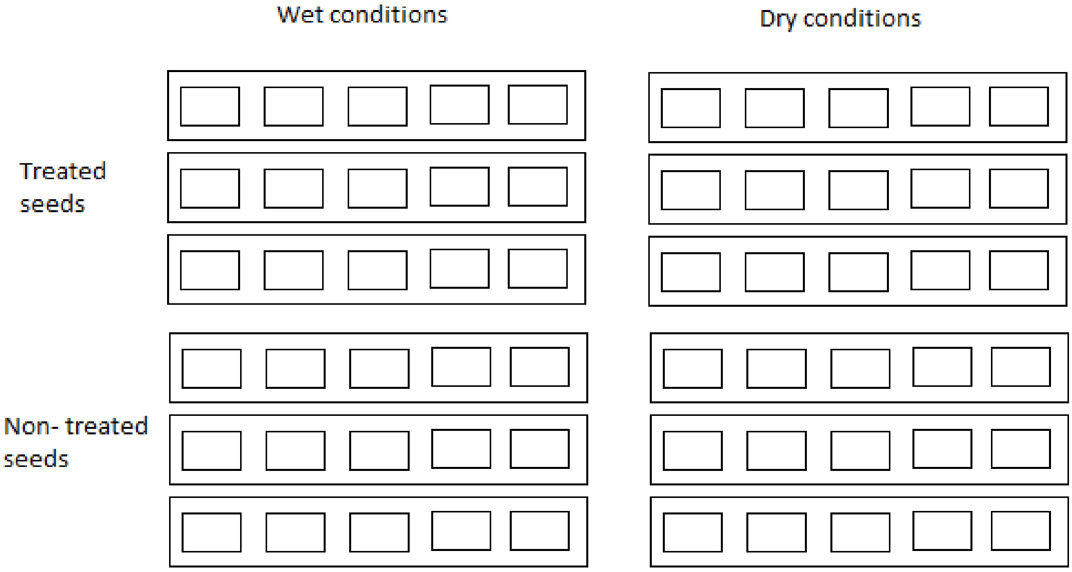

Fig. 15: The schema showing the distribution of 60 flower pots on 12 plates and division into 4 groups (source: Drška, 2016)

3.1.3 Conditions in the growth chamber

The plants grew in a fully automatic growth chamber Fytoscope (PSI, Brno, Czech Republic). The laboratory conditions were stable and correlated with the central European field conditions in which the canola usually germinates and grows in the end of August. Figure 16 shows the development of conditions during a day period.

Fig. 16: The table expressing the conditions for cultivation (source: Drška, 2016; according to Ryplová et al., 2015)

Phases of day	Temperature (°C)	Relative humidity (%)	Irradiation (µmol m^{-2} s^{-1})
Dawn – 2 hours	17 → 24	80 → 65	0 → 286
Day – 10 hours	24	65	286
Dusk – 2 hours	24 → 17	65→80	286 → 0
Night – 10 hours	17	80	0

3.1.4 Water regime

Only the first watering, immediately after the sowing has been made from above, using a sprayer, to fasten the germination process, because the seeds have been sown dry, not previously swollen. Next times water has been poured from the bottom side into the plates. The watering for all four variants has been same in the first stages – 1 litre of cold tap water to every plate three times a week, to provide the same conditions for the germination but soon after (day 18) the amount of water for the dry variants has been cut into halves – 0,5 litre to every plate, whereas the watered pots received normally 1 litre per a plate. Gradually as the plants had grown, more water supplies were required and in the measuring period the pouring was conducted according to the figure 17 below.

Fig. 17: The table with the amount of water (in litres) given to all four variants since the 18th day of cultivation (source: Drška, 2016)

	Non-t + w	Non-t + d	T + w	T + d
Monday	1 l	0,5 l	1 l	0,5 l
Tuesday	0,5 l	-0-	0,5 l	-0-
Wednesday	1 l	0,5 l	1 l	0,5 l
Thursday	0,5 l	-0-	0,5 l	-0-
Friday	1,5 l	1 l	1,5 l	1,5 l

3.1.5 Principles of measuring fluorescence

All the fluorescent measurements have been identified by a pocket fluorometer FluorPen FP 100 (see figure 18). This device detected the parameter Qy (standing for quantum yield), expresses the photosynthetic efficiency as a ratio of Fv/Fm (see figure 10). The plants in pots were removed from the growth chamber and covered with the paper boxes for at least 20 minutes. In the dark adapted second leaves the reaction centres of PSII opens, oxidize, similarly as the other parts of electro-transport chain.

Fig. 18: FluorPen FP 100 (source: Drška, 2015)

3.1.6 Detection of chlorophylls according to Lichtenthaler

For the determination of photosynthetic pigments content the commonly used method described by Lichtenthaler and Wellburn (1983) was chosen. The pigments were extracted in 80 % acetone and then determined by using spectrophotometry. The spectrophotometer Shimandzu, showed in figure 20, was used. The sample consisted of 6 leaf cores, which were perfectly homogenized and diluted into 10 ml of solvent. Three second leaves from all four studied variants were selected and 6 leaf cores with a diameter of 0,8 cm have been cut by cork borer. Figures 21 and 22 are showing the second leaves during the first and the third measurement in dry, non-treated group. Additionally figure 22 shows the used dissolvent and the leaf cores. The extract was centrifuged for 10 minutes at 3000 rpm/min. Then the spectrophotometer detected the absorbance at three wavelengths (663, 646 and 470 nm). On the basis of Lichtenthaler´s formula for determining the pigments in 80% acetone (see figure 19), the content of chlorophyll a, chlorophyll b, and carotenoids in units (micro-mol/ml) was calculated in program Excel. The figures 24 and 25 were taken during the detection of photosynthetic pigments.

$$C(\text{chl } a) = 12{,}21\times A_{663} - 2{,}81\times A_{646} \quad [\text{mg.l}^{-1}]$$

$$C(\text{chl } b) = 20{,}13\times A_{646} - 5{,}03\times A_{663} \quad [\text{mg.l}^{-1}]$$

$$C(c+x) = (1000\times A_{470} - 3{,}27\times C(\text{chl } a) - 104\times C(\text{chl } b)/227 \quad [\text{mg.l}^{-1}]$$

Fig. 19: Formula for calculation the pigments content (source: Lichtenthaler and Wellburn,, 1983)

Fig. 20: Spectophotometer Shimandzu (source: Drška, 2015)

Fig. 21 and 22: The second leaves during the first (left) and the third measurement (right in dry non-treated group. (source: Drška, 2015)

Fig. 23 (left): The centrifuge tubes closed by a cork, wrapped in aluminium foil, placed in a refrigerator. (source: Drška, 2015)
Fig. 24 (right): The empty cuvette – 2 sides are transparent and 2 are matte (source: Drška, 2015)

Fig. 25: The centrifuge (source: Drška, 2015)

4 Results

4.1 Fluorescence in leaves

Measurement of the chlorophyll a fluorescence is a quick, precise and non-destructive technique, widely used in investigating damage or repair caused in the photosynthesis plant system by various types of stresses (Schreiber et al., 1988; Govindjee, 1995).

The first measurement has been made on the 22nd of October, which was the 34th day of cultivation. Sooner measuring was not possible because the leaves had to be big enough for photosynthetic measurement which came along with the fluorescence measurement. The next reason for that was the fact, that plants were exposed to drought since the 18th day of cultivation. In all four variants 10 second leaves were detected and the average values are shown in the following graphs.

Gained data have been processed in programs EXCEL and STATISTICA and subsequently evaluated by two-ways ANOVA (Analysis of variance) and then by HSD Tukey test all on significance level Pv < 0,05.

During **the first measurement** we found out that there was a significant statistic difference in normally watered variants (see figure 26). The test proved that after 15 days of exposing to stress of drought, plasma treated plants had higher Fv/Fm ratio in dry conditions than in wet conditions (DF = 36, F (1, 36)=11,129, p = 0,00198).

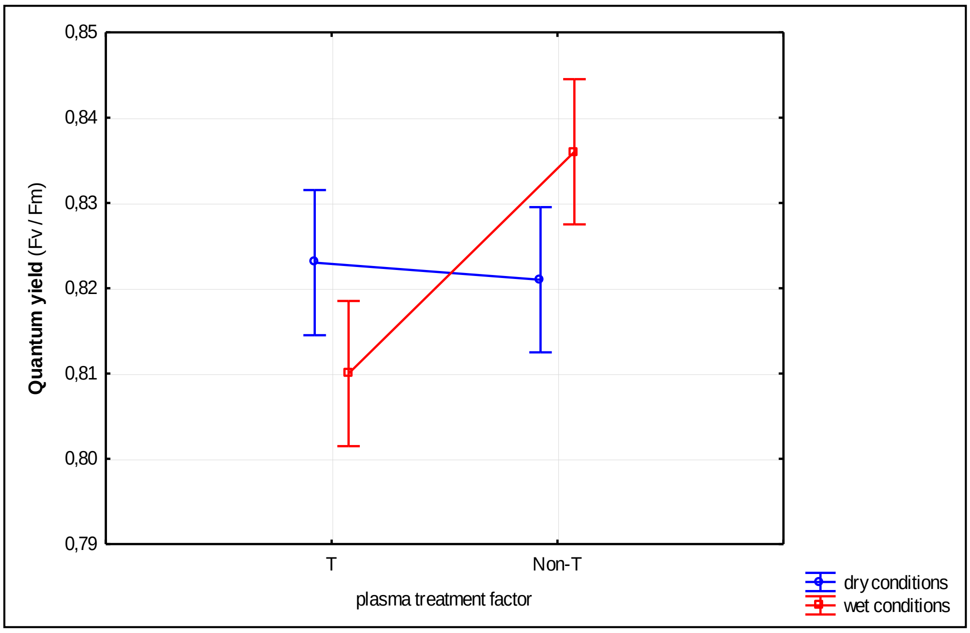

Fig. 26: *Graph of the maximum photochemical efficiency of photosystem II. The (Fv/Fm) on the 34th day of cultivation and the statistically significant interactions. The data were processed by Tukey test (P < 0,05). The test discovered that the treatment factor significantly influenced wet variants (p = 0,000678).*

The second measurement was carried out on the same principle as the first one, a week later (October, 29 = 41st day of cultivation) and the following data were obtained.

Out of the figure 27 it is visible that the water deficit showed as a significant factor and both dry variants (treated and non-treated) demonstrated lower measured Fv/Fm values. The significant difference on the p value < 0,05 was observed between dry variants: (F (1, 36) = 4,4433; p = 0,04207; DF = 36).

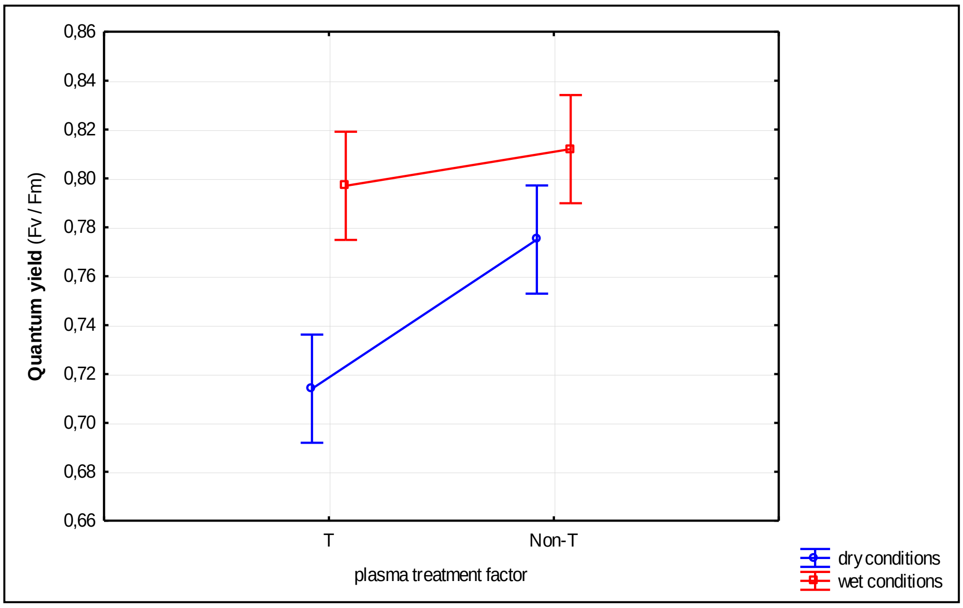

Fig. 27: *Graph of the maximum photochemical efficiency of photosystem II. The (Fv/Fm) on the 41ˢ day of cultivation and the statistically significant interactions. The data were processed by Tukey test (P < 0,05). The test discovered that the treatment factor significantly influenced dry variants (p = 0,001992).*

The Tukey test also proved that plants treated with cold plasma grown in dry conditions significantly differed from all 3 other variants on the P < 0,05. This measurement showed that after exposing to drought for 27 days, the cold plasma treated plants had lower Fv/Fm ratio than the other three variants.

The third measurement was carried out on the same principle as the preceding ones, 8 days after the second measurement (November, 6 = 49th day of cultivation) and the following significant differences in interactions of the tested factors (figure 28) were obtained (F (1, 36) = 5,0932; p = 0,03019).

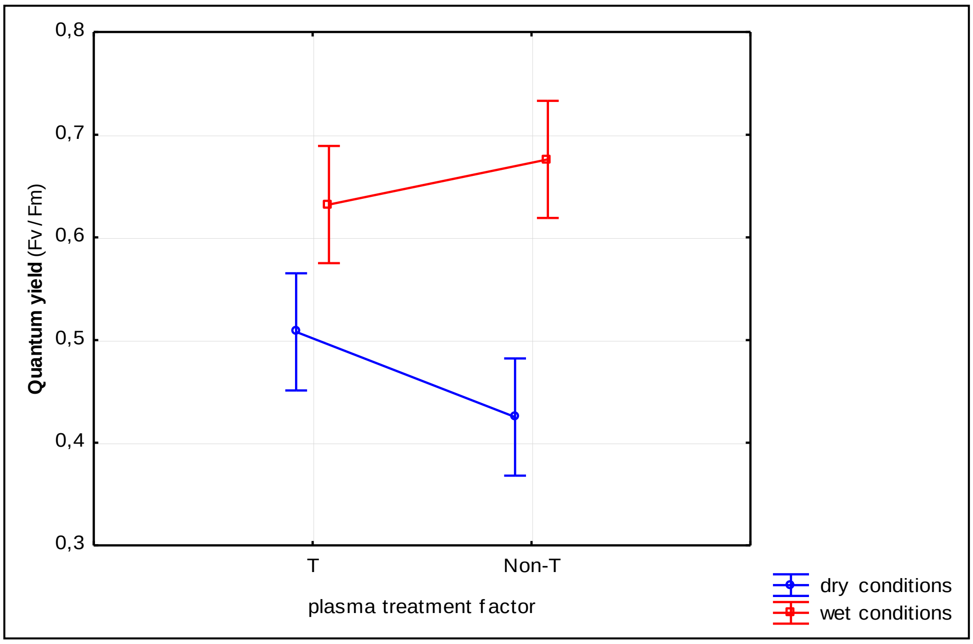

Fig. 28: *Graph of the maximum photochemical efficiency of photosystem II. The (Fv/Fm) on the 49thday of cultivation and the statistically significant interactions. The data were processed by Tukey test (P < 0,05). The test discovered that the water regime significantly influenced both treated plants (p = 0,018102) and non-treated plants (0,000159).*

Although the test did not prove the influence of plasma treatment in dry conditions, but the p reached the value 0,18, which is nearly on the edge and mainly the treated dry plants for the first time reached higher Fv / Fm ratio than non-treated dry ones (see figure 28).

The figure 29 below shows all three measurements on the time scale and the development of the Fv / Fm ratio. In wet conditions (see red part of the figure 29) the decline is very similar in both treated and non-treated conditions, whereas in dry conditions (see blue part of the figure 29 and figure 30) the decrease of quantum yield is greater in non-treated variant (- 0,396) than in treated variant (- 0,315). However his trend is not statistically supported.

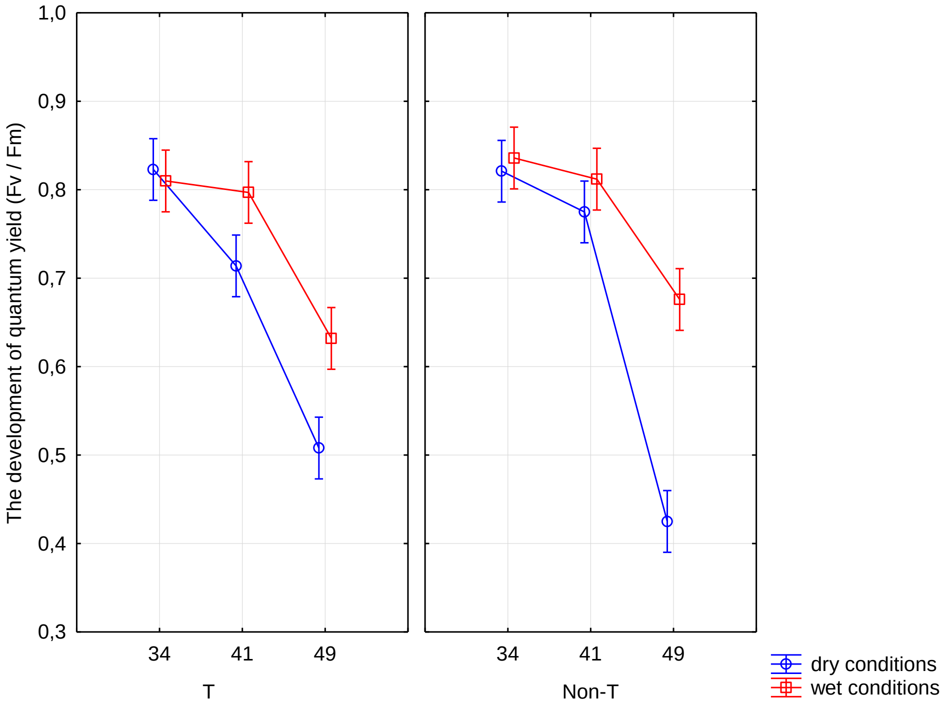

Fig. 29: *The development of Fv/Fm ratio on the time scale - from the 34th to 49th day of cultivation in treated and non-treated variants.*

This test takes in account the role of measurement as the continuous variable. The interaction between the factors of plasma, drought and measurement was proven on the level of significance p = 0,00313; F = 6,0868.

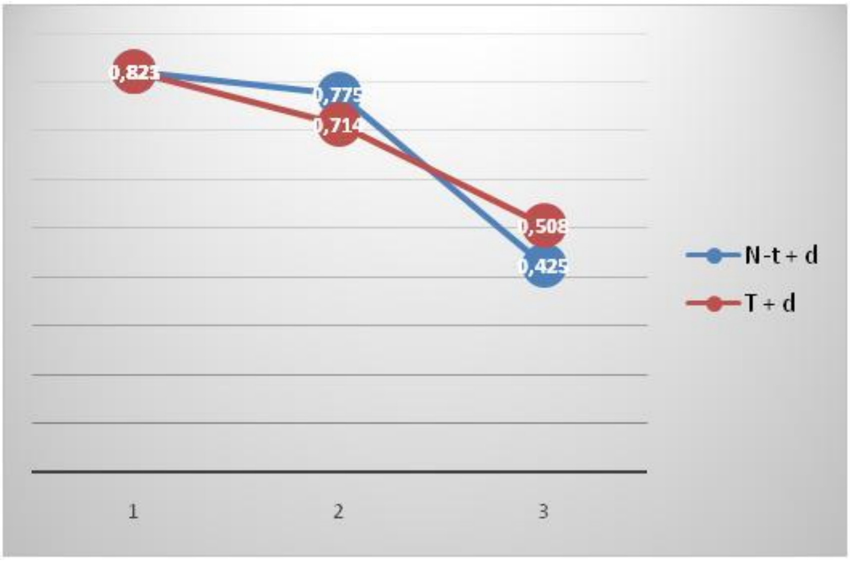

Fig. 30*: The development of fluorescent values in dry conditions*

4.2 Pigments in leaves

On the basis of Lichtenthaler´s formula for determining leaf pigments in 80% acetone measured pigment values were firstly calculated and then transformed to the more commonly used units (in grams per square meter of leaf area) in the program EXCEL and the following tables (with AM standing for average means and STD expressing standard deviations) and graphs with the basic chlorophylls and their ratios were created.

The gained data were then tested in program Statistica and the photosynthetic pigments, their ratios and the significant differences between all four variants were observed.

4.2.1 Evaluation of Chlorophyll a

According to the above mentioned formula for determining the leaf pigments the calculated values in grams per square metres were put into the figure 31, which summarizes all three measurements.

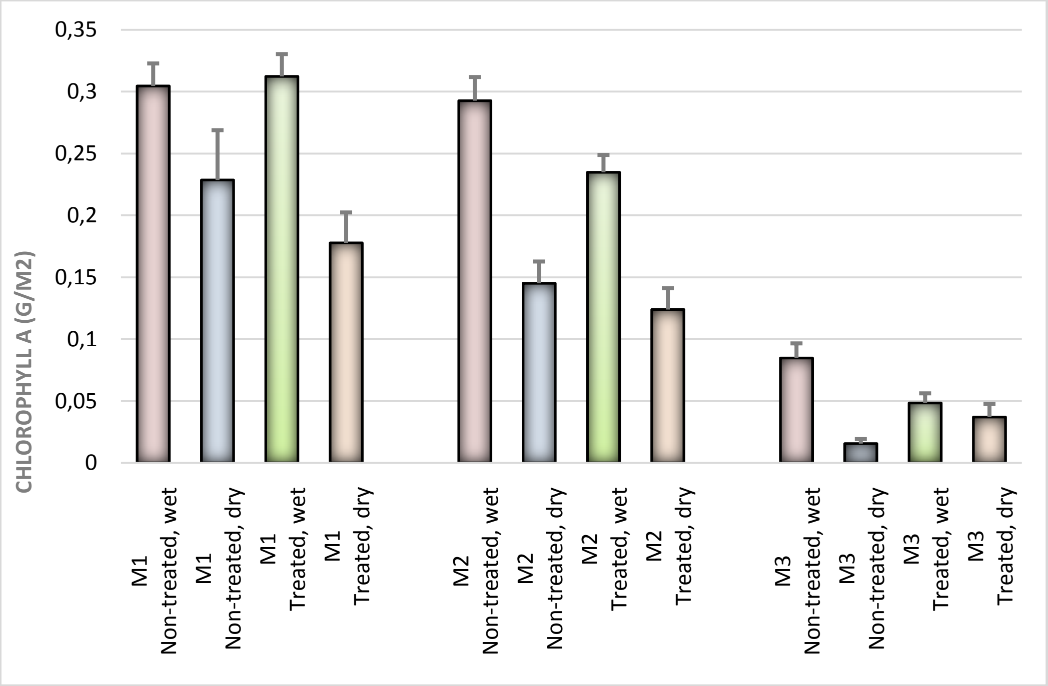

Fig. 31: *The development of chlorophyll a per m^2 of leaves during the three measurements M1, M2, M3 (34th, 41st and 49th day of cultivation). The error bars indicate positive standard deviations.*

These data were then evaluated by ANNOVA test and the important interactions between drought factor and plasma factor on the P < 0,05 are shown in figure 32.

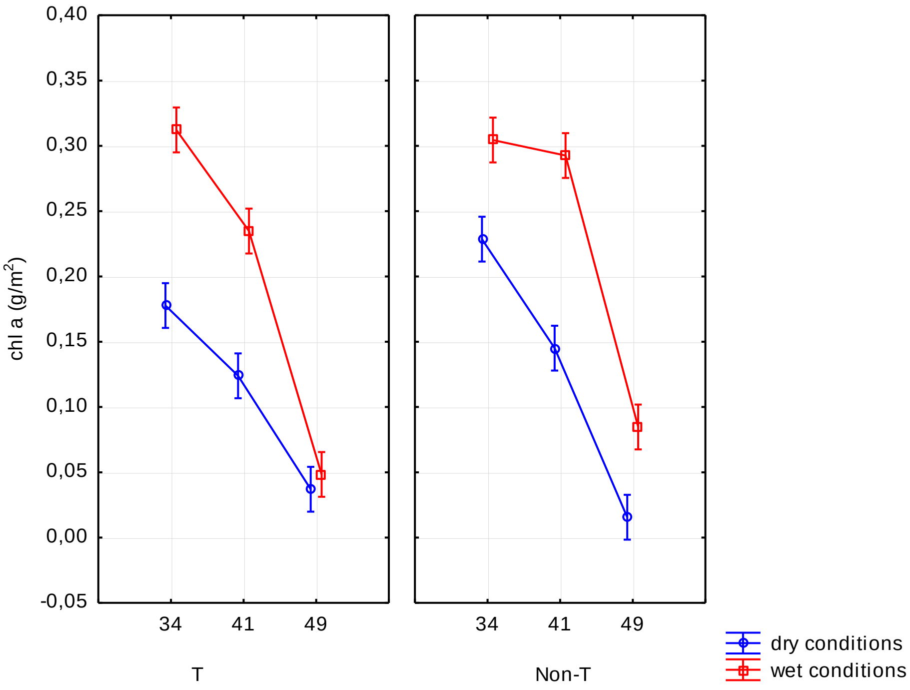

***Fig. 32**: The significant interactions between conditions, plasma treatment and the timing of measurement evaluated in program Statistica (F (2, 48) = 13,167, p = 0,00003)*

The graph portraits the influences of tested factors – type of conditions and factor of treatment according to the days of measurement. The graph suggests slightly faster decrease of chlorophyll a in treated wet conditions than in non-treated wet. In dry conditions the role is vice versa. Cold-plasma treated plants reached lower values in the first measurement but they decreased much slower and in on the 49th day of cultivation, the level of chlorophyll a was higher in plasma treated variant than in non-treated.

The first measurement proved the significant differences in dry conditions (p = 0,038818; F (1, 16) = 5,9465; DF = 16), by using Tukey test. Non-treated dry plants showed higher level of chlorophyll a than treated dry plants (see figure 32).

The second measurement showed the significant difference in wet conditions (p = 0,000502, F (1, 16) = 5,7413; DF = 16). Non-treated wet plants had again the bigger amount of chlorophyll a in second leaves (see figure 32).

The third measurement indicated the significant differences both in wet and in dry conditions (see figure 32). In wet conditions, the non-treated plants contained more of chlorophyll a than in treated variant (p = 0,000221). In dry conditions the role is vice versa. Treated plants showed significantly more of chlorophyll a than the non-treated ones (p = 0,008319; F (1,16) = 51,467; DF = 16). All three measurements proved that plasma treatment influences the amount of chlorophyll a (*F(2, 48) = 13,167, p = 0,00003; DF = 16* – see figure 32).

4.2.2 Evaluation of Chlorophyll b

The same bar graph is used for determination of chlorophyll b (see figure 33).

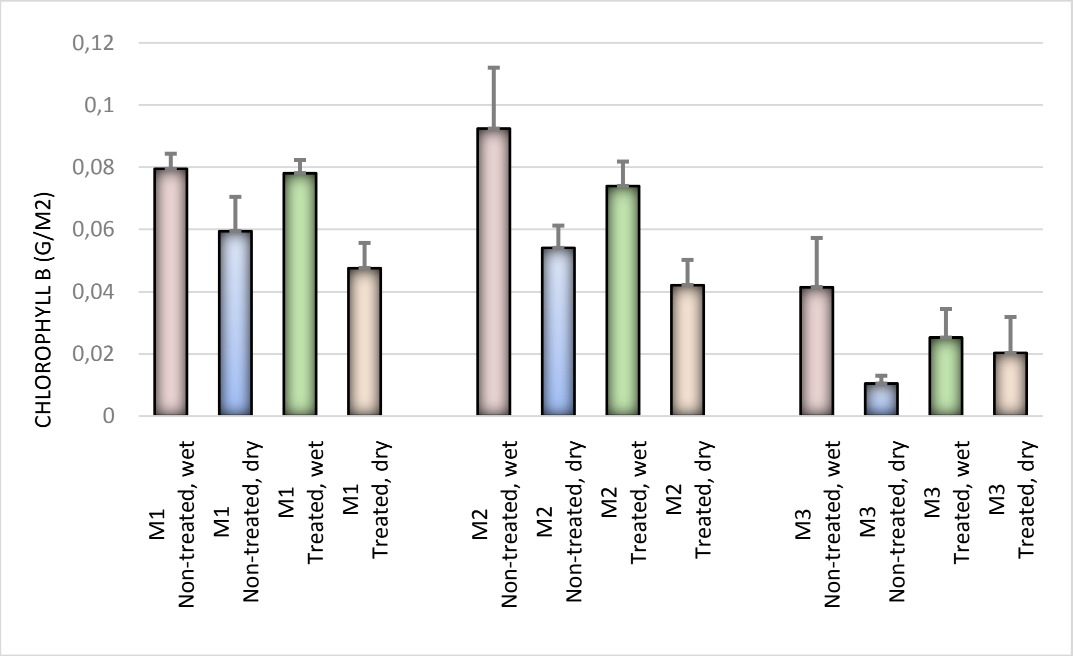

***Fig. 33**: The development of chlorophyll b per m^2 of leaves during the three measurements M1, M2, M3 (34th, 41st and 49th day of cultivation). The error bars indicate positive standard deviations.*

It is possible to observe the differences in the third measurement, which is supported by Tukey test, in which during the first and second measurement the interaction between tested factors was high (P > 0,05), but during the third measurement the decline of this pigment statistically differed according to the conditions (see figure 34) and p = 0,01660, F (1, 16) = 7,1563; DF = 16.

The third measurement confirmed that non-treated wet variant significantly differed from non- treated dry (p = 0,001984) and from treated dry (p = 0,033545) variants. This test revealed the influence of drought but not the influence of plasma treatment.

The interesting fact is that only treated wet plants were no different from the dry ones. It is visible from the figures 33 and 34 that in wet conditions, the values of treated variant are always lower than in non-treated plants. In the dry conditions, this trend is valid only for the first and second measurement. In the third measurement the non-treated dry plants contained less of chlorophyll b than treated dry ones, but this was not statistically supported.

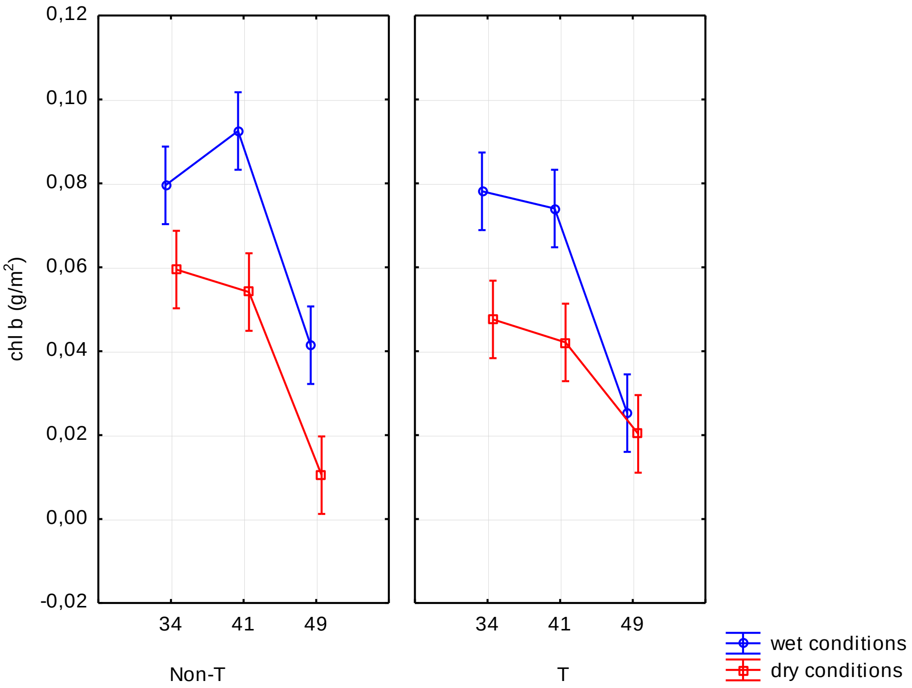

Fig.34: *The significant interactions between* conditions, plasma treatment and the timing of measurement evaluated in program Statistica (F(2, 48) = 3,9494, p = 0,02583)

The co-interaction of conditions and treatment and treatment and measurement was not significantly proven. We can observe that the treatment influenced in the similar way the dry and the wet plants.

4.2.3 Evaluation of carotenoids

On the same principle as the chlorophyll pigments the amount of carotenoids was firstly observed and then also in the ratio chl. (a + b) / car., which appeared to be better indicator. Some literature sources (Yin et al., 2005; Qaderi et al., 2006) proved the increased carotenoid content in leaves in stressed conditions. Our experiment cannot assure this hypothesis, because no statistically significant differences were observed and the interaction of co-acting factors has always been too high – see figure 36 ($P > 0,05$). But by only watching the changes between the second and the third measurement we can reliably observe the changes. The Tukey test did not highlight this deviation, because the checked values were nearly the same. Although, looking at the figure 35, we see that in the third measurement, the amount of carotenoids is higher than in the wet variants, which correlates with the thesis that dry plants suffered from the stress more than wet plants.

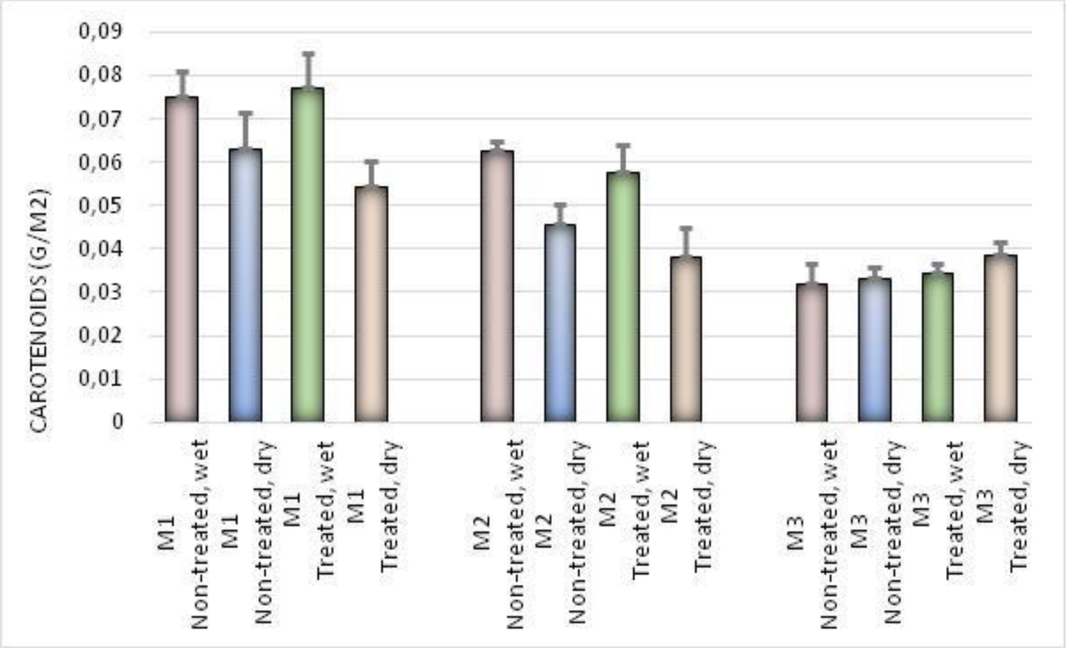

Fig. 35: *The development of carotenoids per m^2 of leaves during the three measurements M1, M2, M3 (34th, 41st and 49th day of cultivation). The error bars indicate positive standard deviations.*

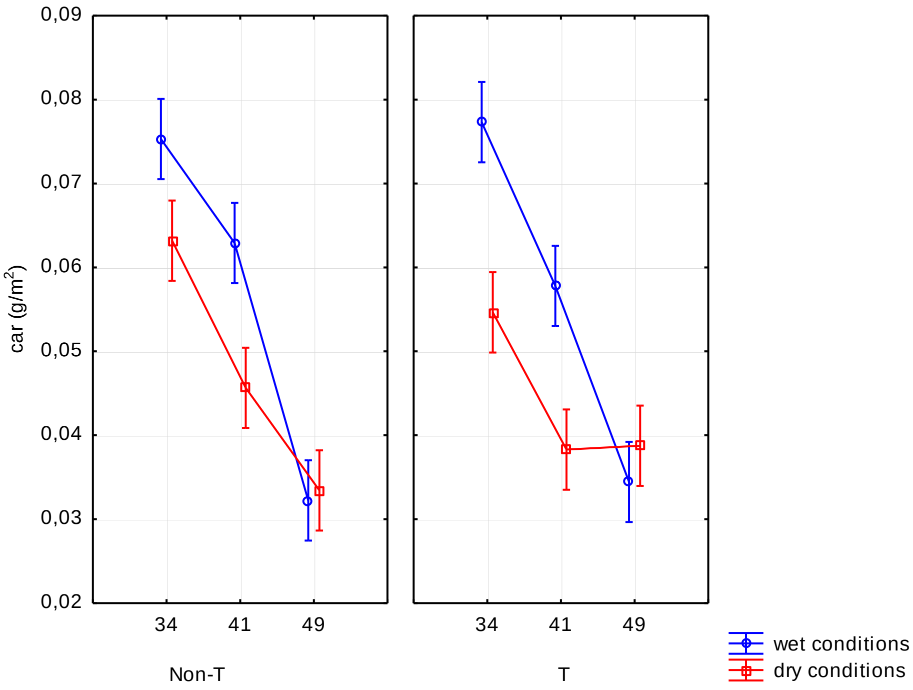

Fig. 36: *The significant interactions between conditions, plasma treatment and the timing of measurement evaluated in program Statistica* (F(2, 48) = 2,1003, p = 0,13352).

4.2.4 Evaluation of the ratio chlorophyll a / chlorophyll b

Figure 37 highlights the ratios in all four variants. The trend of lower ratio is observed in non-treated dry variant. Lower ratio means that there is a relatively high number in the denominator. It points out the relatively high level of chlorophyll b, which is situated in antenna, whereas the higher ratio means high level of chlorophyll a in react centres. Looking at the figure 38, we see two more or less parallel lines, suggesting that there is no statistically significant interaction between studied factors. According to the Tukey test, the drought influence treated and non-treated plants in a similar way and also the treatment influence in a similar way dry and wet plants.

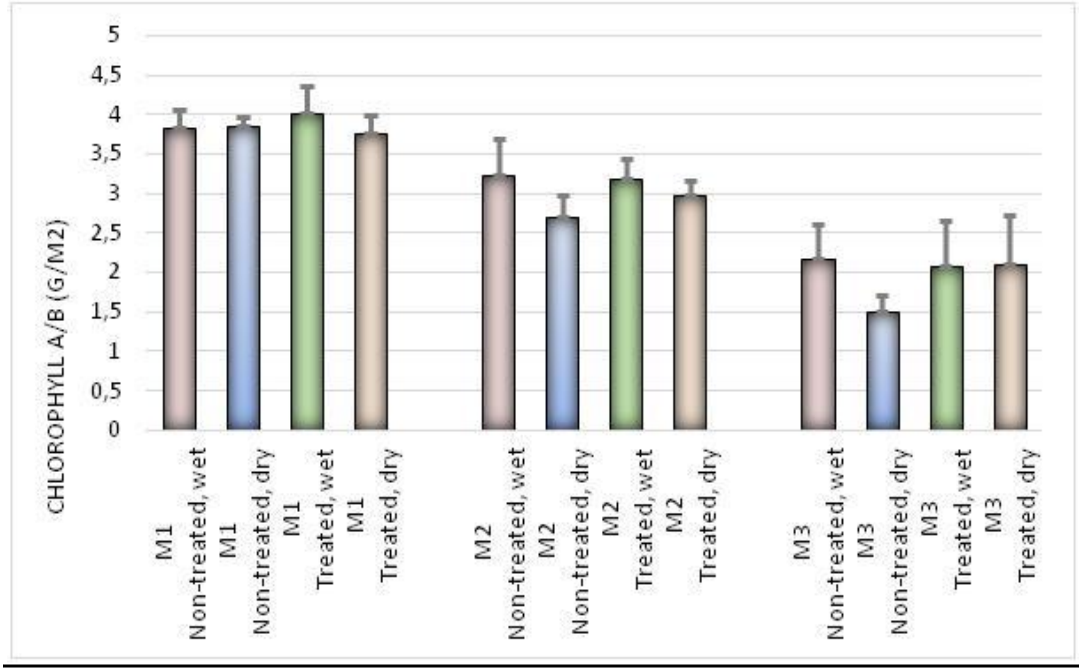

Fig. 37: *The development of ratio chlorophyll a / chlorophyll b per* m^2 *of leaves during the three measurements M1, M2, M3 (34th, 41st and 49th day of cultivation). The error bars indicate positive standard deviations.*

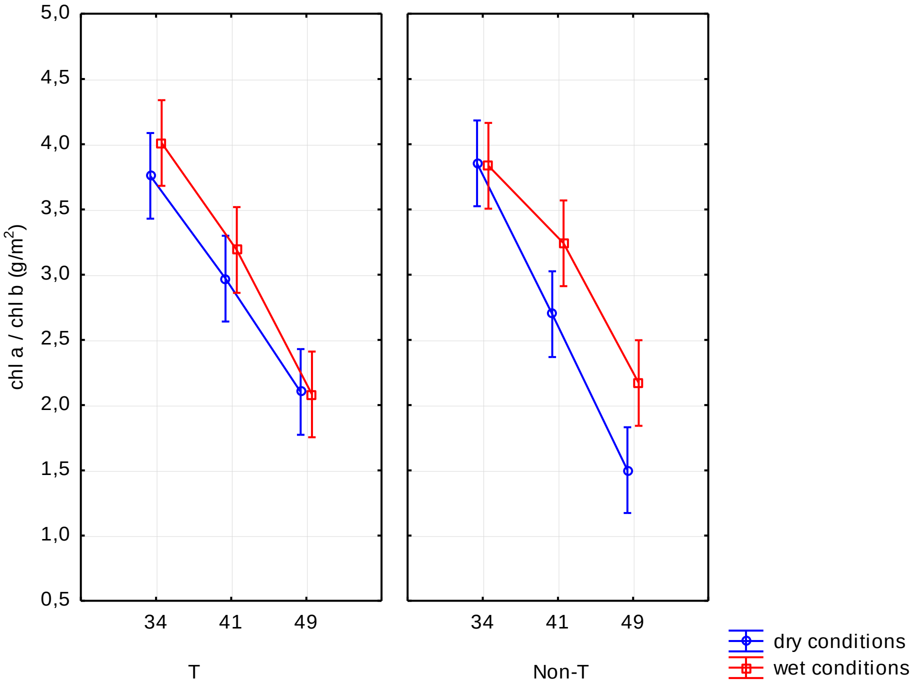

Fig. 38*: The significant interactions between conditions, plasma treatment and the timing of measurement evaluated in program Statistica* (F(2, 48) = 2,1837; p = 0,12369)

4.2.5 Evaluation of ratio chlorophylls a+b / carotenoids

As mentioned in the theory section, normal values in sun leaves lies between 4,2 - 5. In shade leaves the ratio can reach up to 7. The values lower than 4,2 are signs of aging, stress exposure and damage of the photosynthetic apparatus. These values are visible during the third measurement in both dry variant and also in treated wet variant (see figure 39). These leaves appeared yellowish green (see figure 22) and for that stressed plants it is typical that the chlorophyll pigments decrease faster than carotenoids, which was also supported by our experiment.

The Tukey test revealed that during **the first measurement**, the statistically significant difference was firstly between treated dry variant (with the ratio of 4,1) and treated wet variant (the ratio of 5,08) on the significance level p = 0,002471 and secondly between treated dry variant and non- treated wet (with the ratio of 5,12).

The second measurement indicated that non-treated wet variant (with the ratio of 6,13) significantly differed from the non-treated dry (with the ratio of 4,36) on the P = 0,008872, and also from treated dry (ratio of 4,45) on the P = 0,012821). The difference between wet variants (treated and non- treated) was not evaluated as statistically significant.

The third measurement showed (similarly as the second one) also the statistically significant differences between non-treated wet and both dry variants on the P< 0,05 but this time it has been proven that among the wet variants the variance was significant. Treated wet plants had significantly lower ratio (2,13) than non-treated wet plants - 3,89 (see figures 39 and 40).

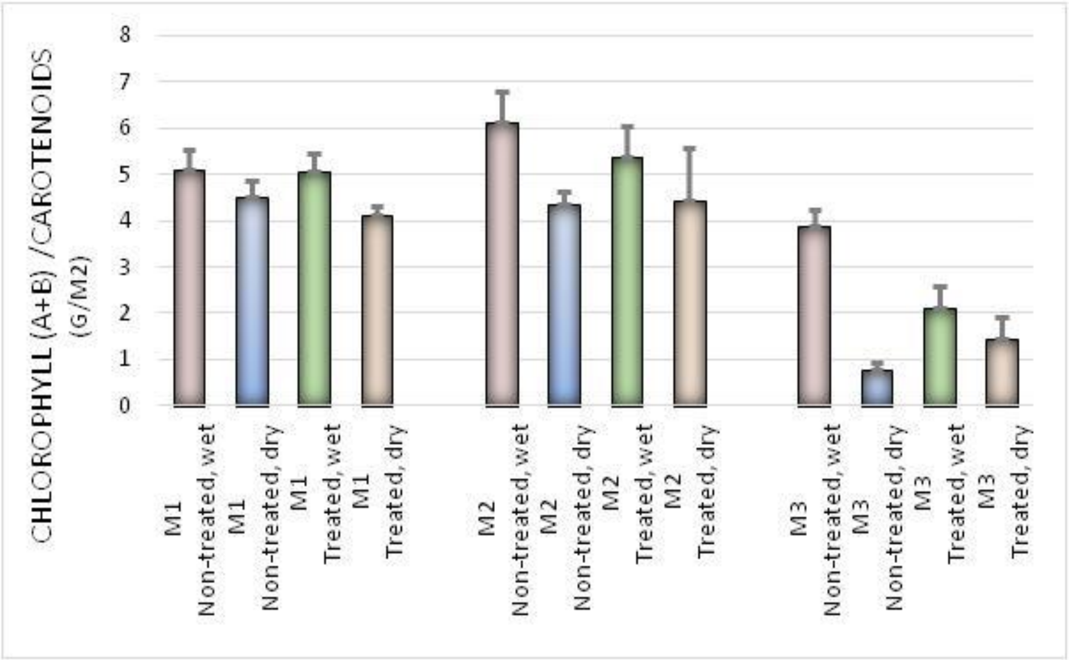

Fig. 39: *The development of ratio chlorophyll (a+b) / carotenoids per* m^2 *of leaves, during the three measurements M1, M2, M3 (34th, 41st and 49th day of cultivation). The error bars indicate positive standard deviations.*

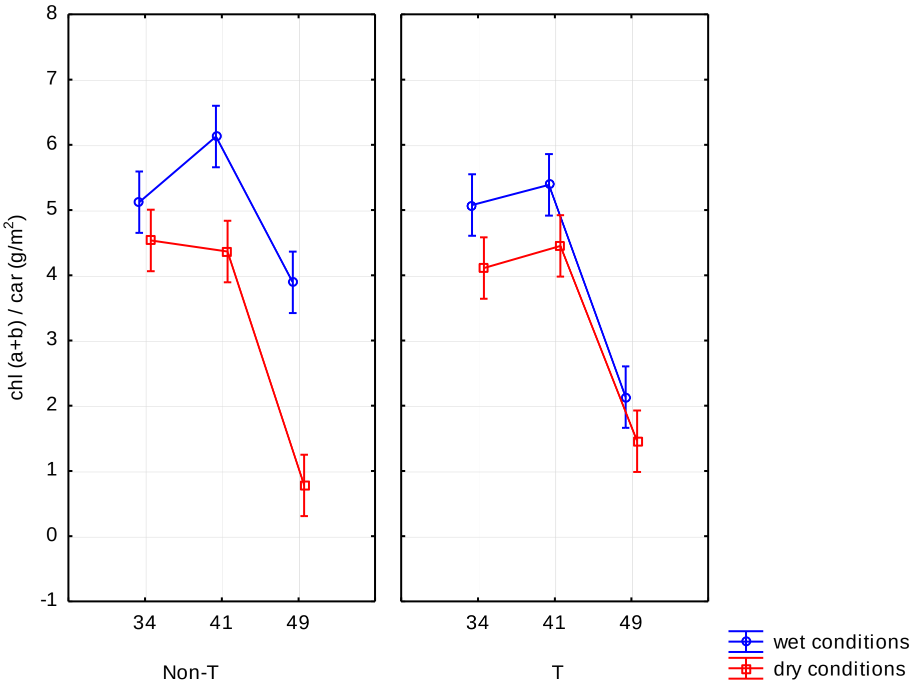

Fig. 40*: The significant interactions between conditions, plasma treatment and the timing of measurement evaluated in program Statistica*_(F(2, 48) = 9,0902, p = 0,00045)

5 Discussion

The initial hypotheses are after our experiment partly answered, the low temperature plasma really influences the growth of plants. When talking about short term stresses from water deficit, the influence has been watched on **fluorescent values of Qy**. The treated dry plants showed statistically significant lower values than non-treated dry during second measurement. This difference is statistically significant but from the measured values (0,71 : 0,77), we can see that the plants during the second measurement did not suffer as much from water shortage in comparison with the third measurement in which the values were 0,508 : 0,450 but this time higher for treated- dry than non-treated dry. It is interesting that this difference was not statistically proven.

When talking about long term stresses from water deficit, the influence of cold-plasma treatment in dry conditions was well observed in total **amount of chlorophyll a** (during the third measurement), which proved the positive influence of treatment. According to Mielke et al. (2003) the treatment can enhance the stimulation of growth in dry conditions by preventing against the oxidative damages, which are caused by the accumulation of reactive oxygen species (superoxide, hydrogen peroxide etc.). Cold-plasma also causes the accumulation of soluble sugars and proteins (proline), which stable the turgidity, increase absorptive ability and protect membranes during drought (Ling, 2015). These physiological processes might be the reason, why the senescence was slowed down in treated dry plants, which showed slower breakdown of chlorophylls than carotenoids. The slower decrease of chlorophylls than carotenoids hence supports the theory that low temperature plasma helps to survive the long term stresses.

The evaluation of **carotenoids** revealed their higher content in both dry variants when exposed to long-term stress, which is supported by many literature sources (Qaderi et al., 2006; Yin et al., 2005). The significant difference on the ($P < 0,05$) was not proven between the dry variants but we can see slightly higher carotenoids content in treated, which does not assure the theory that cold-plasma helps to survive in dry conditions, because plants with high carotenoid content are mostly more stressed and these pigments protect their photosynthetic apparatus against photodamages. Anyway, the carotenoids were increased in both dry variants which show that the drought factor was significant for the growth.

The ratio of **chlorophyll a/chlorophyll b** informed us about the amount of pigments in antenna and in react centres. In dry variants the situation again differed in first and the third measurement. For a long-term water shortage the higher ratio was valid for plasma treated plants, meaning that plasma treated contained more of chlorophyll a and less of chlorophyll b than in the non-treated variant.

The ratio of **chlorophyll (a+b) /carotenoids** again showed that the long-term water deficit influenced both dry variants, but the non-treated more fatally. These leaves seemed more yellow and fade, anyway the statistically significant difference was observed between the wet variants in which the role was vice versa. Non- treated wet plants had much higher ratio (3,9), meaning the sum of chlorophylls was relatively high, and the amount of carotenoids relatively low. In wet treated variant the situation was totally different, the plants showed the ratio only of 2,1 which reliably indicates the higher content of carotenoids – higher need for photoprotection and lower amount of chlorophylls indicating faster senescence.

The experiment really helped to clarify this question, we detected some promising results but the next work in this field is required. It is possible to study the together influence of abiotic stresses, because in real field conditions we do not observe only drought, it is often joined with high temperatures, the excessive light and high level of carbon dioxide in the atmosphere. These factors have been tested in one Canadian study and more results in this field would be welcomed. The next factor is the duration of process in which the seeds were treated with cold plasma and eventually the other significant agricultural crops could also be tested by using this method. When cultivating the plants we figured out some suggestions for the future experiments. We could see now for example that we could sow sprouting seeds to achieve larger sample of observed plants and to fasten the whole process. It also would be good to provide a separate flower pot for every single seed to avoid the situation in which in one pot there were 5 plants and in the other there was only one. It is clear that this only plant has better conditions – more space, light and does not need to compete for minerals in soil and water as much as the plants growing together. This would be optimal but on the growth chamber is not as large so this would on the other hand eliminate the number of observed plants, which is also not ideal. The next idea lies in watering with rain water instead of tap water. Then when measuring pigments in leaves, some non-destructive methods would be optimal and the next suggestion lies in doing the measurements on the third leaves which live longer so the measurements 4 or 5 could be done. We saw the trend to slower decrease in photosynthetic pigments in dry treated plants but we could not assure this theory because the next measurement on the second leaves was not possible. What is missing in our pigment analyses is the absorbance in the wavelength 750 nm, because in this part of light spectra the spectrophotometer could tell whether the centrifuged sample was turbid or clear enough. We did the measurements as right as possible but this parameter would even specify the data.

Our experiment revealed totally different fluorescent and pigment values for the first and third measurement. The plants show different characteristics for short-term and long-term stresses. It appears to be clear that the influence of cold-plasma exists but to tell whether it is positive or negative more of these experiments are required.

6 Conclusion

The aim of my bachelor thesis was to prove **whether there is any influence of cold – plasma treatment on photosynthetic apparatus of rape.** This impact was proven in many cases. During the second fluorescent measurement, the significantly higher decline in Fv/Fm was detected in drought for treated than for non-treated plants. After the long-term exposure to stress of water shortage the reverse was truth and it was possible to observe the trend of slightly better fluorescent efficiency at treated variant than in non-treated (not statistically supported. Regarding chlorophyll a, the effect of drought was confirmed on the first two measurements and the third one partly answered the second hypothesis: **Whether there is any influence of cold plasma treatment of seeds on rape seedlings response to limited water supplies.** The third measurement demonstrated higher chlorophyll a content for treated variant in drought meaning better fitness of these plants and therefore a positive effect of treatment on surviving in dry conditions. Increased content of carotenoids revealed that the dry plants were more stressed regardless of the factor treatment. The very important parameter proved to be the ratio of chlorophylls to carotenoids that exhibited during the first two measurements a similar trend and the values were kept in the optimal range. However, when measuring a third time, the variant wet non-treated remained at optimum values, while others dropped significantly, from which one can watch the remaining three variants were more vulnerable to stress. For both dry variants this conclusion may be expected, however in the wet treated plants the low ratio was surprising and probably caused by more rapid aging of plants. The structure of photosynthetic apparatus thus significantly changed, in antenna complexes more carotenoids were present while in react centres less of chlorophyll a was detected in that variant. Hence, the seed treatment with cold plasma had a significant impact. In **wet conditions** rapid decline of chlorophyll and also the overall ratio of chlorophyll to carotenoids was demonstrated, showing of the **negative effect.** In **dry conditions** the treatment **initially** showed to have a **negative effect** and this was initially confirmed during the first measurement of chlorophyll a and during the second measurement of fluorescent parameter Qy but **then**, when the plants were exposed to prolonged drought **the positive effect** of treatment was observed with significantly higher levels of chlorophyll a. During the third measurement trend of higher values of fluorescence parameter Qy was observed, similarly as the ratio of total chlorophyll a/b, which confirms the higher chlorophyll a content to chlorophyll b. By the conclusion of this work is meant the further clarifying of the issue of seed treatment with cold plasma. This experiment generally revealed different level of pigments in dependence on the duration of the stress. Many promising results were found but further research in this area is needed.

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C. Sources of used pictures

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8 Attachments

8.1 Photos taken during working on the experiment

Fig. 41: Five days after the establishment of the experiment (source: Drška, 2015)

Fig. 42: Twelve days after the establishment of the experiment (source: Drška, 2015)

Fig. 43: 19 days after the establishment of the experiment (source: Drška, 2015)

Fig. 44: 25 days after the establishment of the experiment (source: Drška, 2015)

Fig.45: 25 days after the establishment (source: Drška, 2015)

Fig. 46: The last day of cultivation (source: Drška, 2015)

Fig. 47: Plants after removing from flower pots (from left to right: non-treated wet; treated wet; non-treated dry; treated-dry). The different distribution of the root system is evident (source: Drška, 2015)

Fig. 49: Rapeseed during flowering on the field near Horní Třebonín (source: Drška, 2015)