THE EFFECTS OF SIMULATED ACID RAIN ON GROWTH AND BIOCHEMISTRY PROC ESS IN GRASS (*LOLIUM PERENNE*)

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The effects of simulated acid rain (pH 3 and 4) and control (rain with pH 5.7) on phisiological (germination and grown) and biochemistry process (chlorophyll a and b, carotenoid pigments, peroxidase, catalase) in Lolium perenne were studied. Simulated „rain" (by adding sulphuric acid) and normal rain was applied by spraying daily for 10 days from the beginer of the experiment. The results indicated that under stress of simulated acid rain, the germination and the grown decrease with the declining of pH values of acid rain. The green pigments from grass exposed to simulated acid rain with pH 3 were 18,35 mg/g leaves, in comparison with the control, where the content of green pigments were 23,076 mg/g leaves. Similar data was obtained when we measurements the carotenoid pigments. The peroxidase activity was enhanced to the end of experiment, in the case of the samples spraying with acid rain with pH 3 respectively 4, comparative with control. The change in activity of peroxidase was higher than catalase activity, which showed that peroxidase was more sensitive to acid rain stress than catalase.

***Key words**: acid rain, chlorophyll, peroxidase, catalase*

The rain water is slightly acidic, it has a pH range normally from about 5.5 to 5.7. The pH has changed in many areas in recent years due to the increase in substances like carbon dioxide (CO_2), sulfur dioxide (SO_2), nitrogen oxides (NOx) combining with water and oxygen in the air to lower the pH. The release of sulfuric dioxide and nitrogen oxides into the atmosphere really lowers the pH of rainwater. Acid rain (pH < 5.5) is one of air pollutants.

Acid rain is currently an important controversial subject, due to its wide ranging effects and its ability to spread beyond the initial formation zone. Amongst its negative interactions, we mention: structure decay, destruction of crops and forests, threatening of terrestrial and aquatic fauna, as few species can withstand such conditions, so, in general, the destruction of ecosystems.

Air quality is of fundamental importance for all living organisms. Plant, animal and human health depends very much on clean athmosphere. Polluting elements SO_2, NO_x, CO_2 and HF, which are released in to the air as a result of human activities result in acid rain, following complex phisico-chemical reactions, sometimes aided by the very presence of light.

Oxidative stress is one of the major perils for plants exposed to the polluting factors in the environment. The mechanism by which free radicals act is yet unclear. They can directly cause destruction in cells, tissue, organs and organisms. Cloroplasts are especially sensitive to reactive oxygen species (ROS), suffering effects to their photosynthesis rate, which leads to diminished plant growth [4]. The reactions which generate ROS are counterballanced by increasing internal oxidative processes. Plant cells possess protective mechanisms (enzymatic and non-enzimatic) to remove these polluting agents before a critical cell decay level is reached. Amongst these mechanisms, there are several enzymes – peroxidase, aminoxidase, catalase, superoxide dismutases (SOD) or anti-oxidants – ascorbic acid, vitamin E, β- carotene, glutathione, phenolic compounds, etc., which play an important role in maintaining an adequate redox potential in cells and can protect the cell membranes from active oxidizing agents from exogenous sources [1].

There are many studies concerning air polluting factors on several enzyme systems in plants. These studies made it possible to understand the mechanisms of pollutant toxicity in order to detect injury provoked by vegetation pollution before the emergence of visible phytotoxicity symptoms [5]. Pollutants like SO_2, NO_x, H_2O_2 or O_3 initiate the formation of toxic free radicals.

Plants, the primal producers, are very affected by pollution. Numerous studies have shown that acid rain has serious negative effects on the vegetative organs of plants but it also induces modification the physiology and cellular biochemistry of plants. Biological effects of acid rain on plants are vast and complex and include visible symptoms (chlorosis and necrosis) and non-visible effects, such as diminished photosynthesis, leaf nutrient loss, variations in enzyme activity (especially concerning antioxidant enzymes like catalase and peroxidase). The effect of simulated acid rain (pH 2) on fir seedlings, investigated by [3], reported severe damage, such as visible foliar injury and depression of growth.

Peroxidase is an enzyme involved in numerous physiological mechanisms and it is commonly reported that peroxidase activity is directly involved in plant response to oxidative stressors [8].

The effects of acid rain (pH 2.5-5) on germination and enzymatic activity of peroxidase and catalase were studied by [10] in three different species (*O. sativa, T. aestivum, B. chinensis*). Their results have shown that modifications in enzymatic activity of peroxidase are much more pronounced than in the case of catalase, which demonstrates that peroxidase is much more sensitive to the stress induced by acid rain.

Another study performed by [7], regarding the effects of acid rain on vitamins A, E and C from adult strawberry fruit has shown that the concentration of vitamins in the treated fruit decreases proportionally with the decreasing pH, compared to the control, untreated samples. The authors have found two explanations for these results: i) the increase of reactive oxygen species in plants and fruit as a result of acid rain, leads to the plants using non enzymatic systems in order to resist the induced stress, which in turn leads to a drop in vitamin levels; ii)

different metabolic pathways involved in vitamin biosynthesis suffer inhibition in the presence of an acid environment.

The objectives of this study were: 1) to determine how acid rain with pH 3 and 4, can influence the germination and growth of grass; 2)to determine the chlorophyll contents of grass after exposure to acid rain; 3)to determine the effect of acid rain on enzymatic activities of peroxidase and catalase, which play an important role in protecting plants againts injury of oxidative stressors.

MATERIAL AND METHOD

This study was conducted in the Chemistry and Biochemistry Laboratory of the Faculty of Environmental Protection from the University of Oradea in 2009.

Experimental design

Depending on the pH of used treatment solutions, we have established the following experimental variants:

- V_1 – grass sample treated with rainwater (pH 5.7),
- V_2 – grass sample treated with H_2SO_4 (pH 4.0),
- V_3 – grass sample treated with H_2SO_4 (pH 3.0).

The seeds of grass were planted in sockets filled with lawn soil. For a better distribution of the seeds within the socket, a bottle cap was filled with seeds and distributed in a homogenous fashion (0.8 g seeds/socket). The layer of seeds was then covered with a layer of soil. Each experimental variant was then watered with the corresponding solution, with 20 ml/socket/day, until the germination of the blade of grass. From the moment in which the blades of grass begun to spring, the experimental variants were treated every day (for 10 days) with their corresponding solutions: rainwater (V1), H_2SO_4 with pH 4.0 (V_2), and H_2SO_4 with pH 3.0 (V_3). In this period, we calculated the germination percentage, and also the length of leaves and roots. After 10 days of treatment, we harvested 2 cm of the apical of the blade of grass, and we determined the chlorophyll, and enzymatic activities of peroxidase and catalase.

Measurement of growth

The growth of grass was evaluated by measurements of the blade and root length, during the 10 days from the start of the experiment. Also, in the 5^{th} day, we determined the percentage of germination.

Determination of chlorophyll pigments

The extraction of chlorophyll pigments from the grass was performed with absolute ethanol. Shortly, 50 mg from leaves of each sample (treated or untreated) were collected, and were blended with 5ml ethanol and then cooled at 4^0C for 72 hours. Supernatants were separated and the pigment contents were determined using a spectrophotometer (Shimatzu), at 664nm wavelength for chlorophyll a, at 647 nm for chlorophyll b and at 480 nm for carotenoids.

The data read at the spectrophotometer were mathematically processed using formulae proposed by [6]:

Chlorophyll a (mM) = 12,7 A_{663} – 2.69 A_{645}

Chlorophyll b (mM) = 22.9 A_{645} – 4.68 A_{663}

Carotenoids (mM) = (A_{480} +(0,114 x A_{663}) – (0,638 x A_{645})/ 112,5

where: A_{480} – the value read with a 480 nm filter; A_{645} – the value read with a 645 nm filter; A_{663} – the value read with a 663 nm filter.

Determination of peroxidase and catalase activity

In order to determine the enzymatic activity of peroxidase and catalase in the 10^{th} day after germination, approximately 2 cm were cut from the apical area of the

blade of grass. Determinations were realized in triplicate. Enzymatic activity of peroxidase was measured with a spetrophotometer, the principle of the method being the oxidation of p-phenylenediamine by the peroxidase enzymes present in a vegetal extract, as a result of which a violet-colored solution is obtained. There is a direct proportion between the color intensity of the solution, measured with the spectophotometer at 483 nm wavelenght (UV-VIS Spetrophotometer UVmini-1240, Shimadzu) and the activity of the peroxidase. Peroxidase activity was measured in the blade of grass both in the control lot (experimental variant V_1) and in the samples treated with sulphuric acid of different pH values (3.0 and 4.0).

Enzymatic activity of catalase was determined via the titrimetric method, using permanganometric titration.

RESULTS AND DISCUSSIONS

After determination of the germination percentage (*fig. 1*) and after applying the Student *t* statistic test, the following data was obtained:

- The germination percentage in the control sample was 88%, compared to 78% in V_2 (samples treated with sulphuric acid solution with pH 4.0), respectively 74% in V_3 (samples treated with sulphuric acid solution with pH 3.0);
- From a statistic standpoint, samples treated with solution pH 4.0 registered significant differences ($p<0.05$) compared to the control sample, whereas samples treated with solution pH 3.0 yealded very significant values ($p<0.001$) compared to the control samples.

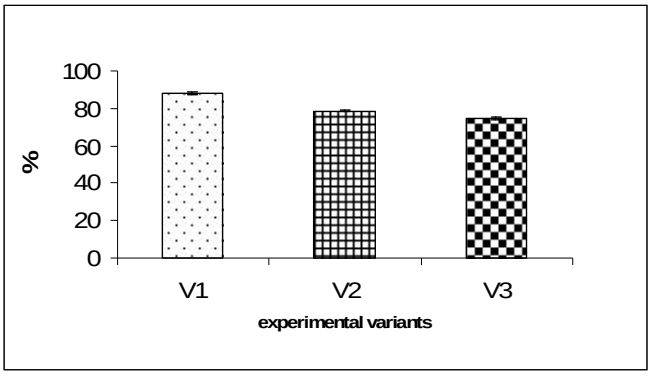

Figure 1 **Determination of the germination percentage**

In order to study the effect of acid rain on the growth in lenght of the blades of grass, we measured their height, both in control samples and treated ones, on the *6, 7, 8 and 9th* day from planting the seeds. Yealded results are shown in *fig. 2.*

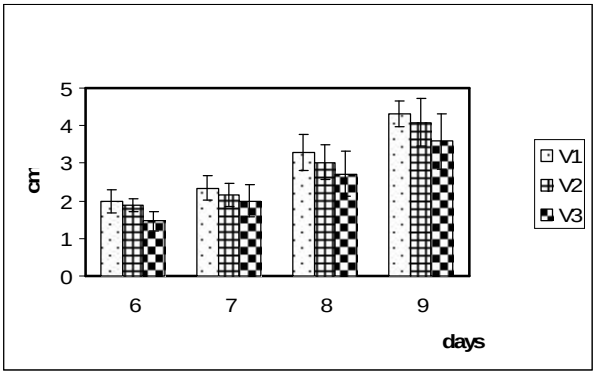

Figure 2 **Biometric measurements of the blade of grass**

The data we obtained (*fig. 2*) demonstrate that the drop in pH levels leads to a decrease in blade lenght. In all 4 days of measurement it is plain that the highest lenght is obtained in the control sample, followed by experimental variant V2 (sample treated with solution pH 4.0). The lowest value was observed in samples treated with solution pH 3.0.

From the moment of germination of the seeds of grass, the samples were watered by spraying dayly with 20 ml rainwater and sulphuric acid solutions with pH 3.0 and 4.0, respectively. In the 10^{th} day from the beginning of the experiment, 2 cm were harvested from the apical area of the blade of grass and measurements were performed by using the extraction and determination of assimilating pigments procedure described in the Materials and Methods. Results are shown in *fig.3.*

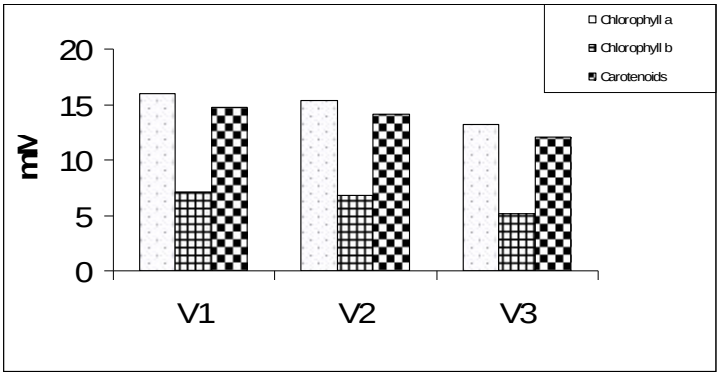

Figure 3 **Assimilating pigment content in blades of grass after treatment with acid rain**

From the green pigment content viewpoint (clorophylle a + clorophylle b), the can be observed that the treatment with sulphuric acid solutions of different pH values, 4.0 and 3.0 respectively, resulted in a decrease, compared to the control. In the case of experimental variant V_2 we observed a decrease in green pigment content of only 3,89%, whereas in the case of V_3 we observed a 20,47% drop in

levels. Concerning the carotenoid pigments content, results resemble those obtained in the case of green pigments (*fig. 3*). A decrease in carotenoid pigment content can be observed in experimental variants V_2 and V_3, compared to the control (V_1), thus, in the case of V_2 the decrease ammounts to 4.1%, whereas in V_3 we registered a decrease by 18.1%.

Of the three experimental variants, the highest enzymatic activity was registered in the case of blades treated with sulphuric acid solution pH 3.0. Considering the control samples' enzymatic activity values (V_1) as 100%, under acid rain pH 4.0 stress we observed an increase of 59.86%, while the blades treated with sulphuric acid solution pH 3.0 suffered an increase of enzymatic activity of 76.16% (*fig. 4A*). This data is similar to that obtained in references [2, 9, 10], the decrease in rain pH leading to an increase in peroxidase enzymatic activity. These results demonstrate the implication of peroxidase enzyme in the defense of the plant against oxidative stress derived from formation of reactive oxygen species.

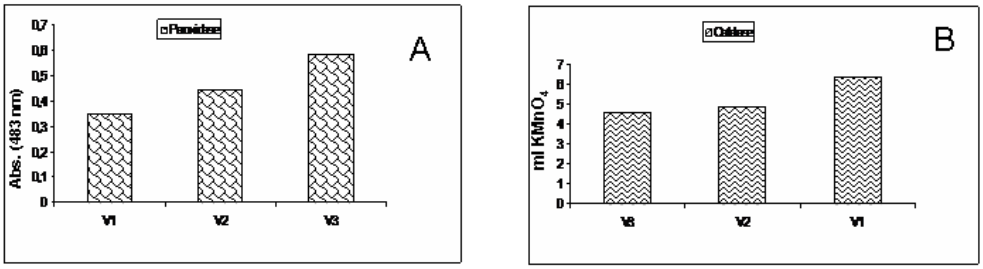

Figure 4 **A. Peroxidase enzymatic activity obtained in all three experimental variants**
B. Catalase enzymatic activity obtained in all three experimantal variants

From results obtained after the determination of catalase activity (*fig. 4, B*) via the titrimetric method, the fact that the control sample presented the highest enzymatic activity can be observed, whereas in the case of V_2 a 23,44% decrease in enzymatic activity can be observed and further more, in the case of V_3 a 28,12% decrease is registered. This data indicates that catalase enzymatic activity is influenced by the environment's pH, being inhibited by low values of pH and is not involved in the removal of reactive oxygen species induced by acid rain stress.

CONCLUSIONS

Following investigations of the effects of acid rain on phisiological and biochemical parameters of the blade of grass, the following general conclusions can be derived:

The germination percentage of the grass seeds is highly influenced by the environmental pH.

Based on the results obtained after biometric analisys, a decrease of solution pH leads to an inhibiting effect on blade growth.

From a green pigment content standpoint (clorophylle a plus clorophylle b), we observed that the treatment with sulphuric acid solutions of different pH values

(4.0 and 3.0 respectively) lead to a decrease in levels, compared to the control sample.

The alteration of pigments involved in photosynthesis is linked to the alteration of the photosynthesis process itself, with drastic consequences on the anabolism metabolism of plants.

Considering the two studies enzymes (peroxidase and catalase) we have shown that peroxidase is the enzyme involved in plant detoxification processes against reactive oxygen species induced by acid rain stress, whereas the enzymatic activity of catalase is influenced by environment pH values, being inhibited by low values, and is not involved in the removal of reactive oxygen species induced by acid rain stress.

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