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# The Effect of Soil Properties on the Transformation of Climbazole and Its Accumulation in Lettuce Plants

Diploma Thesis

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***Declaration***

I declare that this diploma thesis work on the effect of soil properties on the transformation of climbazole and its accumulation in lettuce plants is my own work and all the sources I cited in it are listed in References.

Prague, 2021 . . . . . .

Milena Dulović

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# The effect of soil properties on the transformation of climbazole and its accumulation in lettuce plants

**Summary**

Climbazole is an antifungal agent and as it is used in personal care products, it is continuously entering the sewage system. Wastewater treatment plant (WWTP) processes are not optimized for its removal and, therefore, climbazole finds its way to the environment through WWTP effluents and sewage sludge. Climbazole's toxic effect on organisms, especially aquatic ones, has been well documented in scientific literature. In this study, the effect of climbazole on lettuce plants (*Lactuca sativa* L., variety May King), soil microbial activity and its transformation and accumulation in soil and plant biomass was examined. The study is conceived as a pot experiment with three factors: concentration of climbazole, type of soil, and presence of plants. Three types of soil were used: soil that was treated long-term with farm-yard manure (FYM), soil treated long-term with mineral fertilizers (NPK) and control soil that wasn't fertilized (CON). Climbazole was applied to the soils in concentrations: 0, 0.1, 1, 10, 100 and 1000 μg/kg of dry soil. The effect on plants was assessed through the germination rate of plant seeds and biomass yield, but no significant effects of climbazole in applied concentrations were recorded. The only concentration that had statistically positive effect on germination rate was 1 μg/kg in CON soil, which wasn't replicated in other ones. The possible effect of climbazole on soil microorganisms was assessed through dehydrogenase activity, on which climbazole didn't have a significant effect, except a positive one at 0.1 μg/kg concentration in NPK soil. However, significant stimulating effect of soil fertilization on germination rate, biomass yield and dehydrogenase activity were documented compared to the control. Both FYM and NPK soil affected germination rate and dehydrogenase activity, while FYM soil positively affected biomass yield. All plants wilted in NPK soil and about half of planned number in CON soil. The plants had symptoms of root rot, which indicates climbazole's inability to control soil pathogens in applied concentrations. The highest removal of climbazole was recorded in FYM soil with plants – 83.03 % after 56 days of growth, while the lowest was in NPK soil with plants – 74.28 %. Detected transformation products of climbazole were: hydroxy-climbazole (oxidized form) and climbazole-alcohol found in two diastereomers (reduced form). Climbazole's bioconcentration factor in lettuce's roots was 2.91 and translocation factor was 0.08.

**Keywords:** Pharmaceuticals, personal care products, climbazole, lettuce, degradation, accumulation

# Table of Contents

# 1. Introduction

Pharmaceuticals and personal care products (PPCPs) are emerging contaminants and can be found in sewage water and other parts of the environment, including soil and plants. Many of these compounds are used in large quantities and some studies indicate that a number of them is persistent, bioactive and have potential for bioaccumulation (Mackay and Barnthouse, 2010). A number of studies examining pharmaceutical toxicity and occurrence in the environment has been published, but less attention has been put on assessing potential risks of personal care products when they are released into environment (Brausch and Rand, 2011). Proper monitoring of individual PPCPs and their interactions in the soil – plant relationship have to be intensively studied.

Climbazole is an imidazole antifungal agent, which is widely available, is used in personal care products primarily as an anti-dandruff agent, but also as a preservative in certain cosmetic products. Safety of climbazole for humans is established based on the series of studies and they are well documented by Scientific Committee on Consumer Products (SCCP, 2009). Based on those studies, the use of this antifungal drug in cosmetic products is restricted by the Commission regulation (EU) 2019/698 published by European Commission. Following the human use, climbazole is through the sewage system and WWTP effluents released to the aquatic system, where it can negatively affect organisms, especially after prolonged exposure (Richter et al., 2013). Sewage sludge is a byproduct of WWTP and in pursuit of sustainable agriculture, it can be used as a fertilizer on agricultural land. However, since it often contains compounds that could be harmful to the ecosystem, such as heavy metals and organic pollutants, it requires extensive waste characterization (Lamastra et al., 2018). For this reason, ecotoxicity of climbazole to soils should be studied in more detail.

Despite its high consumption rates and the fact that it is among the frequently found PPCPs in the environment, the knowledge about climbazole's behaviour in the environment is limited. Climbazole's environmental fate received less attention than other antimycotic pharmaceuticals (Zhang et al., 2015).

# 2. Scientific hypothesis and objectives

## 2.1. Hypothesis

It is expected that different soil properties will affect the degradation of climbazole in the soil, as well as its accumulation in the plants.

## 2.2. Objective

The investigation of the effect of different soil climbazole concentrations on lettuce growth and microbial activities in the soil.

The determination of the climbazole degradation in soil during the incubation experiment and its accumulation in the lettuce plants.

# 3. Literature Review

## 3.1. Lettuce (*Lactuca sativa* L.)

Lettuce (*Lactuca sativa* L.) is one of the most important and widely grown crops in the group of leafy vegetables. It is a part of the family Asteraceae (alt. Compositae). Family Asteraceae is thought to be the largest family of plants and it comprises between 23 000 and 30 000 different species (Bayer and Starr, 1998). *L. sativa* is characterized by high genetic diversity resulting from its polyphyletic origin and a complex domestication process (Kesseli et al., 1991), as it is believed that lettuce has been domesticated in the Mediterranean region from the wild species *Lactuca serriola* L. (Harlan, 1992) and has been cultivated for at least 4 500 years (Lindqvist, 1960).

Lettuce is grouped into seven different types which have significant genetic variation in nutritional and phytochemical content (Mou, 2005). Those types are: (1) Crisp-head, Iceberg or Cabbage, (2) Butterhead, (3) Cos, (4) Leaf or Cutting, (5) Latin, (6) Stem or Asparagus, and (7) the Oilseed Group, and they are recognized by International Code of Nomenclature for cultivated plants (Davey et al., 2007).

Lettuce is produced all over the world, but the largest areas dedicated for that purpose are in the USA (91 000 ha) and in Europe (80 000 ha). Significant areas under lettuce can be found also in southeastern Australia, Japan, China, Israel, northern Mexico, Chile, Argentina, Brazil and Peru (Pink and Keane, 1993).

There are a number of characteristics that make this particular species suitable for genetic studies, namely: it has relatively shorth life cycle, plants don't require a lot of space for their growth, it is self-fertile with a high rate of natural self-pollination and it is possible for it to carry out a large number of crosses on one plant (Pink and Keane, 1993).

Seeds of any species have certain optimum values of environmental factors at which germination is at its highest. The requirements for germination of lettuce seeds are: an adequate supply of water, temperature between 1 °C and 25 °C and good soil aeration. These requirements may vary between different lettuce types. However, for most varieties number of germinated seeds drops

if the temperature reaches higher value than 25 °C, and almost entirely stops if the temperature is higher than 30 °C (Borthwick and Robbins, 1928).

### 3.1.1. Nutrient composition

Nutrient content in plants can be affected by numerous factors, such as characteristics of the species and type itself, growing conditions (for example: soil properties, fertilization and irrigation regime, growing season, quality and quantity of light), as well as postharvest handling and storage (Kim et al., 2016).

Kim et al. did a review of individual papers and compared nutritional values and bioactive compounds reported by the authors. Water content of lettuce is quite high and it stands around 95 % (Mou, 2005; USDA, 2015). Although low in fat, lettuce contains polyunsaturated fatty acids and its content in different types of lettuce ranges from 0.7 to 1.6 mg/g FW (14-32 mg/g DW). Additionally, lettuce is low in saturated fatty acids (USDA, 2015). As far as carbohydrates are concerned, lettuce provides carbohydrates with lower digestibility, such as sugar alcohols and dietary fiber. Total dietary fiber content in different types of this vegetable ranges from 9 to 21 mg/g FW or 180-420 mg/g of DW (USDA, 2015). Lettuce is not a rich source of protein, but it contains various non-caloric nutrients like minerals and vitamins (Kim et al., 2016).

Content of minerals in the tissue of lettuce plant can vary greatly depending on the type. Reported values from studies can be significantly different, which could be the consequence of different factors, namely difference in mineral composition of the soil that is used in individual studies (Pinto et al., 2014).

In the following Table 1 are given optimum nutrient ranges for lettuce leaves reported by a number of different authors. The information was compiled by Hartz and Johnstone in 2007. Concentrations are given in g/kg and mg/kg of dry weight units. Contents are shown together for iceberg and romaine lettuce.

*Table 1 Comparison of macronutrient and micronutrient optimum ranges for lettuce leaves published by different authors (Hartz and Johnstone, 2007)*

| | Early heading | | | Preharvest | | | |
|---|---|---|---|---|---|---|---|
| Nutrients | Hartz and Johnstone (2007) | Ludwick (2002) | Hochmuth et al. (1991) | Hartz and Johnstone (2007) | Ludwick (2002) | Jones et al. (1991) | Hochmuth et al. (1991) |
| N [g/kg] | 43-56 | 30-40 | 40-60 | 33-48 | 25-30 | 35-50 | 20-45 |
| P [g/kg] | 4.5-7.5 | 4.0-8.5 | 3.5-8.0 | 3.5-7.5 | 3.5-8.0 | 4.5-8.0 | 2.5-6.0 |
| K [g/kg] | 33-64 | 30-40 | 50-70 | 29-78 | 30-50 | 55-90 | 25-60 |
| Ca [g/kg] | 4.5-7.5 | 14-30 | 10-30 | 6-11 | 14-30 | 15-28 | 14-30 |
| Mg [g/kg] | 2.5-4.0 | - | 2.5-5.0 | 2.5-4.5 | - | 3.6-8.0 | 2.5-7.0 |
| S [g/kg] | 2.5-3.5 | - | >3 | 2.0-3.5 | - | - | >3.0 |
| B [mg/kg] | 19-31 | - | 15-45 | 24-36 | - | 23-60 | 15-45 |
| Zn [mg/kg] | 21-75 | - | 20-50 | 25-73 | - | 20-250 | 20-50 |
| Mn [mg/kg] | 37-73 | - | 15-40 | 45-74 | - | 11-250 | 15-40 |
| Fe [mg/kg] | 86-232 | - | 50-150 | 115-257 | - | 40-50 | 50-150 |
| Cu [mg/kg] | 5.6-8.2 | - | 5-10 | 5.0-8.6 | - | 5-25 | 5-10 |

Additionally, in the following Table 2 are reported actual contents of nutrients in lettuce leaves found in studies compiled by Kim et al. in 2016. Contents are shown together for different types of lettuce.

*Table 2 Reported contents of nutrients found in lettuce reported by different authors (Kim et al., 2016)*

| | USDA (2015) | | Koudela and Petříková (2008) | | Baslam (2013) | | Kawashima and Soares (2003) | |
|---|---|---|---|---|---|---|---|---|
| Nutrients | mg/g FW | mg/g DW | mg/g FW | mg/g DW | mg/g FW | mg/g DW | mg/g FW | mg/g DW |
| P | 0.2-0.3 | 4-6 | - | - | 0.05-0.13 | 0.9-2.5 | - | - |
| K | 1.4-3.2 | 48-72 | 2.4-6.5 | 48-130 | 2.7-4.4 | 53.7-87.6 | 2.4-3.2 | 48-72 |
| Ca | 0.2-0.4 | 4-8 | 0.2-0.8 | 4-16 | 0.2-1.0 | 4.1-20.6 | - | - |
| Na | 0.05-0.3 | 1.0-6.0 | 0.04-2.2 | 0.8-44 | 0.04-0.21 | 0.8-4.1 | 0.05 | 1.0 |
| Mg | 0.07-0.14 | 1.4-2.8 | - | - | 0.11-0.48 | 2.1-9.5 | 0.2 | 4 |
| | µg/g FW | µg/g DW | | | µg/g FW | µg/g DW | µg/g FW | µg/g DW |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Fe | 4.1-12.4 | 82-248 | - | - | 3.0-5.6 | 59.9-112.4 | 5 | 100 |
| Zn | 1.5-2.3 | 30-46 | - | - | 1.8-2.5 | 35.7-50.5 | 3.3 | 66 |

## 3.2. Soil properties and the relationship with the behaviour of pollutants

When it comes to evaluating potential risk of any pollutant, it is not important only to know the properties of that pollutant, but it is also necessary to know the environmental factors, such as soil properties, climatic conditions and characteristics of biocenosis on that particular area.

### 3.2.1. Importance of soil solution

Soil solution is the liquid phase of the soil and it is in quasi-equilibrium with the solid phase. Growth and yield of plants is tightly connected to the concentrations of nutrients in the soil solution. The reason for this is that these concentrations have a significant role in the mechanisms of nutrient supply and uptake by the plant roots (Smethurst, 2000). Soil solution is important because terrestrial organisms come into contact with chemical pollutants mainly through soil solution. Also, soil liquid phase is the most responsible for the transport of pollutants in the soil, to the surface water or groundwater (García-Valcárcel and Tadeo, 2012).

### 3.2.2. Factors that affect the behaviour of pollutants in soil

The behavior of organic pollutants in the soil, referring to their mobility, bioavailability and their transfer to water and atmosphere depends on physical, chemical and biological processes that include: sorption-desorption, volatilization, chemical and biological degradation, uptake by plants, run-off and leaching (García-Valcárcel and Tadeo, 2012).

When it comes to factors that have an influence on degradation rate of pesticide residues, they include: stability of the compound, frequency and rate of pesticide application, weather conditions (sunlight, temperature, humidity and wind), quantity and quality of micro-organisms present, soil characteristics, pH of soil and water, characteristics of plant species (Fantke and Juraske, 2013). Generally speaking, pesticide dissipation in plants follow first-order kinetics, while there are some exceptions (Fantke and Juraske, 2013).

## 3.3. Azole antifungals

Azole antifungals are divided in two groups: imidazoles and triazoles, the difference between them being the number of nitrogen atoms in the azole ring: imidazoles have two and triazoles have three N atoms (Maertens, 2004). In the Figure 1 are given chemical structures of selected 12 imidazoles and triazoles (Campestre et al., 2017).

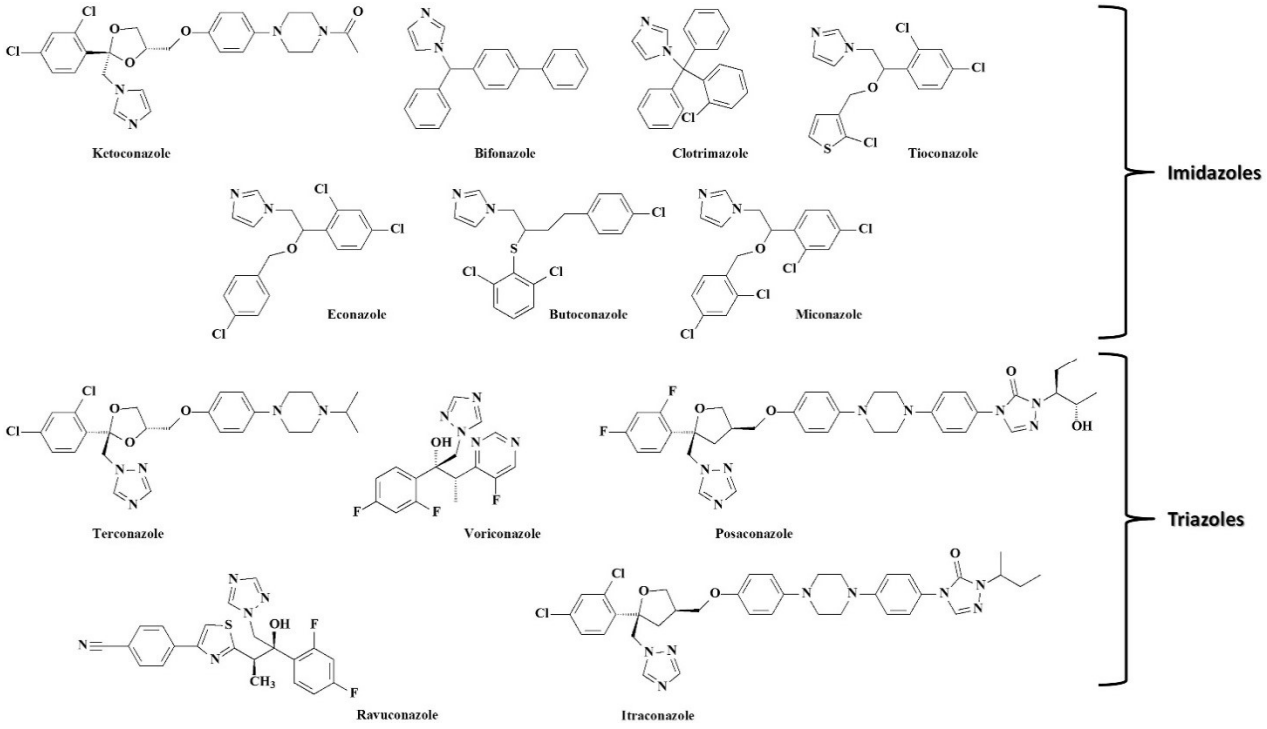



*Figure 1 Examples of imidazoles and triazoles (Campestre et al. 2017)*

### 3.3.1. The use of azoles as pharmaceuticals

The research of antifungal activity of azole compounds began in 1940s with imidazoles, when Wooley described benzimidazole (Wooley, 1944). It continued in 1950s and 1960s with the introduction of topical compounds: chlormidazole, clotrimazole, miconazole and econazole (Fromtling, 1988) for the use in medicine. These pharmaceuticals had a number of unfavorable or unacceptable side effects when used for treatment in humans, which caused miconazole to even be withdrawn from the use (Maertens, 2004). Ketoconazole is imidazole that was a standard treatment for systemic fungal infections since its approval by the FDA in 1981 (Heeres et al.,

1979), until the approval of triazole antifungal agent – fluconazole in 1990 (Maertens, 2004). Fluconazole was deemed more effective and safer option for treatment by a number of studies (Brammer et al., 1990; Arndt et al., 1988; Humphrey et al., 1985). 70-80 % of fluconazole is excreted from the body in the urine in unchanged form (Humphrey et al., 1985), which is one of the pathways for it to end up in the environment. That is one of the reasons why pharmaceuticals in general and their effects should be studied more broadly.

### 3.3.2. The use of azoles as fungicides in agriculture

Apart from being used as treatments for humans, from 1970s, azoles have been used also as agricultural fungicides (Maertens, 2004) and they represent one-third of agrochemical fungicides that have been in use (Lamb et al., 1999). As a matter of fact, for the purpose of plant protection globally, thousands of tons of azole antifungals have been sold every year. In the instructions for the use given by manufacturers it is indicated that about 100 g/ha should be used in the field, which means that 1 $m^2$ of plant surface gets approximately 10 mg of azoles, sometimes multiple times per year if needed.

The work on N-substituted imidazoles and triazoles for the use against pathogenic fungi and yeast began in the middle of 1960s. 1,2,4-triazole derivates were found to be superior to the corresponding imidazoles for the control of phytopathogenic fungi. Consequently, fluotrimazole, as a non-systemic fungicide, was developed for the control of powdery mildew on cereals and fruits. Together with fluotrimazole, miconazole and imazalil are part of the first generation of azole fungicides that were introduced to the market (Büchel, 1986). In 2013, imazalil was the most commonly used fungicide for controlling postharvest fungal pathogens in citrus (Altieri et al., 2013). Triadimefon is one of the first azole derivates developed as systemic fungicide in plants. It is particularly good against powdery mildew and rust fungi in cereals and fruit. Triadimefon is climbazole's triazole analogue (Büchel, 1986).

### 3.3.3. Characteristics of azoles

Some of the advantages that azoles have over other types of antifungal agents are that they are inexpensive, stable and that they have broad spectrum of antifungal activity. They are effective against numerous diseases in plants caused by fungi, for example: mildews, rusts and leaf spots

in various species. Moreover, they can be used in both: prevention, as well as treatment, thanks to their systemic action against fungi. Many azoles can remain active in soil and water with only slight changes in their chemical structure over the period of several months (Hof, 2001). Apart from being found in soil and water, azoles have been traced in various food items, for instance, grapes (Cairns et al., 1989), strawberry (Yamazaki and Ninomiya, 1998) and peppermint (Garland et al., 1999) in low concentrations of up to 0.8 mg/kg. In some instances, high levels of azole residues have been detected in carrots and apples, with concentrations of up to 2.16 mg/kg in a single apple (Hamey and Harris, 1999).

### 3.3.4. Mechanism of action

Azoles work as antifungal agents mainly by inhibiting fungal growth and replication (Koltin and Hitchcock, 1997), which comes as a result of depletion of ergosterol and consequent accumulation of 14-α-methylated precursors, with some secondary effects as well. Ergosterol has a function in fungal membranes. Its depletion alters fluidity of the membrane, and the activity of several membrane-bound enzymes, such as those that have a role in nutrient transport and chitin synthesis (Georgopapadakou and Walsh, 1996). Mechanism of action of azoles is prevention of the synthesis of ergosterol, the major sterol component of fungal plasma membranes, through inhibition of the fungal cytochrome P450-dependent enzyme CYP51 – lanosterol 14-α-demethylase (Como and Dismukes, 1994).

There are two problems when the use of azoles as antifungal agents is concerned, and those are the evolving spectrum of fungal pathogens and the development of azole-resistance (Maertens, 2004). Some fungi have ingrained resistance to such compounds due to ergosterol not being required for their cell wall and membrane formation (Hof, 2001).

### 3.3.5. Safety of azoles

Azoles are deemed safe for human use because they, at therapeutic concentrations, bind more tightly to fungal cytochrome P450-dependent enzyme lanosterol 14-α-demethylase than to mammalian enzyme (Sheehan et al, 1999). Apart from the inhibition of CYP51, azoles also inhibit CYP19 – aromatase, which may cause endocrine disruption in vertebrates through the reduction of estrogen biosynthesis from androgens (Trösken, 2009). Another concern for human health can

be possible influence of azoles on natural fungal flora in humans (Hof, 2001), although it has been reported that azoles don't reach toxic levels through dietary consumption (Hamey and Harris, 1999).

### 3.3.6. Dissipation of azoles from the soil

Fantke and Juraske concluded after their review of selection of available papers on the topic that the geometric mean of reported dissipation half-life values of triazoles is 5.1 days, or in the range from 2 to 12.8 days (Fantke and Juraske, 2013).

In 2013, the study was conducted in China by Chen et al., where the concentrations of climbazole and two other imidazoles (clotrimazole and miconazole) were studied in soils treated one time with biosolids and soils with repetitive treatments. Biosolid in this case was dewatered sludge obtained from wastewater treatment plant in Beijing. The concentration of climbazole, clotrimazole and miconazole in biosolids were as follows: climbazole 165 +/- 6 ng/g, clotrimazole 492 +/- 21 ng/g and for miconazole 427 +/- 25 ng/g. Among other things, it is found that during storage there was no significant loss of these azoles. In samples from the soils with one treatment, established concentrations were for climbazole between 0.6 and 4.3 ng/g, for clotrimazole 2.2 and 8.3 ng/g, and lastly for miconazole between 4.6 and 12.5 ng/g. Soils that had multiple treatments of biosolids, in line with that contained higher concentrations of all three azoles. Namely, determined concentrations of climbazole were from 3.0 to 17.3 ng/g, of clotrimazole from 15.3 to 41.0 ng/g, and for miconazole found concentrations were the highest: ranging between 27.0 and 64.5 ng/g. The concentrations of these three biocides were found to be related to organic carbon content, but not to the clay content (Chen et al., 2013).

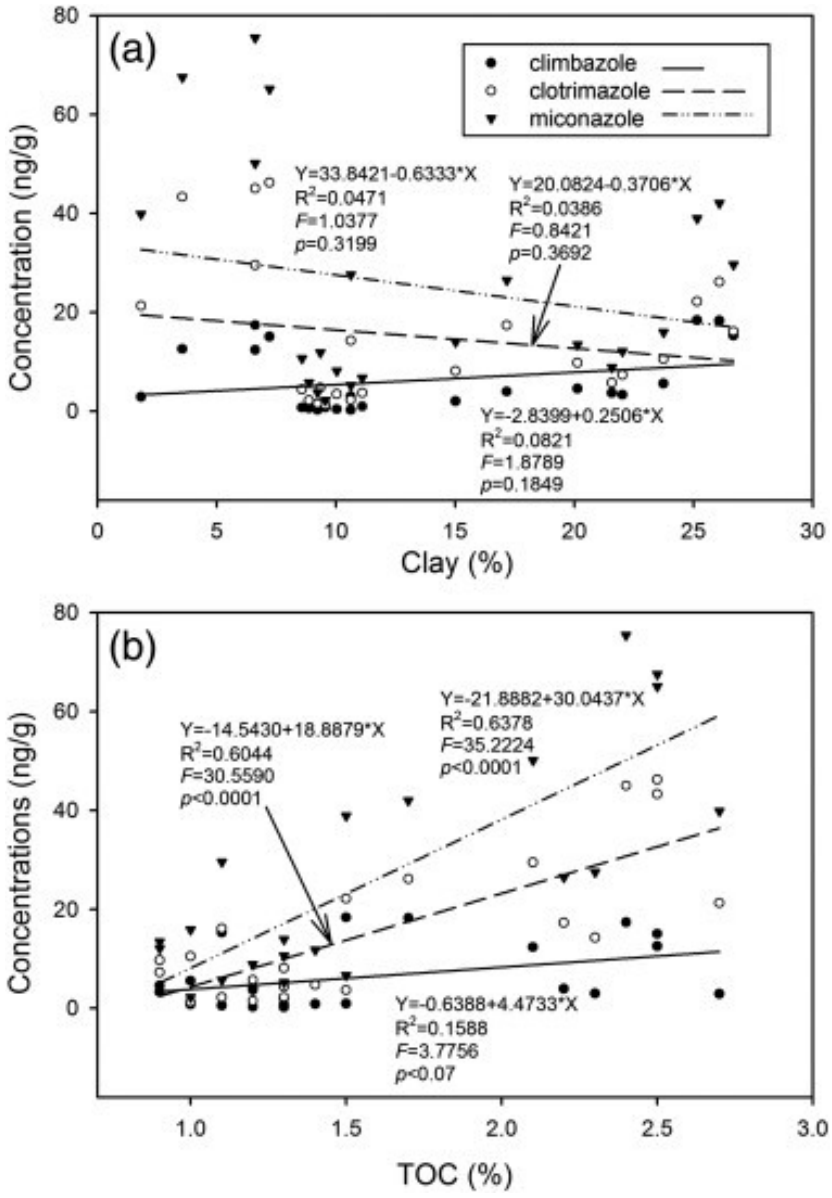



*Figure 2 Correlation analysis between concentrations of azoles and clay and total organic carbon (TOC) content in soil (Chen et al., 2013)*

The dissipation of the three azoles from the soil was followed for a year. It was concluded that climbazole didn't show any significant dissipation – the average concentration fell from 35.3 ng/g in March to 33.9 ng/g in October of the same year. For the other two, the data showed very slow dissipation trend (Chen et al., 2013).

Sorption of some organic compounds, other than the three imidazoles from the previously mentioned study, is also found to be closely corelated with the organic carbon content, such as herbicide atrazine in the study conducted by Moorman et al. in 2001. In this research, adsorption

($K_d$) values were in positive correlation with organic carbon content in the soil. However, soil texture and pH values didn't prove to have any influence on atrazine adsorption (Moorman et al., 2001).

Degradation of difenoconazole and propiconazole in soil was followed in the study by Zhang et al. in 2015. In contrast with the one conducted by Chen et al., these two azoles (triazoles) showed fairly fast degradation rate. Half-lives of difenoconazole in soil were between 4.9 and 5.8 days and for propiconazole they were from 6.1 to 8.4 days. Both azoles showed the degradation rate of more than 95 %, 28 days after the final application (Zhang et al., 2015).

In the study conducted by García-Valcárcel and Tadeo it was found that clotrimazole (imidazole) is characterized by very slow desorption, which supports findings of Chen et al. Namely, desorption from the soil continued even after 26 days without reaching an equilibrium. On the other side, almost half of the adsorbed fluconazole (triazole) was desorbed after only 3 days and a constant desorption rate was reached after 12 days with almost 90 % of initial content of fluconazole being desorbed (García-Valcárcel and Tadeo, 2012).

The observed desorption rate of fluconazole was similar to the ones reported by Singh for some other triazole fungicides: hexaconazole, triadimefon and penconazole. In that study, the amount of triazoles retained in soil after one washing was 30 – 60 %. Additionally, the high correlation between adsorption coefficient ($K_f$) and organic carbon was observed and it is determined that it is the most important parameter affecting the adsorption of triazoles. pH value didn't have high correlation. When it comes to silt and clay content, it was found that clay content had an important role in the adsorption of penconazole. Moreover, the combination of organic carbon and clay content was even better indicator of adsorption of penconazole (98 %). Similarly, the combination of organic carbon and silt content proved to be a good indicator of adsorption of triadimefon and hexaconazole with the contribution to the variability of 79 % and 95 % (Singh, 2002).

The intensity of volatilization as a way of dissipation depends on values of vapor pressure of a compound. Clotrimazole and fluconazole have low vapor pressure and consequently low

volatilization (García-Valcárcel and Tadeo, 2012). There is no available information for vapor pressure of climbazole.

The effect of soil moisture on dissipation of azoles was investigated in numerous studies. Data shows that dissipation rate of organic compounds is higher in soils with higher moisture content than in drier soils. That can be explained by the connection of soil moisture with the desorption of organic compounds from the soil – desorption is higher if the soil moisture content is higher (Lennartz and Louchart, 2007). Furthermore, soil moisture can intensify abiotic degradation, as well as biological degradation through the increase of biological activity in wetter environment (García-Valcárcel and Tadeo, 2012). Leaching also can have a significant role.

### 3.3.7. Uptake of azoles by plants

Uptake of azoles by plants and their subsequent accumulation was found to be mainly affected by the physicochemical properties of azoles. Hydrophobicity of the azole was observed to be the factor which has a strong effect on its translocation from nutrient solution to the aerial part of a plant. Different azoles show tendency to accumulate in different parts of a plant, which is in line with their $K_{ow}$ values. For example: fluconazole, which has lower $K_{ow}$ (log $K_{ow}$ = 0.25) and is more hydrophilic, is readily transported and tends to accumulate in leaves and stems of *Valerianella locusta* L. (lamb's lettuce); whereas the more lipophilic compound – clotrimazole, with the log $K_{ow}$ value of 4.1, was found in the highest concentration in roots of *V. Locusta* which indicates low translocation (García-Valcárcel et al., 2016).

Matrix solid phase dispersion method was used for the extraction of azole fungicides from plants in the study carried out in 2016 by García-Valcárcel et al. in which they analyzed uptake of commonly found azoles in the water, namely: clotrimazole, fluconazole and propiconazole by *Valerianella locusta* L. Plants were being grown in the hydroponic system where azoles were being added to the nutrient solution. Clotrimazole was added to the level of 25 ng/ml and 50 ng/ml, and fluconazole and propiconazole to the level of 100 ng/ml and 200 ng/ml. The reason for the lesser concentration of clotrimazole is its lower solubility in water (García-Valcárcel et al., 2016).

Symptoms of phytotoxicity were observed on the plants that were treated with clotrimazole, those symptoms being bleaching, necrotic spots, leaves deformation and mosted importantly: stunted growth. Lesser growth of the roots was espetially noticable, with the weight of them being 70-78 % lower that the control. On the contrary, plants exposed to fluconazole and propiconazole expirienced a slight boost in the growth in the beggining, which equilized with the control by the end of the experiment (García-Valcárcel et al., 2016).

The behavior of fluconazole, propiconazole and clotrimazole related to their accumulation in leaves and roots of lamb's lettuce differs. The point in time when the concentration in leaves is the highest is not the same for the examined azoles: for fluconazole and propiconazole that point is around 14th day of exposure and for clotrimazole, it is in the beggining of the experiment. After reaching the maximum concentration, values for propiconazole and clotrimazole droped, while that of fluconazole remained the same. The same trend was observed in the roots as well. The drop of clotrimazole concentration can be partialy explained by necrosis of the roots which it caused and, the drop of propiconazole could be explained by its degradation in plants and short half-life in wheat straws that has been noticed in some of the previous studies (Garland et al., 2004; Zhang et al., 2015). In the study conducted by Zhang et al., both propiconazole and difenoconazole had relatively short half-life in wheat straw of 5.1 to 6.9 days and 3.6 to 5.5 days, respectively. Half-life values were observed to be shorter in wheat straw than in the soil ilustrating faster degradation (Zhang et al., 2015). Additionaly, it is reported that the detected concentration of clotrimazole in the leaves of lamb's lettuce wasn't influenced by its initial concentration in the nutrient solution, which wasn't the case for the two others, where positive corelation was detected (García-Valcárcel et al., 2016).

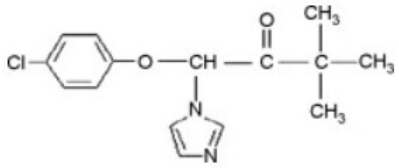
In different plant species, diffetent rates of degradation of the same azole were observed. The example of this can be half-life values of difenoconazole, as reported by different authors in their studies. The values were ranging from 1.4 to 2.6 days in rice plants (Wang et al., 2012), in Chinese cabbage half-life was reported to be 6.6 – 7.8 days (Wang et al., 2008), in chili fruit 4.7 – 8.1 days (Mukhopadhyay et al., 2011) and lastly the longest half-life values were reported for apple, where they were ranging from 26.2 to 81.7 days (Guo et al., 2010).

#### *3.3.7.1. Transport of azoles in plants*

For the evaluation of the transport of azoles from nutrient solution to different parts of the plant it is useful to determine the root concentration factor and the transfer factor (García-Valcárcel et al., 2016). The root concentration factor (RCF) expresses the capability of roots to accumulate contaminants which comes from the direct contact between roots and aqueous environment and it is calculated as a ratio of concentration of the contaminant roots and the concentration of it in nutrient solution (Kang et al., 2010). Transfer factor (TF) is an index that describes the transmission of contaminants from soil to plant tissues. It is calculated as a ratio of concentration of a contaminant in plant tissue to the concentration of it in the soil (Mirecki et al., 2015).

## 3.4. Climbazole

Chemical name of climbazole is 1-(4-chlorophenoxy)-1-(imidazole-1-yl) 3,3-dimethylbutan-2-one. Empirical formula of it is $C_{15}H_{17}ClN_2O_2$ and structural formula is as follows:




Same as other imidazoles, climbazole has a heterocyclic planar ring and occurs in 2 enantiomers: R-Climbazole (figure 3) and S-Climbazole (figure 4).

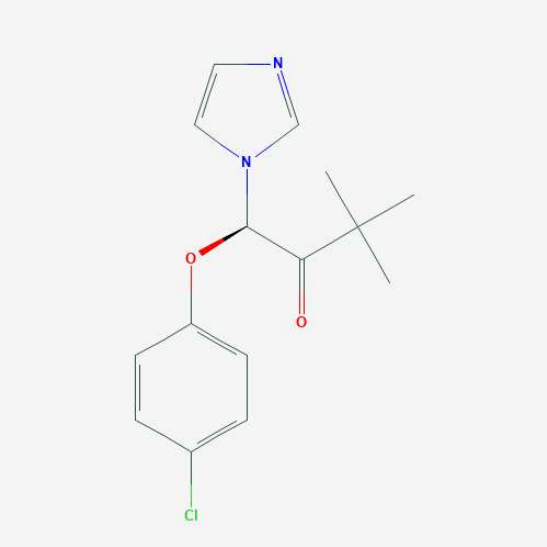



*Figure 3 R-Climbazole*

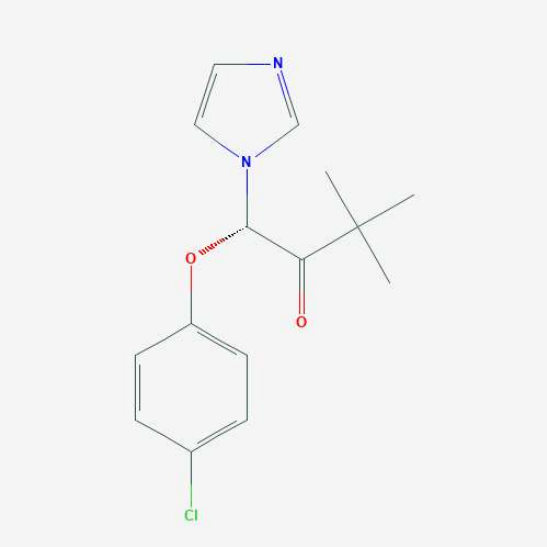



*Figure 4 S-Climbazole*

Molecular weight of climbazole is 292.76 g/mol.

The value of log $K_{ow}$ of this molecule is 3.76. In the situation where the substance has a higher concentration in sediment than in the surface water, high value of $K_{ow}$ can partially explain such occurrence (Creusot et al., 2020).

The value of $pK_a$ when it refers to organic chemicals can strongly affect their toxicokinetics and sometimes also toxicodynamics (Neuwoehner and Escher, 2011). $pK_a$ value of climbazole is 7,50 (Liu et al. 2016), which means that the molecule is a weak base.

### 3.4.1. Use of climbazole

Climbazole is an imidazole antifungal agent, which is widely available and is used in personal care products primarily as anti-dandruff agent, but also as a preservative in certain cosmetic products. Dandruff is a common scalp disorder affecting almost half of the population (Elewski, 2005). The reason why an antifungal agent is successfully used as a treatment for dandruff is that lipophilic yeast belonging to the genus *Malassezia* plays a significant role in its occurrence (Saint-Leger et al., 1988): the levels of *Malassezia* increase by 1.5 to 2 times compared to its normal level when there is occurrence of dandruff (Ranganathan and Mukhopadhyay, 2010).

### 3.4.2. Solubility

Climbazole is not soluble in water (about 5.5 ppm) at the temperature of 21°C. In other solvents, also at the room temperature, it is soluble to varying degree, namely: in 2-(2-ethoxy-ethoxy) ethanol its solubility is 30%, in phenoxyethanol – 45%, in denaturated 96% vol. ethanol – 50%, in benzyl alcohol – 55%, in parfum oils its solubility is up to 50%, while in isopropanol, cyclohexanone, polyethylene glycol 200 and 400 and DMSO it is completely soluble (SCCP, 2009).

The data compiled by Paz-Alvarez et al. shows that climbazole has high solubility in propylene glycol and Transcutol P at the temperature of 32+/-1 °C, but not so much in octyl salicylate despite the lipophilic nature of the molecule (Paz-Alvarez et al., 2018). The solubility of climbazole in alcohols increases as the temperature increases and as the alcohol chain length decreases (Kim et al., 2014).

### 3.4.3. Degradation of climbazole

#### *3.4.3.1. Photodegradation*

In the experiment done by Castro et al: ultrapure water model solutions, and wastewater samples were exposed to UV radiation with the goal of removing climbazole. It is found that the efficiency of the removal depends on the intensity of UV radiation. Half-life ($t_{1/2}$) values of climbazole with the usage of two lamps in the photoreactor were 1.7 minutes for ultrapure water and 2.2 minutes for wastewater. With one lamp, $t_{1/2}$ value for ultrapure water was 3.95 minutes and for wastewater – 3.63 minutes. It is found that climbazole was relatively labile under the experimental conditions. The main transformation routes of climbazole are dechlorination, hydroxylation and intramolecular cyclization (Castro et al., 2016).

In another similar experiment done by Lui et al., climbazole was almost completely degraded under UV – 225-425 nm after the 4-minute exposure to it. Comparatively, under UV – 254 almost complete degradation happened after 60 minutes with $t_{1/2}$ value of 9.78 minutes. The pH value didn't have significant influence on the rate of photodegradation. In the same study, it is found that transformation products have lesser ecotoxicity than climbazole (Lui et al., 2016). UV treatment could be a good solution for removing of climbazole from wastewaters.

In Table 3 are listed climbazole transformation products that are detected in spiked ultrapure water solutions exposed to 254 nm light. UV-exposure experiments revealed a significant number of them (Castro et al., 2016).

*Table 3. Database of transformation products (TPs) generated from climbazole under UV exposure of spiked ultrapure water samples (Castro et al., 2016)*

| Precursor species | Transformation product code (TP code) | Retention time (min) | $[M+H]^+$ | Proposed empirical formula $[M+H]^+$ | Mass error (ppm) | Double bond equivalents (DBE) |
|---|---|---|---|---|---|---|
| Climbazole (CBZ) | / | 25,23 | 293,1048 | $C_{15}H_{18}ClN_2O_2$ | -1,0 | 8 |
| | CBZ-TP1 | 22,44 | 275,1389 | $C_{15}H_{19}N_2O_3$ | -0,4 | 8 |
| | CBZ-TP2 | 20,80 | 259,1436 | $C_{15}H_{19}N_2O_2$ | -1,9 | 8 |
| | CBZ-TP3 | 12,30 | 167,1168 | $C_9H_{15}N_2O$ | -6,6 | 4 |
| | CBZ-TP4 | / | | 4-chlorophenol | / | / |
| | CBZ-TP5 | 22,83 | 293,1050 | $C_{15}H_{18}ClN_2O_2$ | -0,3 | 8 |
| | CBZ-TP6 | 21,62 | 293,1049 | $C_{15}H_{18}ClN_2O_2$ | -0,6 | 8 |
| | CBZ-TP7 | 22,25 | 293,1051 | $C_{15}H_{18}ClN_2O_2$ | -0,1 | 8 |
| | CBZ-TPs 8, 9 | 20,70, 21,40 | 293,1052 | $C_{15}H_{18}ClN_2O_2$ | 0,3 | 8 |
| | CBZ-TP10 | 22,74 | 259,1427 | $C_{15}H_{19}N_2O_2$ | -5,4 | 8 |
| | CBZ-TP11 | 18,87 | 259,1434 | $C_{15}H_{19}N_2O_2$ | -2,7 | 8 |
| | CBZ-TP12 | 19,53 | 259,1436 | $C_{15}H_{19}N_2O_2$ | -1,9 | 8 |
| | CBZ-TP13 | 19,41 | 275,1388 | $C_{15}H_{19}N_2O_3$ | -0,7 | 8 |

#### *3.4.3.2. Thermodegradation*

In the study conducted by Coiffard et al. in 1999, it is found that climbazole is thermostable. They examined aqueous solutions at temperatures of 50 °C, 70 °C and 90 °C. The degradation followed

first order kinetics and degradation rate constant and half-life values were as follows: for the temperature of 50 °C, degradation rate constant was 3.03 x $10^{-3}$ and half-life value was 228.7 days. For the temperature of 70 °C, the values were 4.94 x $10^{-3}$ and 140.6 days, lastly for the temperature of 90 °C the values were 7.62 x $10^{-3}$ and 91.1 days (Coiffard et al., 1999).

#### *3.4.3.3. Biodegradation*

Manasfi et al. in 2020 ran an experiment in which the biodegradation of climbazole and two antibiotics by *Trichoderma harzianum* and *Trichoderma asperellum* was analyzed. *Trichoderma spp.* have antibiosis ability and mycoparasitism ability thanks to the fact that they produce a large mixture of antifungal enzymes (Harman, 2006). Climbazole was chosen because of its common occurrence in wastewater effluents (Richter et al. 2013) and its low biodegradability in activated sludge processes. Concentrations of analyzed compounds were measured at the beginning and at the end of the experiment and the difference between them was attributed mainly to the biodegradation processes by the authors. There was a significant difference in the level of achieved degradation of climbazole between the two strains that were used. *T. harzianum* achieved almost complete removal of 91 %, compared to only a slight biodegradation of 14 % achieved by *T. asperellum* after 13 days of incubation. Additionally, there are 3 transformation products of climbazole that were identified in this study, two of them being climbazole-alcohol and 4-chlorophenol. These transformation products were also being successfully biotransformed by *T. harzianum*, same as climbazole itself. The main transformation pathway, as reported by authors, was reduction of the carbonyl moiety, which is followed by hydroxylation pathway. O-dealkylation reactions with the resulting formation of 4-chlorophenol were also observed (Manasfi, 2020).

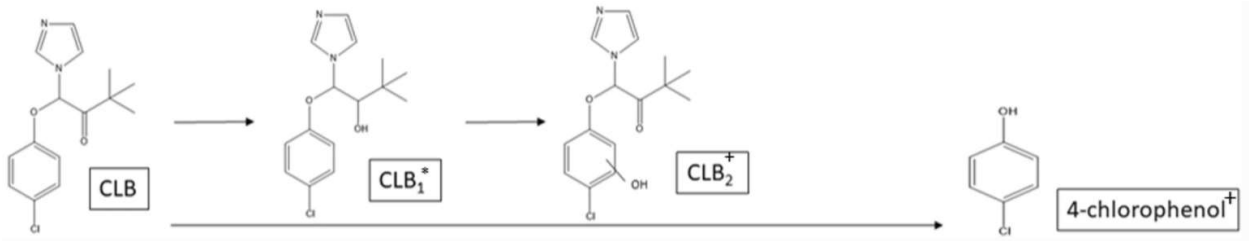



*Figure 5 Schematic display of proposed transformation pathway of climbazole by Manasfi, 2020. (* - confirmed by a standard; + - new, proposed in the study)*

### 3.4.4. Toxicity of climbazole

A series of studies were performed to establish safety of this azole antifungal drug for humans and they are well documented by Scientific Committee on Consumer Products (SCCP, 2009). These studies include acute, subacute, subchronic and chronic toxicity assessments after repeated administration, skin irritation and sensitization, eye irritation, dermal and percutaneous absorption, reproductive and developmental toxicity, genotoxicity and mutagenicity (SCCP, 2009).

#### *3.4.4.1. Climbazole's way to the environment*

Climbazole is mostly used in personal care products, which are widely used by humans. Personal care products include soaps, hair and skin care products, toothpastes, perfumes and sunscreens, and they are widely used by humans and most of them, unlike pharmaceuticals, have little biological activity. Therefore, most of them enter the sewage system unaltered (Daughton and Ternes, 1999). As climbazole is mainly used in personal care products, it is entering sewage system as well. Wastewater treatment plant (WWTP) processes are not always efficient in removing them, therefore personal care products can be found in wastewater treatment plants' effluents and enter the environment that way. Additionally, digested sludge is used as fertilizer on agricultural land (Ternes et al., 2004). The use of sludge for agricultural purposes in EU is mainly regulated by the outdated Council Directive 86/278/EEC, in which there are set limits for heavy metals in sludge. However, several European countries have adopted additional, more strict regulations for heavy metals, synthetic organic compounds and microbial contamination. Limit values for certain synthetic organic compounds such as halogenated organic compounds, phthalates, nonylphenol, PAHs and PCBs were included in the third draft of the Working Document on Sludge (European Commission 2000a) (García-Valcárcel and Tadeo, 2012; Hudcová et al., 2019).

When antimycotic drugs arrive at sewage treatment plants, they are distributed between liquid and solid (sludge) phases based on their polarity. The efficacy of their removal is poor during primary and biological treatments. The behaviour of climbazole during oxidative treatments hasn't been explored. The presence of it in liquid and solid phases at Sewage treatment plants

can be explained by its limited biodegradability and its medium polarity of log $K_{ow}$ 3.49 (Castro, 2016). The aqueous removal rate of climbazole in WWTPs is around 70 % (Cai et al., 2021). It was reported by Zhang et al. in 2015 that the estimated usage of climbazole in China is 345 tons and that 245 t could be discharged into the environment after wastewater treatment. According to the multimedia fate modeling, approximately 93 % of climbazole is discharged into water compartment and 7 % is discharged into the soil compartment (Zhang et al., 2015). The main technology for the removal of organic pollutants in WWTPs is activated sludge process which uses anaerobic, anoxic and aerobic treatment processes. In the study conducted in 2021 by Cai et al. it was found that climbazole can be degraded in the aerobic activated sludge. However, there are few other reports of degradation mechanism of azole fungicides in activated sludge process and it is unknown if their removal is due to the adsorption by sludge or degradation by microorganisms (Cai et al., 2021).

Although personal care products are introduced to surface waters at very low concentration (ng/l), they can still pose a problem to the aquatic organisms if they are exposed to it long-term and as complex mixtures (Boxall et al., 2012). Among other biocides, climbazole was found in influents of two Waste water treatment plants located in Germany with the concentrations of up to 1350 +/- 70 ng/l, as well as in activated sludge with the concentrations of up to 1160 +/- 80 ng/g TSS (ng per g of total suspended solids). This is regarded as the first time for climbazole to be found in the sludge. Furthermore, climbazole was the biocide found in the highest concentration in samples taken from a small German river Wickerbach with maximum concentrations of 530 +/- 70 ng/l (Wick et al., 2010). In WWTPs in Beijing, climbazole has been found in influents with the concentration of 610 – 940 ng/l and, after elimination of 30 - 51 %, in effluents – 300 – 615 ng/l (Qi et al., 2015). Climbazole was also found in surface water (Zhang et al., 2015) and in the sludge (Chen et al., 2013; Casado et al., 2015).

#### *3.4.4.2. Genotoxicity of climbazole*

Genotoxicity is defined as destructive effect on a cell's genetic material, either DNA or RNA, affecting its integrity. Genotoxins cause mutations and there are three primary effects: they can be carcinogens, mutagens or teratogens (Shah, 2012). Climbazole is found not to be genotoxic *in*

*vivo* and it poses a limited carcinogenic risk in the study conducted by Pérez-Rivera et al. in 2007. In the study, a complete battery of genotoxicity assays is done, which are given in requirements and guidelines for testing. Climbazole did not induce gene mutation in bacterial assays in which *S. typhimurium* and *E. coli* strains were used. A small increase in mutation frequency is noticed in mouse lymphoma cells in the absence of S9-mix after the exposure of 24 hours to climbazole, but those results were not replicated in micronucleus assay in human lymphocytes, same as *in vivo* micronucleus assay in rodents after systemic exposure (Pérez-Rivera et al., 2007).

#### *3.4.4.3. Ecotoxicity of climbazole*

Ecological information on climbazole which refers to its aquatic toxicity is provided by the supplier. The data shows that it has a negative effect on the growth of the green algae *Pseudokirchneriella subcapitata* at very low concentrations. Based on the fact that median effective concentration was 0.087 mg/l, climbazole was classified as very toxic to aquatic organisms (Richter et al., 2013).

In the study conducted by Richter et al. in the year 2013, ecotoxicity of climbazole was analyzed. Effective concentrations (EC) for 5 aquatic and 5 terrestrial species were determined in laboratory tests following international standard guidelines. pH dependence of the aquatic toxicity was also examined, as well as the influence of soil pH and organic matter content on the terrestrial phytotoxicity. For aquatic biotests, dilutions of the different level of the stock solution were used. And for terrestrial biotests, climbazole was added directly to the test soil to obtain needed concentrations for the tests. Two soil substrates were used: a natural sandy loam (LUFA 2.3) and an artificial soil with 5 % peat (Richter et al., 2013).

Effects of climbazole on aquatic organisms were tested on species: *Lemna minor* (herbaceous plant), *Navicula pelliculosa* (algae), *Pseudokirchneriella subcapitata* (algae), *Danio rerio* (fish) and lastly *Daphnia magna* (crustacean). All of these listed species were negatively affected by climbazole, the most affected being *L. minor*. Negative effect on biomass yield and growth rate were concentration-dependent, covering 10 – 50 % effect (Richter et al., 2013).

Species used for tests of toxicity of climbazole on terrestrial organisms are herbaceous plants *Avena sativa* and *Brassica napus*, bacteria *Arthrobacter globiformis*, segmented worm

*Enchytraeus bigeminus* and springtail *Folsomia candida*. No effects on the reproduction *F. candida* and *E. bigeminus* were noticed up to concentration of 1000 mg/kg of dry soil. Climbazole's low toxicity on *A. globiformis* was recorded with EC50 of 456 mg/kg needed for the inhibition of dehydrogenase activity. In the study it was also concluded that climbazole had significant effect on growth of plant species *A. sativa* and *B. napus*, which was concentration-dependent. The lowest determined EC50 for biomass yield was recorded in LUFA 3.2 soil for *A. sativa* (18.5 mg/kg) and it was slightly higher for *B. napus* (30.7 mg/kg). In artificial soil values were 45.5 mg/kg and 83.8 mg/kg respectively. Emergence was not significantly affected until 100 mg/kg for plants in LUFA 2.3 soil and 316 mg/kg for plants in artificial soil. Climbazole effects on seedling emergence, shot length and biomass of both species, but especially *B. napus* differed greatly in different soils, especially when comparing soils with the different pH. Plant growth was very poor in more basic soils. On the other side, peat content didn't have a significant effect on the phytotoxicity of climbazole (Richter et al., 2013).

### 3.4.5. Regulations for the use of climbazole in personal care products

The use of climbazole in cosmetic products is restricted by the Commission regulation (EU) 2019/698 published by European Commission. The use of it as a preservative is limited only to face creams, hair lotions, foot care products and rinse-off shampoos. Therefore, in face creams, hair lotions and foot care products its maximum concentration is 0.2 %, while in rinse-off shampoo maximum concentration is set to be 0,5 %. Additionally, in the regulation it is cited that when climbazole is not used for preservative purposes, it can be used only as anti-dandruff agent in rinse-off shampoos, and in that case its concentration must not be higher than 2 %, which is equivalent to approximately 15 g/l (European Commission, 2009). Products that are produced with these allowed concentrations in mind are considered safe for human use. However, simultaneous use of 3 or 4 of them is not considered safe, as well as the combination of hair lotion and face cream, and face cream and foot care product (SCCP, 2017). It is found that climbazole from a 2 % climbazole shampoo does not penetrate human skin after a contact that lasts for 30 minutes (Dossier on climbazole, 2007).

The safety of concentration limits is verified by the calculation of margins of safety in this case and the results are given in Addendum to the scientific opinions on climbazole (P64). Calculations are based on values for: dermal absorption through human skin, skin area surface for which the product is intended for, frequency of application of the finished product, typical human body wight, systemic exposure dose and no observed effect level which is obtained by oral application to rats for 90 days (SCCS, 2018).

# 4. Material and Methods

## 4.1. Soil

Soil used in the experiment was taken from a field located in Humpolec, which is a part of long-term field fertilization experiment established in 1979. The purpose of mentioned experiment is investigation of the effects of long-term application of nitrogen (N), phosphorus (P), potassium (K) and farmyard manure at different rates, as well as different intensity of liming and plant density. Eight-year crop rotation is utilized at the site in order: (1) oat with clover, (2) clover, (3) winter wheat, (4) silage maize, (5) winter wheat, (6) spring barley, (7) potato, and (8) spring barley (Madaras et al., 2010). Samples are collected after the year in which potato was grown.

In FAO classification system, this soil is classified as Haplic Cambisol on paragneiss. Soil type of it is sandy loam. Altitude of the site is 525 m a.s.l., average annual temperature is 6.7 °C and average annual precipitation is 681 mm (Madaras et al., 2010). Some characteristics of the experimental site and soil are shown in the following Tables.

*Table 4 $C_{ox}$ – organic carbon content, pH – soil reaction, clay – particles under 0.002 mm, CEC – cation exchange capacity (Madaras et al., 2010)*

| $C_{ox}$ [%] | pH kCl | Clay [%] | CEC [mmol(+)/kg] | P available [mg/kg] | K available [mg/kg] | K aqua regia [mg/kg] |
|---|---|---|---|---|---|---|
| 1.7 | 6.1 | 12.9 | 62 | 27 | 212 | 10 710 |

*Table 5 Soil mineralogical composition: relative presence in range from +++++ (dominant mineral) to + (present at the limit of detectability) (Madaras et al., 2010)*

| Quartz | Feldspars | | Mica group | Chlorite / vermiculite | Kaolinite | Mixed-layer phyllosilicates |
|---|---|---|---|---|---|---|
| | Plagioclase | K-spar | | | | |
| +++++ | +++ | ++ | +++ | + | +++ | ++/+++ |

Soils used in the experiment had different long-term fertilization treatments previously. Namely: soil FYM has been treated with farmyard manure (40 t/ha in years when silage maize and potatoes are grown during crop rotation) and mineral P, K and Mg (Table 6); soil NPK has been treated with NPK mineral fertilizer (Table 7); and soil CON hasn't been treated with fertilizers

since the beginning of the experiment in 1979 and is considered for the control soil in this experiment.

*Table 6 Fertilization rates for soil FYD during crop rotation*

| Year | FYM [t/kg] | P [kg/ha] | K [kg/ha] |
|---|---|---|---|
| 1 | - | 60 | 100 |
| 2 | - | - | - |
| 3 | - | 40 | 70 |
| 4 | 40 | 50 | 150 |
| 5 | - | 40 | 70 |
| 6 | - | 40 | 50 |
| 7 | 40 | 50 | 150 |
| 8 | - | 40 | 50 |

*Table 7 Fertilization rates for soil NPK during crop rotation*

| Year | N [kg/ha] | P [kg/ha] | K [kg/ha] |
|---|---|---|---|
| 1 | 40 | 60 | 100 |
| 2 | - | - | - |
| 3 | 100 | 40 | 70 |
| 4 | 175 | 50 | 150 |
| 5 | 125 | 40 | 70 |
| 6 | 90 | 40 | 50 |
| 7 | 150 | 50 | 150 |
| 8 | 60 | 40 | 50 |

## 4.2. Experimental design

The experiment is conceived as a pot experiment. It was undertaken in controlled conditions in the greenhouse of the Department of Agro-Environmental Chemistry and Plant Nutrition. Temperature during the day was 23 °C and during the night it was 18 °C. Direct sunlight was being shadowed as needed. Species of plant used in the experiment was butterhead lettuce (*Lactuca sativa* L.), variety May King. Plants were grown in double plastic pots (one transparent and one white) filled with 350 g of dry soil.

The experiment was conducted in randomized factorial design. In the experimental design there were 180 pots with 3 factors: type of the soil, presence of plants and concentration of climbazole. Three types of soil were used, each in the equal number of pots (60). Climbazole was added in 6 different concentrations to each soil: 0, 0.1, 1, 10, 100 and 1000 μg/kg of dry soil, and mixed thoroughly. Furthermore, half of the treatments had plants sown in them, and the other half didn't. Each treatment was replicated five times. Detailed experimental design is shown in the Table in Appendix 1.

### 4.3. Crop cultivation

Pots were prepared and climbazole treatments diluted in 10 ml of demi water were applied to the soil on July 3, and sowing was done a week later, on July 10. Twenty seeds of *Lactuca sativa*, var May King, were sown in each of 90 pots allocated for it. Other 90 pots were left without plants. Sowing was done at the depth of 1 cm, as indicated on seed packaging. After sowing, pots were irrigated with 50 ml of demi water to promote germination of the seeds.

Irrigation has been done regularly and as needed during the whole experiment. Water used was demi (demineralized) water. The amount of water that will be added for every irrigation was calculated based on soil water holding capacity. The upper limit for irrigation was set at 60% of soil water holding capacity, and during irrigation water was being added only until that point is reached. All of the pots, both with plants and without, were being irrigated. The leachate was captured by the double pot and returned back to the soil in order to prevent losses when appeared.

The number of germinated seeds was counted periodically 4 times, every 3 days, starting from $7^{th}$ day after sowing. Thinning of seedlings was done 3 times: 10, 13 and 16 days after sowing. In the end, 4 plants were left in every pot respectively. After the last counting, there were a few seeds that germinated (~5), but they are deemed not viable.

It was noticed that some plants started to wilt starting from day 10 after sowing. Aeration of the soil was done twice in order to mitigate wilting.

## 4.4. Soil sampling

Soil samples from the pots were taken 3 times. The first soil sampling was done on July 10, a week after the treatment of soil with climbazole. Approximately 2 g of soil were taken from pots in which plants were not planned to be grown, and for each concentration a composite was made (~10 g). The second sampling was done during the experiment, on July 31, when ~2 g of soil was taken from the pots in which plants haven't been grown (treatments without plants). They were put in plastic bags and stored in freezer. Second sampling was done at the end of the experiment, immediately after the harvest of the lettuce on September 4, when 18-20 g of soil was taken from each of 180 pots. Samples from each pot were divided in two plastic bags, for the storage in both fridge (+4 °C) and freezer (-42 °C).

## 4.5. Harvest

Harvest of the lettuce was done on September 4, after the vegetation period of 56 days. Plant biomass was separated from the roots. All of the plant parts were thoroughly washed in demineralized water, dried on open air with the help of filter paper and their weight was measured and noted. After this, plant parts were securely wrapped with aluminum foil, put in liquid nitrogen and consequently stored in the freezer at the temperature of -42 °C.

## 4.6. Dehydrogenase activity

The activity of dehydrogenase was assessed following the methodology described by Thalmann (1968). 2.5 g of soil was taken from all soil samples (180) stored in the fridge (4 °C) and mixed in tubes with 2.5 ml of TTC solution. As type of the soil is sandy loam, TTC solution was prepared by dissolving 0.3 g of triphenyltetrazolium chloride (TTC) per 100 ml of Tris buffer. Tris buffer was prepared by dissolving 12.1 g of (hydroxy methyl)-aminomethane in 1000 ml of distilled water and was adjusted with HCl to pH of 7.8, as assessed soil was acidic (pH ~5.9). Tubes with soil samples and TTC solution were consequently sealed and incubated for 24 h at 30 °C. After the incubation, 10 ml of acetone was added to each tube, shaken thoroughly and incubated in dark at the room temperature for 2 hours. The soil suspension was then filtered and the optical density

of supernatant was measured against the blank at 546 nm using DR3900 Laboratory Spectrophotometer by Hach Lange.

For the calculation it is needed to make a calibration curve with the solutions of following concentrations of triphenylformazan (TPF): 0, 5, 10, 20, 30 and 40 µg/ml. To 0, 0.5, 1, 2, 3 and 4 ml of TPF respectively was added 8.3 ml of Tris buffer (pH 7.6) and that was brought up to 50 ml with acetone. TPF concentrations were read from the calibration curve, corrected for the control value (only Tris buffer) and calculated with the formula:

$$\text{Dehydrogenase activity } TPF\ (\mu g)/dwt\ (g) = \frac{TPF\ (\mu g)/\text{ml} * 25}{dwt * 2.5}$$

Where dwt is the dry weight of 1 g of soil sample, 2.5 is the weight in g of soil sample used and 25 is the volume of solution added to the soil sample.

## 4.7. Dry weight of soil

For the determination of the dry weight of soil, 2 – 2.1 g of fresh soil from each soil sample (180) was transferred to aluminum dishes. The weight of aluminum dishes was previously recorded. Prepared samples were then dried in the oven overnight at the temperature of 105 °C. Samples were than reweighted. The weight of the dry soil is then calculated by subtracting the weight of the aluminum dish from the weight of the sample after drying.

## 4.8. Climbazole extraction from soil, biomass and roots

Before extraction, it was needed for soil, lettuce biomass and lettuce roots to be freeze-dried. From the soil samples that were stored in the freezer, soil was put in previously weighted falcon tubes and weighted again. The same was done for biomass and roots. Tubes were then covered with paper tissues, and put in the freeze dryer. After 2 – 4 days samples were taken out and weighted. Lastly, mass of freeze-dried soil samples, biomass and roots was calculated by subtracting the weight of falcon tubes from the weight that is recorded after freeze-drying. Lettuce biomass and roots were than milled with laboratory electric mill. The same procedure was followed for butterhead lettuce bought from the store (Kaufland) needed for validation of the method.

Soil samples that were examined were the highest climbazole treatments from the second and third sampling. Biomass and root samples that were examined were from the highest climbazole treatment in FYM soil. For the extraction, 0.1 g of freeze-dried sample was precisely measured and transferred to falcon tubes. At this point spiking with the solution of internal standard (Climbazole-D4) was done as needed (100 ng/g). To the samples prepared in this way, 5 ml of MeOH was added and they were vortexed. Then they were put in the sonic bath for 15 minutes on 50 °C. Following that, they were put in centrifuge for 10 minutes on 4500 rpm. Supernatant was then transferred to new falcons. Another 5 ml of MeOH was added to the samples and procedure was repeated. Obtained solution after two extractions was then filtered through a syringe filter (recycled cellulose) and 1 ml is transferred to vials with the pipette.

Possible extraction solutions were: MeOH (methyl alcohol), MeOH + 0,5 FA (formic acid), MeOH + $H_2O$ (1:1) and MeOH + $H_2O$ (1:1) + 0,5 FA. After comparing results obtained from the extraction of the identical samples, MeOH was deemed to be the best extracting solution for climbazole.

Following the extraction, contents of climbazole and its transformation products were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The presence of individual analytes was confirmed by the retention time and the presence of quantification and confirmation MRM transitions. Quantification of individual compounds was based on calibration curve prepared by serial dilution of the stock solution. For the stock solution standards of individual compounds (Santa Cruz Biotechnology) were diluted in MeOH (200 ng/ml). Standard of climbazole-alcohol was the same as described in Sochacki et al. (2020). Stock solution was also used for spiking before and after the extraction to get the matrix suppression and analyte recoveries. The compounds were quantified using internal standard (isotopically labelled climbazole – D4 purchased from Santa Cruz Biotechnology). Blanks and control spiked samples were included in each run.

## 4.9. Bioconcentration and translocation factors

The calculation of bioconcentration factors (BCF) of climbazole and its transformation products in lettuce biomass and roots is performed based on the following formula:

$$\mathrm{BCF} = \frac{\text{concentration in lettuce biomass/roots}}{\text{concentration in soil}}$$

Obtained values are then used in the following formula for the calculation of translocation factors (TF) of climbazole and its transformation products in lettuce plants:

$$\mathrm{TF} = \frac{\text{BCF of biomass}}{\text{BCF of roots}}$$

## 4.10. Statistical methods

For the calculation of means, standard deviations, bioaccumulation and translocation factors, as well as for analysis of variance and significant differences among means ($P \leq 0.05$) with ANOVA tests, *post hoc* analyses and T-tests was used software MS Excel 2019 with Analysis ToolPak.

# 5. Results

## 5.1. Germination rate

Comparison of average numbers of germinated seeds for every treatment variant are shown in Figure 6. Obtained results are shown in detail in the Table in Appendix 2, together with standard deviations.

In the soil FYM, seeds sown in control pots where no climbazole was added had the highest germination. Seeds in soil treated with 0.1, 1 and 100 µg/kg of dry soil behaved very similarly and their germination rate was a little bit lower than the control. Next concentration was 1000 µg/kg and the concentration where the lowest germination rate was recorded was 10 µg/kg. In the soil NPK, control had the same germination rate as soils treated with 0.1 and 100 µg/kg. Highest concentration was recorded in soils treated with 1 and 10 µg/kg, and the lowest in soils treated with 1000 µg/kg. In the soil CON, in contrast to the soil FYM where control had the highest germination rate, control together with the concentration 0.1 had the lowest one. In rising order, next concentrations were 10, 100 and 1000 µg/kg, while the highest germination rate was recorded in the soil with climbazole treatment of 1 µg/kg, which was the only treatment that had statistically significant difference compared to the control.

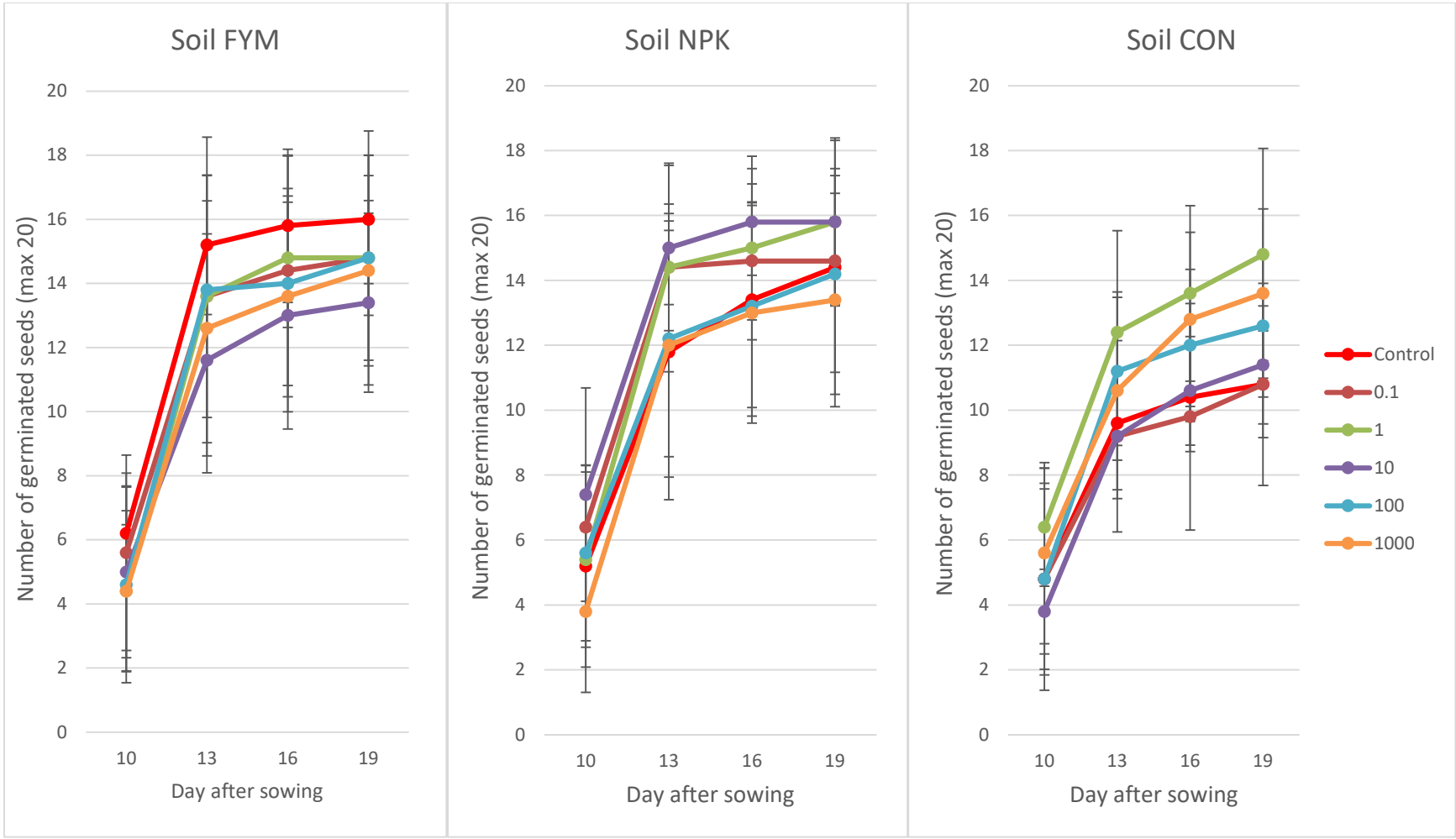



*Figure 6 Average number with standard deviation of germinated lettuce seeds treated with different concentrations of climbazole, compared between farmyard manure (FYM) soil, mineral (NPK) soil and control (CON) soil. Maximal possible number of germinated seeds is 20. All of the variants had 5 replications.*

In the following table, there are shown average germination rates for all of the different climbazole treatments. All of the variants show the most significant rise in numbers between 1st and 2nd counting or between 10 and 13 days after sowing. The highest germination rate of 75.7 % or 15.1 out of 20 germinated seeds was recorded in the soil that was treated with climbazole in the concentration of 1 µg/kg, but it isn't statistically significantly higher. All of the other variants, including control, had similar germination rate ranging between 67 and 69.3 % (13.4 and 13.9 out of 20 seeds).

*Table 8 Comparison of germination rate [%] between different treatments of climbazole in all soils together.*

| | Day after sowing | | | |
|---|---|---|---|---|
| Concentration [µg/kg] | 10 | 13 | 16 | 19 |
| Control | 27.0 | 61.0 | 66.0 | 68.7 |
| 0.1 | 28.0 | 62.0 | 64.7 | 67.0 |
| 1 | 27.0 | 67.3 | 72.3 | 75.7 |
| 10 | 27.0 | 59.7 | 65.7 | 67.7 |
| 100 | 25.0 | 62.0 | 65.3 | 69.3 |
| 1000 | 23.0 | 58.7 | 65.7 | 69.0 |

With the following table, average numbers of germinated seeds are shown for 3 different soils used in the experiment. In all of the soils, the most significant rise in numbers is between 1st and 2nd counting or between 10 and 13 days after sowing. The lowest germination rate all throughout was recorded in control soil and 19 days after sowing was 61.7 % or 12.3 out of 20 seeds. In other two soils germination rate was practically the same, without statistically significant difference and 19 days after sowing it amounted to 73.5 % or 14.7 out of 20 seeds. Soil CON had statistically significant negative effect on the germination rate starting from 13th day after sowing when compared to FYM and NPK soil.

*Table 9 Comparison of germination rate [%] between farmyard manure (FYM) soil, mineral (NPK) soil and control (CON) soil.*

| | Day after sowing | | | |
|---|---|---|---|---|
| Type of soil | 10 | 13 | 16 | 19 |
| FYM | 25.17 | 67.00 | 71.33 | 73.50 |
| NPK | 28.17 | 66.50 | 70.83 | 73.50 |
| CON | 25.17 | 51.83 | 57.67 | 61.67 |

## 5.2. Biomass yield

Mean values of fresh biomass weight and number of plants per pot for all climbazole concentrations and soil type variants are shown in the following Figure 7.

When number of plants per pot in different soils is concerned, there are very noticeable differences. A number of plants wilted, especially in the mineral (NPK) soil, where only 2 plants survived in total. In the control soil, there were 56 plants left in total at the end of experiment. Soil fertilized with farmyard manure (FYM) suited plants the best: 117 plants were left in the pots out of 120 that were planned (4 plants per pot).

The average weights of biomass for 5 replications per each climbazole treatment in FYM soil is significantly higher than in CON soil, which in turn is significantly higher than in NPK soil. For FYM soil, climbazole treatments had progressively higher positive effect on the weight of biomass, however, that effect is statistically insignificant. The control had the average value of 7.51 g and the highest recorded one was for the treatment of 1000 µg/kg (8.53 g). When CON soil is concerned, the treatment of 0.1 µg/kg had the lowest value of 1.59 g, while the treatment of 1 µg/kg had the highest biomass weight of 3.78. In NPK soil, all of the climbazole treatments had no biomass yield at the end of vegetation period of 56 days except for 10 µg/kg, which had 0.51 g. There are no climbazole treatments with statistically significant difference when compared to control.

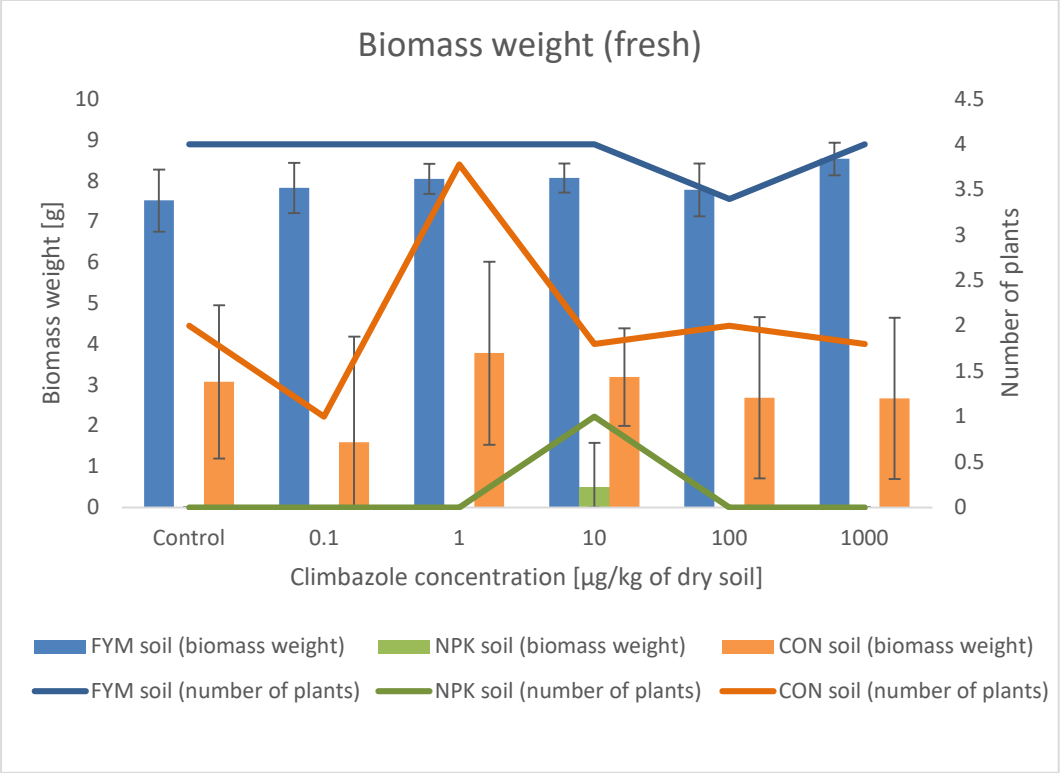



*Figure 7 Average fresh biomass weight with st. deviation and average number of plants per pot after vegetation period of 56 days for climbazole treatment and soil variants. Number of replications is 5*

Average fresh root weight for all climbazole treatments in different soils is compared in the following Figure 8. The difference between all of the soils is significant. When compared to lettuce biomass, the ratio between FYM soil and control soil is higher, which indicates that farmyard manure had stronger positive effect on growth of roots compared to growth of biomass. Average values for FYM soil were the highest and they were ranging from 3.51 g for the climbazole concentration of 10 µg/kg to 3.75 g for the concentration of 1000 µg/kg of dry soil. Average values for CON soil were ranging from 0.11 g, which was recorded for 0.1 µg/kg concentration to 0.37 g, which was recorded for the climbazole treatment of 1 µg/kg. In soil NPK, the only group that had root weight was the treatment of 10 µg/kg and it amounted to 0.51 g average.

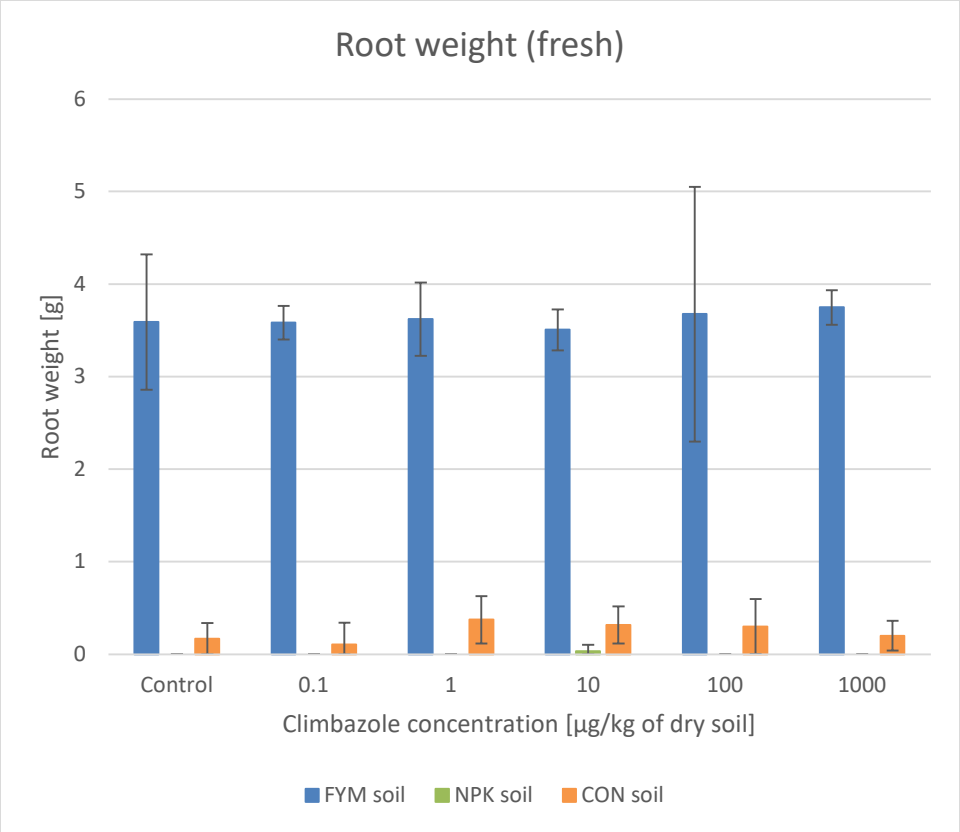



*Figure 8 Average lettuce root weight (fresh) with st. deviation after vegetation period of 56 days for climbazole treatment and soil variants. Number of replications is 5*

## 5.3. Microbial activity in soil

Average values of dehydrogenase activity in soil in 5 replications per treatment variant are shown in detail in the Table in Appendix 4. Dehydrogenase activity is compared between different concentrations of climbazole treatments, between different used types of soil (control, soil fertilized with farm-yard manure and soil fertilized with mineral NPK fertilizer), as well as between soils in which were or were not plants grown.

The highest value of dehydrogenase activity in soil where plants were grown was 10.93 µg TPF $g^{-1}$ (dw) $day^{-1}$ which was measured in NPK soil for climbazole treatment of 0.1 µg/kg of dry soil. The lowest value of 6.12 µg TPF $g^{-1}$ (dw) $day^{-1}$ was measured in control soil for climbazole treatment of 10 µg/kg. When compared to control, treatments of climbazole had different effect on dehydrogenase activity in different soils, but there isn't any statistically significant difference. The only treatment that had a statistically significant positive effect was 0.1 µg/kg in NPK soil,

but that wasn't replicated in other ones. In NPK soil, climbazole treatments had slightly positive effect compared to the control. In FYM and control soil, climbazole treatments had slightly negative effect on dehydrogenase activity compared to control (Figure 9).

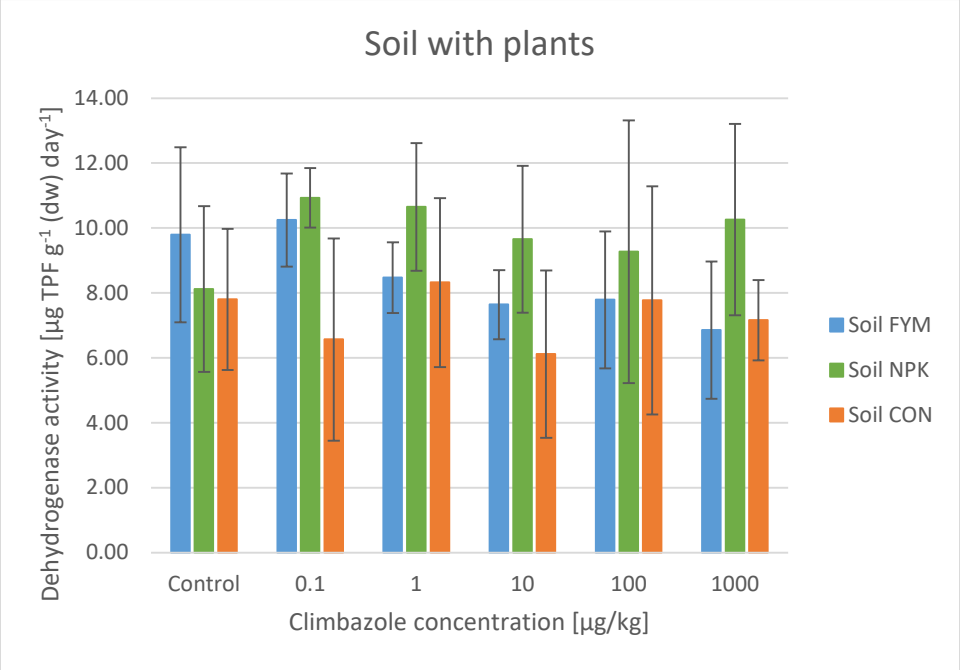



*Figure 9 The level of dehydrogenase activity in farmyard manure (FYM), mineral (NPK) and control (CON) soil with plants, compared between different climbazole treatment concentrations. Averaged values of 5 replications with standard deviations are presented*

The highest dehydrogenase activity in the soil where plants were not grown was recorded in NPK soil which was treated with climbazole concentration of 100 µg/kg and it amounted to 10.77 µg TPF $g^{-1}$ (dw) $day^{-1}$. While the lowest activity was recorded in FYM soil treated with the climbazole concentration of 10 µg/kg and it was 7.73 µg TPF $g^{-1}$ (dw) $day^{-1}$. Except a couple, climbazole treatments in all soils had slightly negative effect when compared to the control (Figure 10).

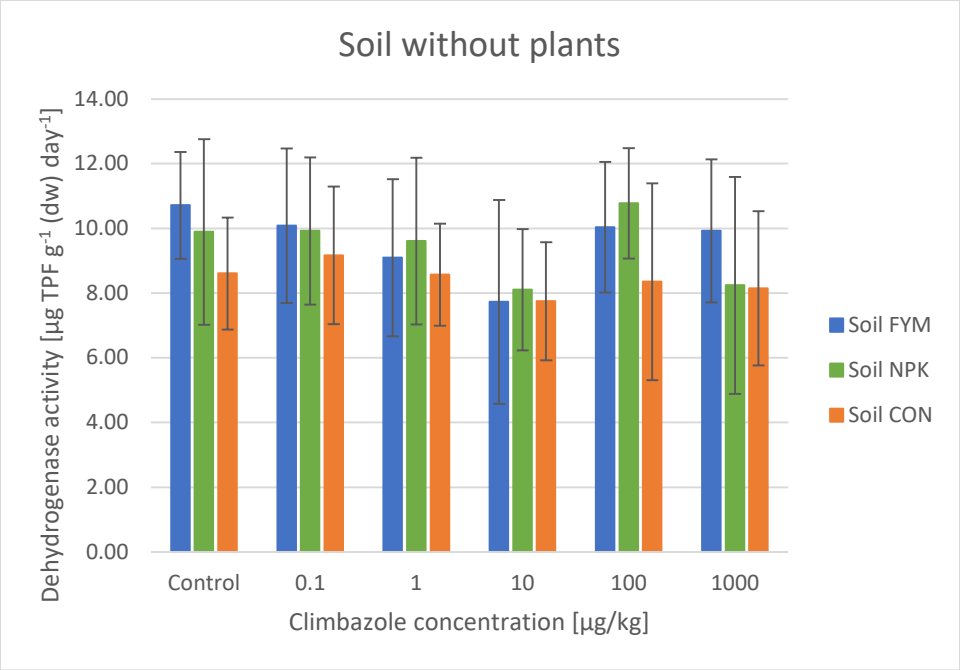



*Figure 10 The level of dehydrogenase activity in farm yard manure (FYM), mineral (NPK) and control (CON) soil without plants, compared between different climbazole treatment concentrations. Averaged values of 5 replications with standard deviations are presented.*

In the Table 10 are compared average values of dehydrogenase activity between FYM soil, NPK soil and CON soil, with and without plants growing in them. There are statistically significant differences between all of the soils when they had plants growing in them, with control soil being the lowest with 7.29 µg TPF $g^{-1}$ (dw) $day^{-1}$, while mineral NPK soil had the highest positive impact on dehydrogenase activity (9.81 µg TPF $g^{-1}$ (dw) $day^{-1}$). When there were no plants growing in the soil, control soil was again the lowest with 8.43 µg TPF $g^{-1}$ (dw) $day^{-1}$, while the highest average value was recorde in FYM soil (9.60 µg TPF $g^{-1}$ (dw) $day^{-1}$) and it was statisticaly significantly higher than control soil. The significant difference was also noticed in FYM soil, where plants had a slightly negative effect on dehydrogenase activity.

*Table 10 Comparison of average dehydrogenase activity between farmyard manure (FYM) soil, mineral (NPK) soil and control (CON) soil, with and without plants growing*

| | Presence of plants | |
|---|---|---|
| Type of soil | With plants | Without plants |
| FYM | 8.47 (2.08) | 9.60 (2.35) |
| NPK | 9.81 (2.58) | 9.43 (2.48) |
| CON | 7.29 (2.51) | 8.43 (2.02) |

## 5.4. Degradation of climbazole in soil

After analysis by LC-MS/MS, climbazole and its reduced and oxidized form are detected in soil samples. Average values of concentration for every compound, during time and with variation for presence of plants are given in detail for every soil in Tables in Appendix 5. No significant difference was noticed in degradation of climbazole between soils that had plants growing in them and those that didn't.

Applied concentration of climbazole to the analyzed FYM soil was 1000 μg/kg of dry soil. 28 days after application, in soil without plants, climbazole concentration was 299.88 μg/g and 64 days after application it was 191.01 μg/g. In the soil with plants after 64 days it was 169.75 μg/g. The concentration of both diastereomers of climbazole-alcohol were rising during time, while the concentration of hydroxy-climbazole was falling. After 64 days, both diastereomers of climbazole-alcohol had slightly higher concentration in the soil with plants than in the soil without them. The situation for climbazole-alcohol was the opposite.

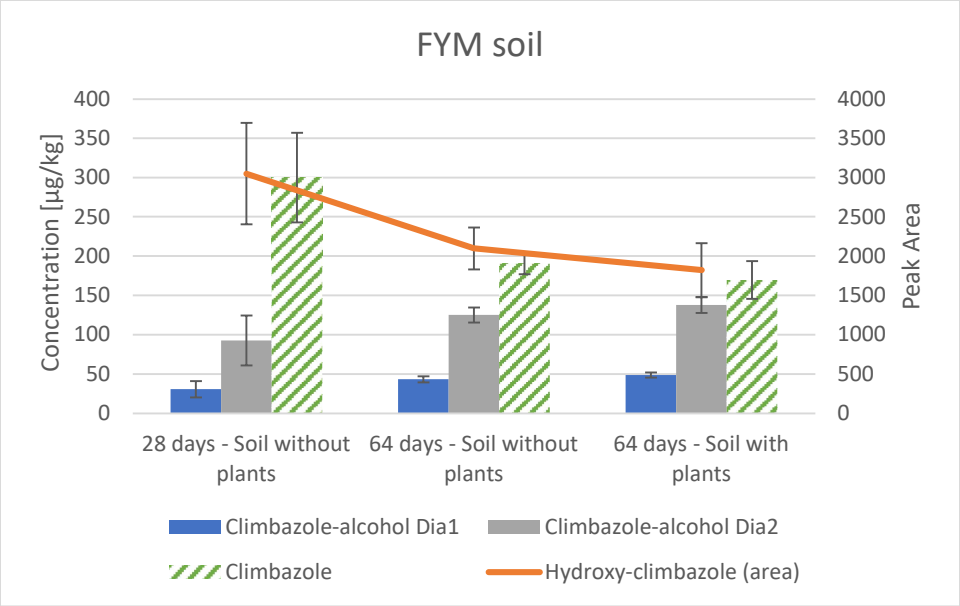



*Figure 11 Comparison of concentrations of climbazole and two climbazole-alcohol diastereomers (Dia1 and Dia2) and peak area of hydroxy-climbazole in farmyard manure (FYM) soil during time (28 and 64 days after treatment) and in soil with or without presence of plants. Average values of 5 replications with standard deviations are shown.*

To the analyzed NPK soil was applied 1000 µg of climbazole per kg of dry soil. In the soil without plants 28 days after application, climbazole concentration was 304.79 µg/g and 64 days after application it was 226.57 µg/g. In the soil that had plants, after 64 days it was 257.23 µg/g. The concentration of both diastereomers of climbazole-alcohol were rising during time, while the concentration of hydroxy-climbazole was falling. After 64 days, both diastereomers of climbazole-alcohol had slightly lower concentration in the soil with plants than in the soil without them. The situation for climbazole-alcohol was the opposite.

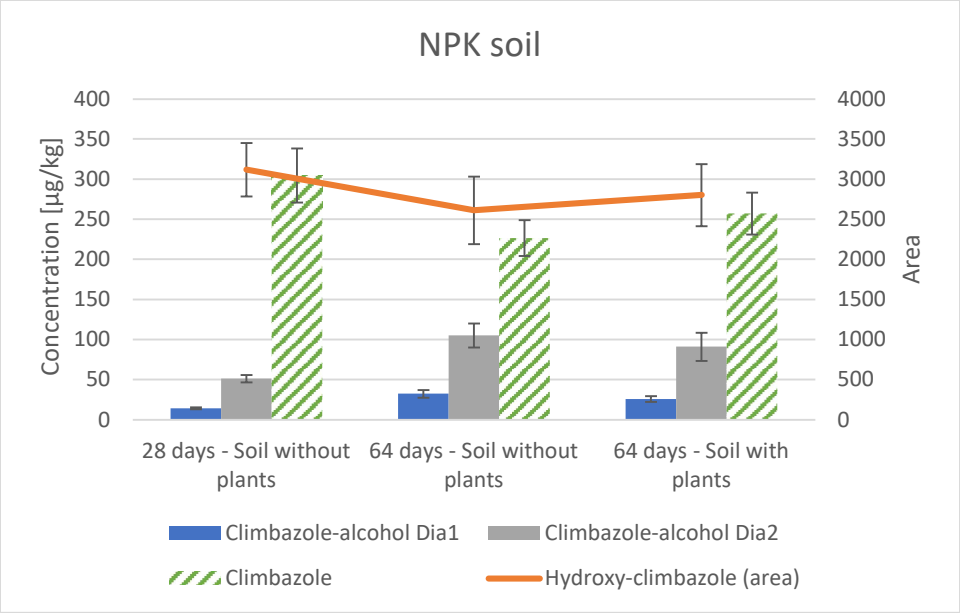



*Figure 12 Comparison of concentrations of climbazole and two climbazole-alcohol diastereomers (Dia1 and Dia2) and peak area of hydroxy-climbazole in mineral (NPK) soil during time (28 and 64 days after treatment) and in soil with or without presence of plants. Average values of 5 replications with standard deviations are shown.*

Applied concentration of climbazole to the analyzed CON soil at the beginning of the experiment was 1000 µg/kg. Recorded concentration of climbazole 28 days after application, in soil without plants, was 297.21 µg/g and 64 days after application it was 216.25 µg/g. In the soil with plants after 64 days the concentration was 201.81 µg/g. The concentration of both diastereomers of climbazole-alcohol was rising during time, while the concentration of hydroxy-climbazole was slowly falling. At the end of experiment, 64 days after climbazole application, both diastereomers of climbazole-alcohol had higher concentration in the soil with plants than in the soil without them, while for climbazole-alcohol was the opposite.

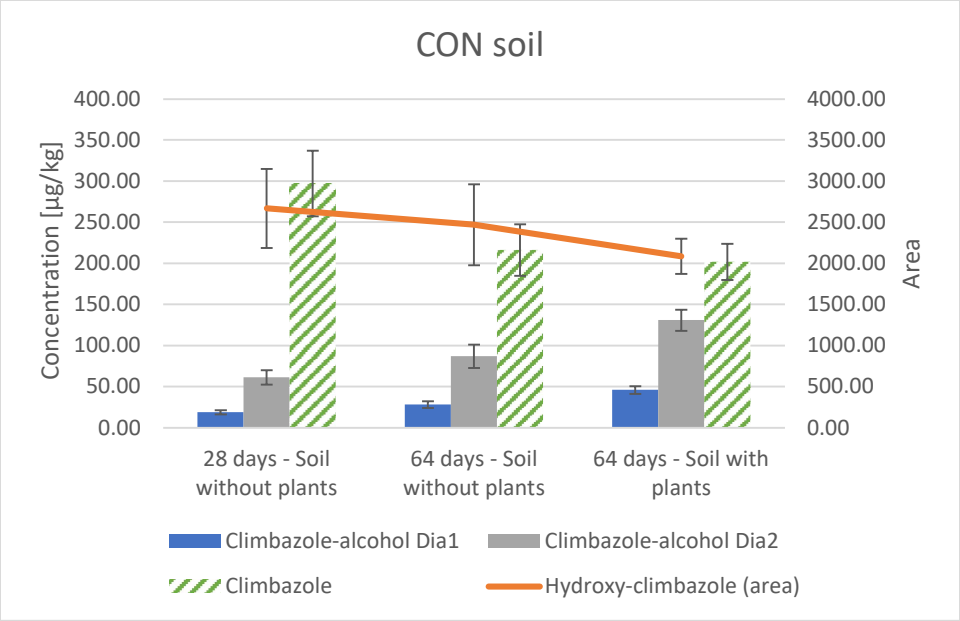



*Figure 13 Comparison of concentrations of climbazole and two climbazole-alcohol diastereomers (Dia1 and Dia2) and peak area of hydroxy-climbazole in control (CON) soil during time (28 and 64 days after treatment) and in soil with or without presence of plants. Average values of 5 replications with standard deviations are shown.*

## 5.5. Accumulation of climbazole in plants

Average concentrations for climbazole and climbazole-alcohol detected in lettuce biomass and roots are given in the Table 10, as well as peak area for hydroxy-climbazole. Applied treatment to the soil was 1000 µg/kg. Climbazole is found to be taken up by lettuce plants and it was detected and quantified in both plant roots and plant biomass. Climbazole transformation products were also found in both plant biomass and roots: oxidized form of climbazole which is hydroxy-climbazole and reduced form found in two diastereomers, which is climbazole-alcohol. The exception is hydroxy-climbazole, which wasn't detected in plant biomass. All compounds have significant differences in their content in biomass when compared to their content in roots, with content in roots being much higher. Hydroxy-climbazole couldn't be quantified.

*Table 10 Concentration of concentrations of climbazole and two climbazole-alcohol diastereomers (Dia1 and Dia2) and peak area of hydroxy-climbazole in biomass and roots of lettuce grown in the soil treated with climbazole at rate of 1000 µg/kg of dry soil. Average values of 5 replications with standard deviations are presented.*

| | Compound | Biomass | Roots |
|---|---|---|---|
| Concentration [ng/g] | Climbazole-alcohol-Dia1 | 45.76 (4.60) | 162.91 (21.33) |
| | Climbazole-alcohol-Dia2 | 91.87 (10.44) | 416.31 (54.20) |
| | Climbazole | 38.13 (3.26) | 494.15 (57.75) |
| Area | Hydroxy-climbazole | 0 (0) | 639.92 (89.35) |

To evaluate the content of contaminants in organisms bioconcentration factor can be used, while the translocation factor can be used to measure the amount of contaminant transferred from one organ to another. Bioconcentration factor (BCF) is calculated as the concentration of contaminant in plant organ divided by the concentration of contaminant in soil. Translocation factor is calculated by dividing BCF of biomass with BCF of roots. Diastereomer 1 of climbazole-alcohol has the highest values for BCF in lettuce biomass and roots, as well as the highest TF. It is followed by Dia2 of climbazole alcohol, climbazole and lastly hydroxy-climbazole with the lowest values for all of the factors.

*Table 11 Bioconcentration factors (BCF) for lettuce biomass and roots and translocation factors (TF) for climbazole, two climbazole-alcohol diastereomers (Dia1 and Dia2) and hydroxy-climbazole.*

| Compound | BCF biomass | BCF roots | TF |
|---|---|---|---|
| Climbazole-alcohol-Dia1 | 0.94 | 3.34 | 0.28 |
| Climbazole-alcohol-Dia2 | 0.67 | 3.02 | 0.22 |
| Climbazole | 0.22 | 2.91 | 0.08 |
| Hydroxy-climbazole | 0.00 | 0.35 | 0.00 |

# 6. Discussion

## 6.1. Germination rate

Fungicides are often used as seed treatment for a few reasons: to extend life of the seed during storage, to reduce pathogen transmission via seed, as well as infestation by soil fungi (Embrapa, 2008). However, secondary effects on the germination and growth, both stimulating and inhibitory, are also likely to occur. In the study conducted by Dhanamanjuri et al. in 2013, the effect of fungicides Captan, Bavistin, Domarck, Blitox and Sitara in concentrations of 1 ppm, 10 ppm, 100 ppm and 1000 ppm on the germination of seeds and growth of seedlings was investigated. They concluded that germination percentage and biomass production were slightly affected by fungicide treatments. Fungicide Carbendazim (Bavistin) has shown the highest stimulating effect on the seed germination and plant growth at 10 ppm for *Cicer arietinum* and at 1 ppm for *Zea mays*, while tetraconazole and copper oxychloride have shown the highest only inhibitory effect (Dhanamanjuri et al., 2013). However, in another study conducted by Saeidi and Mirik in 2006, fungicides Carbendazim and Captan didn't have any significant effect on germination (Saeidi and Mirik, 2006). Accoring to this, both inhibiting and positive effect of climbazole on plants could be expected, as well as no effect. In this experiment, almost no significant, either positive or negative effect of different concentrations of climbazole on the seed germination rate was observed. No clear overall trend was observed: same climbazole concentrations in different soils had different effect compared to control, although not significantly different. Compared to the control, germination rates of climbazole treatments in farmyard manure soil were lower, in mineral soil (NPK) were both higher and lower and in control soil were higher. The only concentration that had statistically significant positive effect on germination rate was 1 µg/kg when applied to control soil. This finding could indicate the existence of hormetic effect of climbazole on lettuce seeds. However, it wasn't replicated in other soils.

When germination rate in 3 differently treated soils that were used in this experiment are compared, it can be observed that germination rate in control soil that hasn't been fertilized is significantly lower (61.7 %) than in the other two which have been fertilized long-term with either

farmyard manure or mineral NPK fertilizer (73.5 %). This finding suggests that both farmyard manure and NPK mineral fertilizer have a positive effect on seed germination. This result is supported by the study conducted in 2011 by Jalaluddin and Hamid, where they found that addition of manure and triple phosphate showed increase in germination rate of sunflower seeds compared to control (Jalaluddin and Hamid, 2011), as well as by the study by Shahzaman et al. in 2017, where both mineral (Diammonium phosphate, urea) and farmyard manure had positive impact on germination rate (Shahzaman et al., 2017).

## 6.2. Biomass yield

In the study conducted by Richter et al. in 2013 was concluded that growth of the plants *Avena sativa* and *Brassica napus* is very sensitive to climbazole. Observed symptoms in plants were stunted growth of the leaves and shoots especially, as well as darker green color. The lowest effective concentration 10 % (EC10) for biomass yield was 4.9 mg/kg of dry soil was recorded for *A. sativa* in LUFA 2.3 soil (Richter et al., 2013). However, in this study, none of the applied concentrations of climbazole showed a significant effect on biomass yield of lettuce plants. With the highest applied concentration being 1 mg/kg of dry soil, the reason for that can be that it was too low of a dose to produce a significant effect on lettuce plants.

A number of plants wilted early on in the experiment. The wilting wasn't distributed evenly in different soils. Namely: wilting started first in NPK soil and by the end of the experiment only 2 out of planned 120 plants were left, in CON soil about half of the plants wilted, while plants in FYM soil were unaffected. All of the pots had the same irrigation rate, which indicates that overwatering was not the reason. Plants had symptoms of root rot. The reason for plant wilting can be contamination of NPK and Control soil with some sort of pathogen, but no tests were performed to determine the presence of it in the soil. No pesticides were used to mitigate root rot, because in that case it wouldn't be clear if any recorded effects were coming from climbazole or the pesticide. Aeration was performed, but it wasn't effective. The data shows no significant difference in number of plants for different climbazole treatments in the same soil, therefore, it can be concluded that climbazole didn't cause wilting of the plants and that it wasn't effective in

controlling the possible soil pathogen. This would be in line with the fact that climbazole is not used as fungicide in agriculture, but as antifungal agent in personal care products.

Both organic and mineral fertilizers are well documented to stimulate the growth of plants. In the study conducted by Jalaluddin and Hamid in 2011, the data clearly shows significantly higher growth of *Helianthus annus* L. when the soil was fertilized with both organic fertilizer and triple phosphate when compared to the control (Jalaluddin and Hamid, 2011). Jammu et al., in 2017 had the same finding when applying DAP, Urea and farmyard manure to the soil (Jammu et al., 2017). This is replicated in this study as well, in the case of farmyard manure. Plants grown in the soil fertilized with FYM had significantly higher biomass yield than those grown in control soil. NPK soil couldn't be compared because of the fact that plants in it didn't survive.

### 6.3. Microbial activity in soil

Dehydrogenase activity is a good indicator of overall microbial activity in soil and it can be a good indicator of soil condition. The most important soil factors that are stimulating dehydrogenase activity are soil moisture, soil aeration state, organic matter content, pH and temperature. Factors that are inhibiting are depth of the soil profile, fertilization and pesticide amendment and presence of heavy metals. Considering the number of factors, it is not a straightforward task to conclude which one of them is causing any noticed effect. The decreased microbial activity can be caused by direct toxicity and reduced pH. When measured, pH of FYM soil was 5.85, of NPK soil was 5.87 and that of CON soil was 5.93. Therefore, in this study pH was quite stable among the different soils, so it may not have a significant effect.

A number of studies confirm that organic fertilizers have higher positive impact on soil enzymatic activity than mineral fertilizers. Chu et al. confirmed this with the study conducted in 2007. The study found that both organic and mineral long-term fertilization greatly increased soil microbial biomass and dehydrogenase activity, and that furthermore organic manure had a significantly greater impact compared to mineral fertilizers (Chu et al., 2007). The positive impact of both organic and mineral fertilization was confirmed in this study with both mineral (NPK) and farm-yard manure (FYM) fertilized soils having statistically significantly higher dehydrogenase activity than the control soil. But, contrary to the study by Chu et al., mineral fertilizer had a slightly more

positive effect than farmyard manure. However, this outcome may be influenced by the fact that a number of plants wilted early on during experiment which caused the variability of conditions in the group of pots where the plants should have been grown. Namely, there was a significant difference in a number of surviving plants between soils, but also in biomass yield of those plants that survived. In NPK soil there were only 2 plants left, in CON soil 56 and in FYM soil there were 117 plants. Furthermore, plants in FYM soil were much bigger than in the other two soils. This resulted in difference in evapotranspiration, aeration of the soil, level of root exudes and possibly other factors that affect dehydrogenase activity in soil in different ways.

Wolińska and Stępniewska reported that a decrease of dehydrogenase activity by pesticides was dependent on the pesticide doses (Wolińska and Stępniewska, 2012). Conversely, some other authors have reported a slight rise of dehydrogenase activity as a result of applied Prochloraz fungicide (Tejada et al., 2011) and Glyphosate herbicide (Andrea et al., 2003). The reason for this increase of activity possibly can be that a pesticide is used as a source of energy and nutrients by microorganisms and that it might stimulate soil oxidative processes. However, in this study, no significant effect of climbazole on dehydrogenase activity was noticed. Climbazole didn't show neither positive nor toxic effect. The only treatment that had a statistically significant positive effect was 0.1 µg/kg in NPK soil with plants, but that result wasn't replicated in other variants. Possible reason for this can be that applied concentrations of climbazole (1mg per kg of dry soil being the highest) were too low to show toxic effect, which is supported by the study of Richter et al. from 2013, where 10% effective concentration of climbazole (EC10) was 400 mg/kg of dry soil for the inhibition of dehydrogenase activity in *Arthrobacter globiformis* (Richter et al., 2013).

## 6.4. Degradation of climbazole in soil

The original plan was for all of the taken soil samples to be analyzed. However, due to restrictions in access for students to the Faculty building and, consequently, to the laboratories for prolonged period of time and high total number of samples, we weren't able to do it. Therefore, only samples of soil with the highest treatment of climbazole (1000 µg/kg of dry soil) were analyzed. It would've been better if samplings were done more frequently during the course of experiment, so that the degradation rate of climbazole can be better assessed during time.

The rate of climbazole removal during time was comparable between different soils. Achieved removal of climbazole in soil without plants was the highest in FYM soil where it was 80.90 %, which was closely followed by CON soil with 78.38 % and lastly by NPK soil with 77.34 %. In this case neither FYM soil nor NPK significantly affected climbazole removal. When the soil with plants is concerned, both FYM and NPK soil had statistically different removal compared to the control soil (79.82 %), with 83.03 % and 74.28 % respectively. One of the reasons for this could be the difference in number of plants growing in these different soils, with NPK having almost no plants, and CON having about half the number of FYM soil. This finding could support positively corelated relationship between presence of plants and degradation of climbazole in the soil by Sochacki et al. in 2020. However, there was no significant difference in climbazole degradation between FYM soil where plants were growing and the same soil where they were not, although a significant uptake of climbazole by plants was recorded. Apart from direct uptake, vegetation could positively affect removal of climbazole by stimulation of the microbiota by root exudes or oxygen transfer of sorption (Paz et al., 2019). Photodegradation had insignificant role in the degradation of climbazole and its transformation products in soil. Manasfi et al. in 2020 reported that *T. harzianum* achieved almost complete removal of climbazole of 91 % after 13 days, which indicates that some microorganisms are successful in removal of climbazole. That could point to biodegradation as the significant path of climbazole degradation in this experiment.

Detected transformation products of climbazole were reduced form of climbazole – climbazole-alcohol and climbazole's oxidized form – hydroxy-climbazole. Climbazole-alcohol was detected in two diastereomers. Hydroxy-climbazole could not be quantified due to the lack of analytical standards and climbazole and climbazole alcohol were quantified. The data points to negative correlation between the concentration oxidized and reduced form of climbazole.

## 6.5. Accumulation of climbazole in plants

Originally, the analysis of all biomass and root samples was planned. However, due to restricted access for students to the Faculty building for prolonged period of time, we weren't able to do it. Therefore, only samples of plants that were treated with the highest concentration of climbazole (1000 µg/kg of dry soil) in FYM soil were analyzed for concentration of climbazole and its

transformation products. Biomass and roots from FYM soil were chosen rather than those from CON soil because in FYM soil there were 5 complete replications, contrary to CON and NPK soil.

Quite high bioconcentration factor for lettuce roots that was found in this experiment is in line with higher $K_{ow}$ value of climbazole, which is 3.76. Compounds with higher $K_{ow}$ values are more lipophilic and those compounds will concentrate in tissues with high lipid content. This finding is similar to the finding of García-Valcárcel et al. in 2016, where clotrimazole which has similar log $K_{ow}$ value of 4.10 and propiconazole with log $K_{ow}$ value of 3.65 had high concentration in roots of Lamb's lettuce compared to biomass and consequently low TF (García-Valcárcel et al.,2016). Climbazole's TF was in this study determined to be 0.08, which is supported by the study conducted by Sochacki et al. in 2020, where determined TF of climbazole was 0.1. However, translocation factors of climbazole-alcohol in this study of 0.28 for diastereomer 1 and 0.22 for diastereomer 2, are significantly lower than values established by Sochacki et al., which were 5.6 and 1.9 respectively (Sochacki et al., 2020). High concentration in roots compared to biomass indicates low translocation of the compound, which is the case for both climbazole and its oxidized and reduced form. The median ratio of climbazole-alcohol diastereomers in lettuce biomass recorded in this study was 0.50, while in roots it was 0.39. The median ratio recorded in the soil where these plants were grown was 0.35, in favor of diastereomer 2. Observed values for soil and roots are close, which indicates that climbazole alcohol was probably taken up from soil. This is supported by Sochacki et al., with the similar finding in their study. The change of ratio in plant biomass compared to the roots can be a sign of preferential transport of Dia1. However, it cannot be ruled out that a part of climbazole wasn't transformed to climbazole-alcohol in plants.

# 7. Conclusion

Based on the results of this study, it can be concluded that climbazole in applied concentrations didn't have a significant effect on lettuce's seed germination and biomass yield. Likewise, no significant effect on dehydrogenase activity of microorganisms in the soil was recorded. However, differently fertilized soil did have an effect on all of these indicators. Both soil that was treated long-term with farm-yard manure and soil treated long-term with mineral NPK fertilizer stimulated seed germination, biomass yield and dehydrogenase activity in the soil when compared to the control soil that hasn't been fertilized.

The data shows that most of the climbazole was removed from the soil during the course of the experiment. The highest removal of climbazole was recorded in FYM soil with plants – 83.03 %, while the lowest was in NPK soil with plants – 74.28 %. Detected transformation products of climbazole were: hydroxy-climbazole (oxidized form) and climbazole-alcohol found in two diastereomers (reduced form). Climbazole's bioconcentration factor in lettuce's roots was 2.91 which indicates its lipophilic nature and the ability of lettuce plant to uptake this antifungal agent. The translocation factor of climbazole was determined to be 0.08 which shows weak transport ability from underground parts of plant to the ones above. Climbazole's behaviour in the environment and its potential risks and toxicity should be studied further.

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# Supplementary material

## Appendix 1 – Experimental design

| Type of soil | Presence of plants | Concentration of climbazole [µg/kg] | Pot Number |
|---|---|---|---|
| FYM soil | Plants | Control | 1 |
| | | | 2 |
| | | | 3 |
| | | | 4 |
| | | | 5 |
| | | 0.1 | 6 |
| | | | 7 |
| | | | 8 |
| | | | 9 |
| | | | 10 |
| | | 1 | 11 |
| | | | 12 |
| | | | 13 |
| | | | 14 |
| | | | 15 |
| | | 10 | 16 |
| | | | 17 |
| | | | 18 |
| | | | 19 |
| | | | 20 |
| | | 100 | 21 |
| | | | 22 |
| | | | 23 |
| | | | 24 |
| | | | 25 |
| | | 1000 | 26 |
| | | | 27 |
| | | | 28 |
| | | | 29 |
| | | | 30 |
| NPK soil | | Control | 31 |
| | | | 32 |
| | | | 33 |
| | | | 34 |
| | | | 35 |
| | | 0.1 | 36 |
| | | | 37 |
| | | | 38 |
| | | | 39 |
| | | | 40 |
| | | 1 | 41 |
| | | | 42 |
| | | | 43 |

| Type of soil | Presence of plants | Concentration of climbazole [µg/kg] | Pot Number |
|---|---|---|---|
| FYM soil | No plants | Control | 1 |
| | | | 2 |
| | | | 3 |
| | | | 4 |
| | | | 5 |
| | | 0.1 | 6 |
| | | | 7 |
| | | | 8 |
| | | | 9 |
| | | | 10 |
| | | 1 | 11 |
| | | | 12 |
| | | | 13 |
| | | | 14 |
| | | | 15 |
| | | 10 | 16 |
| | | | 17 |
| | | | 18 |
| | | | 19 |
| | | | 20 |
| | | 100 | 21 |
| | | | 22 |
| | | | 23 |
| | | | 24 |
| | | | 25 |
| | | 1000 | 26 |
| | | | 27 |
| | | | 28 |
| | | | 29 |
| | | | 30 |
| NPK soil | | Control | 31 |
| | | | 32 |
| | | | 33 |
| | | | 34 |
| | | | 35 |
| | | 0.1 | 36 |
| | | | 37 |
| | | | 38 |
| | | | 39 |
| | | | 40 |
| | | 1 | 41 |
| | | | 42 |
| | | | 43 |

| Type of soil | Presence of plants | Concentration of climbazole [μg/kg] | Pot Number |
|---|---|---|---|
| | | | 44 |
| | | | 45 |
| | | 10 | 46 |
| | | | 47 |
| | | | 48 |
| | | | 49 |
| | | | 50 |
| | | 100 | 51 |
| | | | 52 |
| | | | 53 |
| | | | 54 |
| | | | 55 |
| | | 1000 | 56 |
| | | | 57 |
| | | | 58 |
| | | | 59 |
| | | | 60 |
| CON soil | | Control | 61 |
| | | | 62 |
| | | | 63 |
| | | | 64 |
| | | | 65 |
| | | 0.1 | 66 |
| | | | 67 |
| | | | 68 |
| | | | 69 |
| | | | 70 |
| | | 1 | 71 |
| | | | 72 |
| | | | 73 |
| | | | 74 |
| | | | 75 |
| | | 10 | 76 |
| | | | 77 |
| | | | 78 |
| | | | 79 |
| | | | 80 |
| | | 100 | 81 |
| | | | 82 |
| | | | 83 |
| | | | 84 |
| | | | 85 |
| | | 1000 | 86 |
| | | | 87 |
| | | | 88 |
| | | | 89 |
| | | | 90 |


| Type of soil | Presence of plants | Concentration of climbazole [μg/kg] | Pot Number |
|---|---|---|---|
| | | | 44 |
| | | | 45 |
| | | 10 | 46 |
| | | | 47 |
| | | | 48 |
| | | | 49 |
| | | | 50 |
| | | 100 | 51 |
| | | | 52 |
| | | | 53 |
| | | | 54 |
| | | | 55 |
| | | 1000 | 56 |
| | | | 57 |
| | | | 58 |
| | | | 59 |
| | | | 60 |
| CON soil | | Control | 61 |
| | | | 62 |
| | | | 63 |
| | | | 64 |
| | | | 65 |
| | | 0.1 | 66 |
| | | | 67 |
| | | | 68 |
| | | | 69 |
| | | | 70 |
| | | 1 | 71 |
| | | | 72 |
| | | | 73 |
| | | | 74 |
| | | | 75 |
| | | 10 | 76 |
| | | | 77 |
| | | | 78 |
| | | | 79 |
| | | | 80 |
| | | 100 | 81 |
| | | | 82 |
| | | | 83 |
| | | | 84 |
| | | | 85 |
| | | 1000 | 86 |
| | | | 87 |
| | | | 88 |
| | | | 89 |
| | | | 90 |

## Appendix 2 – Germination

| | Day of sowing | 10 | | | 13 | | | 16 | | | 19 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Soil type | FYM | NPK | CON | FYM | NPK | CON | FYM | NPK | CON | FYM | NPK | CON |
| Concentration [µg/kg] | Control | 6.2 (1.5) | 5.2 (3.1) | 4.8 (2.8) | 15.2 (2.2) | 11.8 (4.6) | 9.6 (1.1) | 15.8 (2.4) | 13.4 (3.6) | 10.4 (1.7) | 16 (2.0) | 14.4 (3.9) | 10.8 (1.6) |
| | 0.1 | 5.6 (3.1) | 6.4 (0.9) | 4.8 (3.4) | 13.6 (3.8) | 14.4 (1.1) | 9.2 (3.0) | 14.4 (3.6) | 14.6 (1.8) | 9.8 (3.5) | 14.8 (3.2) | 14.6 (1.3) | 10.8 (3.1) |
| | 1 | 4.4 (2.1) | 5.4 (2.7) | 6.4 (1.8) | 13.6 (2.0) | 14.4 (3.2) | 12.4 (3.1) | 14.8 (2.2) | 15 (2.8) | 13.6 (2.7) | 14.8 (1.8) | 15.8 (2.6) | 14.8 (3.3) |
| | 10 | 5 (3.1) | 7.4 (3.3) | 3.8 (1.3) | 11.6 (3.5) | 15 (2.6) | 9.2 (1.9) | 13 (3.5) | 15.8 (1.6) | 10.6 (1.7) | 13.4 (2.8) | 15.8 (1.6) | 11.4 (1.8) |
| | 100 | 4.6 (3.1) | 5.6 (2.7) | 4.8 (3.0) | 13.8 (4.8) | 12.2 (3.6) | 11.2 (2.3) | 14 (4.0) | 13.2 (3.1) | 12 (2.4) | 14.8 (4.0) | 14.2 (3.0) | 12.6 (2.2) |
| | 1000 | 4.4 (2.5) | 3.8 (2.5) | 5.6 (2.8) | 12.6 (4.0) | 12 (4.1) | 10.6 (3.1) | 13.6 (3.1) | 13 (3.4) | 12.8 (2.7) | 14.4 (3) | 13.4 (3.3) | 13.6 (2.6) |

## Appendix 3 – Biomass yield

| | | Fresh weight of biomass [g] | | | Fresh weight of roots [g] | | | Number of plants | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Soil type | FYM | NPK | CON | FYM | NPK | CON | FYM | NPK | CON |
| Concentration [µg/kg] | Control | 7.51 (0.76) | 0 (0) | 3.08 (1.88) | 3.59 (0.73) | 0 (0) | 0.17 (0.17) | 4.00 (0) | 0 (0) | 2.00 (1.41) |
| | 0.1 | 7.82 (0.61) | 0 (0) | 1.59 (2.59) | 3.58 (0.18) | 0 (0) | 0.11 (0.24) | 4.00 (0) | 0 (0) | 1.00 (2.59) |
| | 1 | 8.05 (0.37) | 0 (0) | 3.78 (2.24) | 3.62 (0.4) | 0 (0) | 0.37 (0.26) | 4.00 (0) | 0 (0) | 3.78 (1.52) |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 10 | 8.07 (0.36) | 0.51 (1.07) | 3.19 (1.19) | 3.51 (0.22) | 0.03 (0.07) | 0.32 (0.20) | 4.00 (0) | 1.00 (0.55) | 1.80 (0.45) |
| | 100 | 7.78 (0.64) | 0 (0) | 2.69 (1.97) | 3.68 (1.38) | 0 (0) | 0.30 (0.30) | 3.40 (1.34) | 0 (0) | 2.00 (1.87) |
| | 1000 | 8.53 (0.40) | 0 (0) | 2.67 (1.98) | 3.75 (0.19) | 0 (0) | 0.20 (0.16) | 4.00 (0) | 0 (0) | 1.80 (1.48) |

## Appendix 4 – Dehydrogenase activity [µg TPF $g^{-1}$ (dw) $day^{-1}$]

| | Presence of plants | With plants | | | Without plants | | |
|---|---|---|---|---|---|---|---|
| | Soil type | FYM | NPK | CON | FYM | NPK | CON |
| Concentration [µg/kg] | Control | 9.80 (2.70) | 8.12 (2.56) | 7.80 (2.17) | 10.71 (1.65) | 9.89 (2.87) | 8.61 (1.73) |
| | 0.1 | 10.25 (1.44) | 10.93 (0.91) | 6.57 (3.11) | 10.08 (2.39) | 9.92 (2.27) | 9.17 (2.13) |
| | 1 | 8.47 (1.09) | 10.65 (1.97) | 8.32 (2.60) | 9.10 (2.43) | 9.61 (2.58) | 8.57 (1.58) |
| | 10 | 7.64 (1.06) | 9.65 (2.26) | 6.12 (2.58) | 7.73 (3.15) | 8.11 (1.87) | 7.76 (1.82) |
| | 100 | 7.79 (2.11) | 9.27 (4.05) | 7.77 (3.51) | 10.04 (2.02) | 10.77 (1.7) | 8.36 (3.04) |
| | 1000 | 6.86 (2.11) | 10.26 (2.95) | 7.17 (1.24) | 9.93 (2.21) | 8.24 (3.35) | 8.15 (2.38) |

## Appendix 5 – Degradation of climbazole in soil

56 days after application

| | Presence of plants | With plants | | | Without plants | | |
|---|---|---|---|---|---|---|---|
| | Soil type | FYM | NPK | CON | FYM | NPK | CON |
| Concentration [ng/g] | 2H-Climbazole-Dia1 | 48.81 (3.31) | 25.83 (3.71) | 45.91 (4.59) | 43.30 (3.95) | 32.30 (4.66) | 28.15 (4.09) |
| | 2H-Climbazole-Dia2 | 137.86 (9.99) | 91.04 (17.53) | 130.80 (12.76) | 125.15 (9.48) | 105.06 (14.87) | 87.02 (14.25) |
| | Climbazole | 169.75 (24.01) | 257.23 (26.18) | 201.81 (21.92) | 191.01 (13.80) | 226.57 (22.27) | 216.25 (31.44) |
| Area | OH-Climbazole | 1823.54 (341.52) | 2801.68 (387.50) | 2087.19 (214.20) | 2098.57 (266.09) | 2611.77 (421.63) | 2471.17 (493.07) |

28 days after application

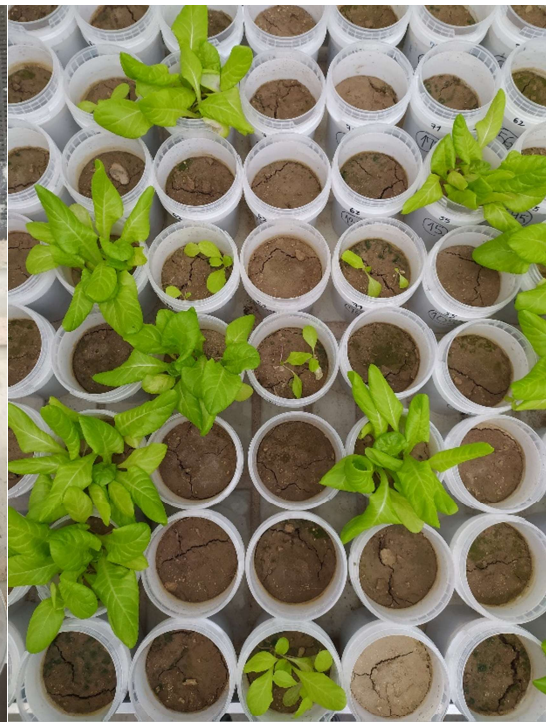
| | Presence of plants | Without plants | | |
|---|---|---|---|---|
| | Soil type | FYM | NPK | CON |
| Concentration [ng/g] | 2H-Climbazole-Dia1 | 30.68 (10.48) | 14.20 (1.15) | 18.88 (2.64) |
| | 2H-Climbazole-Dia2 | 92.74 (31.74) | 51.28 (4.68) | 61.45 (8.76) |
| | Climbazole | 299.88 (56.99) | 304.79 (33.76) | 297.21 (40.02) |
| Area | OH-Climbazole | 3050.92 (645.82) | 3119.42 (333.14) | 2669.20 (481.23) |

## Appendix 6 – Pictures

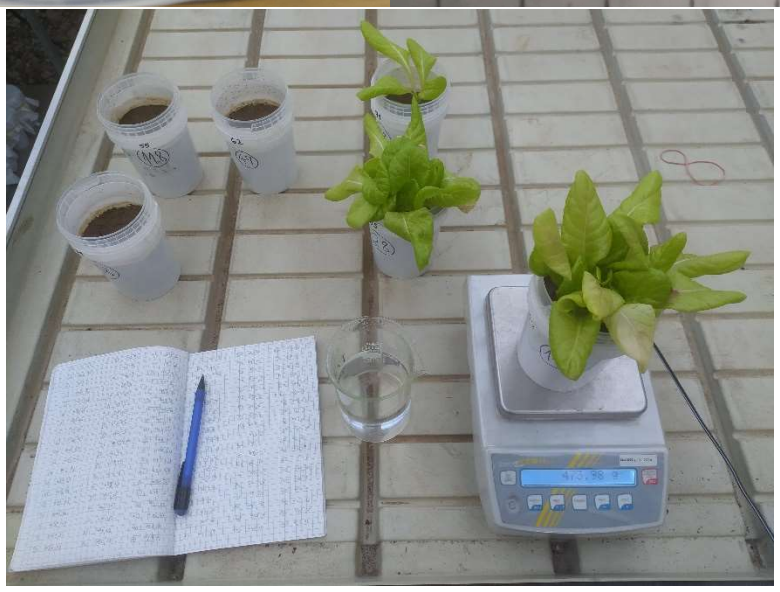
*Picture 1 Applying of climbazole treatments*

*Picture 2 Sowing*

*Picture 3 and 4 Lettuce plants at the beginning and the end of the experiment*



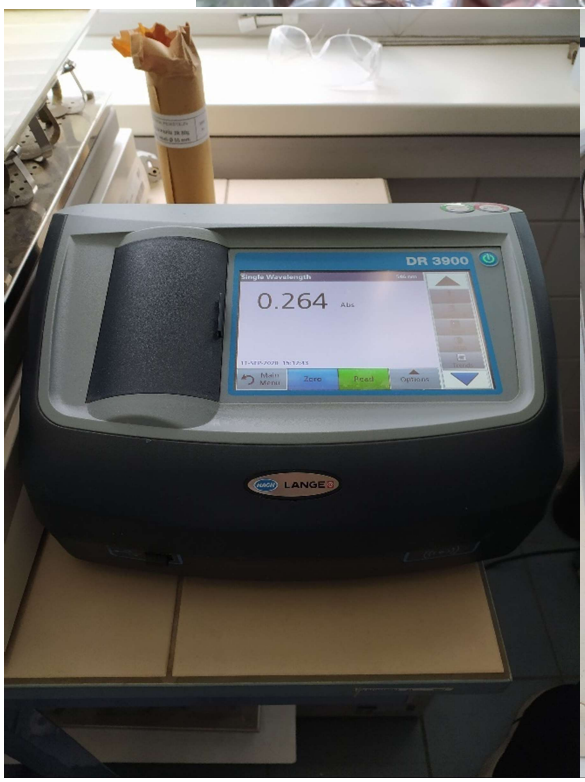
*Picture 5 and 6 Wilting of the plants and root rot*



*Picture 7 Irrigation and recording of the weight*

*Picture 8, 9, 10 and 11 Lettuce harvest*

*Picture 12 Dehydrogenase activity measurement*




*Picture 13 Dehydrogenase activity measurement*

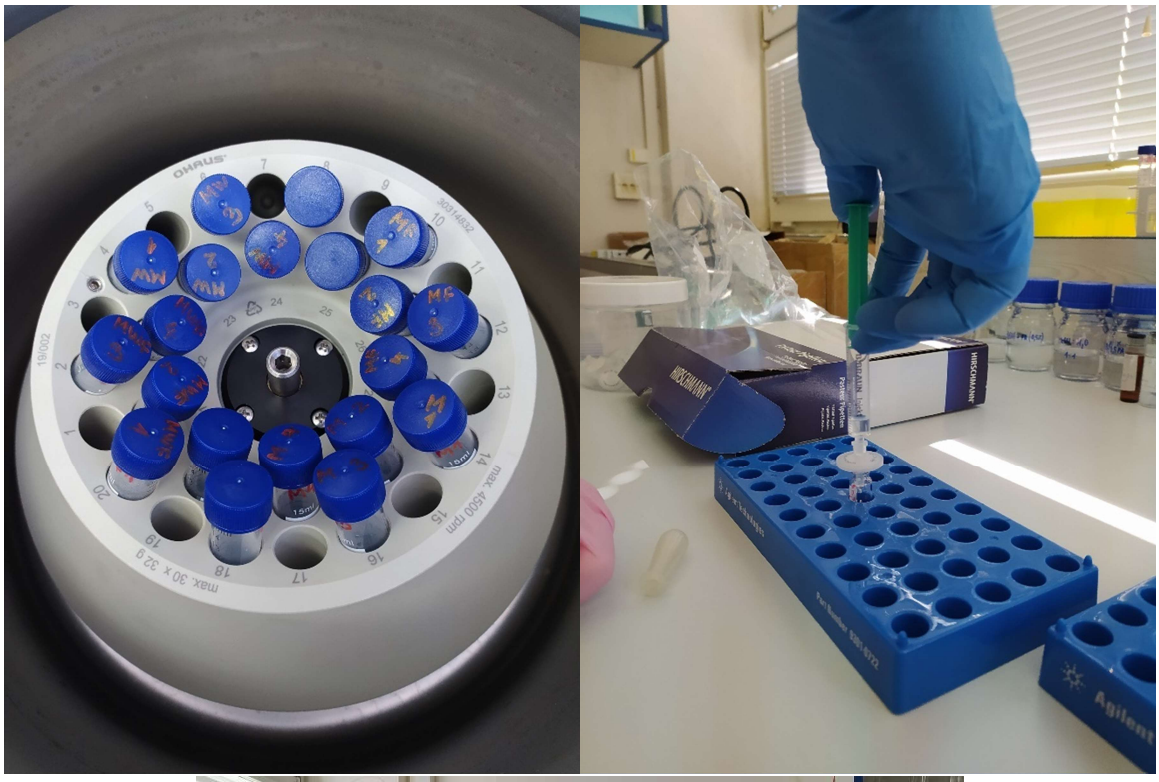

*Picture 14 Drying of the soil samples*

*Picture 15 Freeze-drying of the samples*

*Picture 16 Extraction of climbazoe*

*Picture 17 and 18 Milled freeze-dried lettuce biomass and roots*

*Picture 19 and 20 Extraction of climbazole*



*Picture 21 LC-MS/MS*