The impact of Aspergillus clavatus and Mortierella elongata on the bioactive compounds and yield of lettuce

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THE IMPACT OF ASPERGILLUS CLAVATUS AND MORTIERELLA ELONGATA ON THE BIOACTIVE COMPOUNDS AND YIELD OF LETTUCE

MASTER THESIS

Mia Borojević

Zagreb, September 2024

UNIVERSITY OF ZAGREB
FACULTY OF AGRICULTURE

Environment, agriculture and resource management (INTER-EnAgro)

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MASTER THESIS

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Zagreb, September 2024

UNIVERSITY OF ZAGREB
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STUDENT STATEMENT

ON THE ACADEMIC INTEGRITY

I, Mia Borojević, JMBAG 0178120001, born on 11 February 2000 in Rijeka, Croatia, declare that I independently prepared the Master thesis entitled:

THE IMPACT OF ASPERGILLUS CLAVATUS AND MORTIERELLA ELONGATA ON THE BIOACTIVE COMPOUNDS AND YIELD OF LETTUCE

With my signature I guarantee:

- that I am the sole author of this Master thesis;
- that all literature sources used, both published and unpublished, are appropriately cited or paraphrased and listed in the reference list at the end of the Master thesis;
- that this Master thesis does not contain any parts of work previously submitted to the Faculty of Agriculture or other higher education institutions for the purpose of completing university or specialist study programme
- that the electronic version of this Master thesis is identical to the printed version which has been reviewed by the Committee and approved by the mentor
- that I am acquainted with the regulations of the Code of Ethics of the University of Zagreb (Article 19).

In Zagreb, on _____ _____

UNIVERSITY OF ZAGREB
FACULTY OF AGRICULTURE

REPORT

ON EVALUATION AND DEFENSE OF GRADUATE THESIS

Master thesis of the student Mia Borojević, JMBAG 0178120001, entitled

THE IMPACT OF ASPERGILLUS CLAVATUS AND MORTIERELLA ELONGATA ON THE BIOACTIVE COMPOUNDS AND YIELD OF LETTUCE

was defended and evaluated with the grade _____, on _____.

Committee: signatures:

1. Asst. Prof. Ivana Rajnović, PhD mentor _____
2. Ines Petrić, PhD, scientific adviser co-mentor _____
3. Assoc. Prof. Sanja Radman, PhD member _____
4. Assoc. Prof. Jana Šic Žlabur, PhD member _____

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Content

Summary

Of the Master thesis of student **Mia Borojević**, entitled

THE IMPACT OF ASPERGILLUS CLAVATUS AND MORTIERELLA ELONGATA ON THE BIOACTIVE COMPOUNDS AND YIELD OF LETTUCE

This master's thesis investigates the impact of plant growth-promoting fungi (*Aspergillus clavatus* and *Mortierella elongata*) on the yield components (height, diameter, mass) and content of bioactive compounds (phenols, flavonoids, non-flavonoids, total chlorophyll, chlorophyll a, chlorophyll b, carotenoids, ascorbic acid and antioxidant capacity) in lettuce (*Lactuca sativa* L.). The research was conducted through an open-field experiment, applying different subcultures of these PGP fungi to lettuce plants. Statistical analysis using the ANOVA method and Tukey's post-hoc test confirmed significant differences of bioactive compounds in inoculated treatments in comparision to uninocultaed (control). Higher values indicate that used PGP fungi, significantly enhance lettuce nutritional quality and contribute to the lettuce resilience, contributing to sustainable agricultural practices.

Keywords: *Aspergillus clavatus*, *Mortierella elongata*, lettuce, PGP fungi, sustainable agriculture

Sažetak

Diplomskog rada studenta/ice **Mia Borojević**, naslova

UTJECAJ ASPERGILLUS CLAVATUS I MORTIERELLA ELONGATA NA BIOAKTIVNE SPOJEVE I PRINOS SALATE

Ovaj diplomski rad istražuje utjecaj gljiva koje potiču rast biljaka (*Aspergillus clavatus* i *Mortierella elongata*) na komponentne prinosa (visina, promjer i masa) i sadržaj bioaktivnih spojeva (fenoli, flavonoidi, neflavonoidi, ukupni klorofil, klorofil a, klorofil b, karotenoidi, askorbinska kiselina i antioksidativni kapacitet) u salati (*Lactuca sativa* L.). Istraživanje je provedeno pokusom na otvorenom, primjenom različitih potkultura ovih PGP gljiva na biljke salate. Statistička analiza metodom ANOVA i Tukeyjevim post-hoc testom potvrdila je značajne razlike bioaktivnih spojeva u inokuliranim tretmanima u usporedbi s neionkuliranim (kontrola). Više vrijednosti pokazuju da korištene PGP gljive značajno poboljšavaju nutritivnu kvalitetu salate i doprinose otpornosti salate, doprinoseći održivoj poljoprivrednoj praksi.

Ključne riječi: *Aspergillus clavatus*, *Mortierella elongata*, salata, rast biljaka, održiva poljoprivreda

1. Introduction

Given projections that the world population will reach 9.7 billion by 2050 while the area of arable land continues to decline, the global agricultural sector faces the challenge of ensuring sufficient food production for the growing population while minimizing environmental impact. Today's intensive agricultural systems, which heavily rely on chemical fertilizers and pesticides, are increasingly criticized for their environmental consequences, including soil degradation, water pollution and loss of biodiversity. These challenges are further intensified by the increasing frequency of extreme weather events driven by climate change, such as droughts and floods. These negative impacts are driving the scientific community to seek more sustainable solutions that can increase productivity, enhance crop resilience while reducing the ecological footprint (Popp et al., 2013).

A necessary policy response, led by the European Union, aims to reduce the use of chemicals in agriculture. As part of this effort, the EU is advancing sustainable agricultural practices through initiatives such as the Green Deal (EU Directive 2009/128/EC on the sustainable use of pesticides, 2009). One promising solution in this context is the use of microorganisms, particularly plant growth-promoting fungi (PGPF). These fungi have garnered significant attention due to their numerous beneficial effects on plants, making them an environmentally friendly strategy for both conventional and organic agriculture (European Union, n.d.).

As a part of extreme weather conditions, the project PERSPIRE (Potential of the Rhizosphere Microbiome in Adaptation of Agriculture to Climate Change) explored the role of soil microorganisms, including PGPF, in helping plants cope with stress conditions caused by climate change. The fungal isolates, *Aspergillus clavatus* and *Mortierella elongata*, arriving from this project, were specifically selected with the aim to study their potential to enhance plant resilience, particularly under flood conditions.

Microorganisms like PGPF play a crucial role in enhancing plant growth through various mechanisms. For example, *Aspergillus clavatus* is noted for its ability to act as a hormonal stimulator and to break down complex organic matter, thereby releasing nutrients that plants can absorb (Liao et al., 2019, Lucas et al., 2021). Additionally, these fungi can produce phytohormones such as gibberellins, which mediate stem elongation and other vital processes in plant development, thereby increasing crop yields (Ozimek & Hanaka, 2021). Another promising, yet underutilized, species is *Mortierella elongata*, which is effective in improving phosphorus absorption, a nutrient essential for plant growth (Liao et al., 2019; Zhang et al., 2020; Vacheron et al., 2013). These fungi also contribute to improving soil structure, further enhancing plant health and productivity (Lucas et al., 2021, Ozimek & Hanaka, 2021). However, the full potential of certain PGPF species, including *Aspergillus clavatus* and *Mortierella elongata*, remains largely untapped.

Lettuce (*Lactuca sativa* L.), a crop with high nutritional value and wide commercial application, serves as an ideal model plant for studying the impact of PGPF. The introduction of fungi like

A. clavatus and *M. elongata* in lettuce cultivation systems offers new solutions for sustainable agriculture (Vishwakarma et al., 2017). The fact that plants become more resistant to pathogens and stress conditions, such as drought or low temperatures, further indicates that PGPF could play a key role in future agricultural practices (Asghari et al., 2020). PGPF fungi have the ability to induce systemic resistance in plants, not only enhancing their resistance to pathogens but also improving their nutritional quality, making these fungi vital tools in modern agronomic practices. The interaction of plants with PGPF boosts the synthesis of bioactive compounds, such as phenols, chlorophylls, caratenoids and ascorbic acid, which provide protection to plants and contribute to human health when included in the diet (Alok et al., 2014; Cruz et al., 2012; Seong et al., 2015). The increasemnet of their concentrations in lettuce has become a goal of breeding programs (Fan, 2013).

1.1. The aim

The study focuses on examining the effects of *Aspergillus clavatus* and *Mortierella elongata* on yield components (morphological properties -height, diameter and mass), with particular attention to the accumulation of bioactive compounds, such as phenols, flavonoids, nonflavonoids, photosynthetic pigments (chlorophyll a, chlorophyll b, total chlorophylls and total carotenoids), ascorbic acid, as well as their antioxidant capacity. The driving hypothesis of this study is that the application of *A. clavatus* and *M. elongata* will not only enhance the morphological properties of lettuce but also increase the concentration of bioactive compounds. It is anticipated that inoculated lettuce plants will improve nutrient absorption, and thus greater accumulation of bioactive compounds compared to non-inoculated plants. Through testing this hypothesis, the research aims to provide comprehensive insights into the advantages of utilizing. *A. clavatus* and *M. elongata* in lettuce cultivation. Moreover, this study will contribute valuable data on the interactions between these fungi and lettuce plants, offering a deeper understanding of sustainable agricultural practices. The findings could inform future strategies for optimizing both morphological properties and nutritional quality, advancing efforts towards more resilient and nutritious food production systems

2. Literature view

2.1. Challenges and Strategies in Modern Agriculture

Given the continuous growth of the global population, which is projected to reach 9.7 billion by 2050 (UN, 2015), the demand for food is rapidly increasing. However, the amount of arable land per capita is steadily decreasing—from 0.45 hectares per person in 1961 to 0.21 hectares in 2016 (FAO, 2016). To meet the needs of the growing population, agricultural production will need to double, while at the same time improving efficiency and nutrition quality, despite the decreasing availability of arable land. However, modern agriculture, which largely depends on the intensive use of chemicals pesticides and fertilizers, causes significant environmental problems and contributes to climate change. Although climate change is a global issue, agriculture is one of the sectors most severely impacted (Popp et al., 2013).

Excessive use of mineral fertilizers depletes essential nutrients and reduces soil organic matter, leading to soil compaction, loss of structure, and a decline in water retention capacity.
Additionally, this degradation releases stored carbon from the soil into the atmosphere as CO_2, exacerbating climate change (Lal, 2004). Moreover, increased CO_2 emissions from degraded soils, combined with the intensive use of nitrogen-based fertilizers, can cause soil acidification, further reducing soil fertility. This creates a cycle in which degraded soil requires more chemical inputs to maintain productivity, worsening environmental damage over time. What is more, the excessive use of mineral fertilizers, such as nitrogen and phosphorus, leads to nutrient runoff into water systems, causing eutrophication and reducing water quality and biodiversity (Chandra et al., 2021). Soil degradation and climate changes threats beneficial soil microorganisms, such as bacteria and fungi, reducing their diversity and their ability to provide plants with essential nutrients. (Dubey et al., 2019; Lal, 2004; Singh et al., 2011).

In addition to soil degradation, water pollution and loss of biodiversity, climate changemanifesting through more frequent and severe droughts, extreme rainfall, and rising global temperatures- poses additional stress on agriculture. Droughts limit water availability, while floods suffocate plant roots, making it increasingly difficult to sustain crop productivity. This further disrupts the phenological cycles of plants, shortening growing seasons, and affecting pollination and fruit development. Extreme weather conditions also increase the spread of plant diseases and pests, driving up the costs of crop protection (Chandra et al., 2021). According to a report from the European Environment Agency, Croatia is among the three European countries most affected by weather-related disasters, including storms, floods, and hail (EEA, 2017).

Another pressing challenge in modern agriculture is the growing scarcity of phosphorus, a vital component of mineral fertilizers. Experts predict that exploitable phosphorus reserves could decline by 25% by 2100, while global demand for this crucial nutrient is expected to double. At the same time, nitrogen fertilizer production, despite relying on the abundant supply of atmospheric nitrogen, consumes significant amounts of energy, contributing not only to greenhouse gas emissions but also to rising production costs as energy prices increase (Hale, 2017; Maggio et al., 2012).

The European Union (EU) is aware of these issues and is leading efforts toward sustainable agriculture. The EU has set goals to reduce chemical pesticide use by 50% and artificial fertilizers by 20% by 2030 through the European Green Deal initiative and the Farm-to-Fork strategy. The Common Agricultural Policy (CAP) has been revised to incentivize farmers to adopt sustainable practices. The EU Directive 2009/128/EC on the sustainable use of pesticides further encourages the adoption of biological alternatives (European Union, n.d.).

Economic sustainability in agriculture requires a broad strategy that includes genetic resistance, managing microclimatic conditions, and maintaining ecosystems (Butcher et al., 2013; Long & Ort, 2010; Xiong et al., 2022). Preparations based on microorganisms or their secondary metabolites, including PGPF, provide natural alternatives to chemical inputs for sustainable farming practices (Breza-Boruta & Bauza-Kaszewska, 2023). These preparations improve soil health by enhancing microbial diversity and activities, leading to improved soil structure and fertility (Verma & Prasad, 2020). Additionally, they can help plants develop resistance to biotic and abiotic stresses, supporting crops in overcoming the impacts of extreme weather conditions, diseases, and pests. As a result, they are increasingly recognized as a promising solution, reflected in the rapidly expanding global market for microbial inoculants, which boasts an annual growth rate of around 10% (Berg, 2009).

2.2. Plant-Growth-Promoting Fungi (PGPF)

Plant-growth-promoting fungi (PGPF) are beneficial, non-pathogenic saprophytes that naturally occur in soil ecosystems, contributing to soil fertility and supporting plant development and growth. These fungi not only enhance plant growth but also serve as biocontrol agents, helping crops resist pathogens and environmental stress. Unlike mycorrhizal fungi, which form specific symbiotic structures (mycorrhizae) with plant roots, PGPF do not create such structures but rather function in the rhizosphere zone. Effective PGPF species include *Fusarium, Aspergillus, Penicillium, Trichoderma, Piriformospora, Phoma*, and *Rhizoctonia* (Naziya et al., 2019).

One of the primary ways in which plant growth-promoting fungi (PGPF) enhance plant development is through root colonization. This process not only prevents pathogen invasion but also improves nutrient absorption, such as phosphorus and potassium, resulting in enhanced plant growth. PGPF are capable of solubilizing nutrients, making them more accessible to plants, which further contributes to improved growth. In addition, these fungi produce phytohormones like auxins, gibberellins, and cytokinins, which directly stimulate root growth and overall plant development (Naziya et al., 2019). Beyond their direct effects on plants, PGPF also operate through indirect mechanisms, such as induced systemic resistance (ISR). This process involves the recognition of microbe-associated molecular patterns (MAMPs) by plant pattern recognition receptors (PRRs), which triggers the jasmonic acid (JA) and ethylene signaling pathways. ISR enables plants to prime their defense mechanisms, leading to a faster and stronger response to pathogen attacks without the need for constant high expression of defense genes. This resistance signal spreads throughout the plant, providing protection even to tissues not directly in contact with the fungi. ISR also modulates plant metabolism, increasing the production of bioactive compounds, further strengthening the plant's defenses (Shoresh et al., 2010; Van Wees et al., 2008). PGPF also have the ability to synthesize antimicrobial

compounds and enzymes that directly suppress soil-borne pathogens, thereby contributing to enhanced plant resistance (Naziya et al., 2019).

2.2.1. Applications in Agriculture

To meet the specific needs of various crops, PGPF have been developed into two primary formulations: liquid and dried (powdered) (Vijaykumar, 2023). PGPF formulations are applied through different methods, depending on the agricultural goals and the type of crop being treated.

Seed inoculation is one common method of applying PGPF, where fungi are introduced to seeds to encourage root development, increase seedling vigor, and provide protection against soilborne pathogens. An example of this is the use of *Trichoderma harzianum* in powdered form. It is applied directly to sesame seeds during the seed coating process to improve germination and protect against diseases caused by *Macrophomina phaseolina* and *Sclerotium rolfsii*. Additionally, liquid formulations of *T. harzianum* have been applied to seeds, in combination with biopolymer chitosan and fungicides, further enhancing protection and improving crop resilience (Vijaykumar, 2023).

Root inoculation involves applying PGPF directly to plant roots to promote plant growth, increase root and shoot length, and improve overall plant health. Root inoculation helps increase plant resistance to pathogens and improve nutrient uptake. For example, inoculating tomato plants with *Aspergillus flavus*, *Aspergillus niger*, *Mucor circinelloides*, and *Penicillium oxalicum* has shown to enhance shoot and root length, increase the number of leaves, and improve the content of photosynthetic pigments and carbohydrates. Additionally, this treatment reduced the severity of *Fusarium oxysporum* infection by significant percentages and provided high levels of protection (Attia et al., 2022). Adedayo et al., (2022) have shown that *Penicillium* and *Sordaria* are effective against powdery mildew caused by the pathogen *Oidium neolycopersici*. In cucumber, *Trichoderma* and *Pestalotiopsis* helped suppress downy mildew caused by *Pseudoperonospora cubensis* (Pandit et al., 2022). For pear, the fungi *Cladosporium ramotenellum* and *Phoma spp.* showed a positive effect on plant growth under in vitro conditions, including increases in fresh plant weight, stem length, and root length. Nectarine also benefited from *Cladosporium ramotenellum* and *Phoma spp.*, which improved plant growth and facilitated acclimatization to soil after in vitro cultivation (Cantabella et al., 2020). *Mortierella elongata* shows significant potential in promoting plant growth of various crop species, including bahiagrass, corn, tomato, squash, and watermelon (Zhang et al. 2020).

In hydroponic systems, *Trichoderma harzianum* combined with the bacterium *Pseudomonas chlororaphis* and the fungus *Gliocladium catenulatum* has proven to be highly effective in reducing spore germination of the pathogen *Fusarium oxysporum*, thereby increasing tomato plants' resistance to diseases and improving yield. Additionally, the non-pathogenic variant of the fungus *Fusarium solani* applied to zucchini increased the plants' resistance to the pathogen *Phytophthora capsici*, resulting in better yields and reduced losses due to stem rot. *Trichoderma polysporum*, combined with the bacterium *Streptomyces griseoviridis*, exhibited strong antagonistic properties against *Phytophthora cryptogea* in zucchini, reducing disease severity and enhancing plant resistance (Mourouzidou et al., 2023).

Advanced technology has led to the development of microencapsulation techniques, where PGPF spores are encapsulated in microcapsules that gradually release the fungi into the soil or onto the plant. *Trichoderma ghanense* and *Trichoderma reesei* were used as microcapsules on cocoa against *Moniliophthora roreri*. In vitro inhibition ranged from 73.8% to 85.5%. In field trials, microcapsules applied to 15-day-old fruits showed no external disease symptoms, and in 28-day-old fruits, internal severity was reduced to 60% compared to 81%-100% in control treatments (Avilés et al., 2023). Advanced technologies for the application of fungi enhance their use and expand their application in various fields of agronomy, contributing to the implementation of sustainability across various production systems.

2.3. Aspergillus Clavatus

2.3.1. Biological Characteristics

Aspergillus clavatus (synonyms: *A. apicalis, A. pallidus*) is a part of the section *Clavati* which includes six species (Varga et al., 2007). *A. Clavatus* grows optimally at around 25°C, with a minimum temperature of 5–6°C and a maximum near 42°C (Hocking, 2006). Its thermotolerant and alkaline and acid-tolerant nature allows *A. clavatus* to flourish in various environments, including soil, moist surface, compost piles, and stored grains and nuts, where it can cause spoilage and contamination with mycotoxins (Mokobi, 2021; Varga, et al., 2007). During the growth of *A. clavatus*, the glucose-nitrate mineral media's reaction became acidic, indicating that this species naturally lowers the pH of its environment as it grows (Waksman et al., 1943).

A. clavatus is characterized by its long, smooth conidiophores (Figure 2.1.) that terminate in large, club-shaped vesicles densely covered with phialides (specialized cells that produce asexual spores known as conidia). These conidia are smooth, round to slightly oval, and range in color from light to dark green, often forming long chains that give the fungus its distinctive "clavate" appearance under the microscope. The colonies of *A. clavatus* start out as white or pale and gradually turn green to olive-green as they mature, with the underside of the colonies typically pale to yellowish-brown. On solid media like Potato Dextrose Agar (PDA) or CzapekDox Agar (Figure 2.3.1.1.), colonies reaching a diameter of 4-5 cm within seven days at 25°C and can rise up to 3 mm in height under favorable conditions (Mokobi, 2021).

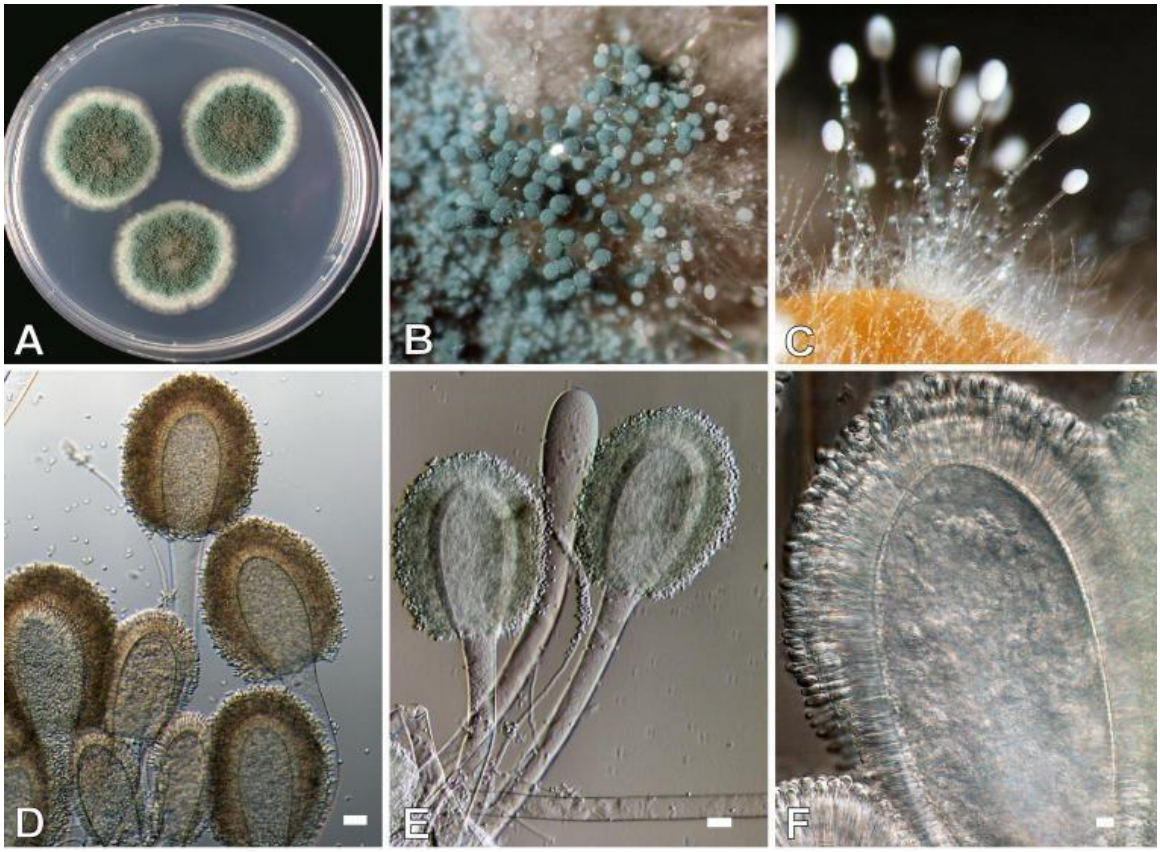


Figure 2.3.1.1. *A.clavatus* colonies after seven days at 25 °C on Czapek-Dox Agar (B-C= macrophotograph of conidiophores, D -F= conidiophores, D and E scale bars = 30 μm, F=10 μm (Source: Varga et al., 2007)

It is well known to produces several mycotoxins that pose significant health risks. One of the most known is patulin, commonly found in moldy apples, pears, peaches, and grapes. Recognized as a potential carcinogen, patulin is regulated in many countries due to its cytotoxic, immunotoxic, and neurotoxic effects (Mahato et al., 2021; Mokobi, 2021; Perrone et al., 2007; Puel et al., 2010). In addition to patulin, *A. clavatus* produces clavacin, an antibiotic distinct from fumigacin, with potent activity against gram-negative and gram-positive bacteria. Clavacin's solubility in ether, chloroform, alcohol, and water highlights its unique chemical properties (Waksman et al., 1943).

2.3.2. Potential Modes of Action on Plant Growth Promotion

One of the most important way *Aspergillus clavatus* promotes plant growth is through the production of gibberellic acid (GA), particularly in stem elongation and seed germination (Lucas et al., 2021).

A. clavatus also produces cellulases, which break down cellulose into simpler sugars like glucose. This glucose can be utilized as an energy source for plants or as a nutrient supplement in the soil. The fungus also produces hemicellulases that degrade hemicellulose, a complex polymer made up of various sugars, such as xylose, enhancing the availability and absorption of these sugars by plants. Pectin-degrading enzymes, such as pectin lyases and pectin esterases, are also produced by *A. clavatus*. These enzymes break down pectin, a substance that binds

cells within plant cell walls, facilitating root growth and improving soil structure. Additionally, *A. clavatus* produces lignin-degrading enzymes, including lignin peroxidases and manganese peroxidases. These enzymes break down lignin, increasing the availability of cellulose and hemicellulose for further enzymatic degradation (Lucas et al., 2021).

2.4. Mortierella Elongata

2.4.1. Biological Charasteristics

Mortierella elongata belongs to the genus *Mortierella* of the family *Mortierellaceae* (Nguyen et al., 2019). *M. elongata* demonstrates a high level of adaptability to a wide range of ecological conditions and it is one of the most common soil taxa, accounting more than 0.5% of the total fungal population in northen Florida agricultural soils. It has been found in various habitats worldwide, including extreme environments such as Antarctica, tundras in Alaska, arid agricultural regions in India, and coastal regions in Mexico. *M. elongata* prefers soil pH levels ranging from 4 to 7. *M. elongata* is capable of forming sporangia on nutrient-poor media, such as water agar, even at low temperatures (Liao, 2020; Zhang et al. 2020).

The colonies of this fungus (Figure 2.2.) are usually pale white or whitish, with a characteristic zonate, rosette-like growth pattern, and they may emit a garlic-like odor during growth (Ozimek & Hanaka, 2021). *M. elongata* is distinguished by its delicate, hyaline mycelium that produces elongated, cylindrical sporangiophores. These sporangiophores bear sporangia containing a single large sporangiospore, typically ovoid to ellipsoid in shape, with colors ranging from light gray to brown. When cultured on potato dextrose agar (PDA) at 20°C, *M. elongata* reaching a diameter of 62–65 mm after just five days of incubation. (Nguyen et al., 2019; Liao, 2020).

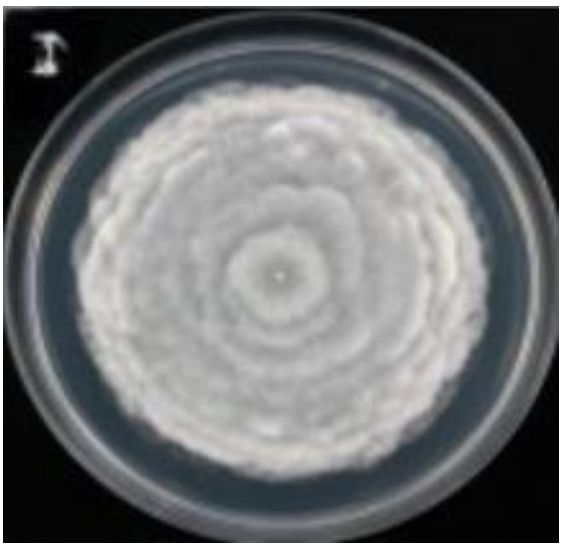


Figure 2.2. *Mortierella elongata* colony on PDA
(Source: Nguyen et al., 2019)

M. elongata is capable of producing lipids, including linoleic, alpha-linolenic, arachidonic, and docosahexaenoic acids, which are vital for various biological functions. These metabolic traits highlight its potential as a candidate for lipid and biofuel production (Liao et al., 2020).

2.4.2. Potential Modes of Action on Plant Growth Promotion

In Figure 2.3. is shown that as an endophyte, *M. elongata* forms biofilms on plant roots, enabling direct interaction with plant cells and facilitating colonization. This interaction enhances nutrient absorption and protection of the plant (Liao et al., 2019).

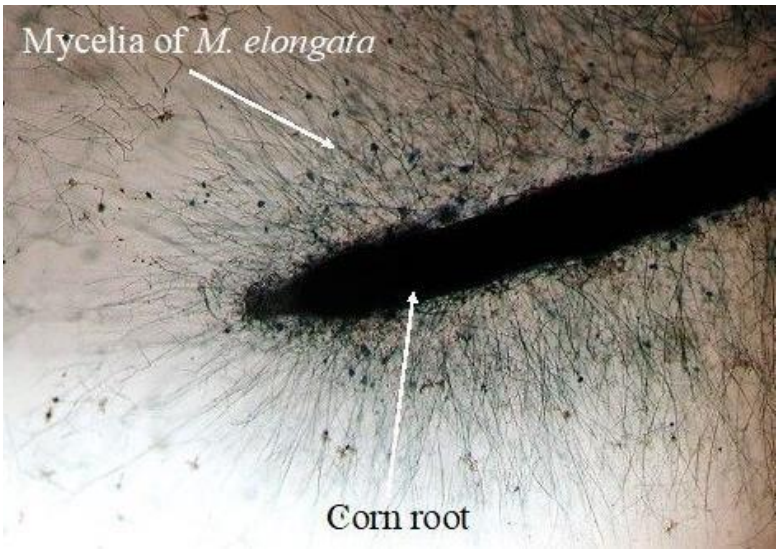


Figure 2.3. Mycelia forming biofilm in association with a corn root tip
(Source: Liao et al., 2019)

M. elongata produces phytohormones, including gibberellic acid (GA), auxin (IAA) and abscisic acid (ABA). Auxin plays a crucial role in cell growth, elongation, and root formation. Abscisic acid is important not only for plant development but also for enhancing resistance to biotic and abiotic stresses, such as drought. Notably, a 40% increase in ABA and IAA was observed in maize roots inoculated with *M. elongata*. (Ozimek & Hanaka, 2021). *M. elongata's* genome includes genes for the biosynthesis of antibiotics like streptomycin, butyrosin, and neomycin, which further enhance its ability to control soil-borne pathogens (Ozimek & Hanaka, 2021; Zhang et al., 2020).

As a saprotroph, *M. elongata* plays a crucial role in breaking down complex organic polymers like cellulose, hemicellulose, and chitin (Ozimek & Hanaka, 2021). *M. elongata's* metabolism primarily relies on simple carbon sources such as D-glucose, D-trehalose, and D-mannose. In its natural environment, it can utilize N-acetylglucosamine, a chitin monomer, as both a carbon and nitrogen source. This capability makes *M. elongata* one of the most important chitin decomposers in the soil, providing essential nutrients like carbon and nitrogen to plants (Liao, 2020 ; Zhang et al., 2020).

The fungus produces enzymes such as phosphatase and beta-glucosidase, which break down organic compounds rich in phosphorus and carbon, thereby increasing the availability of these essential nutrients in the soil. This ability is particularly valuable in nutrient-poor soils, where phosphorus is critical for energy transfer, photosynthesis, and nutrient movement within plants (Liao et al., 2019; Zhang et al., 2020; Vacheron et al., 2013). Iron acquisition is another critical function of *M. elongata*, which produces siderophores that bind iron in the soil and facilitate its transport to plants. Iron is an essential micronutrient but is often poorly soluble in oxidized soils. The siderophores produced by *M. elongata* bind Fe3+, improving its absorption by plants and enhancing their iron nutrition and overall stress resistance (Ozimek & Hanaka, 2021).

2.5. Lettuce

2.5.1. Morphological Properties

Lettuce (*Lactuca sativa* L.) is an annual plant that belongs to the Asteraceae family. Letuce has a taproot that branches out and grows in the upper soil layer, typically at a depth of 30 to 35 cm. The diameter of the root corresponds to the width of the leaf rosette. The lettuce stem consists of nodes and internodes, which are shortened in the early growth stage, while later, the stem elongates, reaching a height of up to 1.5 meters. Leaves grow from the stem, forming a rosette. The outer green leaves of lettuce are richer in vitamins compared to the inner leaves of the head. Lettuce flowers are gathered in flower heads surrounded by bracts. Each flower head contains about 15 yellow, ligulate, bisexual flowers. The fruit of lettuce is an achene and the seeds are elongated or oval, weighing between 0.6 and 1.3 grams per 1000 seeds (Lešić et al., 2016).

2.5.2. Outdoor Cultivation and Growth Conditions

Croatia's varied climate allows for lettuce cultivation throughout the year. Spring varieties, transplanted in late March, are ready for harvest in May and June, while summer varieties mature in July and can be harvested in August if irrigation is available. Winter varieties are planted in autumn and are ready for harvest in April and May after overwintering. Although it is possible to cultivate lettuce as a monocrop, crop rotation is recommended to avoid continuous cultivation on the same land.

Cultivation of lettuce in open fields typically begins with the production of seedlings. Seedlings are grown with a clump of substrate in polystyrene containers. This method of cultivation ensures proper and uniform vegetative space, contributing to consistent growth and high uniformity of the seedlings. During the cultivation of seedlings, air temperature until seedling emergence is at around 20°C (for 3 to 5 days), after which it is reduced to around 15°C. Seedling cultivation lasts 3 to 6 weeks, depending on the length of the day. Planting lettuce in open fields is done when the seedlings reach the stage of 4 to 5 fully developed leaves. Planting is done shallowly, up to the root collar. Spacing between rows is 25 to 35 cm, and between plants in a row, 20 to 35 cm, depending on the cultivar. Immediately after planting, the soil is watered to encourage root establishment. Harvesting is performed by cutting with a knife at the root collar

zone in 2 to 3 rounds, depending on growing conditions. Lettuce yields in open fields with optimal conditions can reach 30 to 40 tons per hectare. (Lešić et al., 2016).

Lettuce performs best in medium-textured soils rich in organic matter, while lighter soils are more suitable for early planting because they warm up faster. Neutral soils with a pH around 7 are ideal (Lešić et al., 2016). According to Fritz and Stoltz (1989), 30 kg of P_2O_5, 150 kg of K_2O, 40 kg of CaO, 14 kg of MgO, and from 80 to 120 kg of nitrogen are needed for a lettuce yield of about 30t/ha. Lettuce is very sensitive to a high concentration of salt in the soil (more than 0,3%), so fertilizing with mineral fertilizers, if there is not enough water, can cause damages like edge burning (Lešić et al., 2016). Parađiković (2009) states that lettuce has higher requirements for P, Mg and B, and due to the shallow roots, it is necessary to fertilize before planting with the application of easily soluble fertilizers.

Crop care includes regular loosening of the soil to destroy weeds and improve soil aeration until the lettuce rosette covers the surface. Irrigation is a critical factor in lettuce cultivation as the plant has a shallow root system, meaning it cannot reach water from deeper soil layers. Therefore, it is necessary to maintain moisture in the top 15 cm of soil above 65% of field water capacity. In spring cultivation, about 150 liters of water per square meter is required (Lešić et al., 2016).

2.5.3. Nutritional and Health Value

Lettuce is mostly used fresh, as this is how its nutritional components are best utilized.
According to nutritional standards, the annual consumption per capita should be 6 kg, while in European countries it is consumed from 2.4 to 4 kg per capita (Lešić et al., 2016)

Table 2.5.3.1. shows that, in addition to being low-calorie, the lettuce s rich in fiber (0.54-1.5%) and minerals (0.43-1.4%), especially calcium and iron (Slamet, 2017, Parađiković, 2009). The veins in the leaf contain more sodium and potassium citrate and fiber than green leaves (Parađiković, 2009). Lettuce contributes to the health of the digestive system thanks to its high levels of dietary fiber, which promotes healthy digestion and maintains the balance of intestinal microbiota. Fiber also helps regulate blood sugar levels (Zhang et al., 2020). Furthermore, it contains citric acid and malic acid, which give it a distinctive taste. The bitter taste of lettuce is caused by lactopicrin, lactucinic acids, lactocerol and neolactucin (Parađiković, 2009).

Table 2.5.3.1. Basic chemical compostion of fresh lettuce leaves (%)

Component	Composition (%)
Water	91.2-95.9
Crude proteins	0.8-2.25
Crude fats	0.1-0.4
Carbohydrates	0.1-0.4
Of which sugars	0.1
Fiber	0.54-1.5
Fats	0.43-1.4
Minerals	0.43-1.4

Souce: Lešić et al. (2016)

Lettuce also contains significant amount of bioactive compounds. Among these, the most prominent are compounds with pronounced antioxidant activity, including phenols, photosynthetic pigments (chlorophylls, carotenoids) and ascorbic acid (vitamin C). Antioxidant activity include ability of removing or inhibiting free radicals (which are extremely reactive due to free electrons) and chelating metal ions that would otherwise lead to the formation of free radicals. Because of significant amount of bioactive compounds, consumption of lettuce has a beneficial effect on human health by slowing aging, lowering cholesterol levels, reducing the risk of cancer, and protecting the heart and blood vessels (Alok et al., 2014; Seong et al., 2015). Increasing of bioactive compounds in lettuce has become a goal of breeding programs (Fan, 2013).

Polyphenolic compounds are secondary metabolites which, as chemical compounds whose synthesis is activated under stress conditions, are crucial for the plant's defense mechanism (Lattanzio 2013). Polyphenolic compounds are mainly divided into two groups: flavonoids, which are based on the common C6-C3-C6 skeleton consisting of two phenyl rings (A and B) linked by a heterocyclic ring (C), and non-flavonoids, such as phenolic acids (C6-C1) (Tutino et al., 2020). Cho et al. (2023) identified chlorogenic acid, chicory acid and coumaroylquinic acid as key phenolic acids in lettuce, also as key nonflavonoids. Phenolic substances are aromatic, which causes them to absorb intensely within the UV spectrum (Van Sumere, 1989). UV radiation primarily stimulates the accumulation of flavonoids, while temperature has a greater influence on the accumulation of certain phenolic acids, including rosmarinic acid, p-anisic acid, and vanillic acid (Sytar et al. 2018). Some flavonoids exhibit strong insecticidal activity or anti-estrogenic effects, which can cause infertility in mammals (Pevalek-Kozlina 2003). Flavonoids are also responsible for the coloration of vegetables (red, blue, purple) (Jatoi et al., 2017). The red lettuce varieties possess higher composition of flavonoids than the green varieties (Mampholo et al., 2016).

The primary porphyrin pigments found in vegetables are chlorophyll a and b (Costache et al., 2012). These pigments are water-insoluble due to their nonpolar structure, in which the porphyrin ring is bound to a magnesium ion. Chlorophyll a and b function as photoreceptors, absorbing light energy for the photosynthesis process, which converts carbon dioxide and water into glucose and oxygen. Chlorophyll pigments have a broad absorption range from blue (400500 nm) to red (600-700 nm). Chlorophyll a, characterized by a CH3 (methyl) group in its side chain, is the main pigment in photosynthesis and appears blue-green. Chlorophyll b, containing a CHO (aldehyde) group, acts as an accessory pigment, absorbing light at wavelengths where chlorophyll a is less efficient and transferring the energy to chlorophyll a. Chlorophyll b is recognizable by yellow-green color (Morna, 2015).

Carotenoid pigments are found in chromoplasts, where they contribute to the color of fruits and vegetables, and in chloroplasts, where they function alongside chlorophyll in the two photosystems (Costache et al., 2012). Carotenoids are a class of orange, yellow, and red pigmented poly-isoprenoid hydrocarbons that concentrate in animal lipids. They are divided into two main categories: carotenes and xanthophylls. Carotenes are linear hydrocarbons, with alfa-carotene, beta-carotene, and lycopene being the most well-known representatives. Xanthophylls, on the other hand, are oxygenated derivatives of carotenoids and include lutein, neoxanthin, violaxanthin, and zeaxanthin (Olatunde et al., 2020). Research by Cruz et al. (2012) shows that in the leaves of *Lactuca sativa* L. var. *capitata* cv. "Four Seasons," beta-carotene and lutein were the most prevalent carotenoids. In the human body, beta-carotene is converted into vitamin A (retinol), which is essential for eye health. While excessive direct intake of vitamin A can be toxic, the body only converts as much vitamin A from beta-carotene as needed (Icahn School of Medicine at Mount Sinai, 2024). Among the pigments found in the macula (region of the retina responsible for producing sharp vision), lutein is present in the highest concentration (University of Rochester Medical Center, 2024).

The content of vitamins in lettuce is shown in Table 2.5.3.2. Vitamin C (ascorbic acid), a watersoluble antioxidant, plays a key role in regenerating vitamin E, which is a lipid-soluble antioxidant. Vitamin E works within the lipid layers of cell membranes, neutralizing lipid peroxyl radicals and preventing cell damage (Traber & Stevens, 2011). Vitamin C content in plants is generally high compared to other compounds, likely due to its sugar-based composition, which is common to all plant species. Plants, fungi, and algae synthesize ascorbic acid in mitochondria and microsomal fractions, starting from D-glucose or D-galactose. In this process, glucose is oxidized to D-glucuronic acid, reduced to L-gulonic acid, converted to Lgulono-1,4-lactone, and then oxidized to L-ascorbic acid (vitamin C). However, humans and other primates cannot synthesize vitamin C due to the absence of the enzyme L-gulono-1,4lactone oxidase, essential for the final step of this biosynthesis (Doseděl et al., 2021). Despite the inability of humans to synthesize vitamin C, it is crucial for numerous bodily functions, including collagen biosynthesis, L-carnitine production, and neurotransmitter synthesis. It is also involved in protein metabolism (Carr & Frei, 1999; Frei et al., 1989). Folic acid is the synthesized form of folate (vitamin B-9) present in fortified foods and has a higher bioavailability than naturally occurring folate. This vitamin is essential for DNA synthesis (Khan & Jialal, 2014).

Table 2.5.3.2. Lettuce Vitamin Content in mg/100 g of fresh matter

Vitamin	Content (mg/100 g of fresh matter)
Carotene	0.16-1.6
Vitamin E	0.5
Vitamin B1	0.04-0.09
Vitamin B2	0.08-0.25
Vitamin B3	0.2-0.5
Vitamin B6	0.036-0.075
Folic acid	0.004-0.054
Vitamin C	6-55

Source: Lešić et al. (2016)

3. Materials and methods

3.1. Used PGP Fungi

As part of the project PERSPIRE (Potential of the Rhizosphere Microbiome in Adaptation of Agriculture to Climate Change, fungi were isolated from the soil of white cabbage (*Brassica oleracea* var. *capitata*) grown under water stress conditions. This project investigated the potential of soil microorganisms to enhance agricultural resilience to extreme weather events such as floods, which are becoming more frequent due to climate change. The soil used for the experiment was sampled from a bare agricultural field in Zagreb (Jakuševac), previously cultivated with wheat. From a total of 86 initial fungal isolates for which PGP properties were tested within the project, *Aspergillus clavatus* and *Mortierella elongata* were selected based on their superior PGP traits, such as enzyme activities (amilase, protease, cellulase) and nutrient solubilization capabilities (phosphorus and potassium solubilization).

After the initial isolation and identification, samples of the selected fungi were preserved at 4°C in the Laboratory for Environmental Microbiology and Biotechnology at the Ruđer Bošković Institute in Zagreb, ensuring their availability for further studies and potential commercial application in sustainable agriculture.

3.2. Subculturing and Preparation of inoculum

Subculturing and inoculum preparation for the lettuce growth experiment were conducted at the Laboratory for Environmental Microbiology and Biotechnology of the Ruđer Bošković Institute in Zagreb. For the experiment, three subcultures of *Aspergillus clavatus* (named K143, K28-2, K39-1) and three subcultures of *Mortierella elongata* (named K13-2, K13-3, K14-2) were used. Each subculture was grown on Potato Dextrose Agar (PDA) nutrient medium. The PDA medium was prepared according to the manufacturer's instructions (Biolab): 39 g of powder was dissolved in 1 L of distilled water and sterilized in an autoclave at 115°C for 10 minutes. Sterile solution was poured into Petri dishes.

The first subculturing was carried out on March 14, 2024. Subculture mycelium samples, previously kept at 4°C, were transferred using a sterile loop onto prepared PDA plates. Incubation of plates was carried out for 3-5 days at 24°C. In subsequent subculturing cycles (April 30, May 9, May 14, May 24, June 1), the fungal samples were transfered from the previously grown mycelium to new PDA plates by cutting the mycelium, along with a portion of the agar, in a circular shape using the wider end of a sterile pipette tip (approximately 9 mm in diameter) (Figure 3.2.1.). Incubation of plates was carried out for 3-5 days at 24°C. All procedures were conducted under a laminar flow hood to prevent contamination. The remaining sample was then used for inoculum preparation.

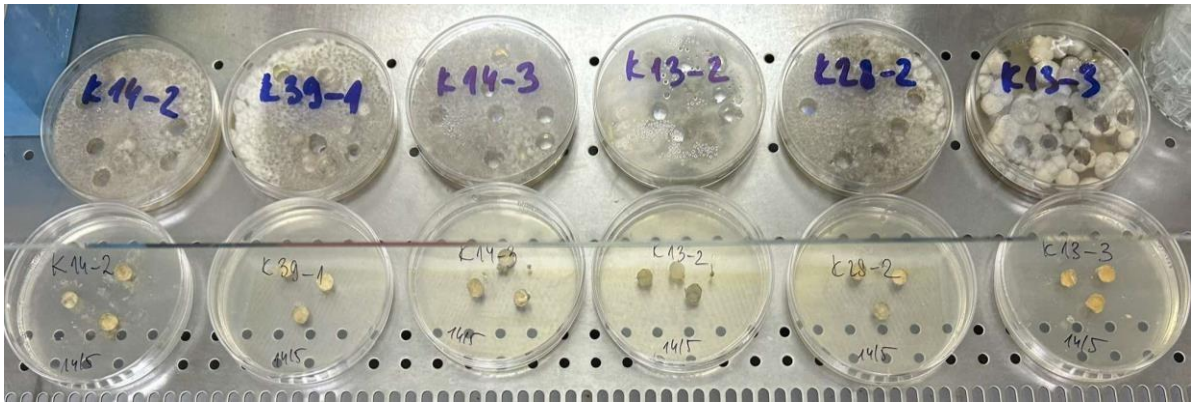


Figure 3.2.1. The Process of transferring samples to a new PDA substrate
(Source: Mia Borojević)

For the production of liquid fungal inoculum, mycellium from the PDA plates was transferred into 100 mL of 0.85% NaCl solution. Transfer was accomplished by adding first minimal amount of NaCl solution to the Petri dish to cover the fungal mycelium, allowing easier removal of the material. Then, several drops of Tween detergent were added to the Petri dishes to facilitate the dissolution of the samples. In the last step fungal material was transferring from the PDA plate with plastic loops into the 100 mL NaCl solution. This freshly prepared inoculum was then ready for application in the field within the few hours after preparation.

3.3. Experimental Design

The experiment was set up as a monofactorial field trial with inoculation as the factor, arranged in a randomized block design with three replications (Figure 3.3.1.). There were seven treatments in total with three replications (on three plots): one control (uninoculated), three treatments with different subcultures of *Aspergillus clavatus* (A1: K14-3, A2: K28-2, A3: K391), and three treatments with different subcultures of *Mortierella elongata* (M1: K13-2, M2: K13-3, M3: K14-2) (Table 3.1.). This comprised a total of 21 plots, with each plot containing 9 lettuce plants.


Figure 3.3.1. Field Experiment on May 29, 2024
(Source: Mia Borojević)

Lettuce sowing for seedling production and following seedling were carried out on March 18, 2024, in an unheated greenhouse at the Maksimir experimental field, under the Department of Vegetable Crops at the Faculty of Agriculture (Figures 3.3.2. and 3.3.3.). Polystyrene trays with 104 cells were used for the sowing. One seed of the Bataille lettuce variety (Nunhems Netherlands BV) was sown in each cell. Sowing was carried out using the commercial substrate KLASMANN POTGROND H. During the cultivation of seedlings, the temperature of the greenhouse was controlled and maintained daily in the range of 20 to 25 °C, and humidification was carried out as necessary.

Figure 3.3.2. Seeds of the bataille lettuce variety (Nunhems Netherlands BV)
(Source: Mia Borojević)

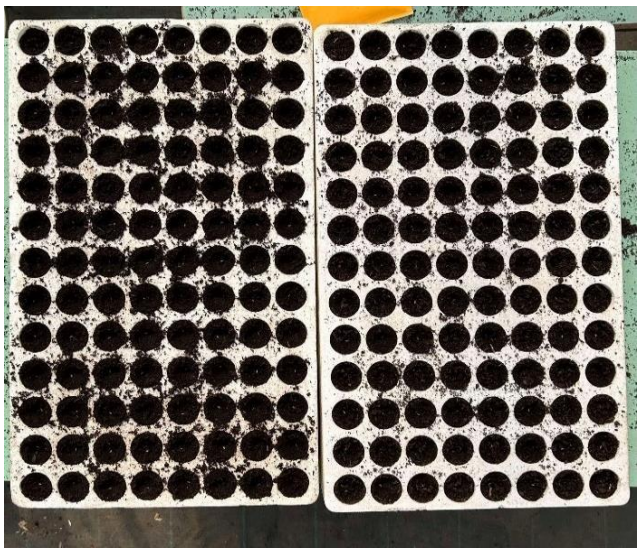


Figure 3.3.3. Seeds sown in a polystyrene container
(Source: Mia Borojević)

The seedlings were transplanted to the open field on April 30, 2024. The planting distance between and within the rows was 0.3 m x 0.3 m, resulting in a planting density of 9 plants/m² (three rows). Lettuce cultivation practices were performed as needed, including weeding on May 29, 2024. Lettuce was regularly irrigated using a drip system.

The fungal inoculum was applied directly to the roots of the lettuce seedlings at the time of transplanting and during the vegetative period on the following dates: April 30, May 9, May 14, May 24, and June 1 (Figure 3.3.4.). Control seedlings were left uninoculated. A pipette was used for the application, with 100 mL of inoculum being evenly distributed to each plant (9 plants x 3 replications), resulting in approximately 3.5 mL of solution being applied to each plant with its specific subculture of *A. clavatus* or *M. elongata.*

Figure 3.3.4. First root inoculation of seedlings
(Source: Mia Borojević)

Lettuce harvest was conducted on June 10, 2024, at the stage of technological maturity. Harvesting was done manually with a knife, by making a cut in the zone of the root neck. Determination of morphological properties, yield and specilized metabolites measurements were measured on 10 representative plants per every treatmant (washed with water). Morphological parameters, including plant diameter and height (cm), marketable plant mass (g) and the marketable yield of lettuce (kg/m²) were determined. The mass was determined by weighing on a laboratory scale, and the width and height with the help of a ruler, as shown in Figure 3.3.5.

Figure 3.3.5. Determination of lettuce diameter and mass
(Source: Mia Borojević)

3.4. Chemical Analysis

To assess the chemical properties of the lettuce samples, several analyses were performed, including determination of dry matter content, total polyphenolic compounds, photosynthetic pigments content, ascorbic acid content and antioxidant capacity. These tests were carried out in the Department of Sustainable Technologies and Renewable Energy Sources at Faculty of Agriculture University of Zagreb.

3.4.1. Dry Matter Content

Dry matter was determined in a halogen moisture meter. The preparation of lettuce leaf samples of each treatment included: washing with distilled water, wiping with paper towels and shredding in a mixer. Five grams of the prepared samples were dried in a halogen moisture meter to a constant weight, and the amount of dry matter was shown as a percentage (%) in the total weight of the sample.

3.4.2. Total Phenols, Flavonoids and Non-Flavonoids Content

The determination of total phenols using the Folin-Ciocalteu method was based on a colorimetric reaction between phenolic compounds and the Folin-Ciocalteu reagent. This reagent, a mixture of phosphotungstic and phosphomolybdic acids, was reduced to blue tungsten and molybdenum oxides upon the oxidation of phenolic compounds. The intensity of the blue coloration that developed was measured spectrophotometrically at a wavelength of 750 nm.

The method consisted of two primary parts. The first part was the preparation of a calibration curve. In this step, 500 mg of gallic acid was weighed and dissolved in 80% ethanol, and the solution was made up to 100 mL in a volumetric flask. From this stock solution, a series of dilutions were prepared by pipetting 0, 1, 2, 3, 5, and 10 mL of the stock solution into volumetric flasks and filling them to the mark with 80% ethanol. These dilutions corresponded to concentrations of 0, 50, 100, 150, 250, and 500 mg/L. From each of these solutions, 0.5 mL was pipetted into separate 50 mL volumetric flasks. Then, 30 mL of distilled water was added to each flask, followed by the addition of 2.5 mL of Folin-Ciocalteu reagent diluted 1:2 with distilled water. After waiting for three minutes, 7.5 mL of a saturated sodium carbonate (Na_2CO_3). solution was added to each flask. The contents were thoroughly mixed, and the flasks were filled to the mark with distilled water. The solutions were then allowed to stand at room temperature for two hours. Once this standing period was complete, the absorbance of each solution was measured at 750 nm, using distilled water as a blank.

The second part of the method involved the extraction of phenolic compounds from lettuce sample. For this part, 10 g of the sample was weighed with an accuracy of ±0.01 g and homogenized with 40 mL of 80% ethanol. This mixture was heated for 10 minutes using a reflux condenser. After heating, the mixture was filtered into a 100 mL volumetric flask using pleated filter paper. The residue, along with the filter paper, was transferred back into the flask, and 50 mL of 80% ethanol was added. The mixture was reheated for another 10 minutes with

the reflux condenser. After the second heating, the second extract was combined with the first in the same 100 mL volumetric flask, and the solution was filled to the mark with 80% ethanol.

From the final extract, 0.5 mL was pipetted into a 50 mL volumetric flask. To this, 30 mL of distilled water was added, followed by 2.5 mL of Folin-Ciocalteu reagent diluted 1:2 with distilled water. After adding 7.5 mL of saturated sodium carbonate solution, the contents of the flask were mixed thoroughly, and the flask was filled to the mark with distilled water. The solution was then left to stand at room temperature for two hours. After this period, the absorbance was measured at 750 nm, using distilled water as a blank.

To isolate flavonoids, formaldehyde is used, which reacts with the C-6 or C-8 positions on flavonoid molecules, forming condensed compounds that can be removed by filtration. After filtration, what remains are the non-flavonoid phenols. These non-flavonoid phenols are then quantified using the described method as for total phenols. For the analysis, 10 mL of the sample is mixed with 5 mL of diluted hydrochloric acid (1:4) and 5 mL of formaldehyde solution. The mixture is purged with nitrogen, sealed, and left at room temperature in the dark for 24 hours. After this, the sample is filtered to remove the condensed flavonoid molecules. The filtrate, containing non-flavonoid phenols, is analyzed. The concentration of flavonoids is calculated by subtracting the non-flavonoid phenols from the total phenols previously determined.

3.4.3. Photosynthetic Pigments Content (Total chlophyll, Chlorophyll a, Chlorophyll b, Total Caratenoids)

The determination of photosynthetic pigments, including total chlorophyll, chlorophyll a, chlorophyll b, and carotenoids, was conducted following the spectrophotometric method described by Holm (1954) and Wetstein (1957). This method involves quantification of concentrations of photosynthetic pigments in the acetone extract of lettuce sample, and convertion of these concentrations into mg/g of fresh matter. The materials used included a scale, mortar and pestle, Büchner funnel, a 300 mL Erlenmeyer flask, a water-jet vacuum pump, a 25 mL volumetric flask, and a spectrophotometer (Shimadzu UV 1650 PC). The chemicals required for the procedure were acetone (p.a.), magnesium carbonate ($MgCO_3$), and quartz sand.

The process of pigment extraction and determination was carried out quickly and under darkened conditions to avoid degradation of the pigments. A 6 g sample of lettuce was weighed and transferred to a mortar. To this sample, approximately half a teaspoon of quartz sand and half a teaspoon of magnesium carbonate ($MgCO_3$) powder, to neutralize acidity, were added, along with 10 mL of acetone. The mixture was ground thoroughly using a pestle in the mortar and then quantitatively transferred into a Büchner funnel with acetone. The acetone-extracted sample was then filtered under vacuum.

Once the macerate was filtered, the filtrate was quantitatively transferred into a 25 mL volumetric flask and filled to the mark with acetone. The absorbance of the obtained filtrate was then measured using a spectrophotometer at wavelengths of 662, 644, and 440 nm, with acetone serving as the blank. The absorbance values obtained (A_{662}, A_{644}, and A_{440}) were

subsequently inserted into the Holm-Weststein equations to calculate the concentrations of photosynthetic pigments in mg/dm³. The numbers in the equations represent molar absorption coefficients according to Holm and Wetstein. The equations are as follows:

Chlorophyll a = $9.784 \times A_{662} - 0.990 \times A_{644}$ mg/L
Chlorophyll b = $21.426 \times A_{644} - 4.65 \times A_{662}$ mg/L
Total chlorophyll (a + b) = $5.134 \times A_{662} + 20.436 \times A_{644}$ mg/L
Carotenoids = $4.695 \times A_{440} - 0.268 \times$ (chlorophyll a + b) mg/L

The formula for calculating the concentration of photosynthetic pigments in mg/g of fresh plant matter is as follows:

$c\ (mg/g) = (c1 \times V) / m$

In this formula, c represents the mass concentration of pigments expressed in mg/g of fresh plant matter, c1 is the mass concentration of pigments expressed in mg/L, V is the volume of the filtrate (volumetric flask) in mL and m is the mass of the sample expressed in mg.

3.4.4. Ascorbic Acid Content

The determination of ascorbic acid (vitamin C) in the lettuce sample is performed using a redox titration method involving 2,6-dichlorophenolindophenol, which acts as both an oxidizing agent and a colorimetric indicator. This method allows for the accurate quantification of L-ascorbic acid by observing the color change as the reagent reacts with the sample. Materials includes an analytical balance, a 100 mL volumetric flask, a 100 mL beaker, a funnel, filter paper, an Erlenmeyer flask, and a burette. The chemicals required are a 2% oxalic acid solution and freshly prepared 2,6-dichlorophenolindophenol solution.

The process begins by weighing 10 g of the lettuce sample on a technical balance with an accuracy of ±0.01 g. The sample is quantitatively transferred into a 100 mL volumetric flask using a 2% oxalic acid solution, ensuring that no residue is left behind. The volumetric flask is then filled to the mark with 2% oxalic acid solution. Once the sample is fully dissolved, the contents of the flask are filtered through filter paper. The resulting filtrate is collected and used for the subsequent determination of ascorbic acid.

Next, 10 mL of the filtrate is pipetted into a 50 mL Erlenmeyer flask. The filtrate is titrated with a freshly prepared 2,6-dichlorophenolindophenol solution until a stable pink color appears, lasting for at least five seconds. The volume of 2,6-dichlorophenolindophenol solution used during the titration is recorded. To calculate the amount of L-ascorbic acid (vitamin C) in the sample, the following formula is applied:

Amount of Vitamin C (mg/100g) = $(V \times F \times 880) / (D \times 100)$

In this formula V represents the volume of 2,6-dichlorophenolindophenol used in milliliters, F is the factor of the 2,6-dichlorophenolindophenol solution, and D is the mass of the sample in grams.

The factor (F) of the 2,6-dichlorophenolindophenol solution is determined by preparing a solution of ascorbic acid. To do this, ±0.0100 g of ascorbic acid is weighed into a 50 mL volumetric flask on an analytical balance, and the flask is filled to the mark with 2% oxalic acid solution. In a separate 50 mL Erlenmeyer flask, 5 mL of the prepared ascorbic acid solution and 5 mL of 2% oxalic acid solution are pipetted. This mixture is titrated with 2,6dichlorophenolindophenol until a stable pink color appears. The factor (F) is then calculated based on the volume of 2,6-dichlorophenolindophenol solution used during this titration.

3.4.5. Antioxidant Capacity

Due to the complexity of oxidation processes, it is often desirable to use several methods simultaneously, because the results obtained by only one method cannot always provide a complete picture of antioxidant activity (Seong et al. 2015). Two different methods were used to measure the antioxidant capacity of the samples: ABTS and FRAP. Both methods are based on measuring the color change caused by the reaction of antioxidants with the corresponding radicals or ions, and the results are recorded spectrophotometrically.

The ABTS method is based on the reduction of the radical ABTS+ (2,2'-azinobis (3ethylbenzothiazoline-6-sulfonic acid)). In this method, antioxidants from the sample transfer electrons to the ABTS radical cation (ABTS•+), which causes a color change. This radical forms a stable blue-green chromophore, which is produced by the reaction of 7 mM ABTS with 2.45 mM potassium persulfate, and the mixture is incubated in the dark at room temperature for 12-16 hours. Before the actual test, the ABTS•+ solution is diluted with distilled water to an absorbance of 0.734 ± 0.02 at 734 nm. In the process of measuring antioxidant capacity, 0.1 mL of lettuce extract is mixed with 3.9 mL of diluted ABTS•+ solution in a cuvette. The decrease in absorbance at 734 nm is measured after 6 minutes, and the decrease in absorbance reflects the antioxidant capacity of the sample. Results are calculated using a Trolox standard curve and expressed as Trolox equivalents (TE) in micromoles per gram of fresh weight. The FRAP method measures antioxidant strength by reducing iron. In this method, Fe3+ ions from the Fe3+-TPTZ complex (2,4,6-tri-pyridyl-s-triazine) are reduced to Fe2+ ions by the action of antioxidant compounds from the sample, which also results in a color change. The FRAP reagent is prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl and 20 mM FeCl3 in a 10:1:1 ratio. To perform the method, 0.1 mL of lettuce extract is mixed with 3 mL of FRAP reagent in a cuvette. The reaction mixture is incubated at 37°C for 4 minutes, after which the absorbance is measured at 593 nm. The antioxidant capacity is quantified on the basis of a standard curve prepared with FeSO4, and the results are expressed as micromoles of Fe2+ equivalents per gram of fresh weight.

3.5. Statistic Analysis

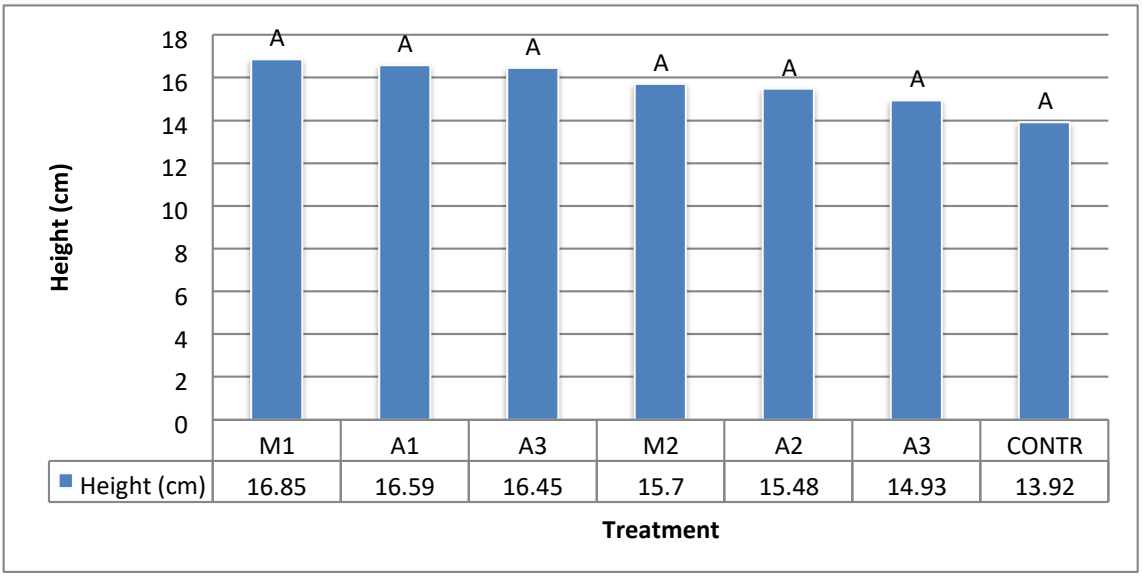
The statistical analysis was performed using SAS software (Statistical Analysis System, version 9.4) to determine significant differences between lettuce treatments inoculated with various subcultures of *Aspergillus clavatus* and *Mortierella elongata* compared to the control. The measured values for each parameter (head diameter, plant height, marketable head mass, yield per unit area, ascorbic acid content, total polyphenols, chlorophyll concentration, and antioxidant capacity) were entered into the SAS program for each treatment's repetitions. SAS then calculated the mean values and performed comparisons between treatments. For easier tracking, the control treatment and treatments with six fungal isolates are numbered from 1 to 7 as follows: CONTRL = control, A1 = K14-3, A2 = K28-2, A3 = K39-1, M1 = K13-2, M2 = K13-3, M3 = K14-2). A one-way analysis of variance (ANOVA) was performed to assess the impact of inoculation on each growth parameter measured, as well as for the 13 traits displayed in the tables. Statistically significant differences were identified with p-values < 0.01. In cases where ANOVA indicated significant differences between treatments, a Tukey HSD (Honestly Significant Difference) post-hoc test was conducted for more detailed pairwise comparisons. Results were presented as mean values ± standard deviations, with significant differences indicated by different group. Figures generated by SAS were used for making graphs in the Excel, version 2016.

4. Results and discussion

This section presents the assessment of different lettuce parameters when treated with fungal isolates of *Aspergillus clavatus* and *Mortierella elongata*, as well as control that is not treated, and assesses whether there are statistically justified differences between the treatments. For easier tracking, the control treatament is represented as CONTR, treatments with *A. clavatus* as A1 (K14-3), A2 (K28-2) and A3 (K39-1) and treatments with *M. elongata* as M1 (K13-2), M2 (K13-3), M3 (K14-2). The group to which the treatment belongs is shown with the corresponding letter above the column. The same group means that there is no statistically justified difference between the treatments of that group. The group overlapping means there is no statistically justified difference between groups.

4.1. Morphological Properties

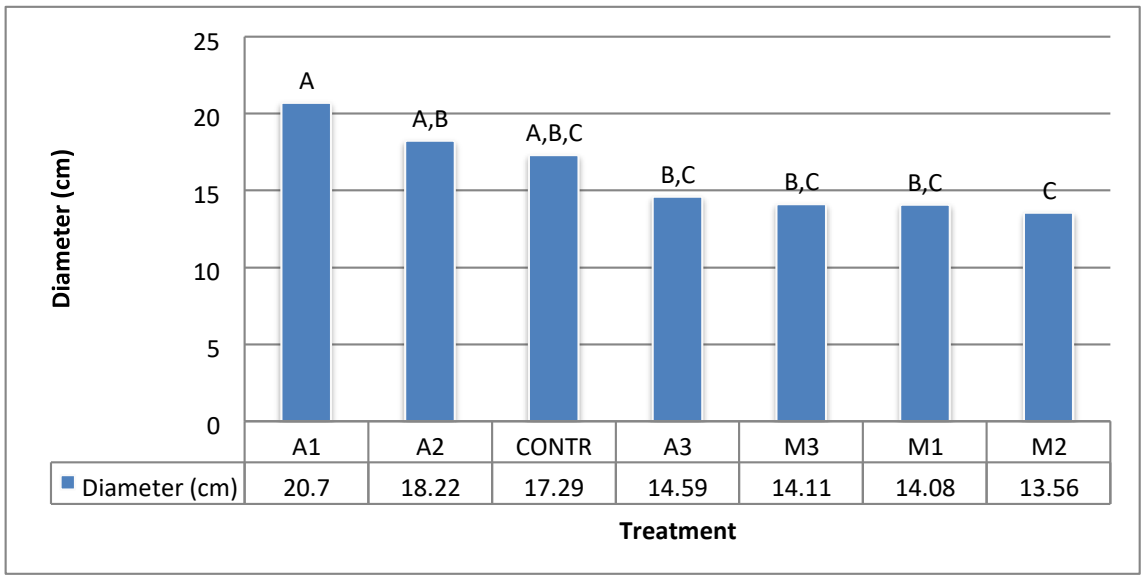
Graph 4.1.1. illustrates the grouping of the height measurements into one group (A). That indicates that differences in height measurements were not statistically justified. The height in treated treatments (A2, A3, A4, M1, M1 and M2) is measured in the range from 14.93 cm to 16.85 cm, while in control (CONTR) is measured the lowest height (13.92 cm). The highest height is acchived in treatment with all *M. elongata* (M1) with height of 16.85 cm, while the highest value of height in treatments with *A. clavatus* is acchived in treatment A1 (16.59 cm).



Graph 4.1.1. Height values and corresponding group for treatments with different fungal isolates; *A. clavatus* (A1, A2 and A3), *M. elongata* (M1, M2 and M3) and control (CONTR)

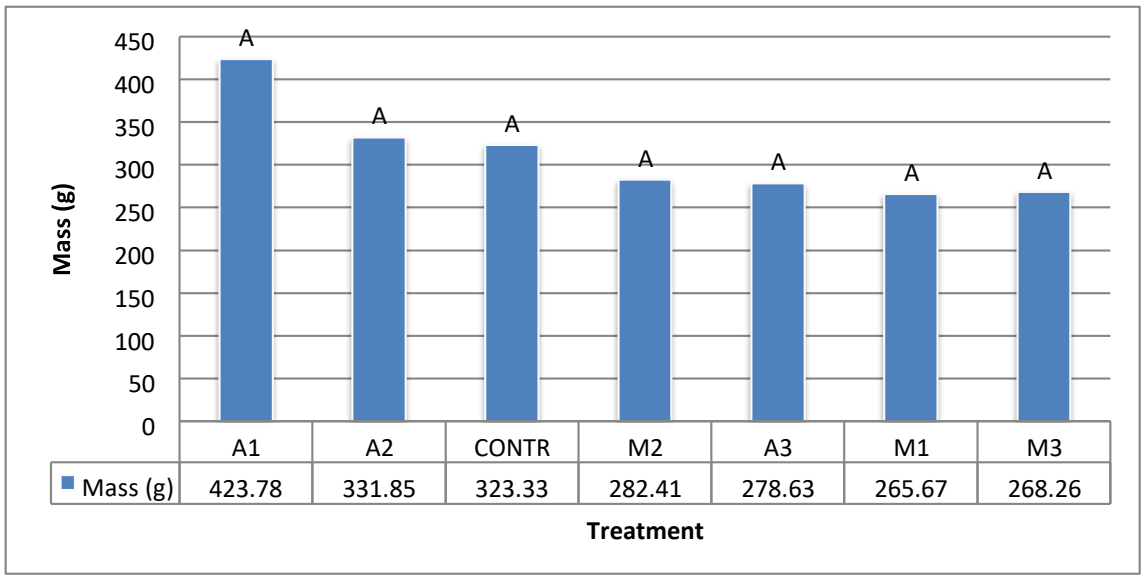
Graph 4.1.2. illustrates the grouping of the diameter measurements into three groups (A, B and C). Differences in diameter measurements were not statistically justified. The highest diameter is measured in all treatments treated with *A. clavatus* (A1, A2, A3) in the range from 14.59 cm to 20.70 cm. The first group contains two treatments treated with *A. clavatus* (A1 and A2) with

20.70 cm and 18.22 cm and untreated control (CONTR) with 17.29 cm. The lowest diameter is measured in treatments treated with *M. elongata (*M1, M2, M3) ranging from 13.56 cm to 14.11 cm).



Graph 4.1.2. Diemeter values and corresponding groups for treatments with different fungal isolates; *A. clavatus* (A1, A2 and A3), *M. elongata* (M1, M2 and M3) and control (CONTR)

Graph 4.1.3. illustrates the grouping of the mass measurements into one group (A). Differences in mass measurements were not statistically justified. The highest masses (331.85 g and 423.78 g) are measured in treatments treated with *A. clavatus* (A1 and A2), followed by the control (CONTR) with 323.33 cm. The mass measurements of all treatments with M. elongata (M1, M2 and M3) were lower than in the control treatment (CONTR), ranging from 265. 67 cm to 282.41 cm.

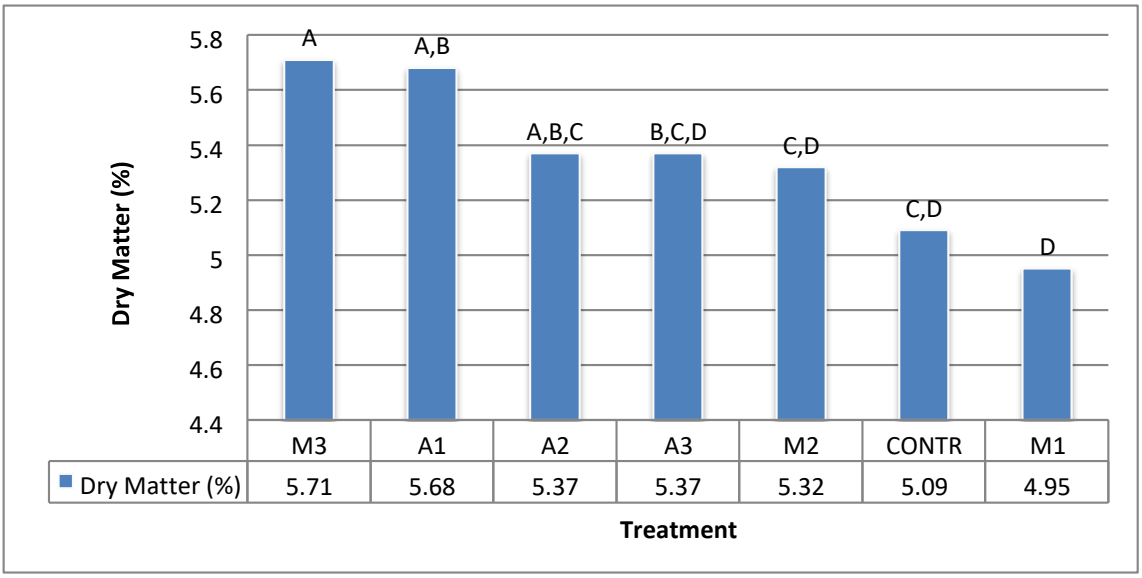


Graph 4.1.3. Mass values and corresponding group for treatments with different fungal isolates; *A. clavatus* (A1, A2 and A3), *M. elongata* (M1, M2 and M3) and control (CONTR)

Although there is a difference in height, weight and mass in the treated treatments from the untreated ones, these differences are not statistically justified. This phenomenon could be due to an increased production of phenolic compounds; under stress, plants may divert resources towards phenolic synthesis as a protective mechanism, which can result in reduced growth (Gurdon et al., 2019).

4.2. Dry Matter Content

Graph 4.2.1. illustrates the grouping of the dry matter contents into four groups (A B, C, D). Differences in dry matter content are not statistically justified. The content of dry matter is in the range from 4.94% to 5.71%, with control (CONTR) having one of the lowest percentages (5.1%), but without statistical justification. The highest average percentage of dry matter was shown to be acchived in treatments treated with *A. clavatus* (A1, A2, A3) in range from 5.32% to 5.68%, while in one treatment with *M. elongata* (M3), the highest percentage of 5.71% was achieved. Results of inocluation with *M. elongata* in treatment M3 (5.71%) showed the biggest deviation with respect to the treatments M2 (5.32%) and M1 (4.95%), which are in the last group together with the control (CONTR).

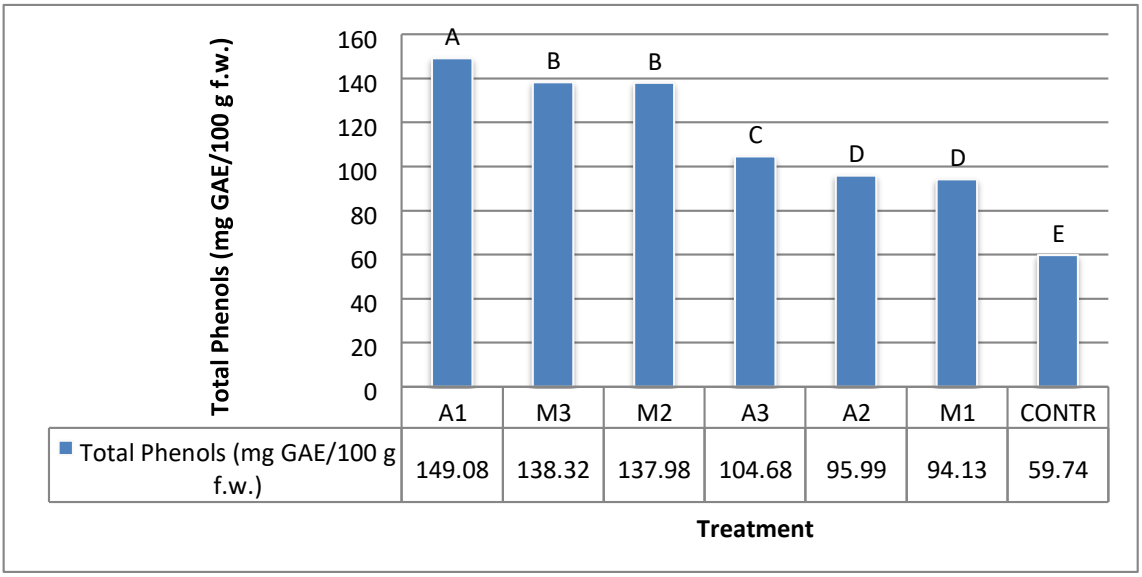


Graph 4.2.1 Dry matter values and corresponding group for treatments with different fungal isolates; *A. clavatus* (A1, A2 and A3), *M. elongata* (M1, M2 and M3) and control (CONTR)

Given that lettuce lacks significant proteins, lipids, and starch, focusing more on sugar synthesis, glycolysis, and plant hormone anabolism during fleshy stem expansion, low dry matter results obtained in this study can be justified (Huang et al., 2022). In the research conducted by Škvorc (2017), where traditional nettle and comfrey preparations were used, it is recorded the highest dry matter content of 4.95%. This content of dry matter is similar or lower than the results of most of the treatments carried out in this research.

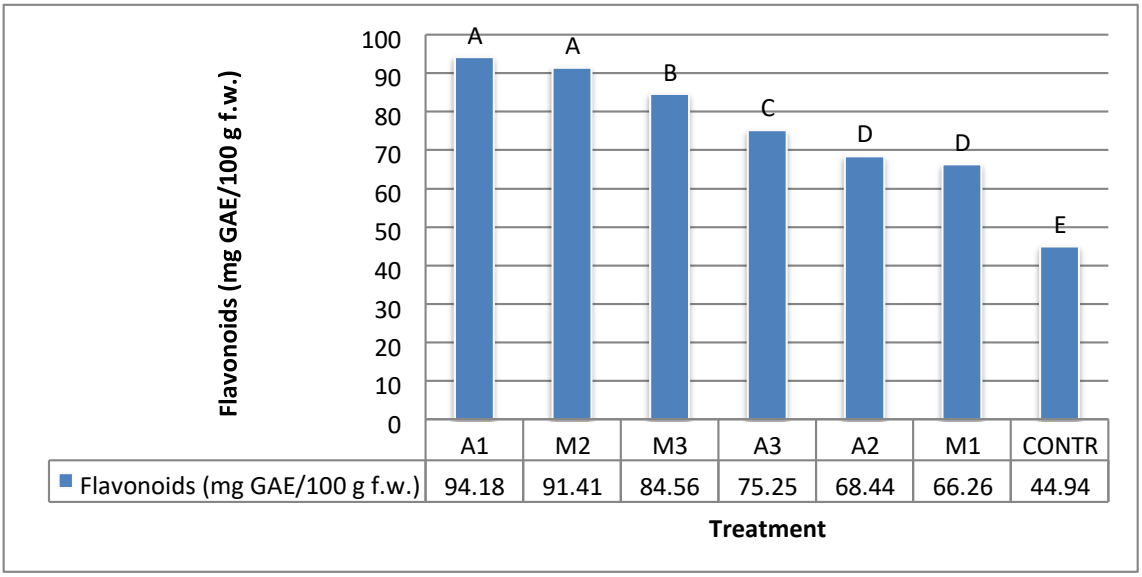
4.3. Total Phenols, Flavonoids and Non-flavonoids Content

Based on the measured levels of total phenols, different treatments are clustered into five groups (A B, C, D and E) and only the control (CONTR) showed the lowest value (59.7433 mg GAE/100 g fw), which is statistically justified (Graph 4.3.1.). The amounts of total phenols in the lettuce treated with isolates of *A. clavatus* (A1, A2 and A3) and *M.* elongata (M1, M2 and M3) ranged from 94.13 to 149.08 mg GAE/100 g fw, as shown in Graph 4.3.1. The higest total phenols content was observed in tretment treated with *A. clavatus* (A1) being statistically justified compared to the next group consisting of tretments treated with *M. elongata* (M3 and M2).



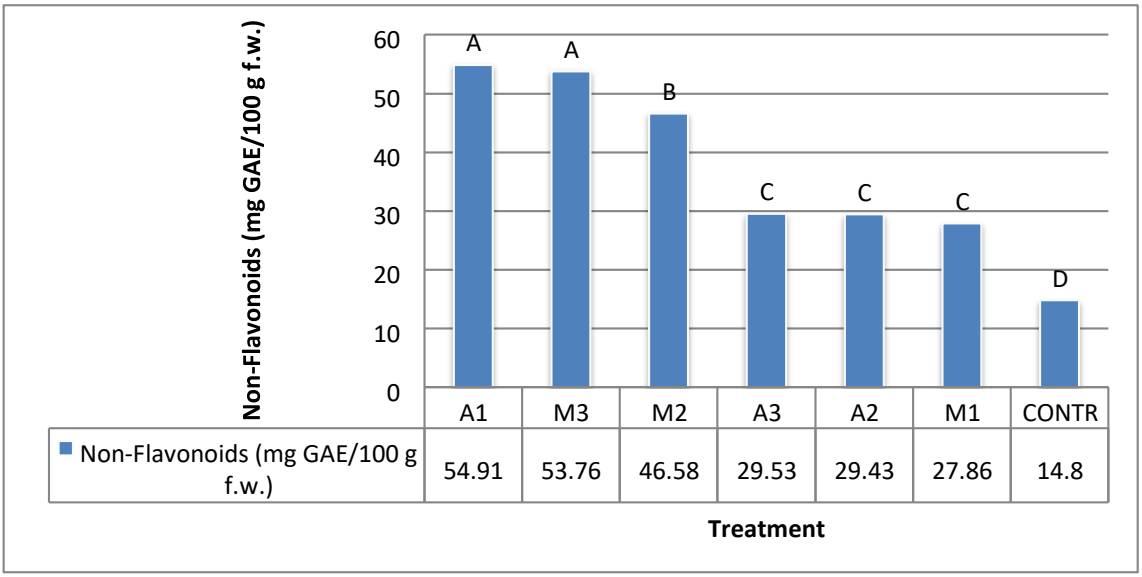
Graph 4.3.1. Total phenols values and corresponding group for treatments with different fungal isolates; *A. clavatus* (A1, A2 and A3), *M. elongata* (M1, M2 and M3) and control (CONTR)

Based on the determined levels of total flavonoids in the letucce, treatments with fungal isolates clustered into five groups (A, B, C, D, and E) and only the control (CONTR) showed the lowest value (44.94 mg GAE/100 g fw), which is statistically justified (Graph 4.3.2). The amounts of total flavonoids in the letucce treated with isolates of *A. clavatus* and *M. elongata* (A1, A2, A3, M1, M2 and M3) ranged from 66.26 to 94.18 mg GAE/100 g fw, as it is shown on Graph 4.3.2. The highest total flavonoid content was observed in the treatments with *A. clavatus* isolate (A1) with 94.18 mg GAE/100 g fw, which is statistically grouped with the *M. elongata* tretament (M2) showing 91.41 mg GAE/100 g fw. These two treatments (A1 and M2) lead to significantly higher flavonoid levels measured in letucce when compared to the other treatments.



Graph 4.3.2 Total flavonoids values and corresponding group for treatments with different fungal isolates; *A. clavatus* (A1, A2 and A3), *M. elongata* (M1, M2 and M3) and control (CONTR)

Based on the determined levels of total non-flavonoids in the letucce, treatments with fungal isolates clustered into four groups (A, B, C and D) and only the control showed the lowest value (14. 80 mg GAE/100 g fw), which is statistically justified (Graph 4.3.3.). The amounts of total non-flavonoids in the letucce treated with isolates of *A. clavatus* and *M. elongata* (A1, A2, A3, M1, M2 and M3) ranged from 27.86 to 54.91 mg GAE/100 g fw, as shown in Graph 4.3.3. The highest total flavonoid content was observed in the treatment with *A. clavatus* (A1) with 54.91 mg GAE/100 g fw, which was statistically grouped with the *M. elongata* tretament (M3) showing 53.76 mg GAE/100 g fw. These two treatments lead to significantly higher flavonoid levels measured in letucce when compared to the other treatments.

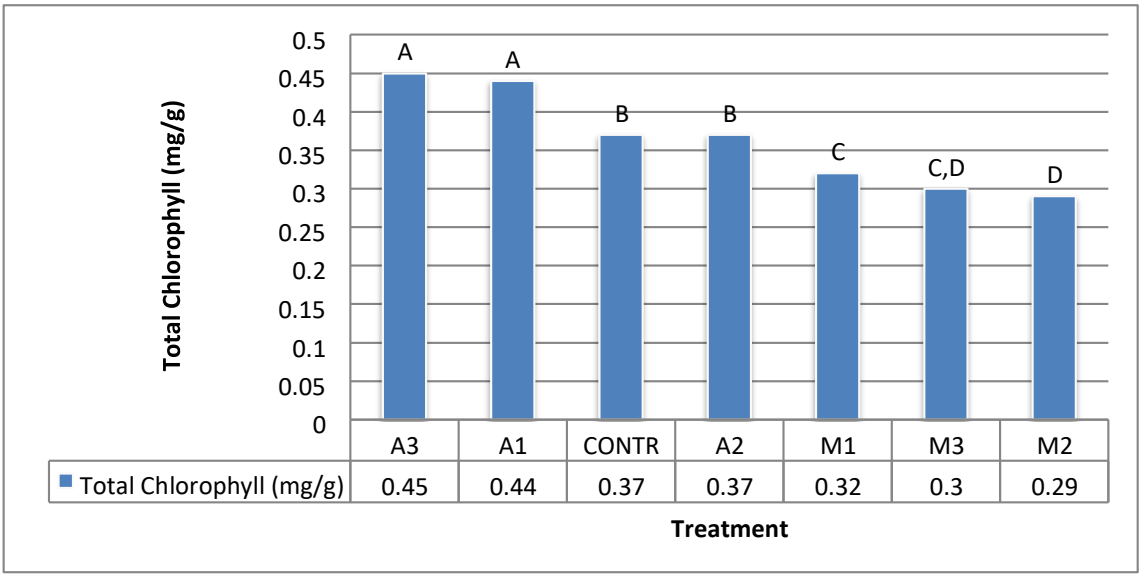


Graph 4.3.3. Total non-flavonoids values and corresponding group for treatments with different fungal isolates; *A. clavatus* (A1, A2 and A3), *M. elongata* (M1, M2 and M3) and control (CONTR)

In the research by Mampoholo et al. (2016) in the green lettuce varieties Multigreen 3, Multigreen 1, and Atlantis, the total phenol contents are 1.12 mg GAE/100 g, 1.14 mg GAE/100 g, and 0.39 mg GAE/100 g. In the research of Fan (2013), the amount of flavonoids in lettuce grown under control conditions (18/14 °C day/night) was between 4.29 and 16.14 mg EK.g-1, while under the influence of temperature stress (28/20 °C day/ night) the amount of flavonoids increased and ranged from 12.89 to 29.24 mg EK.g-1 ST. Heimler et al. (2011) found significant differences between different systems of lettuce cultivation (conventional, organic and biodynamic). The lowest amount of flavonoids (109 mg GAE/100 g of fresh matter) was found in conventionally grown lettuce. Lettuce from organic cultivation was richer in flavonoids, with an amount of 123 mg GAE/100 g of fresh matter, while lettuce from biodynamic cultivation had the highest content of flavonoids, which was 139 mg GAE/100 g of fresh matter.

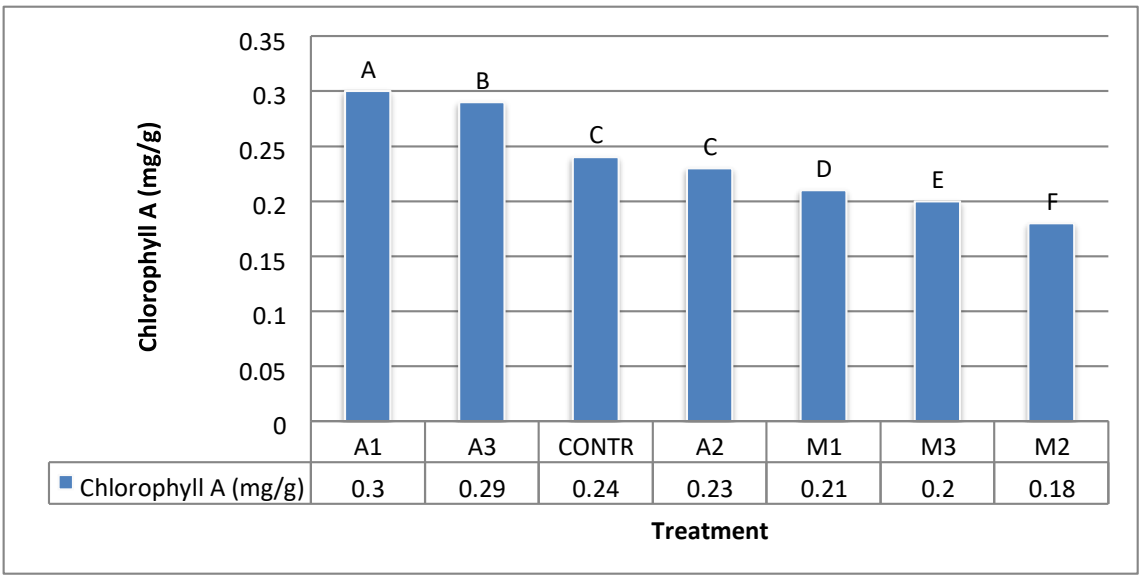
4.4. Photosynthetic Pigments Content (Total Chlophyll, Chlorophyll a, Chlorophyll b, Total Caratenoids)

As shown in Graph 4.4.1., the results of the total chlorophyll (TCh) measurements grouped into four groups (A, B, C and D) which are statistically justified. Total chlorophyll content for different treatment is showed in the Graph 4.4.1. The highest total chlorophyll content was observed in treatments with *A. clavatus* (A3 and A1) with values of 0.45 mg/g and 0.44 mg/g, which form one group, while the next group includes the control (CONTR) with 0,37 mg/g and another *A. clavatus* isolate treatment A2 with 0.37 mg/g. The lowest chlorophyll content is recorded in all letucce treated with *M. elongata* isolates (M1, M2 and M3) measured in range from 0.27 mg/g to 0.32 mg/g.



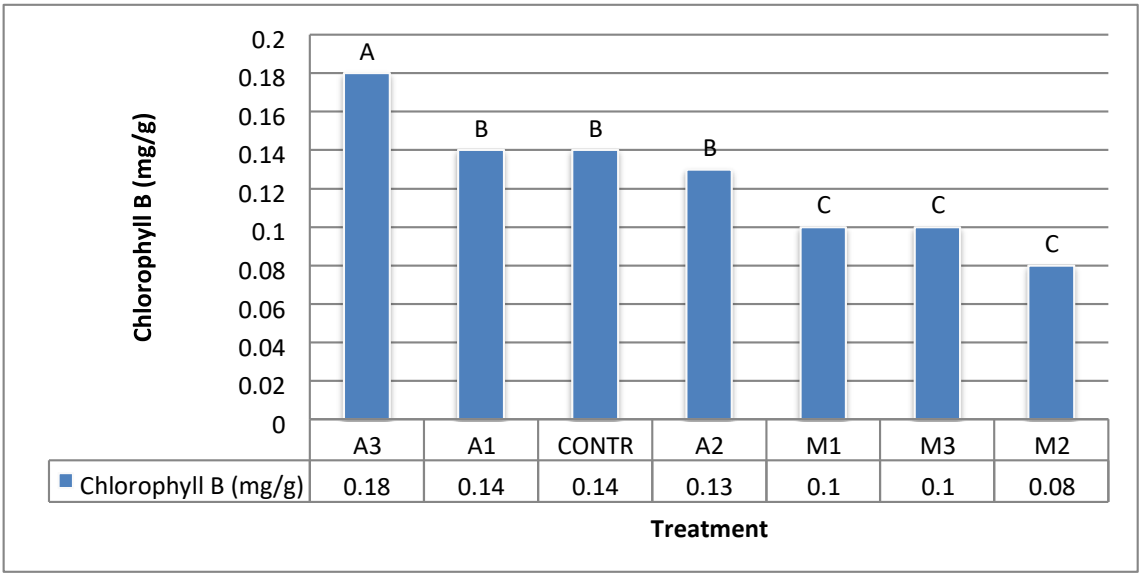
Graph 4.4.1. Total chlorophyll values and corresponding group for treatments with different fungal isolates; *A. clavatus* (A1, A2 and A3), *M. elongata* (M1, M2 and M3) and control (CONTR)

Graph 4.4.2. shows obtained values of chlorophyll a, that is divided into six groups (A, B, C, D, E, F) which are statistically justified. Total chlorophyll a content for different treatment is showed in the Graph 4.4.2. The highest total chlorophyll a content was observed in all treatments with *A. clavatus* isolates (A1, A2 and A3) with value of 0.30 mg/g in the first group, 0.27 mg/g in the second group and 0.24 mg/g in the third group (together with the control (CONTR) with 0.23 mg/g). The lowest content of chlorophyll was observed in all tretaments with *M. elongat*a (M1, M2 and M3) in range from 0.18 mg/g to 0.21 mg/g.



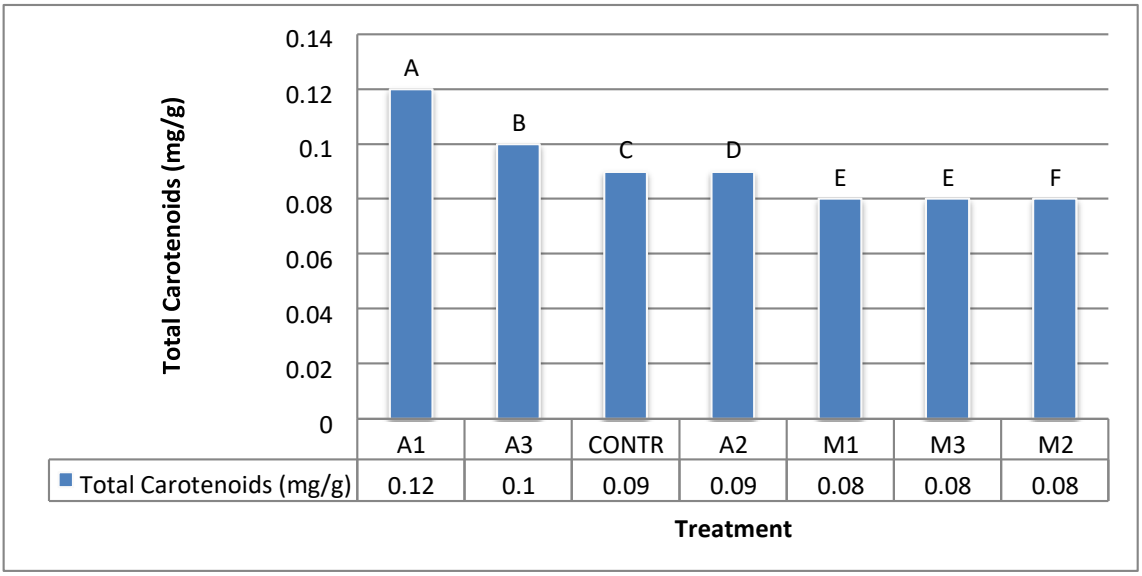
Graph 4.4.2 Chlorophyll a values and corresponding group for treatments with different fungal isolates; *A. clavatus* (A1, A2 and A3), *M. elongata* (M1, M2 and M3) and control (CONTR)

Graph 4.4.3. shows obtained values of the chlorophyll b content grouped into three groups (A, B and C) which are statistically justified. Total chlorophyll b content for different treatment is showed in the Graph 4.4.3. The highest total chlorophyll b content is observed in letucce treated with all *A. clavatus* isolates (A1, A2 and A3) with values in the first group (0.18 mg/g) and in the second group (0.14 mg/g and 0.13 mg/g) (together with the control (CONTR) with 0.14 mg/g). The lowest content of chlorophyll is observed in treatments with *M. elongata* (M1, M2 and M3), ranging from 0.08 mg/g to 0.10 mg/g.



Graph 4.4.3. Chlorophyll b values and corresponding group for treatments with different fungal isolates; *A. clavatus* (A1, A2 and A3), *M. elongata* (M1, M2 and M3) and control (CONTR)

Graph 4.4.4. shows obtained values of the caratenoids content grouped into six groups (A, B, C, D, E and F) which are statistically justified. Lettuce treated with *A. clavatus* isolates (A1 and A3) contains the highest carotenoid content with 0.12 mg/g and 0.10 mg/g, making the first two distincted groups, followed by control (CONTR) with value of 0.09 mg/g (in the third group). The fourth group contains again treatment with *A. clavatus* (A2) with value of 0,09 mg/g. This content is the same as in control (CONTR) treatment. The lowest carotenoid levels are observed in all tretments with *M. elongata* (M1, M2 and M3), with values of 0.08 mg/g (making two last groups).



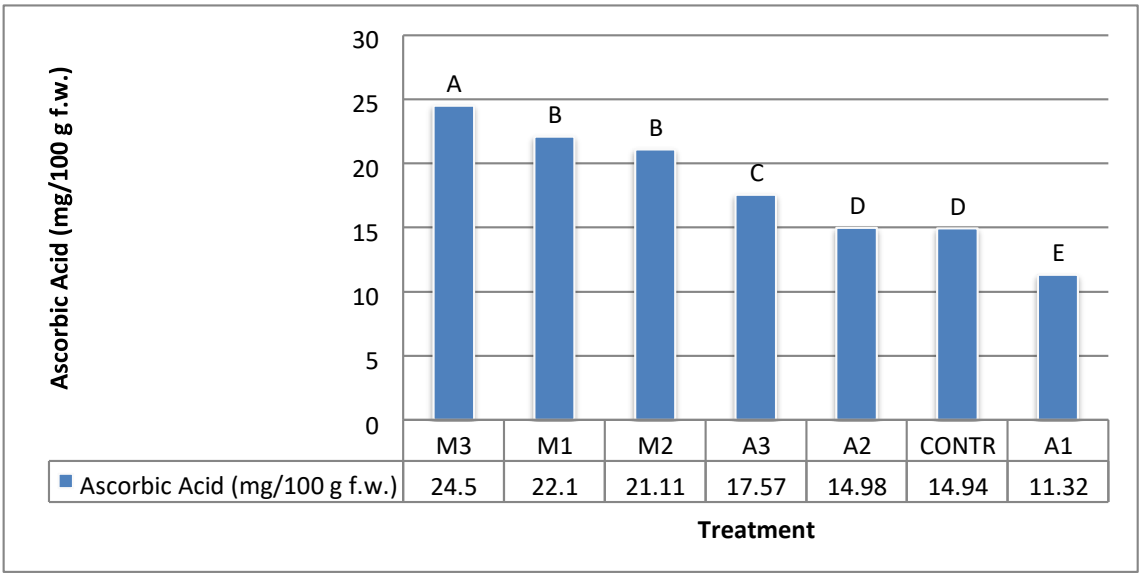
Graph 4.4.4. Total carotenoids values and corresponding group for treatments with different fungal isolates; *A. clavatus* (A1, A2 and A3), *M. elongata* (M1, M2 and M3) and control (CONTR)

Mampholo et al. (2016) determined the amount of chlorophyll a at 0.19 mg/g, chlorophyll b at 0.05 mg/g in Batavia lettuce (for which mineral fertilizers were used for cultivation), while the total amount of chlorophyll (TCh) is 0.23 mg/g. According to Morna (2015), the chlorophyll content in fresh lettuce of the Riga variety varied depending on the applied extraction methods. Two types of organic solvents, methanol and acetone, were used for the extraction of chlorophyll a and b, followed by spectrophotometric analysis at specific wavelengths. Chlorophyll a ranged from 0.12 mg/g to 0.17 mg/g, while chlorophyll b ranged from 0.05 mg/g to 0.08 mg/g, and the total chlorophyll content ranged from 0.20 mg/g to 0.22 mg/g. Most values given in research are mosly the same or higher, including the control.

Morna (2015) determined the amount of total carotenoids in lettuce, which ranged from 0.06 mg/g to 0.06 mg/g fresh weight, which is less than the results obtained in this study (from 0.09 mg/g to 0.12 mg/g). In the research conducted by Cruz et al. (2012), it was estimated that the total carotenoids in the control group of lettuce of *Lactuca sativa* L. *var. capitata* cv. "Four Seasons" amount to 0.10 mg/g, while when applying coffee residues in the soil, this value was from 0.12 mg/g to 0.17 mg/g.

4.5. Ascorbic Acid Content

As shown in Graph 4.5.1., the content of ascorbic acid (vitamin C) measured in different treatments is grouped into five groups (A, B, C, D and E), which are statistically justified. The significantly highest amount of ascorbic acid is found in treatments with *M. elongata* (M3) with 24.50 mg/100 g clusted in the first group and and in treatments M1 (22.10 mg/100 g fw) and M2 (21.11 mg/100 g fw) clustered in the second group. Only treatment with *A. Clavatus* (A1) showed value lower than value of control treatment (14.94 mg/100 g fw).



Graph 4.5.1. Total ascorbic acid values and corresponding group for treatments with different fungal isolates; *A. clavatus* (A1, A2 and A3), *M. elongata* (M1, M2 and M3) and control (CONTR)

In the study by Mampholo et al. (2016), with the aim of selecting lettuce varieties grown in a closed hydroponic system based on their morphological parameters, phytochemical and mineral content for mixed fresh salads or as whole products, results for the content of ascorbic acid (vitamin C) were obtained. The content of ascorbic acid varied depending on the lettuce variety. The highest ascorbic acid content was recorded in the Multired 4 variety, which contained 9.60 mg/100 g of fresh mass, while other varieties like Multigreen 3 and Multigreen 1 had slightly lower contents, at 5.25 mg/100 g and 4.99 mg/100 g. Varieties from the third group, such as Smile, Palmir, Hardy, and other green varieties, had lower ascorbic acid content, below 4 mg/100 g, but had a higher fresh leaf mass. In the study by Aćamović-Djoković et al. (2011), the amount of ascorbic acid is determined in several different lettuce varieties. The highest vitamin C content is recorded in the Levistro variety, amounting to 9.60 mg/100 g of fresh lettuce, while the lowest content was found in the Murai variety, with 3.50 mg/100 g. Other varieties had the following values: Plenti (3.85 mg/100 g), Temptation (4.99 mg/100 g), and Kibou (5.25 mg/100 g). The lettuce is grown using black polyethylene (PE) foil, creating favorable growing conditions, yet the results are still lower than those in this study**.**

4.6. Antioxidant Capacity

As shown in Graph 4.6.1., on the basis of ABTS, the treatments were grouped into three groups (A, B and C) which are not statistically justified. Control (CONTR) showed the lowest value (2494.72 µmol TE/L), but without statistical justification. The highest antioxidant capacity is determined in the treatment with *M. elongata* (M3) with the value of 2519.77 µmol TE/L but without statistically justification. Almost the same value is determined in the treatment with *A. clavatus* (A1) with 2519.40 µmol TE/L.

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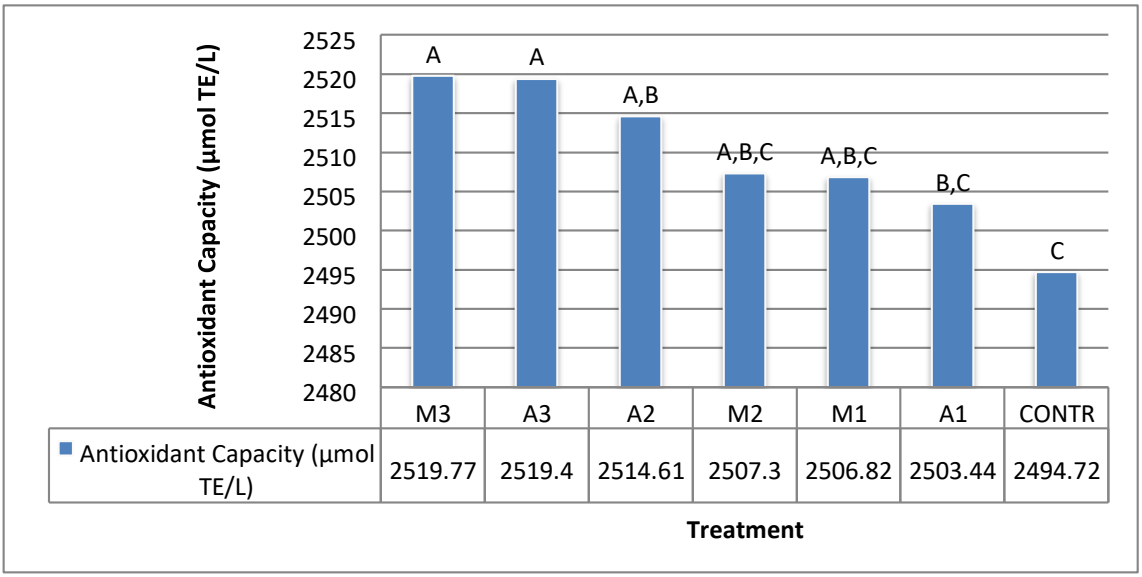
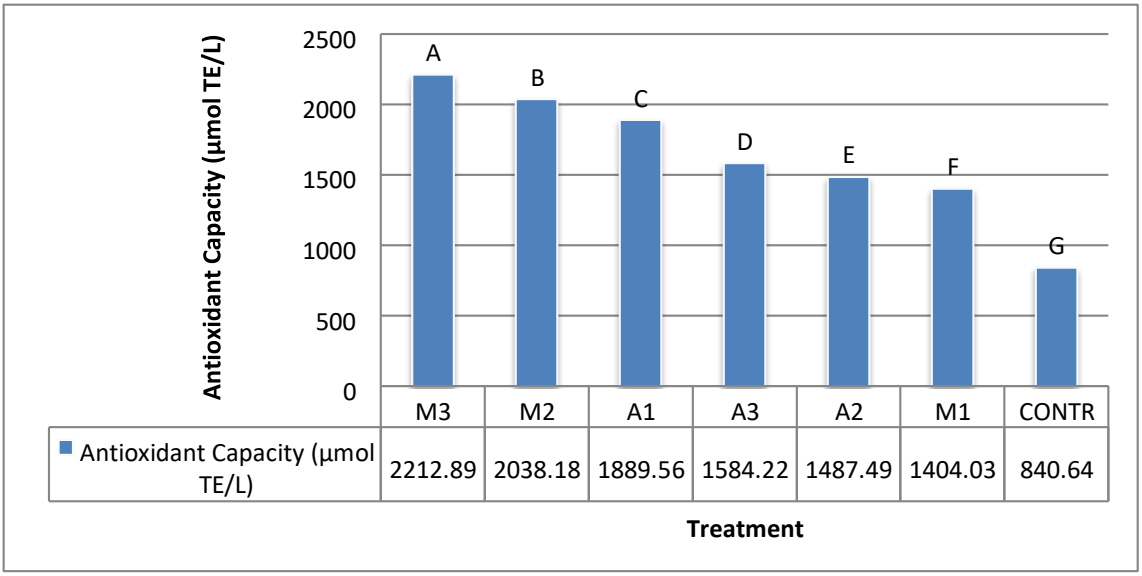


Graph 4.6.1. ABTS values and corresponding group for treatments with different fungal isolates; *A. clavatus* (A1, A2 and A3), *M. elongata* (M1, M2 and M3) and control (CONTR)

As shown in Graph 4.6.2., on the basis of FRAP, the treatments were grouped into seven groups (A, B, C, D, E and F) are statistically justified. The lowest antioxidant capacity measured in the control (CONTR) with the value of 840, being statistically justified compared to other treatments. The highest antioxidant capacity is measured in samples receiving *M. elongata* treatments (M2 and M3) with values of 2038.18 and 2212.89, with statistical justification.



Graph 4.6.2. FRAP values and corresponding group for treatments with different fungal isolates; *A. clavatus* (A1, A2 and A3), *M. elongata* (M1, M2 and M3) and control (CONTR)

In the research of Mampholo and colleagues (2016), who used mineral fertilizers for cultivation, the values of antioxidant activity in green lettuce varieties are reported in the range from 86.68 µmol TE/100 g FW (Monary variety) to 612.93 µmol TE/100 g FW (variety Multigreen 3). Zdravković et al. (2014) state that the high content of phenolic components causes good antioxidant properties in all tested varieties.

5. Conclusion

In this study, application of PGPF (*Aspergillus clavatus* and *Mortierella elongata)*, used as soil inoculums, showed limited effect on yield components (morphological properties- height, diameter and mass) and dry matter content of the lettuce. Although showed differences in height, diameter and mass in the treated treatments from the untreated ones, these differences are not statistically justified. The lowest height is measured in the control. The lowest diameter and mass are measured in the all treatments treated with *M. elongata*. The content of dry matter in the untreated control showed one of the lowest percentages, but without statistical justification.

The contents of total phenols, flavonoids and non-flavonoids in all treated treatments showed significant improvements that are statistically justified. The control showed the lowest value. The treatment with *A. clavatus* (A1) showed the highest value. *A. clavatus* showed values of total phenols 49.44% -88. 05% higher than controls, while *M. elongata* 43. 68% -79.35 % higher than control.

Two treatments (A1 and A3) with *A. clavatus* strains showed significantly improved photosynthetic activity of the lettuce by increasing the total photosynthetic pigments. The treatment with *A. clavatus* (A1) showed the highest increasement of total chlorophyll by 19.51%, chlotophyll a by 22.22 %, chlorophyll b by 25 % and carotenoids by 28.57 %. The treatments with *M. elongata* and treatment with strain A2 of *A. clavatus* showed values of all photosynthetic pigments lower than control. The results are statistically justified.

All treatments with *M. elongata* strains particularly showed increasement of cascorbic acid (vitamin C) content in the lettuce (14.87 % -51.35 % higher than control). The treatment with strain of *M. elongata* (M3) showed the highest value that is statistically justified. In comparison with the control, only lower content of ascorbic acid is showed in one treatment with *A. clavatus* strain (A1). The results are statically justified.

Antioxidant capacity value (both ABTS and FRAP value) of control treatment showed the lowest value. Although one treatment with *M. elongata* isolates showed higher value (M3), statistical justification is showed only when calculating antioxidant capacity based on FRAP. By ABTS *M. elongata* shower increasment for 1.02 %, but it is not statistically justified. By FRAP *M. elongata* increasment for 89.94 % and it is statistically justified.

The positive effects observed indicate that PGPF could play a crucial role in sustainable agricultural practices, enhancing crop productivity and resilience, while potentially reducing reliance on chemical fertilizers and pesticides. This could contribute to more efficient farming methods. In the same time, results showed the importance of selective application of strains depending on agronomic goals – whether the priority is yield improvement or enhancing the bioactive compound content.

6. Reference list

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Biography

Mia Borojević, born on February 11, 2000, in Rijeka, Croatia. Completed secondary education at the Grammar School in Opatija and subsequently relocated to Zagreb, where I pursued an undergraduate degree in Organic Agriculture at the Faculty of Agriculture, University of Zagreb. Further. Enrole the master's program in Environment, Agriculture, and Resource Management (INTER-EnAgro). Throughout studies have been actively engaged in Erasmus+ projects, focusing on permaculture, sustainability, and environmental protection. Gained experience is working on a family farm (OPG), as well as in a company specializing in the cultivation and processing of microgreens.