HULADDUWA MUDIYANSELAGE CHATHURIKA PRIYADARSHANI

GREEN SOLUTIONS: EXPLORING NONSTEROIDAL ANTI-INFLAMMATORY DRUGS' IMPACT ON *CUCURBITACEAE* FOR ENVIRONMENTAL REMEDIATION

UNIVERSIDADE DO ALGARVE

Faculty of Science & Technology

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Erasmus Mundus Joint Master in Applied Ecohydrology (MAEH)

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Declaration of authorship of work

I declare I am the author of this work, which is original and unpublished. The sources consulted have been duly cited in the text and included in the list of references.

Date: 26.08.2024 Signature:

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Sumário

Os anti-inflamatórios não esteróides (AINEs) são fármacos amplamente utilizados em todo o mundo, encontrados como contaminantes na água, no solo e nos sedimentos, representando riscos para os organismos naturais e para a saúde humana. A fitorremediação é uma abordagem eficaz e ecologicamente aceitável para a remediação de poluentes ambientais. As plantas da família Cucurbitaceae têm o potencial de remediar contaminantes no solo. Pesquisas recentes indicam que certos fungicidas podem regular a absorção de compostos orgânicos, modulando proteínas semelhantes a látex (MLP) nestas plantas. Este estudo examina o impacto dos AINEs combinados com benomil na fisiologia, bioquímica e endófitos foliares da planta de curgete (Cucurbita pepo).

As plantas foram cultivadas em substrato de solo OECD sob condições controladas de estufa durante 28 dias. Foram utilizadas seis variantes: controlo, paracetamol (25 mg/L), paracetamol + benomil, diclofenac (2,5 mg/L), diclofenac + benomil e benomil. A água, o fertilizante e o benomil foram adicionados de acordo com um cronograma predefinido. Após a incubação, foram medidos para cada variante a biomassa fresca, o conteúdo de clorofila e as concentrações de compostos fenólicos. A diversidade funcional e estrutural dos endófitos foliares foi analisada através do método Biolog EcoPlate™ e sequenciação do gene 16S rRNA.

O estudo demonstrou que o paracetamol diminuiu a biomassa fresca das raízes, caules e folhas, enquanto o tratamento com diclofenac apresentou uma tendência similar, com a menor biomassa em caules e folhas. O tratamento com paracetamol aumentou o conteúdo de clorofila, enquanto o diclofenac teve um efeito mínimo nos pigmentos de clorofila. Além disso, os compostos fenólicos aumentaram significativamente nas plantas tratadas com paracetamol, mas diminuíram na variante com diclofenac em comparação com o controlo. Ambos os AINEs diminuíram significativamente a atividade metabólica endofítica foliar e a diversidade estrutural microbiana. O benomil, quando aplicado isoladamente, também demonstrou alguns impactos na fisiologia da planta e na comunidade microbiana endofítica foliar. No entanto, o benomil mitigou consideravelmente as consequências prejudiciais das plantas tratadas com AINEs, ao aumentar a biomassa e o conteúdo de clorofila, melhorando a resiliência ao stress oxidativo e promovendo a diversidade microbiana endofítica. Estes resultados contribuem para uma compreensão mais profunda da regulação mediada por fungicidas da fitotoxicidade induzida por AINEs na curgete e destacam o potencial

para o desenvolvimento de estratégias para melhorar a fitorremediação em ambientes contaminados.

Palavras-chave: AINEs, benomil, fisiologia das plantas, endófitos foliares, fitorremediação.

Abstract

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used pharmaceuticals in the world, found as contaminants in water, soil, and sediment, posing risks to natural organisms and human health. Phytoremediation is an effective and ecologically acceptable approach for environmental pollutants. Plants from the *Cucurbitaceae* family have the potential to remediate contaminants in soil. Recent research indicates that certain fungicides can regulate the uptake of organic compounds, modulating major latex-like protein (MLP) in these plants. This study examines the impact of NSAIDs combined with benomyl on the physiology, biochemistry, and leaf endophytes of the zucchini plant (*Cucurbita pepo*).

Plants were grown in OECD soil media under controlled greenhouse conditions for 28 days. There were six variants: control, paracetamol (25 mg/L), paracetamol + benomyl, diclofenac (2.5 mg/L), diclofenac + benomyl and benomyl. Water, fertilizer and benomyl were added according to a predefined schedule. After the incubation, fresh biomass, chlorophyll contents and phenolics concentrations were measured for each variant. Functional and structural diversity of leaf endophytes were analyzed by Biolog EcoPlate™ method and 16S rRNA gene sequencing.

The study demonstrated that paracetamol decreased the fresh biomass of roots, stems and leaves, while diclofenac treatment had a similar trend, with the lowest stems and leaves biomass. Paracetamol treatment increased chlorophyll content, whereas diclofenac had a minimal effect on chlorophyll pigments. Additionally, phenolic compounds increased significantly in plants treated with paracetamol but lowered in the diclofenac variant compared to the control. Both NSAIDs significantly decreased the leaf endophytic metabolic activity and microbial structural diversity. Benomyl, when applied alone, also displayed some impacts on plant physiology and leaf endophytic microbial community. However, benomyl considerably mitigated the detrimental consequences of NSAID-treated plants by enhancing biomass and chlorophyll content, improving resilience to oxidative stress, and promoting endophytic microbial diversity. These findings contribute to a deeper understanding of the fungicide-mediated regulation of NSAID-induced phytotoxicity in zucchini and highlight the potential for developing strategies to enhance phytoremediation in contaminated environments.

Key words: NSAIDs, benomyl, plants' physiology, leaf endophytes, phytoremediation

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List of Abbreviations

NSAIDs	Nonsteroidal anti-inflammatory drugs
MLP	Major latex-like proteins
A	Amines
AA	Amino acids
CA	Carboxylic acids
CCS	Complex carbon sources
CH	Carbohydrates
PC	Phosphate carbon
AWCD	Average well-color development
PCR	Polymerase chain reactions
DNA	Deoxyribonucleic acid
16S rRNA	16 Svedberg ribosomal ribonucleic acid
CFU	Colony forming unit
h	Hours

1. Introduction

Pharmaceuticals are emergent organic compounds that may boost our quality of everyday life. The global consumption of pharmaceuticals continues to grow with spreading diseases, changes in healthcare practices and increasing world population (González Peña et al., 2021). Nonsteroidal anti-inflammatory drugs (NSAIDs) represent pharmaceuticals which contain analgesic (painkilling), antipyretic (fever-reducing), and anti-inflammatory effects (Conaghan, 2012; Fokunang, 2018; Parolini, 2020). Pharmaceuticals from the NSAID group exhibit a broad and complex chemical structure, and most of them are weak organic acids that are extensively metabolized (Tomić et al., 2017; Tyumina et al., 2020). Over 100 NSAIDs have been clinically studied, and more than 50 NSAIDs are available on the global market, making one of the most widely used pharmaceuticals in both human and animal medicine (Fokunang, 2018; Rastogi et al., 2021). NSAIDs are taken by approximately 35 million people worldwide per day, accounting for 5% of all prescribed medications (Bindu et al., 2020; Yan et al., 2021).The most often prescribed NSAIDs worldwide are paracetamol, diclofenac, naproxen, ibuprofen and aspirin (Aguilar-Lira et al., 2022; Mohd Hanafiah et al., 2022). Their chemical structures and formulas are shown in Table 1.1.

Table 1.1: International Union of Pure and Applied Chemistry (IUPAC) names, chemical structures, and chemical formulas some of the most often prescribed NSAIDs in the world.

NSAIDs	Chemical Structure	Chemical Formula
Paracetamol (IUPAC: N-(4-hydroxyphenyl) acetamide)		$C_8H_9NO_2$
Diclofenac (IUPAC: 2-[2-(2,6-dichloroanilino) phenyl] acetic acid)		$C_{14}H_{11}Cl_2NO_2$
Naproxen (IUPAC: (2S)-2-(6-methoxynaphthalen-2-yl) propanoic acid)		$C_{14}H_{14}O_3$

NSAIDs	Chemical Structure	Chemical Formula
Ibuprofen (IUPAC: 2-[4-(2-methylpropyl) phenyl] propanoic acid)		$C_{13}H_{18}O_2$
Aspirin (IUPAC: 2-acetyloxybenzoic acid)		$C_9H_8O_4$

Source: (PubChem, 2024)

1.1. NSAIDs - emerging pollutants of XXI century

Because of their frequent utilization, NSAIDs are currently one of the major types of emerging contaminants in groundwater, surface waters, seawater, stormwater runoff, wastewater, and drinking water globally (Huynh et al., 2023; Lin et al., 2023; Mohd Hanafiah et al., 2022; Parolini, 2020). NSAIDs have been discovered even in thoroughly protected areas like Antarctica, where human activities are limited (González Peña et al., 2021). Among the most regularly discovered NSAIDs in the environment nowadays are diclofenac (35 countries), paracetamol (29 countries), ibuprofen (28 countries), naproxen (21 countries), and included in the top 10 priority pharmaceuticals to be detected in aquatic environments by the European Union. Among all, paracetamol is the most concentrated environmentally discovered NSAID, with a concentration of 230 µg/L (Parolini, 2020; Tyumina et al., 2020; Zhou et al., 2023). Table 1.2 presents environmentally recorded paracetamol and diclofenac concentrations worldwide as selected NSAIDs due to their greater availability and existing environmental effects.

Table 1.2: Reported paracetamol and diclofenac concentrations in different environments around the world.

Environmental Matrices	Paracetamol (ng/L)/ (ng/g)	Diclofenac (ng/L)/ (ng/g)	References
Surface water	117 ng/L (Colorado River, USA) 112-555 ng/L (UK) 65 ng/L (Germany) 227,000 ng/L (Bolivia)	9000 ng/L (Poland) 10,000 ng/L (S. Africa) 34–145 ng/L (Argentina) 364 ng/L (Brazil)	(Al-kaf et al., 2017; Gumbi et al., 2017; Lonappan et al., 2016; Rastogi et al., 2021; Sathishkumar et al., 2020; Taschina et al., 2022)
Groundwater	15 – 1,890 ng/L (USA) 34 ng/L (Spain) 10 ng/L (France)	380 ng/L (Spain) 13480 ng/L (Nigeria) 590 ng/L (Germany) 113.8 ng/L (Taiwan)	(Al-kaf et al., 2017; Jurado et al., 2021; Lu et al., 2016; Sacher et al., 2001; Sathishkumar et al., 2020)
Drinking water	36 – 6500 ng/L (USA) 34 ng/L (Spain) 10 ng/L (France)	1.2 ng/L (USA) 16 ng/L (Japan) 56 ng/L (France)	(Al-kaf et al., 2017; Lonappan et al., 2016; Sathishkumar et al., 2020)
Wastewater treatment (influent)	105,910^{h} ng/L (UK) 8907^{h} ng/L (India) 48,500 ng/L (Poland) 155.3–22,889 ng/L (S. Africa)	31.5 - 86.3 ng/L (China) 27600 ng/L (Malaysia) 12.16–246.3 ng/L (S. Africa) 747 ng/L (New Zealand)	(Huynh et al., 2023; Mohd Hanafiah et al., 2022; Omotola et al., 2022; Yan et al., 2021)

Environmental Matrices	Paracetamol (ng/L)/ (ng/g)	Diclofenac (ng/L)/ (ng/g)	References
Soil	31 ng/g (Spain) 101–257 ng/g (Pakistan)	0.249–0.567 ng/g (S. Africa) 101–257 ng/g (Pakistan)	(Huynh et al., 2023; Phong Vo et al., 2019)
Sediment	33 ng/g (Spain) 1.8 ng/g (Pego-Oliva marsh, Spain) 2.2 ng/g (S. Korea)	278.1 ng/g (Jilin Songhua River, China)	
Sludge	898 ng/g (Australia) 113 ng/g (USA) 13–419 ng/g (Spain)	19 ng/g (Australia)	(Wu et al., 2012; Yang et al., 2016)

[h]-Hospital wastewater influent

NSAIDs reach the environment mainly through industrial and municipal wastewater, leachate from landfills and direct waste disposal (Huynh et al., 2023; Rastogi et al., 2021; Rodríguez-Saldaña et al., 2023). The human body does not entirely metabolize these drugs, and therefore, unutilized chemicals are released unchanged or as complexes into wastewater. In addition, 58-68% of unmodified paracetamol is eliminated from the human body during therapeutic usage and discharged into sewage water. These pharmaceuticals are challenging to remove from the wastewater, and the treatment efficiency of some NSAIDs does not exceed 30%. The quantity of NSAIDs might increase during wastewater treatment because of conjugate release. There are no strict monitoring and discharge limits for NSAIDs. In addition, direct disposal of unused and expired medicine, poor sanitary practices, and lack of wastewater treatment capacity and facilities, these substances continue to be released into the environment globally (Al-kaf et al., 2017; Rodríguez-Saldaña et al., 2023; Tyumina et al., 2020). As a result, NSAIDs frequently reach reservoirs, lakes, rivers, and oceans during rains and tides and deposit and accumulate in sediment (Huynh et al., 2023). Additionally, NSAID concentrations in aquatic ecosystems are higher in winter than in spring-summer due to low mixing, cold temperatures, and limited biological degradation by microorganisms (Yang et al., 2017). NSAIDs were also found in soils caused by using sewage sludge as crop manure in agricultural processes (Opriș et al., 2020).

1.2. Environmental fate and behavior of NSAIDs

NSAIDs ending up in the environment raise significant concerns due to the potential environmental and human health consequences. These compounds have been shown in studies to harm aquatic organisms such as polychaetes, crustaceans, molluscs, fish, and others by causing genotoxicity, metabolic disorders, endocrine failure, reproduction disruption and teratogenic effects, morphological and tissue alterations, changes in organ growth, behavioural changes, and population instabilities. Fish are the most widely investigated group in terms of NSAID toxicity (Marmon et al., 2021; Świacka et al., 2021). Furthermore, some NSAIDs might cause hazardous effects, especially when combined with other environmental contaminants. According to Chopra & Kumar (2020), diclofenac metal complexes are poisonous to many species and have antibacterial behaviours. Ultimately, these impacts affect natural balance and may infiltrate the food chain, posing risks to both wildlife and humans (Świacka et al., 2021). Figure 1.1 shows the sources, distribution, and environmental impacts of NSAIDs.

Paracetamol has been reported to be toxic to fish (*Oryzias latipes*) at a lethal dosage (LC50) of >160 mg/L after 48 hours and crustaceans (*Daphnia magna*) at an effective concentration (EC50) of 26.6 mg/L after 96 hours (Świacka et al., 2021). Sarma et al. (2014) discovered that the population of *Moina macrocopa* and *Plationus patulus* declined when the concentrations of paracetamol increased to 2, 4, 8, 16, and 32 mg/L. *Solea senegalensis* (a marine fish) significantly influenced catalase and cholinesterase activity at diclofenac concentrations at 2000 ng/L (Nunes, 2020). Survival, reproduction, and population in *Daphnia magna* and *Moina macrocopa* were significantly reduced at 25 mg/L and 50 mg/L diclofenac, respectively. *Oryzias latipes* displayed lower hatchability and delayed hatching after being exposed to 0.001 mg/L of diclofenac for 3 months (Lee et al., 2011). In addition to their environmental toxicity, NSAIDs have ability to bioaccumulate in food chains. Huerta et al. (2015) found 12.4 ng/g dry weight of diclofenac in *Hydropsyche sp.* obtained from the River Segre in the Pyrenees region of the Iberian Peninsula. Sathishkumar et al. (2020) discovered that diclofenac had antibacterial activities against numerous bacteria, including *Brucella sp.*, *Escherichia coli*, *Fusobacterium necrophorum*, *Mycobacterium tuberculosis*, and *Salmonella typhimurium*, at dosages ranging from 50 to 100 µg/L.

The degradation of NSAIDs in the environment occurs via two pathways: photodegradation and biodegradation (Lin et al., 2023). NSAIDs and their combinations transform into metabolites

through biodegradation by microbial communities (bacteria and fungi), algae, and plants. Microbes play an essential role in decomposition by NSAIDs used as carbon or energy sources. For example, *Stenotrophomonas sp.*, *Pseudomonas sp.*, *Delftia tsuruhatensis*, *Penicillium sp.*, and *Burkholderia sp.* can utilize paracetamol (Wu et al., 2012). *Klebsiella sp.*, *Rhodococcus ruber*, *Labrys portucalensis*, *Brevibacterium sp.*, *Pseudoxanthomonas sp.*, *Ganoderma applanatum* and *Laetiporus sulphurous* may degrade diclofenac (Lin et al., 2023; Rastogi et al., 2021).

1.3. Impact of NSAIDs of plant health

Apart from the adverse effects on organisms, numerous studies have reported possible impacts of NSAIDs on plants, including inhibition of growth, cell and root damage, and metabolic disorders (Huynh et al., 2023). Furthermore, depending on the concentration, length of exposure, species and the stage of plant development, NSAIDs can induce oxidative stress in plants at high concentrations or impact plant phytohormones at low concentrations (Taschina et al., 2022).

In the study of Hammad et al. (2018), paracetamol and diclofenac reduced the growth and development of maize plants (*Zea mays* L.). The accumulation of paracetamol in maize seeds and roots increased linearly with the dose. Kudrna et al. (2020) found that high concentrations of paracetamol considerably reduced photosynthesis and chlorophyll fluorescence in lettuce plants (*Lactuca sativa* L.) after 14 days of treatment with paracetamol at 5 µM, 50 µM, 500 µM, and 5 mM. Cho & Kim, (2021) reported a growth inhibition in *Arabidopsis* plants exposed to diclofenac from the seedling stage. Seedlings showed smaller leaves and discoloration at 30 µM of diclofenac compared to untreated samples and increased oxidative stress. Kummerová et al. (2016) found biochemical alterations, including a reduction of photosynthetic pigments, in *Lemna minor* plants following ten days of treatment with diclofenac and paracetamol at doses ranging from 0.1 to 100 µg/L. Both drugs significantly caused plant growth by decreasing quantity, dry weight, and leaf area. However, besides the phytotoxicity, plants may uptake and detoxify these pollutants to a certain extent. Thus, they improve the water and soil quality by giving necessary protection against the environmental contamination (Bartha et al., 2010).

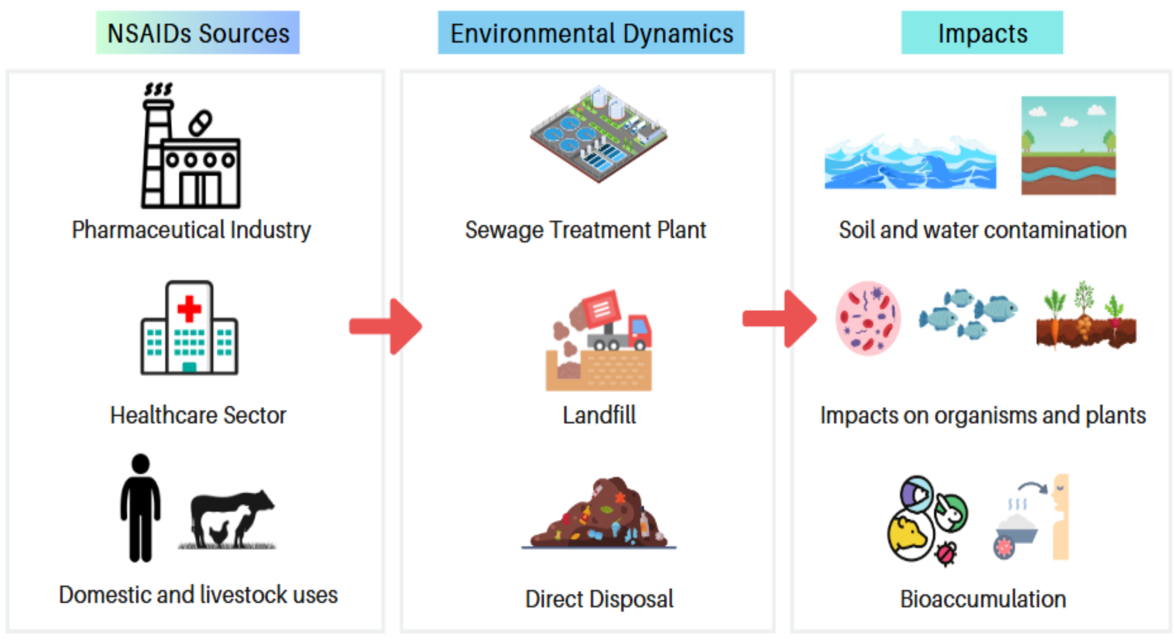

Figure 1.1: Sources, distribution and environmental impacts of NSAIDs.

1.4. Phytoremediation as green approach to Environmental Sustainability

Phytoremediation is the use of plants and associated microbes to remove inorganic and organic pollutants from soil, water, and the atmosphere (Ansari et al., 2015; Singh et al., 2024). It is also an environmentally friendly and cost-effective natural clean-up mechanism for environmental contaminants (Etim, 2012). Phytoextraction (contaminant uptake into plant biomass), rhizofiltration (contaminant removal from water), phytovolatilization (pollutants transformation into volatile forms and release into the atmosphere), Phyto-stabilization (contaminants immobilization by absorption, adsorption, or formation of insoluble compounds), and phytodegradation (degradation of organic contaminants) are main processes involved in phytoremediation (Ansari et al., 2015; Singh et al., 2024).

The interaction between plants and associated microbes (endophytic, phyllo-spheric, and rhizospheric bacteria) facilitates the removal of pollutants and converts them to less-toxic forms via metabolic and enzymatic activities (Karaś et al., 2021; Martínez et al., 2023; Singh et al., 2024). Rhizospheric bacteria degrade contaminants in the plant root zone. Releasing sugars, amino acids, and enzymes by plants promotes microbial development and rhizodegradation of pollutants (Etim, 2012). Endophytes, microorganisms found within plants, can regulate the metabolic processes of

pollutants (Karaś et al., 2021). Furthermore, they can produce biosurfactants and phytohormones in host plants, which improves nutrient and water uptake capability and can promote plant growth and phytoremediation efficiency (Singh et al., 2024).

Plants can uptake NSAIDs and their metabolites directly from the environment. Plants can also convert NSAIDs into plant metabolites. According to Loise et al. (2019), paracetamol can be metabolized into N-acetyl-benzoquinomine, glucuronide, sulphate, mercapturate, and glutathione by plants, while diclofenac-lactam, 40-hydroxy-diclofenac, 50-hydroxydiclofenac, and diclofenac-benzonic acid are diclofenac transformations. Besides, numerous studies were performed on the applications of phytoremediation for NSAIDs using plants and plant-associated microbes. Examples of paracetamol reduction percentages from various plants included *Phragmites australis* (51.7-99.9%), *Typha latifolia* (46.7-99.9%), *Scirpus grossus* (94%), and *Spirodela polyrhiza* (> 95%) (Al-Falahi, 2022; Ranieri et al., 2011; Singh et al., 2024). Plant responses to diclofenac removal involved *Phalaris arundinacea* (52-91%), *Scirpus validus* (80%), *Phragmites australis* (57%), *Typha angustifolia* (55%), and *Cyperus alternifolius* (69.3 ± 0.2%) (Hijosa et al., 2010; Singh et al., 2024; Zhai et al., 2016; Zhang et al., 2012). Therefore, plants play an excellent role in removing paracetamol and diclofenac from the environment. However, there is still limited information on the potential removal of pharmaceuticals. Comprehensive experimental studies are needed to understand the mechanisms of pharmacological uptake and phytoremediation capacity by plants (Zhang et al., 2012).

1.5. Potential for Phyto removal of NSAIDs using *Cucurbitaceae* plants

Cucurbitaceae is a vegetable and fruit crop family, with around 125 genera and 960 species that include cucumbers, melons, and pumpkins (Mukherjee et al., 2022). Apart from their edible and medicinal uses, many studies have shown that *Cucurbitaceae* plants have a significant potential to absorb and remediate environmental contaminants such as heavy metals, persistent and emerging organic pollutants (POPs and EOPs), herbicides, and others (Fujita & Inui, 2021; Mierzejewska et al., 2022c; Mierzejewska-Sinner et al., 2024). Cucurbits, especially, are capable of extracting, translocating, and accumulating extremely hydrophobic and organic chemicals from soil. According to the investigations, cucumber and zucchini are the most effective phytoremediators among the cucurbits. Zucchini has been shown to collect high levels of organic pollutants in its aerial parts (Wyrwicka & Urbaniak, 2016). Otani et al. (2007) identified cucurbits, notably

zucchini, as cleaning crops for phytoremediation in contaminated fields. Cucurbits additionally aid with rhizoremediation by increasing the growth of microorganisms and the decomposition of organic pollutants due to their root exudates such as plant secondary metabolites (Mierzejewska et al., 2023; Urbaniak et al., 2020b). Eevers et al. (2016) explored the possibility of pesticides degradation including 2,2-bis(p-chlorophenyl)-1,1-dichloroethylene by endophyte microbes in zucchini plants.

Rastogi et al. (2021) mentioned the uptake and metabolism of NSAIDs in different edible crops, including cucumber. García & Fernández-López (2022) recognized ibuprofen accumulation in zucchini plant irrigated with wastewater effluent. Fujita & Inui (2021) reported the detection of paracetamol in the aerial parts of zucchini and cucumber plants. However, there is still limited research on the intake, effects, and phytoremediation potential of NSAIDs on cucurbit plants.

1.6. Controlling the phytoaccumulation capabilities of cucurbits toward NSAIDs

Major latex-like proteins (MLPs) in cucurbit plants have a crucial function in enhancing the solubility of organic pollutants by producing MLP-organic pollutant complexes. MLPs originate primarily in the roots, and organic contaminants can transfer from the plant's roots to aerial parts through this mechanism (Goto et al., 2019).

Pesticides binding to MLPs competitively inhibited the interaction between MLPs and organic contaminants in the roots, leading to a decline in the accumulation of pollutants in above-ground tissues (Fujita et al., 2020b). Fujita et al. (2020a) found that using pesticides in zucchini plants dramatically reduced hydrophobic pollutants in aerial portions by reducing MLP gene transcription in the roots. Interestingly, certain fungicides, such as Oryzemate, have been found to upregulate MLP gene expression in the roots and enhance the accumulation of organic pollutants in the aerial tissues of zucchini (Chitose et al., 2024). This regulation can be relevant as MLPs show a strong affinity for binding with aromatic compounds (Fujita et al., 2020b), which could also apply to NSAIDs (PubChem, 2024). Consequently, the phytoremediation potential of NSAIDs within the *Cucurbitaceae* family may be regulated by MLPs in the presence of competing substances such as fungicides.

2. Aims of the research

Pharmaceuticals are already an essential part of human life. While being among the most often used pharmacological classes worldwide, NSAIDs have become a significant hazard to the natural environment, living organisms, and human wellness, resulting in extensive pollution of water, soil, and sediment. As a result, it is critical to investigate sustainable management strategies to remove these pollutants from the environment. Numerous studies have demonstrated that phytoremediation is an effective and ecologically acceptable approach for environmental contaminants. However, the effects and potential remediation of NSAIDs by plants are still limited in the research field. Additional research into the interactions of various plant species, exudates, and related bacteria with these drugs still needs to be investigated. Notably, the *Cucurbitaceae* family has the potential to absorb and remediate contaminants in soil. However, there have been no investigations on the impacts and phytoremediation potential of NSAIDs on *Cucurbitaceae* plants.

In view of the above, the goal of the present study is to monitor the impact of NSAIDs and the fungicide on both the zucchini health and the zucchini-associated bacteria diversity to verify the usefulness of this family of plants in the remediation of NSAIDs-contaminated areas. To achieve the above research goal, the following study objectives have been proposed:

1. To verify the influence of selected NSAIDs and/or the fungicide on zucchini's growth (biomass), physiological status (chlorophyll content), and stress response (phenolic compound concentrations).
2. To check the influence of selected NSAIDs and/or the fungicide on the functional and structural diversity of plant-associated bacteria (leaf endophytes).

3. Materials and Methods

Soil

OECD artificial soil, composed of sand, kaolin and peat, free of contaminants, commonly used in a wide range of soil toxicity tests as a reference soil, was used as substrate media for the experiment (Oleszczuk & Hollert, 2011; Urbaniak et al., 2017).

Plant

Zucchini (*Cucurbita pepo*, Atena Polka F1) was chosen for the experiment considering its potential to uptake of various pollutants based on previous studies (Mierzejewska et al., 2022a; Mierzejewska-Sinner et al., 2024; Urbaniak et al., 2020b). The seeds were purchased from a certified supplier (W. Legutko).

3.1. Experimental Setup

Six variants were tested during the experiment, including control (C), paracetamol (P), paracetamol with benomyl (P+B), diclofenac (D), diclofenac with benomyl (D + B), and benomyl (B). Four pots of each variant were prepared. Three zucchini seeds were introduced per pot. Plants were grown for 28 days in 200 cm^3 pots filled with 150 cm^3 of OECD soil in a growth chamber at 23 ^{0}C ± 0.5 ^{0}C and 60% w/v soil humidity. After one week, two seedlings were removed from the pots, leaving in each pot only one seedling in the same growth stadium. All variants were watered daily, up to the water-holding capacity of OECD soil (Mierzejewska et al., 2022a). After 8 days of incubation, plants were exposed to two selected NSAIDs: 25 mg/L of paracetamol (based on phytotoxicity bioassay) or 2.5 mg/L of diclofenac (Siemieniuk et al., 2021). All the variants were fertilized with 60 mL of Florovit NPK 5-6-8 (10 mL of liquid fertilizer per 1.2 L of distilled water) on the 5th, 12th, 19th and 26th day of cultivation. Benomyl (DRE-C10490000; 250 mg/L) was sprayed on the aboveground part of the plants according to the timeframe shown in Table 3.1.

Table 3.1: The time frame of benomyl spraying during the experiment; experimental variants: control (C), paracetamol (P), paracetamol with benomyl (P + B), diclofenac (D), diclofenac with benomyl (D + B), and benomyl (B).

Variant	0 day	5th day	12th day	17th day	19th day	24th day	26th day
C	-	-	-	-	-	-	-
P	-	-	-	-	-	-	-
P + B	-	-	+	+	-	+	-
D	-	-	-	-	-	-	-
D + B	-	-	+	+	-	+	-
B	-	-	+	+	-	+	-

"+" indicates spraying of benomyl; "-" indicates no benomyl treatment

3.2. Sampling

3.2.1. Leaves sampling for chlorophyll, phenolic compounds and endophytes analysis

Plants were carefully removed from the pots after 28 days of cultivation and separated from the soil. Roots, stems, and leaves of the plants were separated using sterile blades. 2nd, 3rd, and 4th leaf of each plant was taken and cut into small pieces using sterile blades. 0.40 g of leaf sample was weighed, wrapped in aluminium foil, and stored at -20 degrees for phenolics and chlorophyll analysis. Additionally, 2nd, 3rd, or 4th leaf of each plant was randomly taken and placed in 50 mL sterile falcon tubes and stored at 8°C for further endophytes analysis (Mierzejewska et al., 2023; Urbaniak et al., 2020e).

3.3. Plant physiology and biochemical analysis

3.3.1. Determination of biomass

After separation of the plants from the soil, the fresh biomasses of the roots, stems and leaves were weighed according to Urbaniak et al. (2020e).

3.3.2. Determination of chlorophyll contents

Chlorophyll a and chlorophyll b contents were measured using a 96% methanol extraction. 0.2 g of fresh leaves were ground up in a mortar. 2 mL of 96% methanol was added, and the mixture was homogenized again. The suspension was transferred to a 25-mL graduated cylinder. The mortar

and pestle were washed with 4 mL of 96% methanol, and the rinse was transferred to the cylinder. The total volume of the solution was raised to 10 mL with 96% methanol. The extract was shaken for 10 seconds and left for 10 minutes to allow for phase separation. 5 mL of supernatant was filtered through a soft filter into a test tube, and absorbance (A) was measured at 663 and 645 nm using the Thermo Scientific™ Multiskan™ Skyhigh Microplate Spectrophotometer. The chlorophyll a and chlorophyll b contents were calculated using Eq.1 and Eq.2. The values were expressed as µg per g of fresh weight of the plant (Harborne, 1980; Urbaniak et al., 2020e).

$$Chlorophyll\ (a) = 12.7 \times A\ (663) - 2.69\ \times A\ (645) \qquad \text{Eq. 1}$$

$$Chlorophyll\ (b) = 22.9 \times A\ (645) - 4.68\ \times A\ (663) \qquad \text{Eq. 2}$$

3.3.3. Determination of phenolic compound contents

Phenolic compounds were determined in the leaves using an 80% methanol extraction. 0.15 g of fresh leaves were ground up in a mortar. 1 mL of 80% methanol was added, and the mixture was homogenized again. The suspension was transferred to a 2 mL microcentrifuge tube and centrifuged at 5000 rpm for 15 min. The supernatant was transferred to a falcon tube and topped up to 1 mL with 80% methanol. 0.25 mL of plant extract was mixed with 10 µL of 0.1% HCL and 2 mL of 2% HCL. The absorbance (A) of the samples were measured at 280, 320, 360, and 520 nm using the Thermo Scientific™ Multiskan™ Skyhigh Microplate Spectrophotometer. Phenolic contents (Total phenols, phenylpropanoids, flavanols and anthocyanins) were calculated using Eq.3, Eq.4, Eq.5 and Eq.6, and values expressed as mg of standard per 100 g of fresh plant weight (Fukumoto & Mazza, 2000; Urbaniak et al., 2020b).

$$Total\ phenols\ (Chlorogenic\ acid) = A280/0.264/1.15\ \times 100 \qquad \text{Eq. 3}$$

$$Phenylpropanoids\ (caffeic\ acid) = A320/0.887/1.15\ \times 100 \qquad \text{Eq. 4}$$

$$Flavonols\ (quertecin) = A360/0.513/1.15\ \times 100 \qquad \text{Eq. 5}$$

$$Anthocyanins\ (cyanidin) = A520/0.645/1.15\ \times 100 \qquad \text{Eq. 6}$$

3.4. Leaves' endophytes functional diversity analysis

3.4.1. Leaves surface sterilization and extraction

Surface sterilization and extract preparation was done under the laminar flow in sterile conditions. 3.5 g of fresh leaves sample was sterilized using 200 mL of 1x 70% ethanol (1 minute), then with 200 mL of 1x 2.5% NaOCl + 0.1% Tween 20 (1 minute), 200 mL of 1x 70% ethanol (1 minute), and 3x sterile distilled water (30 seconds) respectively. Leaves were placed in 50 mL sterile falcon tubes. The sterility of the last wash was tested by inoculating 100 µl of the third rinsing water on a Petri dish with undiluted LB medium (Mergeay et al., 1985; Plaszkó et al., 2022).

The sterilized fresh leaves samples were homogenized using sterile mortar and pestle and adding 1% NaCl up to 8 milliliters. The extraction was filtered through 100 µm mesh cell filters into a 50 mL falcon tube. The volume was filled up to 35 mL with 1% NaCl to prepare a 10^{-1} dilution. A serial dilution was further prepared using 1% NaCl to obtain 10^{-2} and 10^{-3} dilutions of leaves extracts (Mierzejewska et al., 2022a; Szymańska et al., 2013).

3.4.2. Biolog Ecoplates for leaf endophytes

Biolog Ecoplates analysis was prepared under the laminar flow in sterile conditions. 120 µL of 10^{-2} diluted leaf extraction was poured into each well of the microarray plates using a multichannel pipette. The plates were incubated at 27°C for ten days. The optical density was measured at 590 nm wavelength at 24-hour intervals starting from time "0" using a Thermo Scientific™ Multiskan™ Skyhigh Microplate Spectrophotometer. The microbial activities were analyzed for 31 different carbon sources from 6 carbon groups (A-amines, AA-amino acids, CA-carboxylic acids, CCS-complex carbon sources, CH-carbohydrates, and PC-phosphate carbon) using Microsoft Excel 365 (Mierzejewska et al., 2022a; Szymańska et al., 2013).

3.5. Isolation of endophytic bacteria from zucchini leaves

3.5.1. Leaf endophytes isolation

Endophytic bacteria from leaves were isolated under the laminar flow in sterile conditions. 100 µl of 10^{-1}, 10^{-2}, and 10^{-3} dilutions of each variant were spread on LB medium plates and incubated for 7 days at 27 °C. After the incubation, the number of colonies on each plate was counted. Colony Forming Unit (CFU/mL) was calculated based on the colonies counting in 10^{-2} dilution using Eq. 7 (Bio-Resource, 2011).

$$CFU = ND/V \qquad \text{Eq. 7}$$

N- Number of colonies

D- Dilution factor

V- volume of culture plate (mL)

Distinct colonies from each plate were inoculated into LB medium plates with 50 µL of sterile $MgSO_4$ using a sterile loop. The plates were stored in an incubator at 27°C for 2 to 5 days. Pure colonies were inoculated into 2 mL of sterile Eppendorf tubes containing 1 mL of 869 liquid medium. Eppendorf tubes were incubated on a shaker at 27 °C for 2-10 days. The samples were centrifuged for 5 minutes at 8000 rpm and stored at 8°C (Hassan, 2017).

3.5.2. DNA extraction and detection of bacterial DNA by 16S rRNA gene

Endophytic bacterial DNA was isolated using the GeneMATRIX Bacterial & Yeast Genomic DNA purification Kit Cat. No. E3580 (EURx Ltd. 80-297, Gdansk, Poland). The quality of the DNA samples was checked using Nanodrop 2000 (Thermo Fisher Scientific), and all the concentrations were above 10 ng μL^{-1}. The extracted DNA samples were stored at -20°C. The extracted genomic DNA was subjected to PCR using the thermocycler 2720 Thermal Cycler. Universal primers for prokaryotes were used (27F: 5′- AGAGTTTGATCCTGGCTCAG -3′, 1492R: 5′- GGT TAC CTT GTTACG ACT T-3′) for the analysis. After preparing the samples, PCR was performed for 25 cycles following the temperature gradient in Table 3.2 (Shen & Fulthorpe, 2015).

The PCR amplification was confirmed using 1.5 % agarose gel media stained with Gel Red nucleic acid gel strain (0.5 µg/mL concentration). 5 µL of PCR products were mixed with 1 µL of loading buffer and transferred into wells in the gel media. Electrophoresis was conducted for 60 minutes at 100 V in 1x TBE buffer solution, and the products were visualized under UV light. The amplified products were sequenced using the same primers used for PCR amplification. The taxonomic classification was detected based on the nucleotide alignments using the BioEdit software and the BLAST tool at the NCBI Web BLAST (Nucleotide BLAST, 2024; Shen & Fulthorpe, 2015).

Table 3.2: Temperature gradients for 16S rRNA gene.

Step	Temperature °C	Time
Initial denaturation	98	180 s
Denaturation	98	10 s
Annealing	56	30 s
Extension	72	60 s
Final extension	72	420 s

3.6. Data analysis

The data were analyzed using Microsoft Excel 365 and PAST 4.0 software. Based on the normality of distribution, further statistical analyses were performed. Plant biomass, chlorophyll contents, and phenolic compounds were compared using one-way analysis of variance (ANOVA) followed by Tukey's HSD (honestly significant difference) post hoc test. Biolog® results, including total AWCD, AWCD for six carbon groups, and microbial catabolic diversity indices, were analyzed using the Kruskal-Wallis's test followed by Dunn's post hoc test. All statistical analyses were conducted at a significant level of $p \leq 0.05$. Data are presented as mean ± standard error.

4. Results

4.1. Plant physiology and biochemistry

4.1.1. Fresh biomass

Paracetamol

The average fresh biomass of zucchini plants in response to paracetamol exposure is presented in Figure 4.1, with significant differences observed among control and treated variants ($p < 0.05$). The highest biomass was observed in the control roots (2.78 ± 0.83 g/plant), stems (3.62 ± 0.85 g/plant), and leaves (6.69 ± 1.38 g/plant). Statistically relevant decline was observed for biomass of roots, stems and leaves in other variants as compared to the control. The P variant had the lowest biomass for stems (1.45 ± 0.30 g/plant) and leaves (2.23 ± 1.00 g/plant) compared to other variants, with reductions of 60% and 67%, respectively, relative to the control (Fig. 4.1, Tab. 4.1). Root biomass was also significantly lower in the P variant (1.13 ± 0.41 g/plant) than the control, but the B variant showed the lowest value (0.94 ± 0.20 g per plant). There were no statistical differences in roots, stems, and leaves biomass for the P, P + B, and B variants. However, there was a distinct pattern of increasing biomass in stems and leaves in order to P < P + B < B.

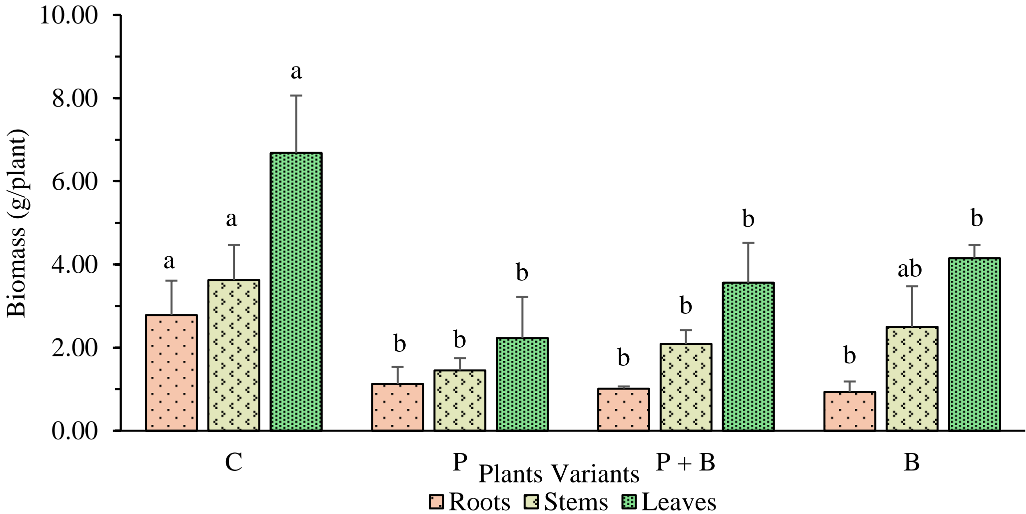

Figure 4.1: Average fresh biomass (± SD) for paracetamol treatment: roots, $F_{(3, 12)} = 13.56$, $p = 0.0004$; stems, $F_{(3, 12)} = 7.103$, $p = 0.0053$ and leaves, $F_{(3, 12)} = 14.24$, $p = 0.0003$ for plants variants control (C), paracetamol (P), paracetamol with benomyl (P + B) and benomyl (B). Error bars indicate standard deviation (n = 4). Lowercase letters indicate the significant difference at $p \leq 0.05$ based on the Tukey post hoc test.

Table 4.1: Roots, stems and leaves biomass reduction percentages for paracetamol treatment in plants variants: paracetamol (P), paracetamol with benomyl (P + B) and benomyl (B) compared to the control.

Plants Variants	Biomass reduction percentages compared to control (%)		
	Roots	Stems	Leaves
P	59	60	67
P + B	64	42	47
B	66	31	38

Diclofenac

The response of average fresh biomass in zucchini plants to diclofenac treatment is illustrated in Figure 4.2. A significant difference was observed among the variants ($p < 0.05$), with the highest biomass recorded in the control roots (2.78 ± 0.83 g/plant), stems (3.62 ± 0.85 g/plant) and leaves (6.69 ± 1.38 g/plant); other variants showed a substantial biomass reduction compared to the control. The D variant had the lowest stems (1.70 ± 0.51 g/plant) and leaves biomass (2.23 ± 0.66 g/plant), with reductions of 53% and 67%, respectively, relative to the control variant (Fig. 4.2, Tab. 4.2). The lowest root biomass was observed in variant B (0.94 ± 0.20 g/plant). Interestingly, the D + B variant demonstrated an increase in biomass of roots, stems, and leaves compared to variant D. Despite these variations, the Tukey post hoc test showed no statistical differences in the leaves biomass among the treated variants.

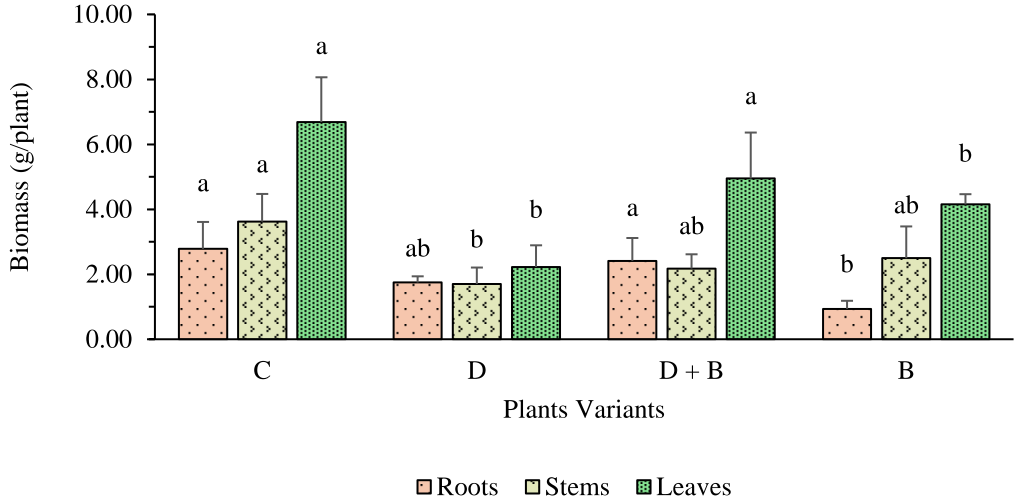

Figure 4.2: Average fresh biomass (± SD) for diclofenac treatment: roots, $F_{(3, 12)} = 8.25$, $p = 0.003$; stems, $F_{(3, 12)} = 5.01$, $p = 0.0176$ and leaves, $F_{(3, 12)} = 12.31$, $p = 0.0006$ for plants variants control (C), diclofenac (D), diclofenac with benomyl (D + B), and benomyl (B). Error bars indicate standard deviation (n = 4). Lowercase letters indicate the significant difference at $p \leq 0.05$ based on the Tukey post hoc test.

Table 4.2: Roots, stems and leaves biomass reduction percentages for diclofenac treatment in plants variants: diclofenac (D), diclofenac with benomyl (D + B), and benomyl (B) compared to the control.

Plants Variants	Biomass reduction percentages compared to control (%)		
	Roots	Stems	Leaves
D	37	53	67
D + B	13	40	26
B	66	31	38

4.1.2. Chlorophyll contents

Paracetamol

The average chlorophyll contents in zucchini leaves in response to paracetamol exposure are represented in Figure 4.3. Significant differences were observed among the variants ($p < 0.05$), with P + B variant demonstrating the highest chlorophyll contents (total chlorophyll: 441.33 ± 25.80 µg/g fresh weight (f.w.); chlorophyll a: 290.93 ± 10.44 µg/g f.w., chlorophyll b: 150.51 ± 15.44 µg/g f.w.). The control variant had the lowest total chlorophyll (262.06 ± 8.60 µg/g f.w.) and

chlorophyll a (141.39 ± 10.49 µg/g f.w.). Besides, the P variant had significantly higher total chlorophyll (349.17 ± 12.20 µg/g f.w.) and chlorophyll a (228.45 ± 22.85 µg/g f.w.) compared to the control. Chlorophyll b was statistically equivalent among the C, P and B variants. However, a significant increase in chlorophyll contents was observed in the P + B variant compared to the P.

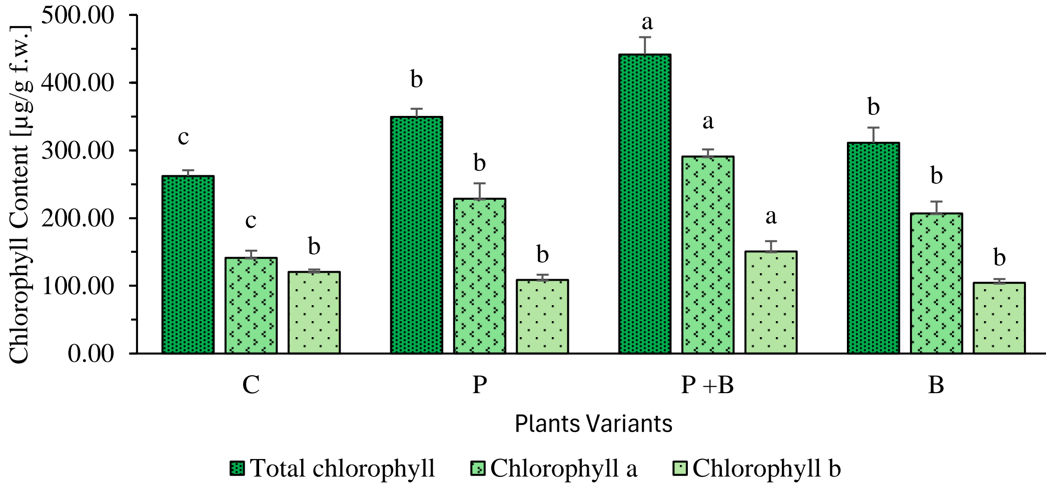

Figure 4.3: Average chlorophyll contents (± SD) in zucchini leaves for paracetamol treatment: total chlorophyll content, $F_{(3, 8)} = 49.45$, $p = 1.65E-05$, chlorophyll a, $F_{(3, 8)} = 43.62$, $p = 2.65E-05$ and chlorophyll b, $F_{(3, 8)} = 15.27$, $p = 0.0011$ for plants variants. Error bars indicate standard deviation (n = 3). Lowercase letters indicate the significant difference at $p \leq 0.05$ based on the Tukey post hoc test.

The chlorophyll a:b ratio in zucchini leaves is presented in Table 4.3, with significant differences observed among the variants ($p < 0.05$). The highest a:b ratios were detected in the P, P + B, and B variants (all the values were ~ 2), and all these values being statistically similar. The lowest chlorophyll a:b ratio (1.17 ± 0.11) was observed in the control.

Table 4.3: Chlorophyll a:b ratio for paracetamol treatment; $F_{(3, 8)} = 48.48$, $p = 1.78E-05$ for the variants. Lowercase letters indicate the significant difference at $p \leq 0.05$ based on the Tukey post hoc test.

Plants Variants	Chlorophyll a:b ratio (± SD, n = 3)
C	1.17 ± 0.11 [b]
P	2.10 ± 0.08 [a]
P +B	1.94 ± 0.13 [a]
B	1.98 ± 0.10 [a]

Diclofenac

The changes in the chlorophyll content in reference to diclofenac treatment are displayed in Figure 4.4. A significant difference was observed between treated variants in term of chlorophyll a and chlorophyll b ($p < 0.05$); however, the total chlorophyll content was statistically similar between all the variants. The highest chlorophyll a content was detected in the B variant (206.90 ± 17.48 µg/g f.w.), while the other variants showed statistically similar values. The highest chlorophyll b content was found in the D + B variant (151.71 ± 20.80 µg/g f.w.), with the remaining variants statistically similar. Chlorophyll contents were statistically equivalent between control and D variant.

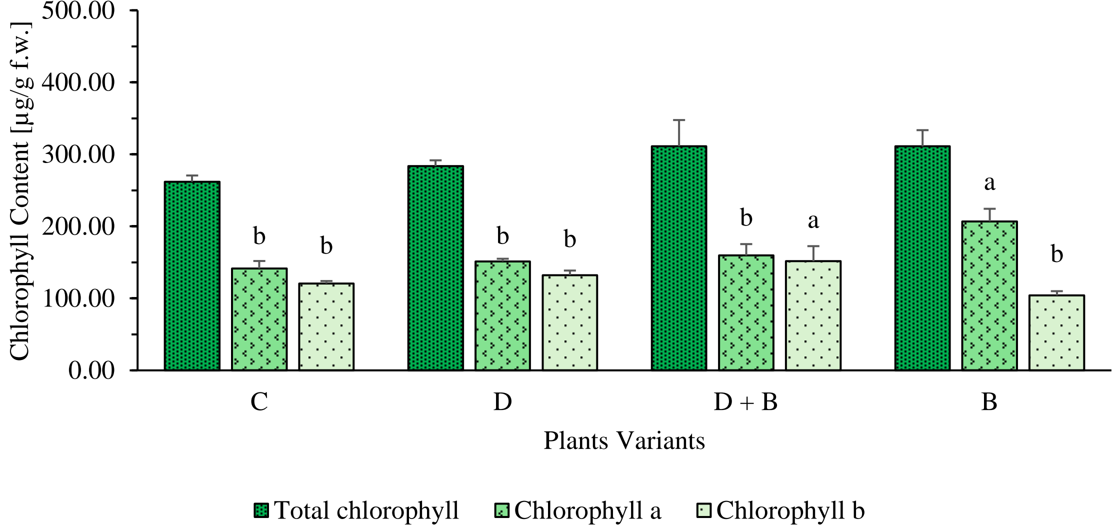

Figure 4.4: Average chlorophyll content (± SD) in zucchini leaves for diclofenac treatment: total chlorophyll content $F_{(3, 8)} = 3.44$, $p = 0.0721$, chlorophyll a $F_{(3, 8)} = 14.92$, $p = 0.0012$ and chlorophyll b $F_{(3, 8)} = 9.27$, $p = 0.0056$ for plants variants. Error bars indicate standard deviation ($n = 3$). Lowercase letters indicate the significant difference at $p \leq 0.05$ based on the Tukey post hoc test.

The chlorophyll a:b ratio in zucchini leaves in response to diclofenac treatment is mentioned in Table 4.4. Significant differences were observed among the variants ($p < 0.05$), with the highest ratio detected in the B variant (1.98 ± 0.10), while the other variants exhibited statistically similar ratios.

Table 4.4: Chlorophyll a:b ratio for diclofenac treatment; $F_{(3, 8)} = 81.55$, $p = 2.44E-06$ for the plant's variants. Lowercase letters indicate the significant difference at $p \leq 0.05$ based on the Tukey post hoc test.

Plants Variants	Chlorophyll a:b ratio (± SD, n = 3)
C	1.17 ± 0.11 [b]
D	1.17 ± 0.05 [b]
D + B	1.06 ± 0.05 [b]
B	1.98 ± 0.10 [a]

4.1.3. Phenolic compound contents

Paracetamol

The average phenolic compound contents in zucchini leaves in response to paracetamol exposure are presented in Figure 4.5. Significant differences occurred in total phenols (chlorogenic acid), phenylpropanoids (caffeic acid), and flavonoids (quercetin) ($p < 0.05$); however, no significant differences were found in anthocyanins (cyanidin). The highest total phenols content was detected in the P (1131.32 ± 5.65 mg/100g f.w.), P + B (1117.04 ± 19.17 mg/100g f.w.), and B (1114.93 ± 12.77 mg/100g f.w.). The lowest total phenols were identified in the control (1042.11 ± 7.94 mg/100g f.w.). The highest and statistically similar values for phenylpropanoid and flavonoid contents were found in the P and B variants, and the lowest contents were detected in the control and P + B.

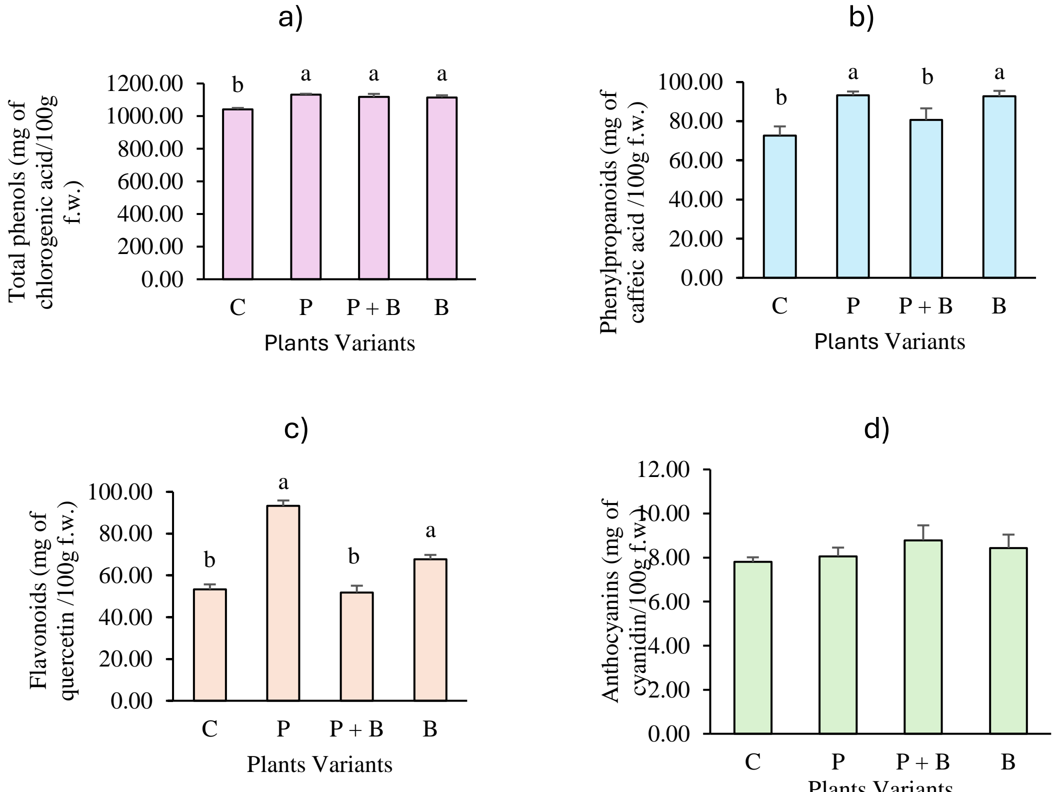

Figure 4.5: Average phenolic compounds (± SD) in zucchini leaves for paracetamol treatment: (a) total phenols F (3, 8) = 30.94, p = 9.45E-05; (b) phenylpropanoids $F_{(3, 8)} = 17.49$, $p = 0.0007$; (c) flavonoids $F_{(3, 8)} = 23.9$, $p = 0.0002$ and (d) anthocyanins $F_{(3, 8)} = 2.163$, $p = 0.1704$ for plants variants. Error bars indicate standard deviation (n = 3). Lowercase letters indicate the significant difference at $p \leq 0.05$ based on the Tukey post hoc test.

Diclofenac

Figure 4.6 displays the average phenolic compound contents in zucchini leaves in response to diclofenac treatment. Significant differences were observed in the total phenols, phenylpropanoids, and flavonoids ($p < 0.05$) among the variants. There was no significant difference in anthocyanins. The B variant contained the highest total phenol content (1114.93 ± 19.17 mg/100g f.w.), while other variants were statistically similar. The highest phenylpropanoid and flavonoid contents were also detected in the B variant, with values being: 92.73 ± 2.79 mg/100g f.w. and 67.69 ± 2.12 mg/100g f.w., respectively. The lowest and statistically similar values were observed for phenylpropanoid and flavonoid contents in the D and D + B variants. However, significantly lowest phenylpropanoid and flavonoid contents were found in D variant compared to control.

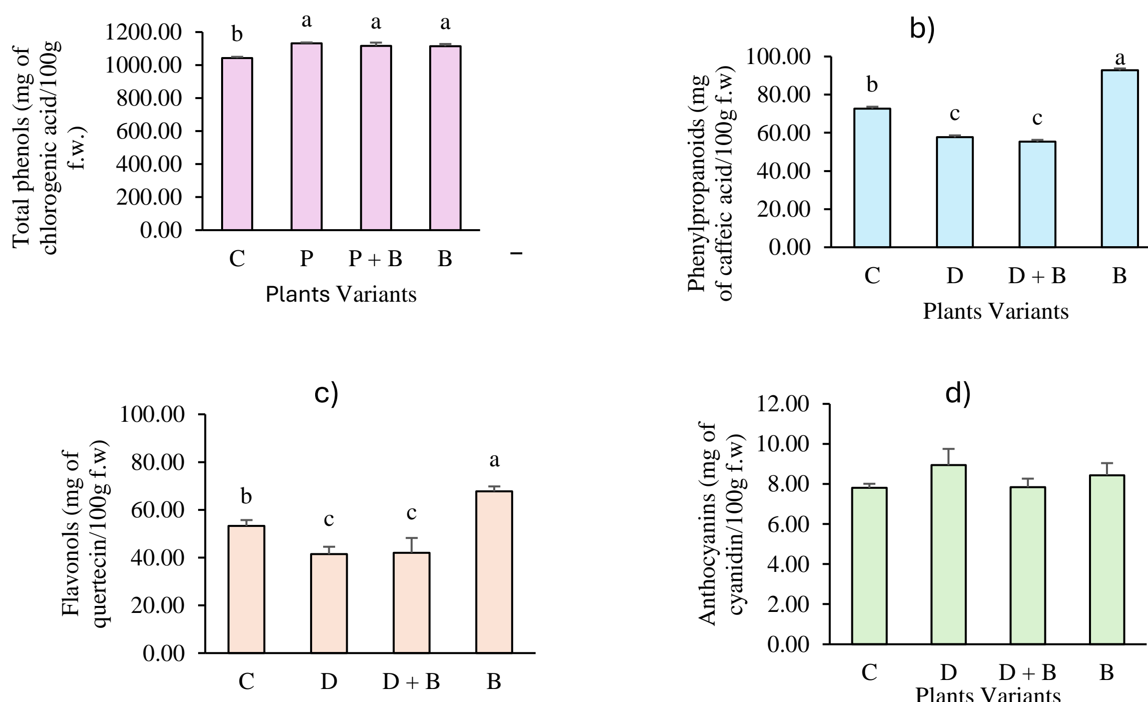

Figure 4.6: Average phenolic compounds (± SD) in zucchini leaves for diclofenac treatment: (a) total phenols, $F_{(3, 8)} = 31.94$, $p = 8.40\text{E-}05$; (b) phenylpropanoids, $F_{(3, 8)} = 39.21$, $p = 3.94\text{E-}05$; (c) flavonoids, $F_{(3, 8)} = 31.37$, $p = 8.97\text{E-}05$ and (d) anthocyanins, F (3$_{(3, 8)} = 2.78$, $p = 0.1095$ for plants variants. Error bars indicate standard deviation (n = 3). Lowercase letters indicate the significant difference at $p \leq 0.05$ based on the Tukey post hoc test.

4.2. Plant endophytes biodiversity

4.2.1. Community-level physiological profiling (CLPP)

Paracetamol

The functional diversity of the leaf endophyte microbial community under paracetamol treatment was evaluated using average well-color development (AWCD). The results are presented in Figures 4.7 to 4.10. Figure 4.7 illustrates the changes in AWCD over a 240-hour incubation period. Control demonstrated the highest AWCD, peaking at 168 hours. The P variant exhibited the lowest AWCD, while the P + B showed a considerable increase compared to the P variant. The AWCD for the P + B variant was the highest after the control, with a gradual rise observed after 192 hours of incubation. The overall optimum carbon substrate utilization occurred at 216 h for all variants. Thus, it was chosen for future investigation.

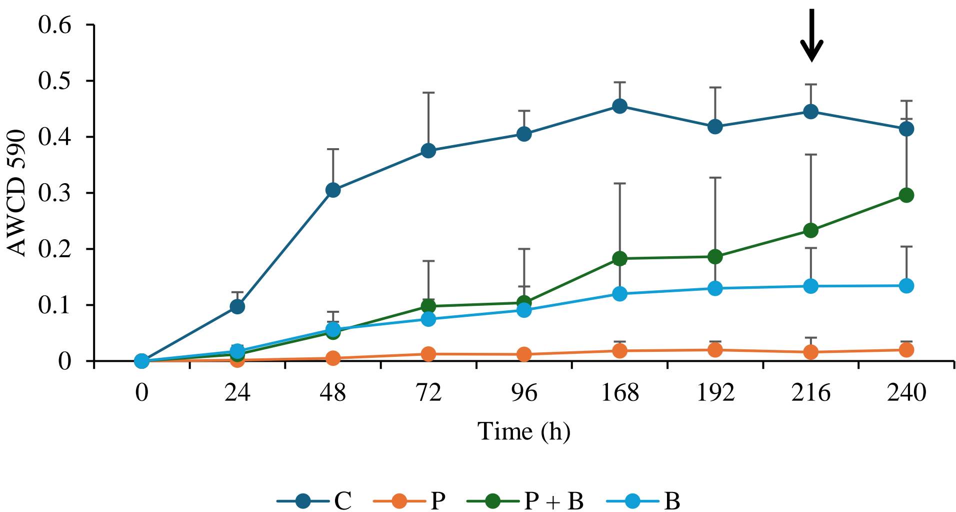

Figure 4.7: Dynamics of the AWCD index for paracetamol treatment over 240 h incubation period. The black arrow indicates at 216 h incubation.

Figure 4.8 represents the total AWCD of all 31 carbon substrates after a 216-h incubation period. Values were significantly different among the variants ($p < 0.05$). The highest total AWCD was observed in the control variant (0.45 ± 0.06), and it was statistically equivalent to P + B variant (0.23 ± 0.16). P had the lowest total AWCD (0.02 ± 0.01), and it was statistically identical to the B variant. Moreover, the P + B showed a significant increase in total AWCD compared to P variant.

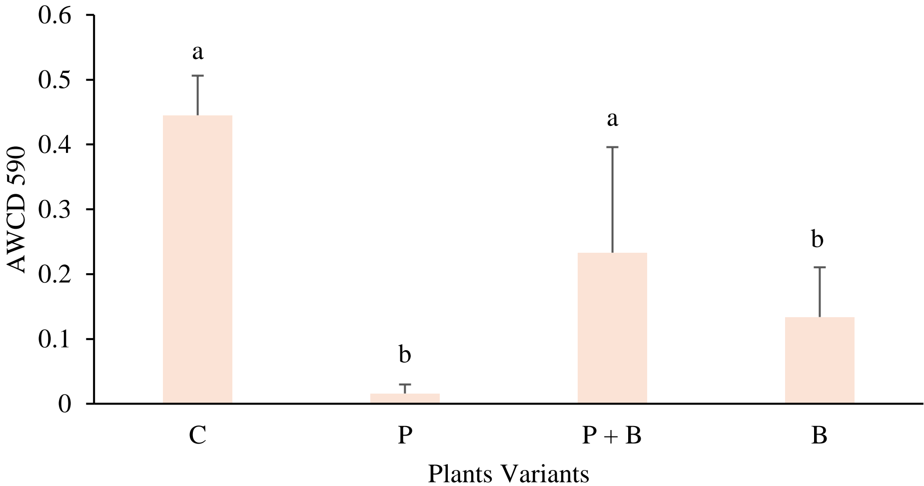

Figure 4.8: Total AWCD for paracetamol treatment after 216 h in the EcoPlate™ assay. Lowercase letters indicate significant differences at $p \leq 0.05$ according to Dunn's post hoc test.

Figure 4.9 displays the microbial consumption of the six carbon groups during the paracetamol treatment after the 216-h incubation. The highest carbon utilization rates were detected for

carbohydrates (AWCD; 0.04-1.39, Fig.4.9e) and amino acids (AWCD; 0.02-1.29, Fig.4.9b), whereas the lowest rate occurred for amines (AWCD; 0.01-0.24, Fig.4.9a). The carbon uptake was significantly different only for carbohydrates ($p < 0.05$). The control exhibited the highest carbohydrate utilization (AWCD; 1.39 ± 0.45, Fig.4.9e), but the C and P + B variants showed statistically similar results. Conversely, P demonstrated the lowest carbohydrate usage (AWCD; 0.04 ± 0.07, Fig.4.9e) and was statistically identical to the B variant. Despite most carbon sources not showing statistically significant differences, the control consistently displayed the highest utilization rates across all carbon groups, whereas the P showed the lowest. Additionally, there was a tendency for increased carbon utilization in the P + B compared to the P variant.

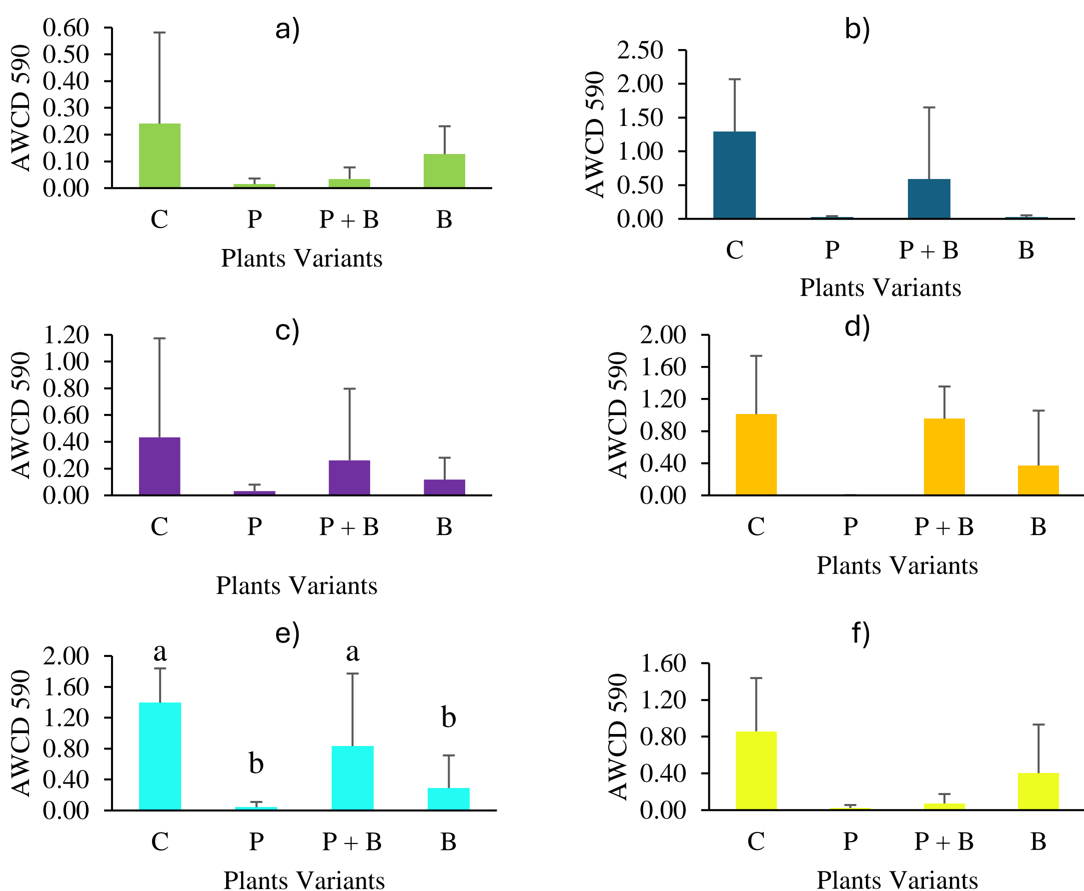

Figure 4.9: The AWCD for the six groups for paracetamol treatment after 216 h in the EcoPlate™ assay: a) amines, b) amino acids, c) carboxylic acids, d) complex carbon sources, e) carbohydrates and f) phosphate carbon. Lowercase letters indicate the significant difference at $p \leq 0.05$ by Dunn's post hoc test.

Figure 4.10 presents the heatmap of 31 carbon substrates following 216 h of incubation for paracetamol treatment. In group A, only the control showed minimal usage of putrescine (AWCD = 0.48). In the AA group, the control and the P + B variants efficiently consumed L-asparagine, with AWCD values of 2.25 and 2.45, respectively. Besides, the control also showed moderate use of L-asparagine, L-serine, and glycyl-L-glutamic acid, with AWCD values of 1.88, 1.18, and 1.22, respectively. The P + B demonstrated minimal use of L-asparagine (AWCD = 0.75). The control in the CA group displayed the highest substrate consumption for gamma-hydroxybutyric acid (AWCD = 2.32), while the P + B variant showed moderate use of D-malic acid (AWCD = 1.63). The lowest substrate consumption was observed in the control for D-glucosamine acid (AWCD = 0.93) and D-galactonic acid gamma-lactone (AWCD = 0.45) and in the P + B variant for pyruvic acid methyl ester (AWCD = 0.94). In the CCS group, substrates were consumed moderately and minimally in both the C and P+B variants. In the CH group, D-cellobiose and alpha-D-lactose showed considerably higher utilization in both the C and P+B variants, while other substrates exhibited moderate to minimal. In the PC group, the control had moderate consumption of D,1-alpha-glycerol phosphate (AWCD = 1.27). Overall, microbial metabolism among the substrates increased in this order: P < B < P + B < C. The P variant exhibited the lowest substrate usage without consuming 18 substrates, indicating minimal metabolic activity. Interestingly, the P+B variant showed enhanced microbial metabolism compared to the P variant.

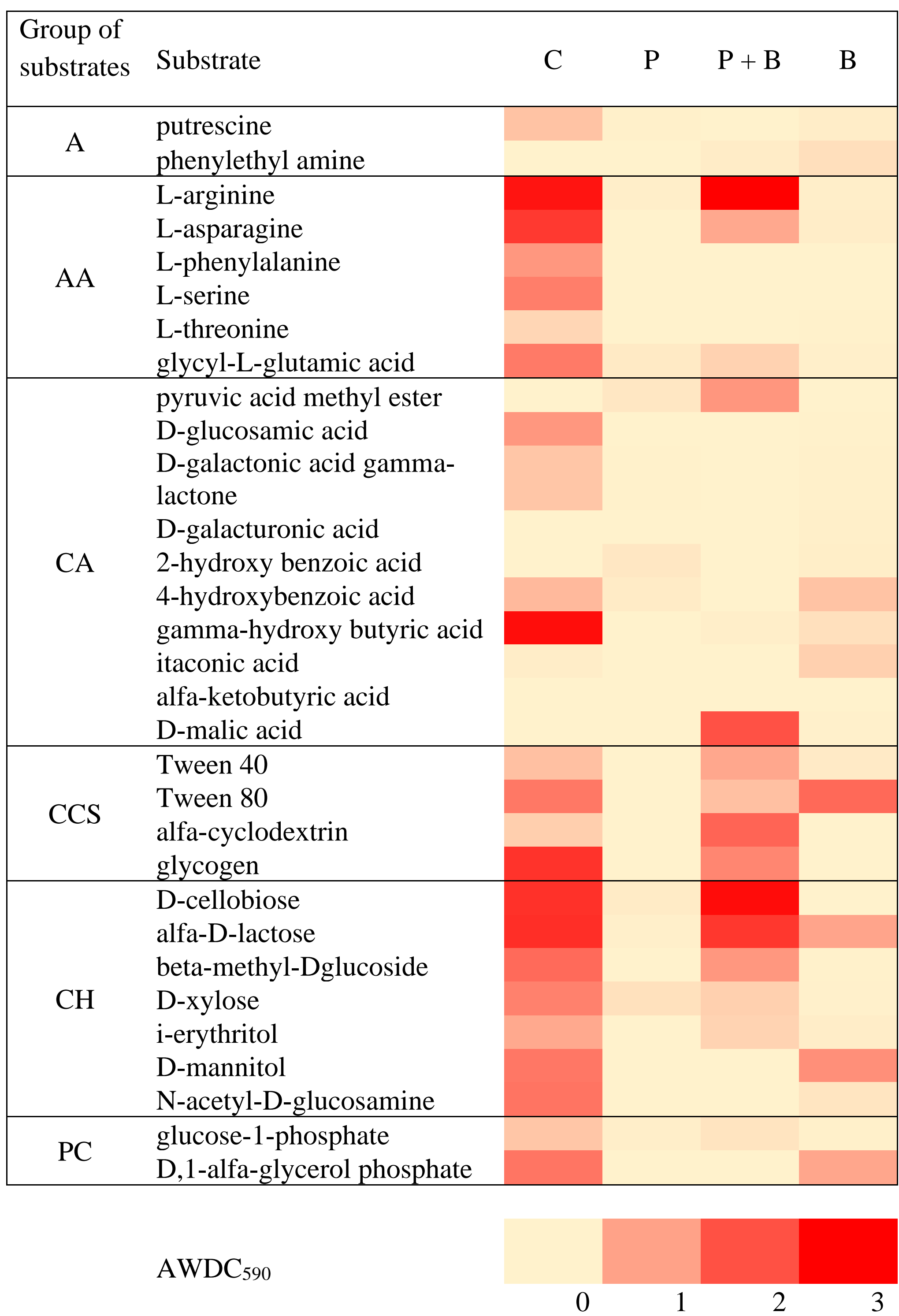

Figure 4.10: The heatmap for 31 carbon substrates following paracetamol treatment after 216 h incubation. Substrate groups: A - Amines, AA - Amino Acids, CA - Carboxylic Acids, CCS - Complex Carbon Sources, CH - Carbohydrates, and PC - Phosphate Carbon.

Diclofenac

Figures 4.11- 4.14 demonstrate the functional diversity of the leaf microbial community under diclofenac treatment. Figure 4.11 depicts the changes in AWCD across a 240-hour incubation period, with the control having the highest AWCD, which peaked at 168 hours. However, the D variant revealed a substantially lower AWCD than the others. The D + B had a considerably higher AWCD than the D and remained lower than the C and B variants. The optimal carbon substrate consumption occurred at 216 hours for all variants, and subsequence calculations were done based on this time.

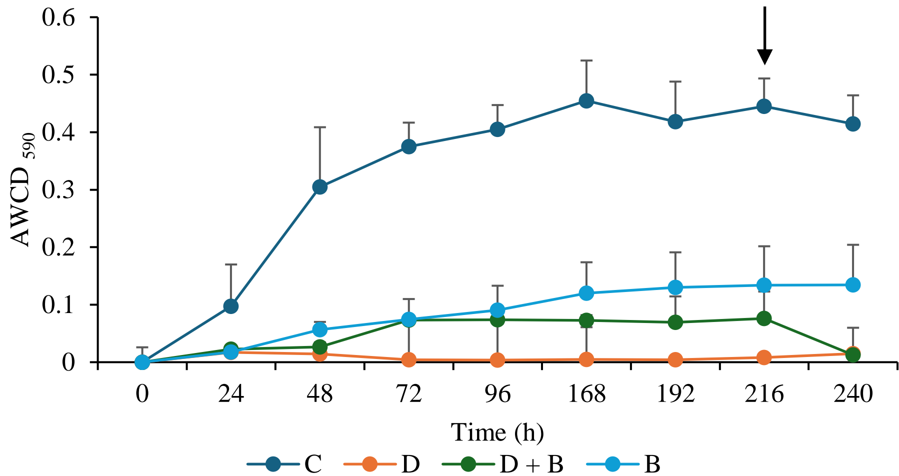

Figure 4.11: Dynamics of the AWCD index for diclofenac treatment over 240 h incubation period. The black arrow indicates at 216 h incubation.

Figure 4.12 represents the changes in total AWCD for diclofenac treatment after 216 h incubation period. Values were significantly different among the variants ($p < 0.05$). The highest total AWCD was exhibited in the control (0.45 ± 0.06), but the C, D + B, and B variants were statistically similar. The lowest AWCD was obtained in D variant (0.008 ± 0.003), with much greater AWCD in D + B.

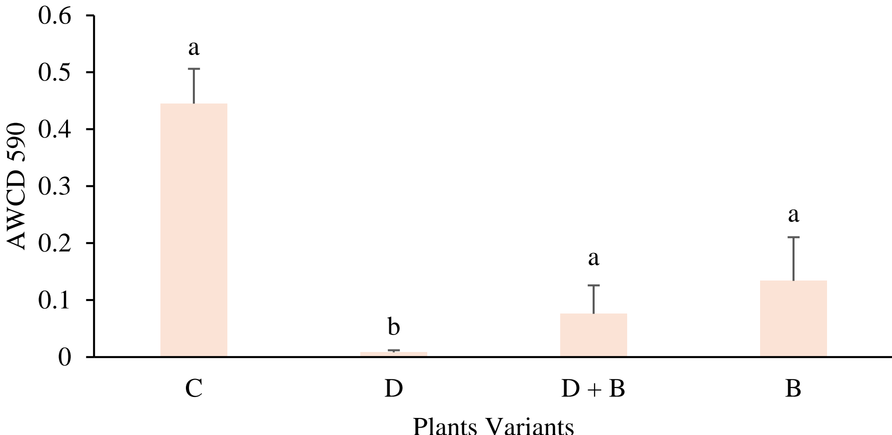

Figure 4.12: The total AWCD for diclofenac treatment after 216 h in the EcoPlate™ assay. Lowercase letters indicate the significant difference at $p \leq 0.05$ by Dunn's post hoc test.

Figure 4.13 illustrates the microbial activity of six carbon groups for diclofenac treatment after a 216 h incubation period. The highest utilization rates were observed for carbohydrates (AWCD; 0.01 –1.39, Fig.4.13e) and amino acids (AWCD; 0.01 –1.29, Fig.4.13b), while the lowest rate was for amines (AWCD; 0.01 –0.24, Fig.4.13a). The carbon uptake was significantly different for carboxylic acids and carbohydrates ($p < 0.05$) among the variants. The control demonstrated the highest carboxylic acids consumption (AWCD; 0.43 ± 0.74, Fig.4.13c); however, the C and B variants were statistically similar. The lowest carboxylic acids utilization occurred in the D variant (AWCD; 0.01 ± 0.02) and was statistically equivalent to the B. The highest carbohydrate use was detected in the control (AWCD; 1.39 ± 0.45, Fig.4.13e) and was statistically similar with D + B and B, while the D variant had the lowest values (AWCD; 0.01 ± 0.01). Although most carbon utilization was not significantly different, the control exhibited the highest carbon consumption, while the D variant had the lowest across all carbon groups. Additionally, there was a trend of increased carbon utilization in D + B compared to the D variant.

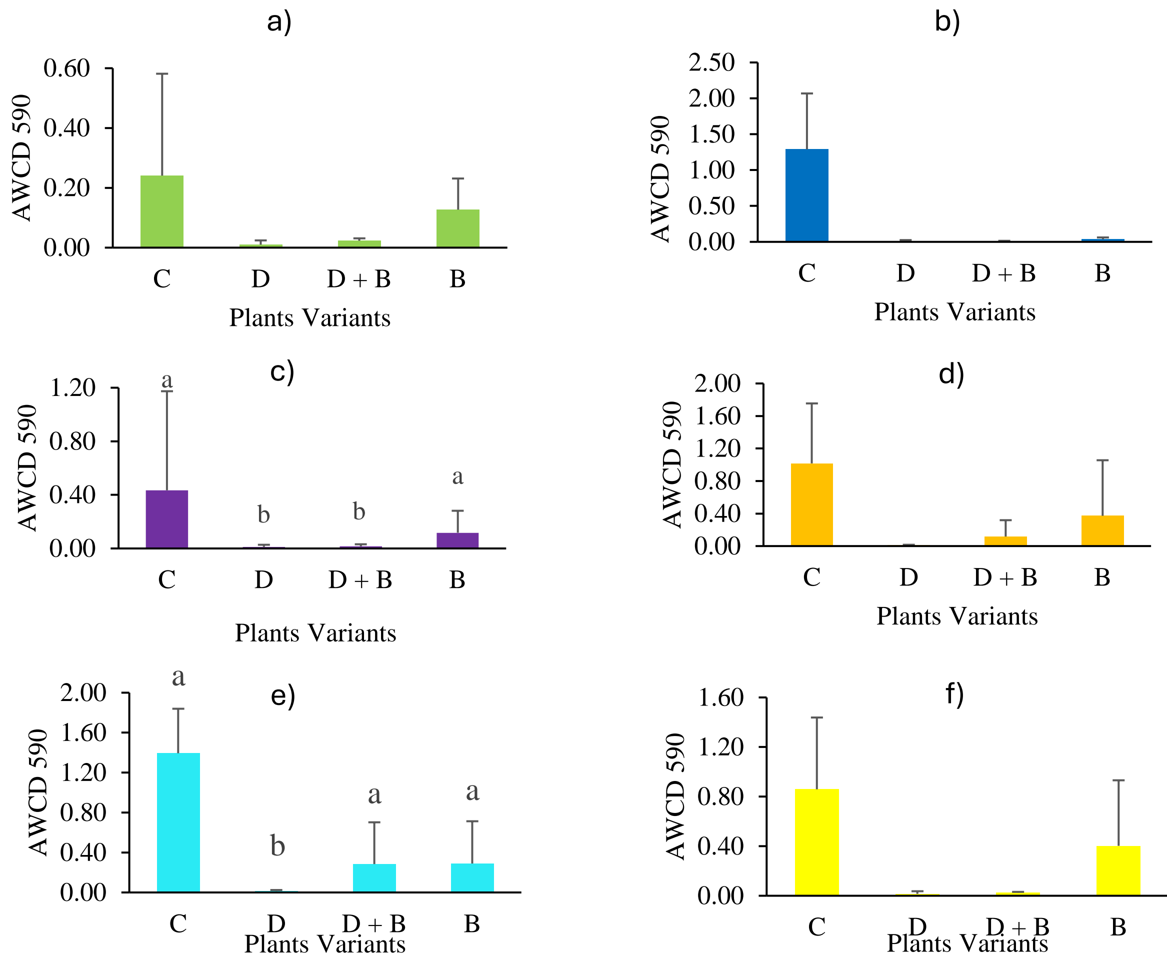

Figure 4.13: The AWCD for the six groups for diclofenac treatment after 216 h in the EcoPlate™ assay: a) amines, b) amino acids, c) carboxylic acids, d) complex carbon sources, e) carbohydrates and f) phosphate carbon. Lowercase letters indicate the significant difference at $p \leq 0.05$ by Dunn's post hoc test.

Figure 4.14 depicts the heatmap for 31 carbon substrates after 216 h incubation for all the variants in the diclofenac treatment. Considering all the substrates, the control demonstrated the highest carbon utilization, with variations and values similar to those mentioned in the paracetamol treatment under Fig.4.10. The D variant had the lowest substrate consumption for all carbon substrates, with AWCD ranging from 0.00 to 0.04. There were no microbes involved for eleven carbon substrates. There was a slight improvement in microbial metabolism in the D + B compared to the D variant, specifically for glycogen (AWCD = 0.42) in the CCS group and for I-erythritol (AWCD = 0.22), D-mannitol (AWCD = 0.61), and N-acetyl-D-glucosamine (AWCD = 1.09) in the CH group. Overall, microbial metabolic activity increased in the following order: D < D + B < C.

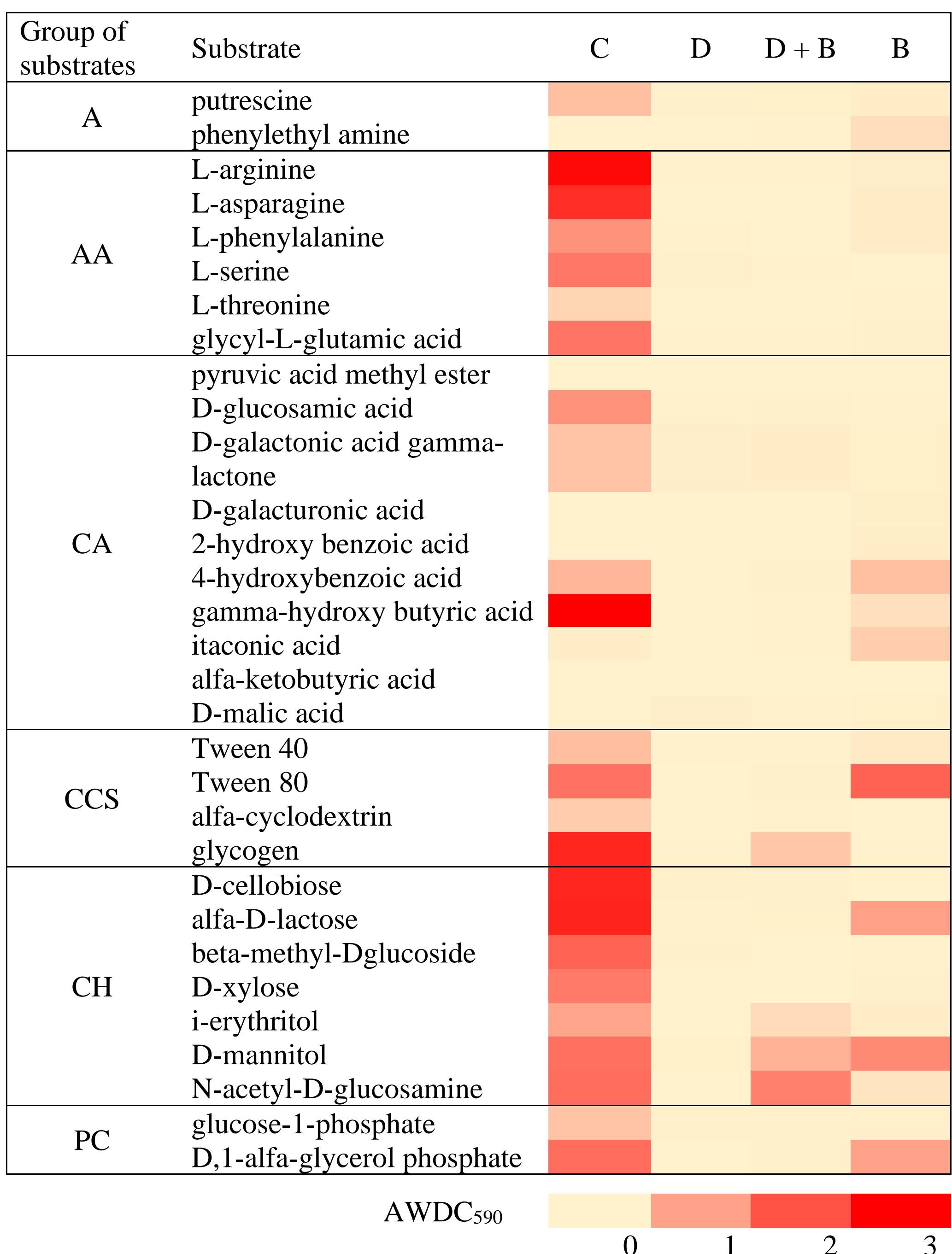

Figure 4.14: The heatmap for 31 carbon substrates following diclofenac treatment after 216 h incubation. substrate groups: A- amines, AA- amino acids, CA- carboxylic acids, CCS- complex carbon sources, CH- carbohydrates and PC- phosphate carbon.

4.2.2. Microbial catabolic diversity

Paracetamol

Table 4.5 presents the microbial catabolic diversity indices derived from EcoPlate™ data for the paracetamol treatment. The Shannon-Weaver diversity (H′) index showed significant differences

among the variants ($p < 0.05$), ranging from 0.17 to 2.30. The control group had the highest H′ index (2.30 ± 0.08), while the lowest was in the P variant (0.17 ± 0.12). The P + B variation had a substantially greater H′ index (1.24 ± 0.90) as compared to the P variant.

The Shannon Evenness index (E) also varied significantly among the variants ($p < 0.05$), ranging from 0.08 to 0.92. The control group exhibited the highest E values (0.92 ± 0.06), whereas the P variant had the lowest (0.08 ± 0.06). Interestingly, the E index also significantly increased in the P + B variant (0.64 ± 0.12) compared to the P.

In contrast, the Substrate Richness index (S) was statistically similar across all variants, with no value reported for the P variant.

Table 4.5: Microbial catabolic diversity indices for the paracetamol treatment after 216 h incubation: Shannon-Weaver diversity index (H′), Shannon evenness index (E) and substrate richness index (S). Lowercase letters indicate the significant difference at $p \leq 0.05$ by Dunn's post hoc test.

Plants variants	H' (± SD, n = 3)	E (± SD, n = 3)	S (± SD, n = 3)
C	2.30 ± 0.08 ^{a}	0.92 ± 0.06 ^{a}	10.67 ± 1.15
P	0.17 ± 0.12 ^{c}	0.08 ± 0.06 ^{c}	0.00 ± 0.00
P + B	1.24 ± 0.90 ^{ab}	0.64 ± 0.12 ^{ab}	6.33 ± 4.62
B	0.83 ± 0.31 ^{bc}	0.32 ± 0.13 ^{bc}	3.67 ± 1.53

Diclofenac

Table 4.6 presents the microbial catabolic diversity indices based on the EcoPlate™ data for the diclofenac treatment. The H′ index showed significant differences among the variants ($p < 0.05$), ranging from 2.30 to 0.12. The control group exhibited the highest H′ index (2.30 ± 0.08), which was statistically identical to the D + B and B variants, while the D variant had the lowest H′ index (0.12 ± 0.04). Additionally, the H′ index significantly increased in the D + B variant (0.50 ± 0.21) compared to the D.

The E index also significantly varied among the variants ($p < 0.05$), with values ranging from 0.05 to 0.92. The control had the highest E value (0.92 ± 0.06), whereas the D variant had the lowest E

value (0.05 ± 0.01). The E value significantly increased in the D + B variant (0.16 ± 0.07) compared to the D.

The S index was significantly different among the variants ($p < 0.05$). The control variant had the highest value (10.67 ± 1.15) and was statistically equivalent to the B variant. There was no value for the D variant. However, the S index significantly increased in the D + B variant compared to the D.

Table 4.6: Microbial catabolic diversity indices for the diclofenac treatment after 216 h incubation: Shannon-Weaver diversity index (H′), Shannon evenness index (E) and substrate richness index (S). Lowercase letters indicate the significant difference at $p \leq 0.05$ by Dunn's post hoc test.

Plants variants	H' (± SD, n = 3)	E (± SD, n = 3)	S (± SD, n = 3)
C	2.30 ± 0.08 [a]	0.92 ± 0.06 [a]	10.67 ± 1.15 [a]
D	0.12 ± 0.04 [b]	0.05 ± 0.01 [b]	0.00 ± 0.00
D + B	0.50 ± 0.21 [a]	0.16 ± 0.07 [a]	1.33 ± 1.15 [b]
B	0.83 ± 0.31 [a]	0.32 ± 0.13 [a]	3.67 ± 1.53 [a]

4.3. Endophytic microbes from zucchini leaves

4.3.1. Colony forming unit (CFU) for leaf endophyte extraction

The Colony Forming Unit (CFU) for the 10^{-1} dilution of leaf extraction for the variants are represented in Table 4.7. The highest CFU was observed in the P + B variant, with a value of 25.5 CFU*10^2/mL, while the lowest CFU was detected in the D variant, with a value of 1.5 CFU*10^2/mL. Additionally, the CFU increased in the D + B compared to the D variant. No colony formation was observed for the P variant.

Table 4.7: CFU for leaf extraction (10^{-1}) for variants; control (C), Paracetamol (P), paracetamol with benomyl (P + B), diclofenac (D), diclofenac with benomyl (D + B) and benomyl (B).

Variants	CFU* 10^2/mL
C	9.5
P	ND
P + B	25.5
D	1.5
D + B	3.5
B	12.5

ND: Not detected

4.3.2. Presence of leaf endophytic bacterial DNA confirmed by 16S rRNA gene

Figure 4.15 exhibits the electrophoresis results after performing PCR to confirm the presence of the bacterial 16S rRNA gene in DNA samples extracted from isolated endophytic microbial colonies. The findings confirmed the presence of the 16S rRNA gene (1465 base pairs) in the C, P + B, D, D + B, and B variants. No microbial colonies were observed in the P variant.

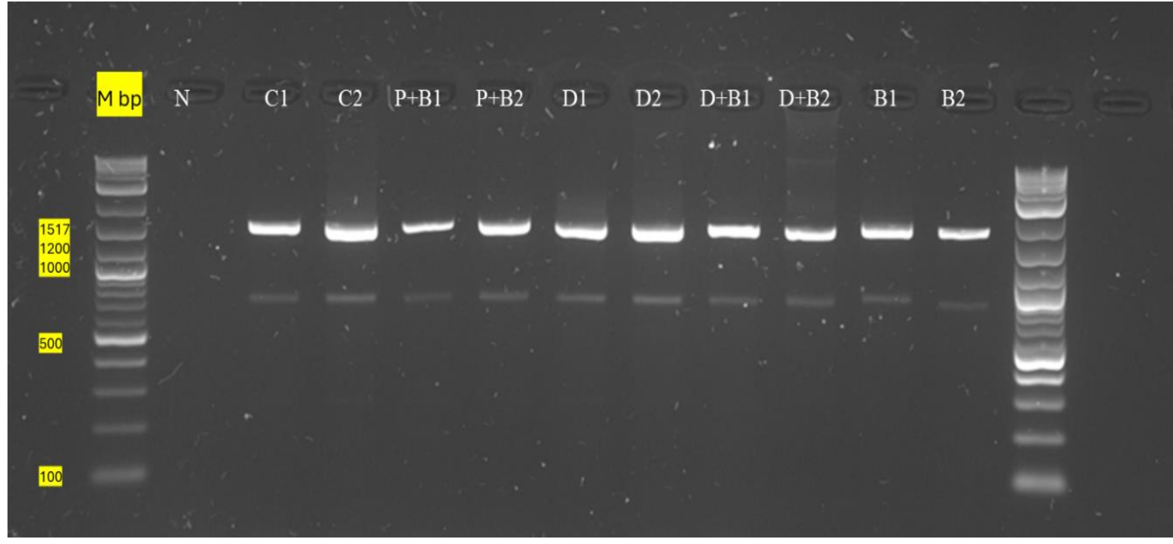

Figure 4.15: Electrophoresis outcomes showing positive results confirming the presence of 16S rRNA gene in the samples at 1465 bp for variants control (C), paracetamol with benomyl (P + B), diclofenac (D), diclofenac with benomyl (D + B) and benomyl (B). N was the negative control.

4.3.3. Identification of leaf endophytic bacteria using DNA sequencing

Figure 4.16 illustrates the distribution of endophytic bacterial phyla across the different variants studied. The analysis revealed three distinct groups: Bacillota, Actinomycetota, and Pseudomonadota. Bacillota was the most dominant, appearing in the C, P + B, and B variants. Actinomycetota was present in the P + B, D, and D + B variants. Pseudomonadota, the least common group, was found exclusively in the C and B variants. Notably, no bacteria were found in the P variant.

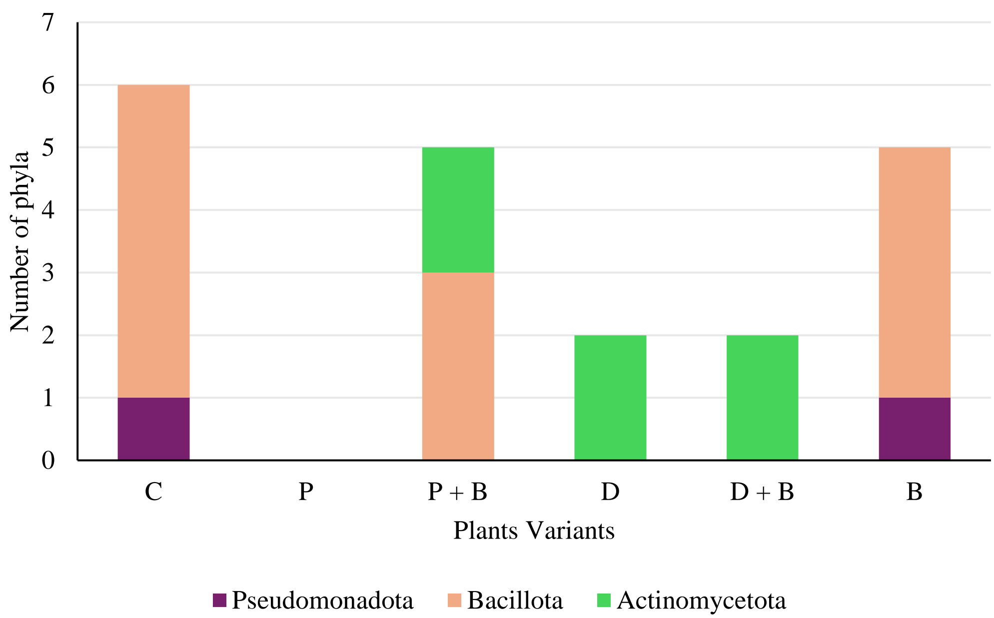

Figure 4.16: Identified bacterial phyla in the endophytic extractions in each variant.

Table 4.8 contains a detailed summary of the identified leaf endophytic bacteria. *Bacillus was* the most prominent bacterial genus in the C, P + B, and B variants. However, *Bacillus* was not detected in the D and D + B variants. The C variant had the most bacterial genera (6 genera), followed by the P + B and B variants (5 genera each). In contrast, only two bacterial genera were identified in the D (*Cellulomonas and Micrococcus*) and D + B (*Rhodococcus and Microbacterium*) variants.

Table 4.8: Identified endophytic bacteria up to genus level, query length and the percent of identity for each variant.

Variants	Phylum	Class	Family	Genus	Query length	Percent identity (%)
C	Pseudomonadota	Gammaproteobacteria	Enterobacteriaceae	*Enterobacter*	803	100
	Bacillota	Bacilli	Bacillaceae	*Bacillus*	817	100
	Bacillota	Bacilli	Bacillaceae	*Bacillus*	801	100
	Bacillota	Bacilli	Bacillaceae	*Gottfriedia*	402	99.5
	Bacillota	Bacilli	Bacillaceae	*Bacillus*	524	100
	Bacillota	Bacilli	Bacillaceae	*Bacillus*	407	90.2
P	ND	ND	ND	*ND*	ND	ND
P + B	Bacillota	Bacilli	Bacillaceae	*Bacillus*	748	100
	Bacillota	Bacilli	Bacillaceae	*Bacillus*	764	100
	Actinomycetota	Actinomycetia	Microbacteriaceae	*Microbacterium*	488	99.6
	Bacillota	Bacilli	Bacillaceae	*Bacillus*	490	97.4
	Actinomycetota	Actinomycetia	Microbacteriaceae	*Microbacterium*	534	99.4
D	Actinomycetota	Actinomycetia	Cellulomonadaceae	*Cellulomonas*	523	99.8
	Actinomycetota	Actinomycetia	Micrococcaceae	*Micrococcus*	558	98.6
D + B	Actinomycetota	Actinomycetia	Nocardiaceae	*Rhodococcus*	678	99.1
	Actinomycetota	Actinomycetia	Microbacteriaceae	*Microbacterium*	581	99.1
B	Bacillota	Bacilli	Bacillaceae	*Bacillus*	753	100
	Bacillota	Bacilli	Bacillaceae	*Bacillus*	813	100
	Bacillota	Bacilli	Bacillaceae	*Neobacillus*	767	100
	Pseudomonadota	Gammaproteobacteria	Erwiniaceae	*Pantoea*	500	99
	Bacillota	Bacilli	Bacillaceae	*Bacillus*	572	99.8

ND: Not detected

5. Discussion

Environmental contamination is a significant global concern. Finding applicable solutions is a considerable challenge. In this context, nature-based solutions are long-term strategies for improving soil, water, and air quality. These solutions align with several UN Sustainable Development Goals, the European Green Deal, and the EU Biodiversity Strategy for 2030 (Fermeglia & Perišić, 2023). Phytoremediation represents a prominent nature-based strategy for ecosystem management, and it is integral as a specific tool in the framework of ecohydrology. This natural process addresses environmental challenges while providing multiple co-benefits (de Beauregard et al., 2002; Zalewski et al., 2021). Therefore, it is crucial to investigate the responses of various plants to environmental pollutants from both micro and macro perspectives. This study examines the influence of the zucchini plant on paracetamol and diclofenac, two common NSAIDs in the environment, and the effect of benomyl on advancing environmental remediation efforts.

5.1. Influence of NSAIDs on plants physiology and biochemistry

Plant biomass is a widely accepted indicator of environmental stressors on plants when assessing potential phytotoxicity. Literature indicates that pharmaceuticals, including NSAIDs, can significantly impact plant growth and biomass in various ways (Siemieniuk et al., 2021). In our experiment, the fresh biomass of the zucchini, including roots, stems, and leaves was decreased in paracetamol (P) (Fig.4.1 and Tab.4.1) and diclofenac (D) (Fig.4.2 and Tab.4.2) variants. A similar decline was observed for paracetamol in the biomass of cucumber at 10mg/L (Sun et al., 2019). Paracetamol did not influence the biomass of *Solanum nigrum* at 0.25 and 0.5 mg/L (Martins & Teixeira, 2021). However, there is a high possibility of declining biomass in some plants with increasing paracetamol concentration of more than 50 mg/L (Badar et al., 2022). Moreover, Siemieniuk et al. (2021) discovered a significant biomass reduction in tomato plants at 2 mg/L diclofenac. These findings indicate that paracetamol and diclofenac can have inhibitory, stimulatory, or neutral effects on plant biomass, depending on the concentration, exposure duration, and plant species. Also, diclofenac can have a more growth-inhibitory effect at lower concentrations than paracetamol. Additionally, these NSAIDs may lead to oxidative stress that harms cellular components in plants, and growth reduction could be an adaptive survival mechanism to tolerate oxidative stress. Besides, NSAIDs may inhibit water and nutrient absorption by shortening root systems, thus limiting plant growth and biomass (Bartha et al., 2010; Cho & Kim, 2021; Zezulka et al., 2019).

Benomyl treatment (B) has decreased plant biomass, with the most substantial reductions observed in the leaves and roots compared to the control (Fig.4.1 and 4.2). Benomyl is a prominent fungicide that prevents plants from fungal infections, and depending on the conditions, its use could impact plant growth and biomass (Kara et al., 2020). Interestingly, we observed an increased biomass in the P+B variant compared to the P variant (Fig.4.1). A similar pattern occurred in the D+B variant compared to the D variant, where the leaf mass was significantly higher in the D+B variant (Fig.4.2). This suggests that benomyl might influence to reduce the phytotoxic effects of NSAIDs on zucchini, though the exact mechanisms remain unclear. In contrast, Fujita et al. (2020a) found that the fresh weight of the aerial parts of zucchini grown in soil contaminated with hydrophobic pollutants did not significantly differ with pesticide treatment.

Chlorophyll pigments are essential for photosynthesis and functional indicators of health and productivity in evaluating the responses of plants to contaminants (Siemieniuk et al., 2021). In the current study, total chlorophyll, chlorophyll a, and the chlorophyll a:b ratio increased under paracetamol treatment (Fig.4.3 and Tab.4.3). Our results agree with the findings of Badar et al. (2022) and Kurade et al. (2021). The studies found enhanced chlorophyll in spinach at 100 mg/L and 200 mg/L paracetamol and lettuce at 500 µM and 5 mM paracetamol. However, increasing chlorophyll levels through paracetamol does not invariably enhance the photosynthetic process. Even with higher chlorophyll levels, certain plants, including spinach, showed a decline in overall photosynthetic efficiency from impaired photosystems I and II and interrupted electron transport by paracetamol. Besides, decreased chlorophyll content was observed in *Pisum sativum*, *Vicia faba*, and *Cicer arietinum* at 0.5 mg/L of paracetamol (Taschina et al., 2022). Nevertheless, some plants have no adverse response to photosynthesis pigments even at a considerably higher amount of paracetamol exposure (Tăşchină et al., 2017). In the case of diclofenac, chlorophyll did not exhibit a significant difference between the D variant and the control (Fig.4.4 and Tab.4.4). Similarly, in maize plants, photosynthetic pigments did not fluctuate in response to 2 mg/L diclofenac (Siemieniuk et al., 2021). However, an extensive reduction was seen in pea and maize treated with 5-10 mg/L diclofenac. An equivalent reduction occurred in spinach and lettuce at 1 mg/L of diclofenac (Opriș et al., 2020). Consequently, raising diclofenac doses can have a detrimental impact on chlorophyll. However, the photosynthetic pigments in response to these NSAIDs vary among plant species and with the drug concentration. At some point, increasing pigments might be an adaptive mechanism to the stress of these pollutants. On the other hand,

NSAIDs can involve decreasing pigments in certain plants, even at lower concentrations, potentially due to enzymatic degradation or inhibition of biosynthesis (Al-Muwayhi, 2018).

We discovered that benomyl boosted the chlorophyll content of zucchini in our experiment (Fig.4.3). Some studies showed increased photosynthesis of benomyl-treated plants, while other showed a net decrease in photosynthetic efficiency at the fungicide's exposure (Gamboa et al., 2005). However, the highest chlorophyll content occurred in the P+B variant (Fig.4.3), and the D+B variant exhibited a significant increase in chlorophyll b (Fig.4.4). These findings suggest that the combination of NSAIDs and benomyl generally enhances photosynthetic pigments. However, the fundamental mechanism regulating this interaction remains unknown.

Phenolic compounds are plants' secondary metabolites, essential for physiological and biochemical functions, including defense against biotic and abiotic stresses. These compounds are potent antioxidants which effectively neutralize oxidative stress, and accumulation may increase in response to environmental stress (Kumar et al., 2020). We observed substantially enhanced total phenols, phenylpropanoids, and flavonoids in P variant (Fig.4.5). Paracetamol exposure disrupted the normal biosynthesis and accumulated phenolic compounds in zucchini. This finding aligns with the study conducted by Kummerová et al. (2016), which demonstrated that *Lemna minor* cultivated at 100 µg/L of paracetamol exhibited a significantly higher polyphenol content. In contrast, 0.5 mg/L of paracetamol did not affect total phenols in *Lens culinaris* and *Vicia faba*, although some other species showed a reduction. In the same study, plants produced lower amounts of flavonoids. In addition, total phenolic compounds might increase during short-term oxidative stress and decrease under long-term stress (Taschina et al., 2022). Besides, the present study indicated no significant differences in total phenols or anthocyanins, and a substantial reduction occurred in phenylpropanoids and flavonoids in the D variant (Fig.4.6). Plants treated with 0.5 mgL^{-1} of diclofenac showed a similar drop in flavonoids (Opriș et al., 2020; Taschina et al., 2022). Furthermore, there is a significant probability of reducing the phenolic compounds in certain plants under NSAID stress. These alterations primarily depend on the concentration of NSAIDs and the potential defense mechanisms of plants against these drugs (Taschina et al., 2022).

Total phenols, phenylpropanoid, and flavonoid concentrations were significantly higher in the B variant (Fig.4.5 and 4.6). The reason could be that fungicides impact plant oxidative stress at certain levels (García et al., 2003). However, the patterns of variation significantly differed when

combining benomyl with NSAIDs. Phenylpropanoid and flavonoid concentrations were considerably lower in the P+B variant compared to paracetamol exposure and statistically equal to the control (Fig.4.5). The D+B variant showed lower phenylpropanoid and flavonoid contents compared to the control (Fig.4.6). These results suggest a potential interaction between NSAIDs and benomyl that might reduce oxidative stress. However, there was no actual scientific evidence about these variations.

Our findings indicate that paracetamol and diclofenac can impact the physiology and biochemistry of zucchini plants, particularly affecting the plant's biomass, chlorophyll content, and phenolic compounds. However, the adverse effects decreased when the plants were under NSAIDs and benomyl treatment. Although there is no direct research on this specific interaction, a potential explanation could involve the relationship between MLPs, organic pollutants, and competitive compounds like fungicides in zucchini, as briefly mentioned in the introduction (see section 1.6). Benomyl, as an aromatic compound (PubChem, 2024), may compete with NSAIDs for binding to MLPs, and this competitive binding might mitigate the phytotoxic effects of NSAIDs by regulating the NSAID accumulation in the above-ground part of the plants (Chitose et al., 2024; Fujita et al., 2020b). However, further studies are required to completely understand fungicide-NSAID-contaminated plant interactions for phytoremediation effectiveness and crop security.

5.2. Influence of NSAIDs on plants endophytic microbial community

Endophyte-assisted phytoremediation has enormous potential for environmental decontamination since many endophytes have pollutant-degrading capabilities. However, there is limited research focusing on endophytic microbes in plant-contaminated pharmaceuticals. The findings contrast with more established topics, such as the general study on endophytes, rhizosphere microorganisms, and the effects of pollutants on microbial communities in soil, water, or pure culture. Most available studies focus on how contaminants affect plant physiology and biochemistry (Kovacs et al., 2022; Nguyen et al., 2019). Our current study might be the initial effort to investigate the impact of NSAIDs (paracetamol and diclofenac) on leaf endophytes in *Cucurbitaceae*. Consequently, direct comparison with findings from other studies is challenging due to the uniqueness of this research focus.

5.2.1. Influence of NSAIDs on functional microbial diversity

The Biolog EcoPlate™ is a widely used indirect method for assessing microbial functional diversity and activity by examining the utilization of 31 different carbon substrates (Dresler et al., 2021). AWCD values represent microbial metabolic activity, where higher AWCD indicates increased metabolic activity (Kovacs et al., 2022). According to AWCD in our study, the higher microbial metabolic activity was in the control group. In comparison, the P and D variants exhibited significantly lower activities, even at the extended carbon substrate utilization period of 216 hours (Fig. 4.7, 4.8, 4.11, and 4.12). The results have proved that the applied doses of paracetamol (25 mg/L) and diclofenac (2.5 mg/L) may have the highest potential to influence leaf endophytic microbial metabolism in zucchini. Supporting this, Cycoń et al. (2016) discovered that specific NSAID dosages can affect microbial metabolism, reducing or eliminating catabolic reactions and damaging cellular integrity. Besides, microbial metabolic activity was lower in the benomyl-treated samples compared to the control (Fig. 4.7 and 4.11). The reason could be fungicide application probably lowered the endophyte microbial community. However, when comparing the B variant to the P and D variants, microbial metabolic activity was higher in the B variant. Thus, NSAIDs might have a higher influence on the endophyte community than benomyl alone. Nevertheless, metabolic activity has improved in P + B and D + B variants related to individual NSAID exposure.

Kovacs et al. (2022) assessed the impact of NSAIDs on the rhizosphere microbiota of *Lycopersicon esculentum,* and the findings indicated a reduction in microbial abundance and a decline in carbon source utilization. However, NSAIDs could be more toxic to rhizosphere microbes than endophytes because of the direct exposure to such substances in soil. In our experiment, we observed a significant reduction in the consumption of carbon sources for P and D variants compared to the control (Fig. 4.9, 4.10, 4.13 and 4.14). Iliev et al. (2022) reviewed that most microbial communities prefer carbohydrates and carboxylic acids due to their ease of digestion. We observed a decline in carbohydrate utilization from endophyte isolations exposed to parcetamol and a similar decline in carboxylic acids and carbohydrates in the D variant (Fig.4.9 and 4.13).

The heat map provides a detailed representation of carbon consumption patterns. In our experiment, the control group displayed a broader range of carbon usage. Conversely, paracetamol and diclofenac showed significantly lower utilization (Fig. 4.10 and 4.14). Kovacs et al. (2022) detected that rhizosphere microbes consumed more carbon during diclofenac exposure but failed to consume

a wider variety of carbon sources. Moreover, carbon consumption from phenolic compounds and amines decreased, whereas the utilization of carbohydrates and carboxylic acids increased towards the end of the assay period. Besides, the P + B endophyte extraction utilized more varieties of carbon sources than the P variant (Fig.4.10). The D + B variant had improved carbon consumption than the D variant but considerably less compared to the control (Fig.4.14). Furthermore, P + B and D + B endophyte communities utilized much more carbohydrates than individual NSAID exposure (Fig.4.10 and 4.14). These findings indicate that exposing endophytes to NSAIDs combined with fungicides considerably increases their functional microbial diversity. All carbon source consumption indices peaked in the control, and the P and D variants had the lowest values (Tab. 4.5 and 4.6). In Kovacs et al. (2022), the control and diclofenac-exposed rhizosphere samples had the highest substrate richness relative to other NSAIDs. Shannon diversity and evenness showed minimal differences between the control and NSAID-contaminated samples.

5.2.2. Influence of NSAIDs on structural microbial diversity

The highest CFU value was observed in the control, while the lowest in the D variant (Tab.4.7). Węgrzyn & Felis (2018) reported similar CFU values for both the control and diclofenac with sulfamethoxazole treatment when studying root endophytes from *Phalaris arundinacea*. Paracetamol usually counts as a non-toxic substance, having an average microbiological toxic concentration of 3435 mg/L. Also, some paracetamol metabolites are more hazardous to microorganisms than the parent compound itself (Kumar & Sharma, 2019). There was no CFU value for the P variant in our experiment, suggesting that 25 mg/L of paracetamol might be harmful to zucchini endophytes. This outcome could also be affected by other conditions, such as changes in the natural environment, cultural conditions or other unidentified factors. However, the P + B variant had the highest CFU, while CFU increased in the D + B variant relative to the D variant. Therefore, a combination of NSAIDs and benomyl could promote the endophytic microbial community in zucchini.

The 16S rRNA gene was detected in all the variants in the experiment, except for the paracetamol one (Fig. 4.15). This finding aligns with numerous studies that have also reported the presence of the 16S rRNA gene in microbial isolates from various parts of plants and rhizosphere microbes exposed to NSAIDs, including diclofenac and paracetamol (Badar et al., 2022; Sauvêtre et al.,

2020d; Węgrzyn & Felis, 2018; Zapata-Morales et al., 2020). However, we couldn't find any report of 16S rRNA detection from NSAIDs-influenced fungicides-treated plants' endophytes.

Phylum Actinobacteria, found in drugs-treated *Miscanthus* × *giganteus* endophytes, can degrade several organic contaminants, including diclofenac (Sauvêtre et al., 2020a). In our study, we found Actinobacteria as the only phylum present in the endophyte extraction in the D variant (Fig. 4.16). Pseudomonaceae, Burkholderiaceae, Bacillaceae, and Enterobacteriaceae are the most abundant endophytic bacterial families in polluted environments (Feng et al., 2017). The Bacillaceae family was predominant and found in the control, P + B, and B variants, while Enterobacteriaceae was detected only in the control (Tab. 4.8). *Bacillus* is well-known as the most prevalent bacterial endophyte (Santoyo et al., 2016), and *Bacillus* was a frequently abundant genus in our study. Overall, we identified that benomyl exposure led to an increase in endophytic bacterial community. Fungicide treatment can enhance the growth of the endophytic bacterial community by reducing competition between bacteria and fungi for carbon and energy sources. However, the reason for increasing microbial structural diversity in combined exposure to NSAIDs and benomyl was still unknown.

6. Conclusion

This study demonstrated that exposure to 25 mg/L of paracetamol and 2.5 mg/L of diclofenac can influence the physiology and biochemistry of zucchini plants, leading to reduced biomass and disrupted biosynthesis of certain phenolic compounds. Additionally, paracetamol increased chlorophyll content, while diclofenac had minimal impact on chlorophyll pigments. Both NSAIDs substantially decreased the functional and structural microbial diversity of leaf endophytes in zucchini, and benomyl, when applied alone, also displayed some impacts on plant physiology and leaf endophytes. However, benomyl minimized several negative consequences of NSAIDs treated plants by enhancing biomass, chlorophyll contents, and oxidative stress resilience while promoting endophytic microbial diversity. This research is the first study to explore the impact of paracetamol and diclofenac on zucchini and the potential for benomyl to improve phytoremediation in NSAID-contaminated cucurbits. Further research is essential to assess the influence of NSAIDs on plants and better understand the interactions between fungicides and NSAID-contaminated plants, focusing on enhancing phytoremediation effectiveness and ensuring crop security.

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