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Original Research Paper

Evaluation of hydrolytic enzymes and antifungal activity of extracellular bioactive compounds of *Desmonostoc alborizicum* and *Neowestiellopsis persica* against plant pathogenic fungi

Bahareh Nowruzi [1,*], Fahimeh Nemati [2]

Abstract

Agriculture requires the extensive use of chemical pesticides to protect crops against pests and diseases. An important mechanism for the biological control of pathogenic fungi is the breakdown of their cell walls. Cyanobacteria are found commonly growing as blooms which provides a competitive advantage to these organisms. This is one of the critical factors responsible for the production of several hydrolytic enzymes with antifungal activity. However, the role of the hydrolytic enzymes of *Neowestiellopsis* and *Desmonostoc*, which are implicated in the fungicidal activity of several biocontrol strains, has not been explored. Therefore in this study, hydrolytic enzymes (chitosanase, protease, FPase, carboxymethyl cellulose, xylanase, cellobiohydrolases and cellobiase) of two cyanobacteria strains were evaluated against a set of phytopathogenic fungi (*Alternaria alternata, Fusarium solani, Fusarium oxysporum, Macrophomina phaseolina, Verticillium dahliae* and *Phytophthora*). The results of statistical analysis showed that the level of protease, FPase and xylanase activity in *Desmonostoc alborizicum* cyanobacterial extract has been significantly higher than in *Neowestiellopsis*. Moreover, IAA hormone activity and soluble protein content were significantly higher in *Desmonostoc alborizicum* cyanobacterial extract. While CMCase, cellobiohydrolases, cellobiase, and chitosanase activity was significantly higher in *Neowestiellopsis persica* A1387 cyanobacterial extract in comparison to *Desmonostoc alborizicum*. Moreover, *Neowestiellopsis*

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persica was observed to be highly potent in terms of its fungicidal activity. Comparative evaluation of the activity of hydrolytic enzymes and antifungal activity revealed that such enzymes might contribute to the fungicidal activity of the cyanobacterial strains, besides other bioactive compounds, including IAA, which are established promising traits for biocontrol agents. This study is a first-time report on the production of hydrolytic enzymes by these two cyanobacteria strains, which can be potential candidates for the development of biocontrol agent(s) against selected phytopathogenic fungi.

Keywords

hydrolytic enzyme, cyanobacteria, plant pathogenic fungi, *Neowestiellopsis persica*, *Desmonostoc alborizicum*

Analiza aktivnosti hidrolitičnih encimov in protiglivne aktivnosti zunajceličnih bioaktivnih snovi cianobakterij *Desmonostoc alborizicum* in *Neowestiellopsis persica* proti fitopatogenim glivam

Izvleček

Kmetijstvo zahteva obsežno uporabo kemičnih pesticidov za zaščito pridelkov pred škodljivci in boleznimi. Pomemben mehanizem biološkega nadzora patogenih gliv je razgradnja njihovih celičnih sten. Cianobakterije pogosto rastejo večjih sestojih na vodni površini, kar je eden od kritičnih dejavnikov, odgovornih za proizvodnjo več hidrolitičnih encimov s protiglivičnim delovanjem. Vendar pa vloga hidrolitskih encimov vrst *Neowestiellopsis* in *Desmonostoc*, ki sta vpletena v fungicidno aktivnost več biokontrolnih sevov, ni bila raziskana. Zato smo v tej študiji ovrednotili delovanje hidrolitskih encimov (hitozanazo, proteazo, FPazo, karboksimetil celulazo, ksilanazo, celobiohidrolaze in celobiazo) dveh sevov cianobakterij proti nizu fitopatogenih gliv (*Alternaria alternata, Fusarium solani, Fusarium oxysporum, Macrophomina phaseolina, Verticillium dahliae* in *Phytophthora*). Rezultati statistične analize so pokazali, da je bila stopnja aktivnosti proteaze, FPaze in ksilanaze v ekstraktu cianobakterije *Desmonostoc alborizicum* bistveno višja kot pri vrsti *Neowestiellopsis persica* A1387. Poleg tega sta bila aktivnost hormona IAA in vsebnost topnih beljakovin znatno višja v ekstraktu cianobakterije *D. alborizicum.* Nasprotno je bila aktivnost CMCase, celobiohidrolaz, celobiaze in hitozanaze bistveno višja v ekstraktu cianobakterije *N. persica* v primerjavi z *D. alborizicum.* Poleg tega je bilo ugotovljeno, da ima vrsta *N. persica* zelo močno fungicidno delovanje. Primerjava aktivnosti hidrolitskih encimov in protiglivične aktivnosti je pokazala, da lahko takšni encimi, poleg drugih bioaktivnih spojin, vključno z IAA, prispevajo k fungicidni aktivnosti cianobakterijskih sevov in imajo obetavne lastnosti za biokontrolo. Ta študija je prvo poročilo o proizvodnji hidrolitskih encimov pri teh dveh sevih cianobakterij, ki sta lahko potencialna kandidata za razvoj biokontrolnih sredstev proti izbranim fitopatogenim glivam.

Ključne besede

hidrolitični encimi, cianobakterije, fitopatogene glive, *Neowestiellopsis persica*, *Desmonostoc alborizicum*

Introduction

Cyanobacteria represent a small taxonomic group of prokaryotes, which are equipped with the power to harm and help plants, animals and humankind and possess tremendous potential for producing a wide range of secondary metabolites (Verma et al., 2022). A preponderance of cyanobacteria was responsible for the inherent and sustained fertility of rice fields, which led to the cyanobacterial populations in soil being evaluated in terms of their diversity and utility as biofertilizers, not only for rice but for other crops, including wheat (Prasanna et al., 2008).

Cyanobacteria are also found commonly growing as blooms in eutrophic lakes, reservoirs and as floating assemblages in marine ecosystems. Such blooms have been notorious and associated with the production of toxins/allelopathic compounds, which provide a competitive advantage to these organisms (Prasanna et al., 2010). This is one of the critical factors responsible for their abundance in diverse environments, especially eutrophic water bodies. However, the chemical potential of these ubiquitous prokaryotes, widely distributed in diverse soil types, aquatic environments and ecologies, in terms of the production of metabolites has not been much investigated. Agriculture requires the extensive use of chemical pesticides to protect crops against pests and diseases. Several of these chemicals pollute our groundwater and drinking water, and therefore some governments have decided to reduce these chemical inputs substantially (Schweitzer and Noblet, 2018). This urges the need for alternative crop protectants. One of these alternatives is the use of biological control agents, among which are microorganisms that can protect plants against diseases (Bonaterra et al., 2022). The colonization and defensive retention of the rhizosphere niche by microorganisms are enabled by the production of allelochemicals, antibiotics, biocidal volatiles and lytic/detoxification enzymes.

Genus *Neowestiellopsis* was originally described by Kabirnataj et al. (Kabirnataj et al., 2018) from Mazandaran (Iran) and belongs to the order Nostocales and family Hapalosiphonaceae. Strains of this genus can be found in both paddy fields and agricultural zones and due to their ability to fix nitrogen, some strains have an important role in agriculture. After that, Nowruzi et al., 2022, found evidence of the poisoning of humans from feeding/due to the ingestion of crop of *Crataegus* plant contaminated with cyanobacterial toxins of *Neowestiellopsis* ca. *persica*, which is most abundant in the agricultural zones of Kermanshah province of Iran using a polyphasic approach (Nowruzi et al., 2022). They recorded the presence of a gene cluster coding for the biosynthesis of a bioactive compound (Nostopeptolides) that is very rare in this family and present toxic compounds (*microcystin*) which might account for the poisoning of humans. Moreover, our previous study revealed that *Desmonostoc alborizicum* strain 1387 is a potentially toxic species isolated from a water supply system in Iran since the *mcy*D and *mcy*G genes of the microcystin synthetase (*mcy*) cluster were successfully sequenced (Nowruzi and Becerra-Absalón, 2022). Using mass spectrometry, detectable amounts of the hepatotoxin microcystin-LR were present in cell extracts of the *Desmonostoc* strain (Nowruzi and Becerra-Absalón, 2022).

Casamatta and Wickstrom (Casamatta and Wickstrom, 2000) reported that the exudates of *Microcystis aeruginosa* were inhibitory towards bacterial plankton communities. A number of nucleosides – tubercidin, toyocamycin and their corresponding derivatives isolated from Scytonemataceae members were observed to be toxic towards *Aspergillus oryzae, Candida albicans, Penicillium notatum* and *Saccharomyces cerevisiae* (Prasanna et al., 2010).

A diverse range of compounds is also known to exhibit a bioregulatory role as a result of their cytotoxic, immunosuppressive and enzyme-inhibiting activities, which are of tremendous pharmaceutical significance (Indumathi, 2016). However, the role of the hydrolytic enzymes of *Neowestiellopsis* and *Desmonostoc*, which are implicated in the fungicidal activity of several biocontrol strains, has not been explored. It is worth noting that the above-mentioned features will specify the furthermore convincing evidence to introduce two toxic species of cyanobacteria as biocontrol agent(s) in agriculture.

Materials and Methods

Cyanobacteria culture and extraction

Desmonostoc alborizicum and *Neowestiellopsis persica* strains were taken from the Cyanobacteria Culture Collection (CCC) affiliated with the Science and Research Branch of the Islamic Azad University, Tehran, Iran. The isolates were maintained in a 250 mL cotton-stoppered Erlenmeyer flask containing liquid Z8 medium at 28±2 °C with periodic shaking (twice a day), illumination of ca. 50-55 µmoL photons $m^{-2}s^{-1}$, and a regime of 14:10 h light: dark cycle (Oudra et al., 2002).

Preparation of cyanobacterial extracellular products

Extracellular products from 1000 ml of two cyanobacteria cultures were prepared (Kwon and Kim, 2008). Biomass was separated by centrifugation (CRU-5000 Centrifuge, Damon/IEC Division, Needham, Massachusetts, USA) for 20 min at 6000 rpm and 4°C. The culture filtrates were concentrated ten times using a rotary evaporator (Heidolph, Kehlheim, Germany) and then sterilized by filtration through 0.2 µm Seitz filters. They were kept refrigerated at four °C until use.

Estimation of fungicidal activity of cyanobacterial extracellular products

Various concentrations of 1, 5, 10, 15 and 20 mg/ml of cyanobacterial extracellular products of studied cyanobacteria strain were tested for their antifungal activity using the paper disk diffusion method against the growth of *Alternaria alternata, Fusarium solani, Fusarium oxysporum, Macrophomina phaseolina, Verticillium dahliae* and *Phytophthora*.

A loop of each slant was mixed in 5 mL of saboraud dextrose agar (SDA) and incubated at 28 °C until 0.5 McFarland standards 2.3 × 10^3 (AL-Abedi et al.). 100 mL of each concentration was dissolved in 1 mL of 5% dimethyl sulfoxide (DMSO). Sterilized paper discs (6 mm) were prepared. The previously saturated discs (6 mm) were placed on the sides of the SDA plates. SDA plates were incubated at 28 °C for five days. The inhibition zones (mm) around discs were measured by the transparent ruler.

Total proteins

The amount of proteins was determined spectrophotometrically according to (Chatterjee and Mukherjee, 2014), with Bovine serum albumin (BSA) as the standard.

IAA activity

IAA activity was measured by the method described by (Lohani et al., 2004). The intensity of the pink colour was measured at 530 nm, and values were compared with those of the standard curve of IAA. The activity of all the hydrolytic enzymes is expressed as a standard International Unit (IU). The culture filtrates of the cultures of the cyanobacterial strains were also scanned in the UV-VIS range using a Specord model spectrophotometer, and the peaks were recorded for the identification of novel compounds.

Measurement of hydrolytic enzymes

Chitosanase activity

The cell-free culture filtrates were analyzed for chitosanase activity (EC 3.2.1.4) by the spectrophotometric method using glycol chitosan as the assay substrate. One unit of chitosanase activity was defined as µsmoles of N-Acetyl glucosamine released per min under assay conditions (Chang et al., 2007).

Protease activity

Protease activity was assayed using casein as a substrate and measured by the liberation of tyrosine from the incubation mixture (Thangam and Rajkumar, 2002). The intensity of the blue colour was measured at 660 nm using a spectrophotometer against the standard curve of Tyrosine and expressed as an International unit (IU), which was calculated as µmoles of tyrosine liberated min–1.

Filter paperase/FPase (exo-β-1, 4-glucanase; EC 3.2.1.91) and CMCase activity (EC 3.2.1.4)

The enzyme activities of both enzymes were assayed spectrophotometrically using filter paper and carboxymethyl cellulose as substrates, respectively (Ezeilo et al., 2022). Reducing sugars liberated were estimated at 575 nm against the standard curve of Glucose. One unit of enzyme represents l µmoles of glucose liberated per ml of culture filtrate per min.

Xylanase activity

The xylanase activity was measured spectrophotometrically at 575 nm using xylan as substrate (Lim et al., 2013) against the standard curve of xylose. One unit of xylanase activity represents I μmole of xylose liberated per ml of culture filtrate per min.

Cellobiase (β-D-glucosidase; EC 3.2.1.21) and Cellobiohydrolases activity

The cellobiase and cellobiohydrolases activity was determined spectrophotometrically at 430 nm by the method outlined by (Wood and Bhat, 1988) against the standard curve of p-nitrophenol.

Statistical analysis

Analyses were performed in at least three independent experiments. Analysis of variance (ANOVA) was applied to verify whether means from independent experiments within each given variant were significant at level $p < 0.05$. Data shown in the figures are means and standard errors (s.e.), using asterisks to present the significant differences.

Results

Antifungal activity of two cyanobacteria strains

Preliminary analyses of the culture filtrates of two cyanobacterial strains against the selected phytopathogenic fungi (as measured by disc diffusion assay) revealed that both strains produced a zone of inhibition of varying diameter, ranging from 20-35 mm at *Desmonostoc alborizicum* and ranging from 32-61 mm at *Neowestiellopsis persica* A1387.

Neowestiellopsis persica was observed to be highly potent in terms of its fungicidal activity, as it produced a much larger zone of inhibition than *Desmonostoc alborizicum*.

The largest and smallest diameter of the zone of inhibition, 61.66 ± 0.6 and 32.07 ± 0.8 mm, were recorded by *Neowestiellopsis persica* against *Macrophomina phaseolina* and *Phytophthora capsici*, respectively. In contrast, the largest and smallest diameter of the zone of inhibition, 35.71 ± 0.7 and 20.01 ± 0.3 mm, were recorded by *Desmonostoc alborizicum* against *Fusarium solani* and *Macrophomina phaseolina*, respectively.

IAA (indole acetic acid) hormone and soluble protein content

According to Table 2, there was a significant difference in the amount of IAA hormone and the content of soluble protein in the extracts of cyanobacteria *Neowestiellopsis persica* A1387 and *Desmonostoc alborizicum* ($p<0.05$). IAA production and soluble protein were also observed in both cyanobacterial strains, which was higher in *Desmonostoc alborizicum* in comparison to *Neowestiellopsis persica* A1387.

Results of hydrolytic enzymes

According to Table 3, there was a significant difference ($p<0.05$) in the two cyanobacterial extracts of *Neowest-*

Table 1. Fungicidal activity of the purified extract of *Neowestiellopsis persica* A1387 and *Desmonostoc alborizicum* in terms of zone of inhibition. Values indicate the diameter of inhibition zone in mm. The results are Means ± SE.

Tabela 1. Fungicidna aktivnost prečiščenih ekstraktov cianobakterija *Neowestiellopsis persica* A1387, *Desmonostoc alborizicum* in cone inhibicij. Vrednosti predstavljajo premer cone inhibicije v mm. Rezultati so povprečne vrednosti ±SN.

	zones of growth inhibition (mm)	
Fungal strain	***Neowestiellopsis persica***	***Desmonostoc alborizicum***
Alternaria alternata	28.57±7.0^b	43.07±7.0^b
Fusarium solani	35.71±7.0^d	33.57±8.0^a
Fusarium oxysporum	33.16±8.0^c	45.57±8.0^c
Macrophomina phaseolina	20.01±3.0^a	61.66±6.0^d
Verticillium dahliae	29.11±7.0^b	41.91±7.0^b
Phytophthora capsici	31.57±8.0^c	32.07±8.0^a

iellopsis persica A1387 and *Desmonostoc alborizicum*. Protease, FPase, and Xylanase enzyme activity were significantly higher in the cyanobacterial extract of *Desmonostoc alborizicum* ($p<0.05$).

While CMCase (1.55 fold), cellobiohydrolases (1.85 fold), cellobiase (1.63 fold), and chitosanase activity (4.21) was significantly higher in *Neowestiellopsis persica* A1387 cyanobacterial extract ($p<0.05$) in comparison to *Desmonostoc alborizicum*.

Totally, protease and chitosanase activity were significantly higher in *Desmonostoc alborizicum* and *Neowestiellopsis persica* A1387, respectively.

Table 2. Mean results of IAA production (µmol/g) and protein accumulation (mg/g) in *Neowestiellopsis persica* A1387 and *Desmonostoc alborizicum*. The results are Means ± SE. Different letters indicate a significant difference at the 0.05 level.

Tabela 2. Povprečne vrednosti za sintezo IAA (µmol/g) in akumulacijo proteinov (mg/g) pri cianobakterijah *Neowestiellopsis persica* A1387 in *Desmonostoc alborizicum*. Rezultati so povprečne vrednosti ±SN. Različne črke predstavljajo statistično značilno razliko pri p < 0,05.

	Neowestiellopsis persica	***Desmonostoc alborizicum***
IAA (indole acetic acid) hormone (µmol/g)	40.78 ± 1.03^{b}	60.63 ± 0.98^{a}
soluble protein content (mg/g)	0.22 ± 0.00^{b}	0.25 ± 0.00^{a}

Table 3. Screening the activity of selected hydrolytic enzymes of *Neowestiellopsis persica* A1387, *Desmonostoc alborizicum*. The results are Means ± SE. Different letters indicate a significant difference at the 0.05 level.

Tabela 3. Pregled aktivnosti izbranih hidrolitičnih encimov pri cianobakterijah *Neowestiellopsis persica* A1387 in *Desmonostoc alborizicum*. Rezultati so povprečne vrednosti ±SN. Različne črke predstavljajo statistično značilno razliko pri p < 0,05.

	***Neowestiellopsis persica* A1387**	***Desmonostoc alborizicum***
Protease activity (mg/g)	5.60 ± 0.13^{a}	4.27 ± 0.01^{b}
Filter papers activity (µmol/hour mg)	2.59 ± 0.10^{a}	2.49 ± 0.20^{b}
CMCase activity (µmol/hour mg)	0.47 ± 0.10^{b}	0.76 ± 0.10^{a}
Cellobiohydrolases activity (µmol/hour mg)	4.69 ± 0.14^{b}	8.70 ± 0.24^{a}
Cellobiase activity (mg/g)	2.99 ± 0.00^{b}	4.88 ± 0.16^{a}
Xylanase activity (mg/g)	3.70 ± 0.12^{a}	2.85 ± 0.50^{b}
Chitosanase activity (mg/g)	1.79 ± 0.23^{b}	7.54 ± 0.65^{a}

Discussion

Cyanobacteria are known to be an important determinant of allelopathic activity, besides leading to severe monetary losses as a result of soil-borne pathogens. Extensive screening programs for bioactive compounds in cyanobacteria have revealed the presence of unique peptides with cyclic structures (cyanopeptolins, depsipeptides), besides linear peptides isolated from a number of strains belonging to genera *Microcystis*, *Nostoc* and *Anabaena* (Welker and Von Döhren, 2006). The analyses of the culture filtrates are currently in progress, as UV scans revealed the presence of novel compounds. Although toxins were the main subject of research on toxic cyanobacterial blooms, the isolation of bioactive compounds other than biotoxins has now been recently stressed (Anderson et al., 2021).

The production of phytohormones, earlier considered as a trait of the plant kingdom, is also widespread among soil and plant-associated prokaryotes (Sergeeva et al., 2002), especially those involved in plant-microbe symbiotic or associative interactions or plant pathogenesis. Microorganisms inhabiting the rhizosphere of various plants are likely to synthesize and release auxin as a secondary metabolite because of rich supplies of substrates exuded from the roots compared with non-rhizosphere soils (Sarwar and Kremer, 1995) (Sureshbabu et al., 2016).

The result of this study showed that the *Desmonostoc* *alborizicum* strain exhibited 1.43 fold higher IAA production than *Neowestiellopsis persica* A1387. In contrast, *Neowestiellopsis persica* A1387 has more effect on interactions with the fungal pathogens.

It has been suggested up to 80% of bacteria isolated from the rhizosphere can produce IAA. The capacity for IAA biosynthesis was found in the representatives of free-living and symbiotic cyanobacteria of the genera *Nostoc*, *Chlorogloeopsis, Calothrix, Plectonema, Gloeothece, Anabaena, Cylindrospermum* and *Anabaenopsis* (Tsavkelova et al., 2006).

Cyanobacteria produce a variety of enzymes (chitosanase, protease, xylanase, and cellobiase) with antifungal activity and twenty-four families of protease inhibitors involved in several human, animal, and plant metabolic pathways (Righini et al., 2022). The culture filtrates are rich in many substances that can display interesting antifungal activity. In particular, several families of metabolites with high antimicrobial activity were isolated from various strains of cyanobacteria (Demay et al., 2019). Culture filtrates of several cyanobacteria species with fungicidal activity produced one or more hydrolytic enzymes, such as proteases, chitosanases, exo-β-1,4-glucanases, and carboxy-methyl cellulase (Righini et al., 2022). For *Anabaena variabilis* ATCC 29413, the gene putatively responsible for chitosanase and antifungal activities was attributed to the glycoside hydrolase 3-like family (Gupta et al., 2010).

All these enzymes are known to be involved in the digestion of fungal or oomycetes cells. For example, chitosan and chitosan-glucan complexes were found in the mycelia of *Aspergillus niger* and *Fusarium moniliforme* (Razak et al., 2018). The use of organic solvents in the extraction process affects the antifungal activity of cyanobacterial extracts (Abedin and Taha, 2008). For example, in the extract of *Microcystis* aeruginosa obtained with diethyl ether were identified the butylated hydroxytoluene and methyl ester of hexadecanoic acid, which has antifungal activity against *Aspergillus* spp., *Fusarium* spp. and *Penicillium* sp. (Deyab et al., 2019). Another important antifungal activity against *A. flavus* was observed in the methanol extract of *Anabaena* spp., *Nostoc* sp. and *Scytonema* sp. The identification of the macrolide scytophycin and the presence of the glycolipopeptide hassallidin extracted from the *Anabaena* strains elucidated the antifungal activity (Shishido et al., 2015).

Phenols and polysaccharides contained in extracts from *Nostoc* spp. are involved in the antifungal activity against *R. solani* (Ismail and Ismail, 2011). In fact, a phenolic compound was isolated and purified from the chloroform extract of *Nostoc muscorum* with strong activity against *Aspergillus niger, A. flavus, Penicillium* sp., and *Fusarium microsporium* (Righini et al., 2022). Aqueous extracts have not been so widely investigated as the extracts obtained with organic solvents, even though they are safer for both humans and the environment. A recent study showed that soluble polysaccharides extracted from *Anabaena minutissima* aqueous extract reduced both colony growth and colony-forming units of *B. cinerea* (Righini et al., 2022). In the same extract, proteins, phycobiliproteins, chlorophylls, carotenoids, and antioxidant activities were also determined and correlated with the antifungal effect against the pathogenic fungus *Podosphaera xanthii* on cucumber detached cotyledons in vitro assay (Pérez-García et al., 2009).

On analyzing the overall performance of the strains in terms of biocontrol traits, strain *Neowestiellopsis persica* A1387 was observed to be the top-ranked strain in terms of diameter of zone of inhibition, CMCase activity, cellobiase, cellobiohydrolase and chitosanase activity, while was top-ranked in terms of cellobiohydrolase activity (8.70±0.2), besides exhibiting high levels of activity of other hydrolytic enzymes and inhibited potentially more the growth of fungi tested. While strain *Desmonostoc alborizicum* has more activity in protease activity, FPase (exo-β-1, 4-glucanase, xylanase activity, moreover the amount of total proteins and IAA activity was higher than *Neowestiellopsis persica* A1387.

Majidi et al., 2011, found maximum cellulase activity for *S. variabilis, K. rosea* and *S. maltophilia* was obtained after 72 h of fermentation with 0.091, 0.089 and 0.084 U mL^{-1} for CMCase and 0.079, 0.074 and 0.072 U mL^{-1} for FPase respectively (Samira et al., 2011). These results are in agreement with those of (Narasimha et al., 2006, and Niranjane et al., 2007), who found that carboxymethyl cellulose was the best carbon source, followed by cellulose for cellulase production. Higher production of cellulase when CMC serves as the substrate may be a result of the induction of the enzyme since cellulose is known to be a universal inducer of cellulase synthesis. The growth profile of the bacterial isolates during fermentation shows that the cellulase was being produced during the growth phase of the *S. variabilis, K. rosea* and *S. maltophilia* (Barzkar and Sohail, 2020).

The excretion of hydrolytic enzymes is known to be a common trait of plant pathogens/symbionts, which promotes a closer association with plant roots/target organisms and improves the stability of such associations. Chitosanases are known to selectively degrade chitosan/chitin by hydrolysis of the β-1, 4-glycosidic bonds that link N-acetyl glucosamine residues of chitin and form the basis for antifungal activity. The chitosanase/chitinase produced by *S. plymuthica* C48 inhibited spore germination and germ-tube elongation in *Botrytis cinerea*. The ability to produce extracellular chitosanases/chitinase is considered crucial for *Serratia marcescens* to act as an antagonist against *Sclerotium rolfsii* and for *Paenibacillus* sp. Strain 300 and *Streptomyces* sp. strain 385 to suppress *Fusarium oxysporum* f.sp. *cucumerinum* (Chaudhary et al., 2013). Although the role of cyanotoxins/peptides/phenolic molecules cannot be ruled out in terms of biocidal activity, it becomes evident from this study that hydrolytic enzymes are definitely contributing to the inhibition of phytopathogenic fungi.

Conclusions

The present investigation, for the first time, illustrates the activity of hydrolytic enzymes in two native cyanobacteria strains and their possible role in biocidal activity against phytopathogenic fungi. The potential of these strains in developing biocontrol agents is immense – as these strains possess abilities for the production of IAA and hydrolytic enzymes. Such multifaceted strains would possess a competitive edge over other rhizosphere microflora against phytopathogenic fungi and need to be explored for developing biocontrol agents.

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ABS

Original Research Article

Economically important species of Asteraceae: An overview of leaf epidermal micro-morphology with respect to water availability

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Abstract

This paper reports the result of a study undertaken with a view to documenting responses of leaf epidermal parameters of three economically important species of the family Asteraceae: *Aspilia africana, Chromolaena odorata* and *Vernonia amygdalina* from Nigeria to seasonal water stress. Obtained quantitative data showed a significant decrease ($p < 0.05$) in stomata density and index on the abaxial surface in *Chromolaena odorata* in dry condition. The increased stomata densities and indices on the adaxial surfaces in *Aspilia africana* and *Vernonia amygdalina* in the wet season suggest that CO_2 was more taken up. The reduction in stomata size (on both leaf surfaces in the three taxa) in the dry season indicated species' adaptations to dry conditions and reduction in stomatal conductance. Turgescent guard cell in the three species in the wet season indicated stomatal opening, which could, though, be a hormonal response to water availability since transpiration needed to be activated. The ability of *Aspilia africana* to reduce water loss and defend against herbivores greatly increased in plants that developed in dry conditions by possessing more multicellular trichomes in comparison to wet conditions, while in *Vernonia amygdalina*, the number increased during the wet season. Water stress had little or no effect on the micro-morphology of anticlinal walls and the shapes and arrangements of the epidermal cells in the three species. This study revealed that investigations into leaf epidermal micro-morphology might be a useful tool to elucidate the multiple mechanisms underlying leaf epidermal structure function in response to water availability.

Keywords

Aspilia africana, *Chromolaena odorata*, Seasonal variations, Stomata, Stomatal conductance, *Vernonia amygdalina*, Water stress

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Ekonomsko pomembne vrste iz družine Asteraceae: Pregled mikro-morfologije listne povrhnjice v odvisnosti od razpoložljivosti vode

Izvleček

Članek poroča o rezultatih študije, ki je bila izvedena z namenom dokumentiranja lastnosti povrhnjice listov treh gospodarsko pomembnih vrst iz družine Asteraceae: *Aspilia africana*, *Chromolaena odorata* in *Vernonia amygdalina* iz Nigerije kot odziv na sezonsko pomanjkanje vode. Pridobljeni kvantitativni podatki so pokazali signifikantno zmanjšanje ($p < 0,05$) gostote listnih rež in indeksa na abaksialni površini pri vrsti *Chromolaena odorata* v sušnih razmerah. Povečane gostote listnih rež in indeksi na adaksialnih površinah pri Aspilia africana in *Vernonia amygdalina* v mokri sezoni kažejo na povečan privzem CO_2. Zmanjšanje velikosti listnih rež (na obeh listnih površinah v treh taksonih) v sušnem obdobju je pokazalo prilagoditve izbranih rastlinskih vrst na sušne razmere in zmanjšanje prevodnosti listnih rež. Turgescentnost celic zapiralk pri vseh treh vrstah v mokri sezoni je pokazala odpiranje listnih rež, kar pa bi lahko bil hormonski odziv na razpoložljivost vode, saj je bilo treba aktivirati transpiracijo. Sposobnost *Aspilia africana* za zmanjšanje izgube vode in obrambe pred rastlinojedci se je močno povečala pri rastlinah, ki so se razvile v suhih razmerah, saj imajo več večceličnih trihomov v primerjavi z rastlinami v vlažnih pogojih, medtem ko se je pri *Vernonia amygdalina* njihovo število povečalo v mokri sezoni. Vodni stres je imel malo ali nič vpliva na mikromorfologijo antiklinalnih sten ter oblike in razporeditve epidermalnih celic pri treh vrstah. Ta študija je pokazala, da so preiskave mikromorfologije povrhnjice listov lahko koristno orodje za pojasnitev več mehanizmov, na katerih temelji funkcija strukture povrhnjice listov kot odziv na razpoložljivost vode.

Ključne besede

Aspilia africana, *Chromolaena odorata*, sezonske spremembe, listen reže, prevodnost listnih rež, *Vernonia amygdalina*, vodni stres

Introduction

Plants growing in the tropical savannah experience a wide range of environmental conditions during the year, especially those related to wet and dry seasons. Different research (Tatrai et al. 2016, Toscano et al. 2016) have been carried out on the effects of seasonal variations or water stress on morphology, anatomy, physiology and biochemistry of different taxa. For example, Thomas et al. (2000) studied the effect of seasons and drought on plant species and suggested that stomata of leaves which have expanded in the dry season were more sensitive to leaf-to-air pressure difference than those which had expanded in the wet season. Thomas et al. (2000), however, concluded that the differences in stomatal density were not significantly different in leaves developed in the two seasons. Furthermore, research conducted by Miller et al. (2010) has also shown that under drought or during dry seasons, tissue dehydration may reach critical levels and interfere with cellular homeostasis and thus changing plant metabolism and growth. Recently, many scholars have provided large quantities of reports on the ability of plants to combat dry conditions. For instance, Nihal et al. (2009) and Zhang et al. (2015) focused on the anatomical structures of plant leaves, while (Soltys-Kalina et al. 2016) focused on the osmotic adjustment of plant tissues. Meanwhile, according to the research conducted by Deikman et al. (2012), the multiple mechanisms by which plants respond to seasonal variations and water stress are a challenge to enhancing plants' drought tolerance but Carswell et al. (2002), as well as Da Rocha et al. (2004), found out that even though precipitation is reduced during the dry season, potential evapotranspiration may be higher in the wet season. According to Malhi et al. (2002), this is due to the increased irradiance that rainforests receive due to reduced cloudiness. Interestingly, an entirely different result was obtained by Carswell et al. (2002), who found no difference in carbon dioxide exchange between seasons in an eastern Amazonian rainforest. Malhi et al. (2002) pointed out that soil water content is lower during the dry season and, in turn, actual evapotranspiration is higher in the wet season. Their findings were in agreement with previous findings of Nepstad et al. (1994) as well as Da Rocha et al. (2004), who claimed that, in the dry season, the shallow soil layer may dry out. However, the deeper layer remains hydrated year-round.

Despite several extensive researches on seasonal variations as well as water stress and consequent effects on plant species, there is a lack of studies performed on the family Asteraceae in Nigeria.

Asteraceae (Aster family) is one of the largest angiosperm plant families among the dicotyledonous. Funk et al. (2005) opined that based on a large number of species (1,620 genera and 23,600 species) representing this family with a cosmopolitan distribution, it constitutes almost 10% of all flowering plants worldwide and divided into 12 subfamilies (Funk et al. 2009). Also, according to Jansen and Palmer (1987), Asteraceae is one of the largest and most economically important families of flowering plants. The family is characterized by a combination of several specialized morphological characteristics (e.g., capitula, highly reduced and modified flowers, inferior ovaries, syngenesious anthers). The aggregation of flowers occurs on a usually flat surface called a receptacle. It is also referred to as the banner of the family (Tadese, 2014).

Species of Asteraceae are of wide economic importance as vegetables (lettuce, artichokes, and endive), sources of oil (sunflower, safflower), insecticides (pyrethrum), and garden ornamentals (chrysanthemum, dahlia, marigold, and many others) (Jansen and Palmer 1987).

Three economically important member species of this plant family, which are present and distributed in Nigeria, were considered in this comparative study, including *Aspilia africana* (Pers.) C.D. Adams, *Chromolaena odorata* (L.) R. M. King and H. Rob; and *Vernonia amygdalina* (Delile) Walp.

Aspilia africana (Pers.) C.D. Adams is a widespread plant in Africa (Hutchinson 1962). It occurs throughout the regions of the savannah and tropical Africa on wastelands (Burkill 1985). The morphological features of *A. africana* have been fully described (Dalziel 1928, Hutchinson 1962). According to Dalziel (1928), *A. africana* is a semi-woody herb, about 1m tall, covered with bristles, and commonly known as a "haemorrhage plant" due to its ability to stop bleeding from wounds (Bep 1960). The taxa have been reported in the literature to possess antimicrobial (Macfoy and Cline 1990), haemostatic (Achonye 1976), anti-inflammatory (Okoli et al. 2007) and anti-fertility (Eweka 2008) activities. Okoli et al. (2007) reported that *A. africana* is one of several medicinal plants used in folklore for wound treatment. In addition, it is also used for the treatment of rheumatic pains (Bep 1960) as well as bee and scorpion stings (Singha 1965). Decoction has been used to remove corneal opacities and foreign bodies from the eyes (Singha1965). The haemostatic (Achonye 1976), antibacterial (Macfoy and Cline 1990), membrane stabilization (Oyedepo et al. 1997) and anti-inflammatory (Okoli et al. 2007) activities of *A. africana* have been reported.

Chromolaena odorata (L.) R.M. King and H. Rob is considered to be one of the top 100 most invasive environmental weeds of wastelands, roadsides and other exposed areas in the world (GISD 2006, Chakraborty et al. 2011, Vijayaraghavan 2016). It is native to North and Central America and was later introduced to parts of Asia, Africa and Australia and has spread throughout the tropical and subtropical areas (Gautier 1992, Omokhua et al. 2016). It has a minimum 1 to 10 years life span, and it is a climbing perennial shrub which grows 2–3 m in height (Henderson 2001, Pandurangan et al. 2015a, Sirinthipaporn and Jiraungkoorskul 2017). Vijayaraghavan (2016) reported that the plant possesses insecticidal properties, and it is used as green manure. Similarly, Ukwueze et al. (2013) reported the plant to be used for the preservation of dead bodies, while Panyaphu et al. (2011) opined that the fresh leaves of *C. odorata* or the decoction had been used by practitioners of traditional medicine for the treatment of human burns, soft tissue wounds, ulcerated wounds, burn wounds, postnatal wounds and also for the treatment of leech bites, indigestion and skin infection. The decoctions of the stems were reported by Pandurangan et al. (2015b) to be effective against skin disease caused by *Propioni bacterium acnes*. It is also used for the treatment of various ailments, such as amenorrhea, catarrh, cold-associated nasal congestion, diabetes, diarrhoea, fever, pertussis and rheumatism, and as a vermifuge (Goodall and Erasmus 1996). Other economic importance, according to Ongkana (2003), includes antimalarial, anthelminthic, analgesic (Chakraborty et al. 2011), anti-inflammatory, antipyretic, antispasmodic (Oludare et al. 2000), antimycobacterial, insecticidal, antioxidant (Phan et al. 2001), anti-gonorrhoeal (Caceres et al. 1995), fungicidal, diuretic (Gopinath et al. 2009), blood coagulating (Triaratana et al.

1991), and antimicrobial effects (Borges et al. 2016).

Vernonia amygdalina Del. is one of the most famous plants found in Africa and Asia (Alara et al. 2017b, Farombi and Owoeye 2011, Ijeh and Ejike 2011). It is the most cultivated species of the genus *Vernonia* which has about 1,000 species (Toyang and Verpoorte 2013, Egharevba et al. 2014). According to Alara et al. (2017b), it is a woody shrub of about 2 to 10 m in height that regenerates rapidly and with bitter-tasting leaves from which its name, "Bitter leaf", springs up. It is popularly used for food and traditional medicine. Its characteristic odour and bitter taste can be reduced either by washing in several changes of water or by boiling before consumption (Toyang and Verpoorte 2013, Alara et al. 2017a). According to Alara et al. (2017b), the Medical Traditional Healer Association in Rukararwe, Uganda, produced a particular greenish powder packed in a sachet and consumed as a tea by patients suffering from malaria (Njan et al. 2008). The plant has also been reported to be used in soup (Ogbono and Orugbo soups) in Nigeria and Cameroun (Ndole dish) (Agbogidi and Akpomorine 2013, Oguwike et al. 2013). The leaves and root decoctions have been used in ethnomedicine to treat hiccups, fevers, kidney problems and stomach disorders (Yeap et al. 2010, Sha 2011, Oduah 2012) in several parts of the world.

A study of the species responses to water availability in their habitats is important, especially in the light of global climate change, and to better understand the variations in leaf epidermal characters in response to seasonal changes of habitat water regime and the structural modifications that occur in relation to these changes. The objective of this paper, therefore, was to characterize leaf epidermal parameters of these three species with special emphasis on seasonal effects by sampling leaves formed in the wet as well as in the dry season, thus explaining the mechanism underlying the observed structure-function responses of the leaf epidermis to water availability and changes in environmental conditions.

Materials and methods

Experimental site and plant materials

The present study was undertaken in a secondary (re-growth) forest located in Alagbado, Ore-Odigbo Local Government Area of Ondo State, Southwest Nigeria (006.76966N, 004.96141E, 67.9M). There are two major seasons in Nigeria – wet and dry. This research was conducted in both seasons. The wet season (April-November) experienced normal precipitation, while the dry season (November- April) was a bit more severe due to unusually low rainfall. The average temperature of the experimental site was 32.2°C in the dry and 22.2°C in the wet, while the average precipitation of the area was 1.7 days in December-February and with at least 0.03 inches (0.000492 litres) in the dry and 24.0 days in July-September and with at least 9.2 inches (0.151 litres) in the wet. (https://weatherspark.com/y/50008/Average-Weather-in-Ore-Nigeria-Year-Round#Figures-Rainfall).

A survey of the experimental site revealed diverse species composition, out of which three species were selected: *Aspilia africana* (Pers.) C. D. Adams, *Chromolaena odorata* (L.) R. M Kings and H. Rob and *Vernonia amygdalina* (Delile) Walp – all Asteraceae. Fresh leaves at the same developmental stage and level of insertion on the stem that formed during wet (July-September) and dry (December-February) seasons were collected for each of the species. Five accessions were considered for each species, and eight mature leaves (replicates) were harvested from each accession.

Epidermal micro-morphology

Sizeable portions (up to 5cm by 5cm) from the leaves of all the accessions were taken from the standard median portion of the leaf lamina (midway between the base and the apex). The epidermis of the leaves was obtained using concentrated nitric acid (HNO_3) following standard procedures as described previously (Adedeji and Jewoola 2008, Ogundare and Saheed 2012, Akinsulire et al., 2018, 2020) and a light microscope (Leica Galen III) was used to examine all the leaf epidermal features including qualitative and quantitative characters such as stomata type, size and density, the shape of guard cells, epidermal cell density, shape and size of the epidermal cell, and appearance of epidermal appendages such as trichomes. Photomicrographs were made with the aid of an Accu-scope Trinocular Microscope (Accu-scope33001 LED Trinocular Microscope with 4.2 MP CMOS Digital Camera).

Stomata index (SI) was calculated for both leaf surfaces using the formula below as proposed by Wilkinson (1979):

Stomata index (SI) = S/ (S+E)*100, where S is the number of stomata per unit area; E is the number of ordinary epidermal cells in the same area.

Data analyses and statistics

Twenty-five measurements (n=25) were taken for each of the quantitative parameters assessed for both seasons (wet and dry) and for both leaf surfaces (adaxial and abaxial). In order to determine the variation in leaf epidermal parameters with respect to water availability, data generated were subjected to Analysis of Variance using SPSS model 9.0, and where significant ($p < 0.05$) differences existed, the species means were separated using Duncan Multiple Mean Separation.

Results

Aspilia africana – wet season (adaxial, abaxial)

On the adaxial surface of the lamina (Fig. 1A), epidermal cells were irregular, anticlinal walls were wavy or sinuous, the periclinal wall was rough, epidermal cells density 16 to 21 mm^{-2}, mean epidermal cell size measured 73.00±2.93 µm in length and 47.00±3.92 µm in width, stomata were categorized as anomocytic, stomata density was 6 to 9 mm^{-2}, guard cells slightly elliptic and flaccid, mean stomata size measured 17.25±0.25 µm in length and 12.50±0.00 µm in width, mean stomata index was 28.32%, multicellular trichome was present, in density 0 to 1 mm^{-2}. On the abaxial surface (Fig. 2A), epidermal cells were largely irregular with sinuous anticlinal walls, epidermal cell density 12 mm^{-2} to 19 mm^{-2}, mean size was 67.75±5.62 µm in length and 45.00±2.42 µm in width, the periclinal wall was pubescent, stomata type in this taxon normocyticytic, stomata were mostly flaccid, occasionally turgid while the guard cells were elliptic in both flaccid and turgid conditions, stomata density was 22 mm^{-2} to 29 mm^{-2}, mean stomata size was 15.00±0.00 µm long and 10.00±0.00 µm wide, mean stomata index was 57.48%, simple unicellular non-glandular trichomes were present, in density 0 to 5 mm^{-2}, multicellular trichomes also present and ranged from 0 to 2 mm^{-2} in density.

Aspilia africana – dry season (adaxial, abaxial)

On the adaxial surface (Fig. 1B), epidermal cells were found to be irregular with sinuous anticlinal walls, and epidermal cells were 19 mm^{-2} to 25 mm^{-2}. Mean size was 65.25±3.32 µm long and 39.50±3.47 µm wide, periclinal walls were pubescent, stomata type was anisocytic, density 5 mm^{-2} to 7 mm^{-2}, guard cells were slightly elliptic and were, slightly turgid, mean stomata size was 12.75±0.25 µm long, and 8.25±0.38 µm wide, mean stomatal index was 20.69%, scale was found on the adaxial surface. On the abaxial surface (Fig. 2B), epidermal cells were largely irregular with sinuous anticlinal walls. Periclinal walls were pubescent, epidermal cell number was 11 to 17 mm^{-2}, mean size was 59.00±3.80 µm long and 41.75±2.24 µm wide, stomata type was anisocytic, guard cells were elliptic and turgid, stomata density was 16 mm^{-2} to 23 mm^{-2}, mean stomata size was 12.50±0.00 µm long and 7.50±0.00 µm wide with stomatal index of 52.36%, simple unicellular non-glandular trichomes were found on the abaxial surface with density 0 mm^{-2} to 4 mm^{-2}. Multicellular trichomes were also present.

Chromolaena odorata – wet season (adaxial, abaxial)

On the adaxial surface (Fig. 1C), epidermal cells were irregular with wavy to sinuous anticlinal walls, periclinal walls rough, epidermal cells ranged from 41 to 53 mm^{-2}, mean epidermal cell size was 20.50±1.93 µm long and 16.50±1.91 µm wide, multicellular trichomes present, frequency 0 to 3 mm^{-2}, stomata anisocytic and sparsely distributed, guard cell elliptic and flaccid, stomata density 0 to 2 mm^{-2}, mean stomata size was 11.25±0.42 µm long and 8.25±0.38 µm wide with a mean stomatal index of 3.18%. On the abaxial surface (Fig. 2C), epidermal cells were largely irregular with sinuous anticlinal walls, epidermal cells frequency ranged between 19 and 23 mm^{-2}, mean epidermal cell size was 22.75±2.62 µm long and 16.75±1.83 µm wide, epidermal surfaces were highly pubescent, covered with numerous multicellular trichomes which ranged between 12 and 18 mm^{-2}, druses were also present on the periclinal walls, stomata type was anisocytic, guard cells were ellipsoidal and flaccid in appearances, stomata density 8 to 16 mm^{-2}, mean stomata size was 11.00±0.41 µm long, and 7.75±0.25 µm wide, mean stomata index was 38.24%.

Chromolaena odorata – dry season (adaxial, abaxial)

On the adaxial surface (Fig. 1D), epidermal cells were largely polygonal. Anticlinal walls were wavy to slightly sinuous. Periclinal walls were slightly striated, epidermal cells 19 to 33 mm^{-2}, mean size was 20.50±1.93 µm long and 16.50±1.91 µm wide, stomata type was anisocytic with elliptic and turgid guard cells, stomata density 0 mm^{-2} to 3 mm^{-2}, mean size was 9.50±0.33 µm long and 7.00±0.33 µm wide, periclinal walls were covered with crystal sands and dense papillae, multicellular trichomes were present but scanty, density 0 mm^{-2} to 2 mm^{-2}, simple unicellular non-glandular trichomes were also present, density 0 mm^{-2} to 2 mm^{-2}. On the abaxial surface (Fig. 2D), epidermal cells were irregular, anticlinal walls were observed to be wavy or slightly sinuous but were straight to undulating along main vein epidermal regions, periclinal walls were generally striated, epidermal cells 15 mm^{-2} to 20 mm^{-2}, mean size was 22.00±2.03 µm long and 13.00±0.97 µm wide, stomata type was anisocytic but scanty, density 2 mm^{-2} to 4 mm^{-2}, mean stomata size was 10.25±0.25 µm long and 6.50±0.41 µm wide, mean stomatal index was 26.27%, guard cells were semi-circular in shape and turgid in appearance, numerous multicellular trichomes were present and occurred majorly on the main vein epidermal regions, density 3 mm^{-2} to 7 mm^{-2}.

Vernonia amygdalina – wet season (adaxial, abaxial)

Epidermal cells on the adaxial surface (Fig. 1E) ranged between pentagonal and octagonal with undulating anticlinal walls, periclinal walls possessed papillae, epidermal cells ranged from 8mm^{-2} to 13 mm^{-2}, mean size was 38.00±2.98 µm long and 25.75±1.58 µm wide, stomata type was anomocytic, guard cells almost straight but occasionally slightly elliptic and flaccid, rarely turgid, stomata density was from 3mm^{-2} to 8 mm^{-2}, mean size was 16.00±0.41 µm long, and 8.50±0.41 µm wide, mean stomata index up to 37.51%, glandular trichomes and scales were both present with 0 to 4 mm^{-2} densities. On the abaxial surface (Fig. 2E), epidermal cells were rectangular to octagonal, anticlinal walls were undulating to slightly wavy, periclinal walls possessed dense epidermal striations and dense papillae, epidermal cells 16 mm^{-2} to 24 mm^{-2}, mean size 40.50±2.54 µm long and 27.75±1.80 µm wide, stomata type was anisocytic with slightly elliptic and flaccid guard cells, density 3mm^{-2} - 6 mm^{-2}, mean size was 20.00±0.00 µm long and 10.00±0.00 µm wide, mean stomata index up to 22.26%, scales and glandular trichomes were both present, densities 0 mm^{-2} to 3 mm^{-2}.

Vernonia amygdalina – dry season (adaxial, abaxial)

Micro-anatomical examination of the adaxial surface (Fig. 1F) revealed that the epidermal cells were rectangular to octagonal in shape with straight or occasionally undulating anticlinal walls, adaxial epidermal cells were about 21 mm^{-2} to 33 mm^{-2}, mean size was 38.00±2.98 µm long and 25.75±1.58 µm wide, periclinal walls possessed dense papillae, stomata type was paracytic, 0 to 6 mm^{-2}, guard cells were straight to ellipsoidal, mean stomatal size was 14.50±0.33 µm long, and 7.50±0.00 µm wide, mean stomata index was up to 9.33%, scale were present but sparsely distributed, 0 to 2 mm^{-2}. On the abaxial surface (Fig. 2F), epidermal cells were triangular to hexagonal, cells 18 mm^{-2} to 4 mm^{-2}, mean size was 40.50±2.54 µm long and 27.25±1.95 µm wide, anticlinal walls were straight to wavy, periclinal walls densely papillate, stomata type was actinocytic while the guard cells were elliptic, guard cells were mostly turgid but occasionally flaccid, stomata density 0 mm^{-2} to 6 mm^{-2}, mean stomatal size was 18.25±0.38 µm long and 10.75±0.38 µm wide, mean stomatal index up to 11.38%. Simple, short, unicellular trichomes were present but sparsely distributed, 0 mm^{-2} to 2 mm^{-2}, scale was also present, 0 mm^{-2} to 2 mm^{-2}.

Discussion

Stomata density, frequency and distribution are inherently hereditary and genetically controlled traits, but plants have high phenotypic plasticity depending on their environments. It was discovered in this study that the decrease in stomata densities and stomata indices observed on the adaxial surfaces of *Aspilia africana* and *Vernonia amygdalina* in the dry season suggest that leaves which were expanded in the dry season tend to possess fewer stomata, and could suggest lower water loss and CO_2 uptake in the species because the more stomata per unit area (stomata density) the more CO_2 can be taken up and more water can be released. This agrees with the previous findings by Bruce et al. (2004). Lack of or inadequate water in species of Asteraceae that developed and collected during the dry season and in such a sunlit environment resulted in reduced stomata densities (on both leaf surfaces) observed across the three species investigated (Tab. 2). Meanwhile, chances are that excessive evaporation

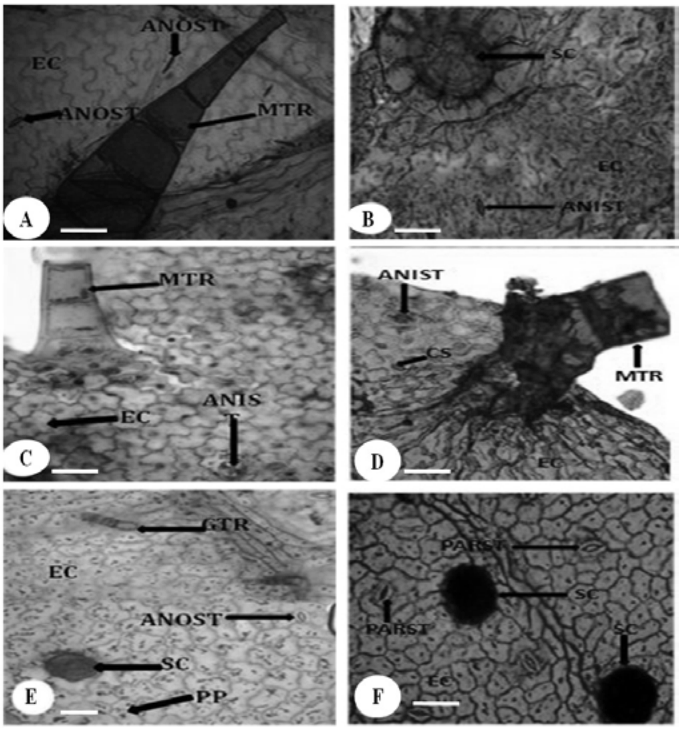

Figure 1. Photomicrographs of the adaxial surface of leaf epidermis in wet and dry seasons.
(A) – *Aspilia africana* in the wet season, (B) – *Aspilia africana* in the dry season, (C) – *Chromolaena odorata* in wet season, (D) – *Chromolaena odorata* in dry season, (E) – *Vernonia amygdalina* in wet season, (F) – *Vernonia amygdalina* in dry season. Abbreviations: EC – epidermal cell, CS – crystal sand, TRB – trichome base, SUTR – simple unicellular trichome, ANIST – anisocytic stomata, ANOST, anomocytic stomata, GTR – glandular trichome, SC – scale, MTR – multicellular trichome, DR – druses, PP – papillae.

Slika 1. Mikrografije adaksialne površine listne povrhnjice tekom mokre in suhe sezone (A) *Aspilia africana* v mokri sezoni, (B) *Aspilia africana* v suhi sezoni, (C) *Chromolaena odorata* v mokri sezoni, (D) *Chromolaena odorata* v suhi sezoni, (E) *Verninia amygdalina* v mokri sezoni, (F) *Vernonia amygdalina* v suhi sezoni. Kratice: EC – epidermalne celice, CS – kristali peska, TRB – baza trihoma, SUTR – preprost enocelični trihom, ANIST – anizocitna stomata, ANOST – anomocitna stomata, GTR – žlezni trihom, SC – merilna skala, MTR – večcelični trihom, DR – drus, PP – papila.

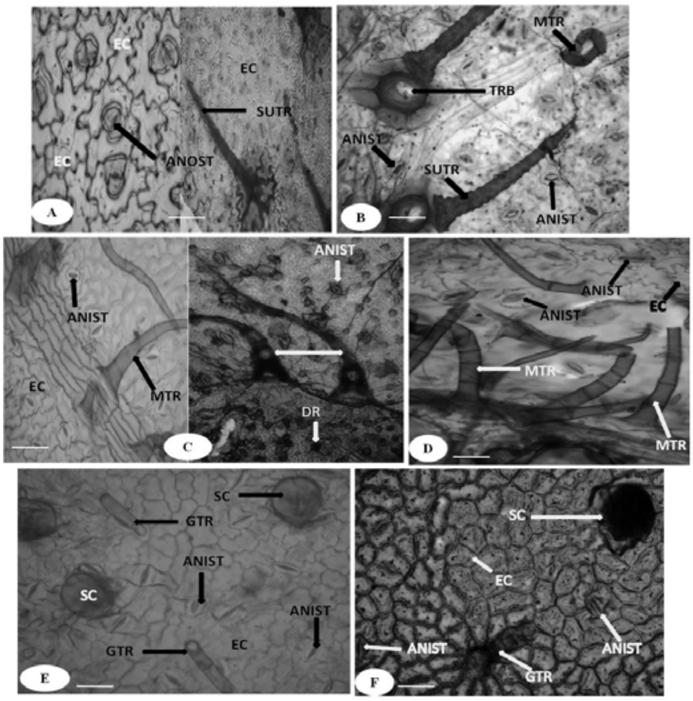

Figure 2. Photomicrographs of the abaxial surface of leaf epidermis in wet and dry seasons.
(A) – Aspilia africana in the wet season, (B) – Aspilia africana in the dry season, (C) – Chromolaena odorata in wet season, (D) – Chromolaena odorata in dry season, (E) – Vernonia amygdalina in wet season, (F) – Vernonia amygdalina in dry season. Abbreviations: EC – epidermal cell, TRB – trichome base, SUTR – simple unicellular trichome, ANIST – anisocytic stomata, ANOST, anomocytic stomata, GTR – glandular trichome, SC – scale, MTR – multicellular trichome, DR – druses, PP – papillae.

Slika 1. Mikrografije abaksialne površine listne povrhnjice tekom mokre in suhe sezone (A) *Aspilia africana* v mokri sezoni, (B) *Aspilia africana* v suhi sezoni, (C) *Chromolaena odorata* v mokri sezoni, (D) *Chromolaena odorata* v suhi sezoni, (E) *Verninia amygdalina* v mokri sezoni, (F) *Vernonia amygdalina* v suhi sezoni. Kratice: EC – epidermalne celice, CS – kristali peska, TRB – baza trihoma, SUTR – preprost enocelični trihom, ANIST – anizocitna stomata, ANOST – anomocitna stomata, GTR – žlezni trihom, SC – merilna skala, MTR – večcelični trihom, DR – drus, PP – papila.

in these species might lead to desiccation and equally severe disruption of photosynthetic function (Bruce et al. 2004). Stomata densities in the three species differed per unit area for both leaf surfaces in both seasons except for *Chromolaena odorata*, which has close values of stomata density only on the adaxial surface in both seasons (Tab. 2). These results further emphasized that though stomata distribution varies per species, can greatly be affected by seasonal variations and water stress. A similar report has previously been published by Ticha (1982), where it was stated that stomatal density might change as a response to one or more environmental factors, including flooding (or wet condition), thus possibly affecting leaf gas exchange. Change in stomata densities due to season explains changes in stomatal conductance (Herrera et al. 2009) since this is directly proportional to the fraction of the leaf area occupied by stomata (Nobel 1974).

It was observed in the dry season that the stomata of the three species were reduced in size (length and width). This is an indication of species adaptations to dry conditions. This corroborates Miller et al. (2010), who claimed that under dry conditions, tissue dehydration might reach critical levels and interfere with cellular homeostasis, while Thomas et al. (2000) opined that it may, in part, be a consequence of anatomical changes to the pathway of water flow. The occasional turgescent guard cells in the dry season were an indication of stomatal opening since transpiration in the species needed to be activated but to a very minimal extent and which was hormonal in the species. Similar reports have been given by Cornic and Fresneau (2002) and Bowne et al. (2012) on wheat cultivars as well as by Easlon and Richards (2009) on tomato (Solanaceae), that, in water-limited conditions, dry season or drought, a growth retardant hormone Abscisic Acid (ABA) is exuded by roots, and which according to Kamanga et al. (2018) act as root-shoot signals, regulating stomatal conductance and transpiration.

The reduction in the mean length and width of epidermal cells of *Aspilia africana* in the dry condition and on both leaf surfaces (Tab. 2) when compared to the wet season may be attributed to the response of the species to inadequate water availability when precipitation was drastically reduced.

The significance of epidermal appendages, trichome presence/absence, density, type and behaviour were critically examined as tools which can successfully be applied when determining leaf epidermal structure-function in response to water availability. *Aspilia africana* possessed more multicellular trichomes on both leaf surfaces in the dry season (Tab. 2). This reflects sunlight and reduced water loss. It could also be the species' adaptation to deter herbivores in such dry conditions through chemical means, as previously reported by Dalin et al. (2008) and Karley et al. (2015) that higher trichome densities may be advantageous to plants as it increases resistance against herbivory. *Chromolaena odorata* and *Vernonia amygdalina* have more of their trichomes (multicellular) during the wet season (Tab. 2). However, the previous research carried out by Adedeji et al. (2007) explained the significant influence of trichome types in different organs of plant body in the delimitation of genera and species of plants.

Qualitatively, there were little or no differences in the shapes of epidermal cells of the species with respect to seasonal variations (wet and dry seasons) (Tab. 1 and Figs. 1-2). Thus, it was concluded that seasonal variations or water availability/unavailability has little or no significance in altering the shapes and/or arrangements of the epidermal cells of the species of Asteraceae investigated; neither does it have effects on the orientation of their anticlinal walls. Pappilate epidermis characterizes the leaf surfaces of *Vernonia amygdalina*, most especially in the dry season (Tab. 1 and Figs. 1F and 2F). Brehm and Krell (1975) and Kay et al. (1981) have previously observed similar features in angiosperms.

On the account of the type of stomata observed in the three species of Asteraceae investigated, *Aspilia africana* possessed anomocytic stomata on both surfaces in the wet (Figs. 1A and 2A), but anisocytic stomata on both surfaces in the dry condition (Figs. 1B and 2B and Tab. 1), while the stomata type remained the same on both leaf surfaces and in both seasons for *Chromolaena odorata* (Figs. 1C and 1D, Figs. 2C and 2D and Tab. 1). Meanwhile, an unusual occurrence of stomata type was found in *Vernonia amygdalina* as it possessed anomocytic stomata on the adaxial surface in the wet season (paracytic in the dry), and anisocytic stomata on the abaxial surface in the wet season (actinocytic in the dry) (Figs. 1E and 1F, Figs. 2E and 2F and Tab. 1). Thus, the overlap in the stomata types in these three taxa may however, be connected to their placement in their respective genus as many researchers have previously used stomata types in the delimitations of different taxa (Akinsulire et al. 2018, Akinsulire et al. 2020).

Table 1. Qualitative leaf epidermal characters of three species of Asteraceae in response to water stress.

Tabela 1. Kvalitativne značilnosti listnega epidermisa treh vrst Nebinovk pod vodnih stresom.

ADAXIAL SURFACE							
Species	**Season**	**Epidermal cell shape**	**Anticlinal wall**	**Periclinal**	**Stomata type**	**Nature of guard cell**	**Type of epidermal appendage**
Aspilia africana	Wet	Irregular	W-S	Rough	Anomocytic	Flaccid	MTR
	Dry	Irregular	Sinuous	Pubescent	Anisocytic	Turgid	Scale; MTR
Chromolaena odorata	Wet	Irregular	W-S	Rough	Anisocytic	Flaccid	MTR
	Dry	Polygonal	W-S	Striated	Anisocytic	Turgid	SUNT; MTR
Vernonia amygdalina	Wet	P-O	Undulating	Papillae	Anomocytic	Flaccid	Scale; GTR
	Dry	R-O	Str.-U	Papillae	Paracytic	Turgid	Scale
ABAXIAL SURFACE							
Aspilia africana	Wet	Irregular	Sinuous	Pubescent	Anomocytic	Flaccid	SUNT; MTR
	Dry	Irregular	Sinuous	Pubescent	Anisocytic	Turgid	SUNT; MTR
Chromolaena odorata	Wet	Irregular	Sinuous	Rough	Anisocytic	Flaccid	MTR; Druses
	Dry	Irregular	W-S	Striated	Anisocytic	Turgid	MTR
Vernonia amygdalina	Wet	R-O	U-W	Papillae	Anisocytic	Flaccid	Scale; GTR
	Dry	Str.-W	Str.-W	Papillae	Actinocytic	Turgid	SUNT; Scale

Abbreviations: P-O - pentagonal to octagonal, R-O - rectangular to octagonal, W-S - wavy to sinuous, Str.-U - straight to undulating, Str.-W - straight to wavy, U-W - undulating to wavy, SUNT - simple unicellular non-glandular trichome, MTR - multicellular trichome, GTR - glandular trichome, (-) - a character not present.

Table 2. Quantitative leaf epidermal characters of three species of Asteraceae in response to water stress (with Duncan Multiple Means Separation).

Tabela 2. Kvalitativne značilnosti listnega epidermisa treh vrst Nebinovk pod vodnih stresom (z Duncanovim post hoc testom).

ADAXIAL SURFACE								
Species	**Season**	**ECD (µm)**	**ECL (µm)**	**ECW (µm)**	**SD (µm)**	**SL (µm)**	**SW (µm)**	**TRD**
Aspilia africana	Wet	17.50±0.39^{b}	73.00±2.93^{c}	47.00±3.92^{c}	7.40±0.31^{c}	17.25±0.25^{c}	12.50±0.00^{b}	0.50±0.17^{a}
	Dry	22.30±0.73^{a}	65.25±3.32^{c}	39.50 ±3.42^{c}	5.60±0.27^{c}	12.75±0.25^{b}	8.25±0.38^{b}	2.20±0.42^{b}
Chromolaena odorata	Wet	46.70±1.04^{c}	20.50±1.93^{a}	16.50±1.91^{a}	1.60±0.37^{a}	11.25±0.4^{a}	8.25±0.38^{a}	1.80±0.39^{b}
	Dry	27.50±1.68^{b}	20.50±1.93^{a}	16.50±1.91^{a}	1.00±0.21^{a}	9.50±0.33^{a}	7.00±0.33^{a}	1.40±0.27^{ab}
Vernonia amygdalina	Wet	10.30±0.63^{a}	38.00±2.98^{b}	25.75±1.58^{b}	5.40±0.54^{b}	16.00±0.41^{b}	8.50±0.41^{a}	2.00±0.47^{b}
	Dry	23.90±1.52^{ab}	38.00±2.98^{b}	25.75±1.58^{b}	3.70±0.75^{b}	14.50±0.33^{c}	7.50±0.00^{ab}	1.00±0.26^{a}
ABAXIAL SURFACE								
Aspilia africana	Wet	15.90±0.74^{a}	67.75±5.62^{c}	45.00±2.42^{c}	25.90±0.75^{c}	15.00±0.00^{b}	10.00±0.00^{b}	2.80±0.37^{a}
	Dry	14.00±0.80^{a}	59.00±3.80^{c}	41.75±2.24^{c}	19.60±0.75^{b}	12.50±0.00^{b}	7.50±0.00^{b}	3.80±0.49^{b}
Chromolaena odorata	Wet	20.90±0.57^{b}	22.75±2.62^{a}	16.75±1.83^{a}	10.80±0.83^{b}	11.00±0.41^{a}	7.75±0.25^{a}	13.90±0.89^{b}
	Dry	18.30±0.63^{b}	22.00±2.03^{a}	13.00±0.97^{a}	3.60±0.27^{a}	10.25±0.25^{a}	6.50±0.41^{a}	5.50±0.40^{c}
Vernonia amygdalina	Wet	19.90±0.82^{b}	40.50±2.54^{b}	27.75±1.80^{b}	4.40±0.34^{a}	20.00±0.00^{c}	10.00±0.00^{b}	1.80±0.39^{a}
	Dry	19.90±0.82^{b}	40.50±2.54^{b}	27.25±1.95^{b}	2.80±0.73^{a}	18.25±0.38^{c}	10.75±0.38^{c}	1.10±0.23^{a}

Abbreviations: ECD - epidermal cell density, ECL - epidermal cell length, ECW - epidermal cell width, SD - stomata density, SL - stomata length, SW - stomata width, TRD - trichome density, (-) - a character not present, n=25

Values are means ± Standard Error

*Values with similar superscript alphabets along the same column are not significantly different ($P < 0.05$)

Conclusion

This study revealed that investigations into the structural parameters of the leaf epidermis are useful when researching the micro-morphological response of plant leaf epidermis to water stress. It is therefore concluded that rainfall patterns have a great significant effect on the structural parameters of leaf epidermal micro-morphology of three studied species of the family Asteraceae.

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ABS

Original Research Paper

Antioxidant effect of methanol extract of plant ebolo *Crassocephalum crepidioides* during paracetamol-induced toxicity in Wistar rats

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Abstract

The effect of methanol extracts *Crassocephalum crepidioides* against Paracetamol (PCM)-induced oxidative stress was investigated. Wistar rats were divided into five groups of six rats. Group 1: control, 10% DMSO, group 2: 250 mg/kg/but PCM, group 3: 300 mg/kg/bwt methanol extract of *C. crepidioides* leaves (MECL), group 4: 250 mg/kg/bwt PCM + 300 mg/kg/bwt MECL and group 5: 250 mg/kg/bwt PCM + 50 mg acetylcysteine (NAC) for two weeks. Groups 4 and 5 were pre-administered with 300 mg/kg/bwt MECL and 50mg NAC, respectively, for one week before the co-administration with PCM. Kidney damage was measured by evaluating serum urea and creatinine, while antioxidant status was assessed by evaluating serum glutathione (GSH) level, glutathione-S-transferase (GST), superoxide dismutase (SOD) and catalase activities. Oxidative stress was determined from malondialdehyde (MDA) levels. PCM had no significant effect on serum urea and creatinine levels but significantly decreased glutathione levels, SOD and catalase activities, while the activity of glutathione-S-transferase and level of malondialdehyde (MDA) was increased significantly, indicating the presence of oxidative stress. Co-administration with MECL or NAC reversed these effects. In conclusion, *C. crepidioides* protects against PCM-induced oxidative stress in the Kidneys of Wistar rats.

Keywords

Antioxidants, *C. crepidioides*, rat kidney, malondialdehyde, paracetamol, toxicity, detoxification, drug

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Antioksidativni učinek metanolnega ekstrakta rastline *Crassocephalum crepidioides* med strupenostjo paracetamola na podgane Wistar

Izvleček

Raziskali smo učinek metanolnih izvlečkov rastline *Crassocephalum crepidioides* na oksidativni stres, ki ga povzroča paracetamol (PCM). Podgane Wistar so bile razdeljene v pet skupin po šest podgan: skupina 1: kontrola, 10 % DMSO, skupina 2: 250 mg PCM /kg telesne teže, skupina 3: 300 mg/kg telesne teže metanolnega izvlečka listov *C. crepidioides* (MECL), skupina 4: 250 mg PCM /kg telesne teže + 300 mg MECL/kg telesne teže in skupina 5: 250 mg PCM/kg telesne teže + 50 mg acetilcisteina (NAC) dva tedna. Skupini 4 in 5 sta pred sočasnim dajanjem PCM en teden prejemali 300 mg MECL/kg telesne teže oziroma 50 mg NAC. Poškodbe ledvic smo merili z oceno koncentracije sečnine in kreatinina v serumu, antioksidativni status pa smo ocenili z oceno ravni glutationa (GSH), aktivnosti encima glutation-S-transferaze (GST), superoksid dismutaze (SOD) in katalaze v serumu. Oksidativni stres je bil določen na podlagi ravni malondialdehida (MDA). PCM ni pomembno vplival na raven sečnine in kreatinina v serumu, vendar je pomembno zmanjšal raven GSH, aktivnosti SOD in katalaze, medtem ko sta se aktivnost GST in raven MDA pomembno povečali, kar kaže na prisotnost oksidativnega stresa. Sočasna uporaba MECL ali NAC je zmanjšala te učinke. Zaključimo lahko, da metabolni ekstrakti rastline *C. crepidioides* zmanjšajo oksidativni stres, ki ga v podganah povzroči PCM.

Ključne besede

antioksidanti, *C. crepidioides*, ledvice podgan, malondialdehid, paracetamol, strupenost, detoksifikacija, zdravilo

Introduction

Nephrotoxicity presents as a defect in kidney-specific detoxification and excretory function. It may be due to the effects of endogenous or exogenous toxins on the kidney (Kim and Moon, 2012). The toxicity of drugs in the kidney is of great concern because patients remain exposed to various drugs that may result in acute and chronic kidney injury (Perazella, 2018). Paracetamol, also called acetaminophen (APAP), is safe when taken at therapeutic doses. However, its overdose can result in renal and hepatic damage (Ishitsuka et al., 2020). Besides the liver, the kidney is the next target organ of PCM toxicity because it receives ample blood supply. It also concentrates, secretes, and metabolically activates certain drugs (Pathan et al., 2013). High doses of PCM reduces the vitality of tubular epithelial cell resulting in kidney damage. ROS are generated by chemicals such as PCM; therefore, oxidative stress is responsible for PCM toxicity (Ucar et al., 2013).

The primary toxicity of PCM lies in its metabolism (Mazaleuskaya et al. 2015). About 90% of PCM is conjugated to glucuronic acid and sulphate, rendering it inactive and easily excreted in the urine. About 2% is excreted unchanged, while about 5-9% is metabolized to reactive N-acetyl-*p*-benzoquinone imine (NAPQI) by cytochrome P450 enzymes. This metabolite is conjugated with glutathione, producing a non-toxic derivative that is excreted in the urine. PCM overdose has long been treated with acetylcysteine (NAC); however, adverse effects such as anaphylactoid reactions, which may include bronchospasm and hypotension, have been shown to occur in some cases during NAC administration (Bateman and Dear, 2019). In PCM overdose, the metabolic pathways of the liver become saturated; thus, large amounts of unmetabolized PCM get to the kidneys. This is activated to NAPQI by renal P450, resulting in nephrotoxicity (Mazaleuskaya et al. 2015). The detoxification of NAPQI by GSH results in its depletion in the kidneys and liver, thereby leading to ROS production and, subsequently, oxidative stress. This eventually results in lipid peroxidation and DNA fragmentation (El-Shafey et al.2015). In the kidney medulla, another enzyme, prostaglandin endoperoxide synthetase (PGES), activates PCM to NAPQI (Mazer and Perrone, 2008). Excessive NAPQI binding to mitochondrial and proximal tubule protein causes mitochondrial dysfunction and cell death (Mazaleuskaya et al. 2015).

Phytochemicals such as flavonoids and tannins with antioxidant properties have been proven to protect the kidneys against the toxic effects of acetaminophen overdose (Tienda-Vázquez et al. 2019). *Crassocephalum crepidioides* (Benth.) S. Moore (known as Ebolo among the indigenous Yoruba-speaking people of southwest Nigeria) is widely grown in tropical Africa, where its leaves and stems are used as herbs and consumed as a vegetable (Omoregie et al. 2015). Arawande et al. (2013) reported that it contains various phytochemicals such as phenol, oxalate, saponin and tannin.

PCM is widely used as a prescription, and it is readily accessible over the counter (Ishitsuka et al., 2020). This, together with the narrow therapeutic index of PCM, makes an overdose fairly common. *C. crepidioides* possesses antioxidant and anti-inflammatory properties; hence it may serve as a suitable solution to PCM-induced nephrotoxicity (Bello et al. 2019), especially as prophylaxis in those who regularly consume it and eventually indulge in the chronic use of PCM or administer it as an overdose due to ailment such as toothache. Hence, the aims of this study are to confirm the nephrotoxic effect of PCM and to provide a remedy to PCM-induced nephrotoxicity through the regular use and consumption of *C. crepidioides* leaves in diets.

Material and Methods

Plant collection and extract preparation

Crassocephalum Crepidioides (Ebolo) leaves were bought from a market in Ilara Mokin, Akure, Ondo State, Nigeria. The plants were authenticated at the Department of Crop, Soil and Pest Management, Federal University of Technology, Akure, Nigeria. The leaves were rinsed with water, sliced into smaller parts, air-dried and kept away from direct sunlight. A sample of *C. crepidioides* leaves weighing 333g was blended and soaked in 3330 ml of 70% methanol (Sigma-Aldrich) for 72 hours, after which it was filtered through Whatman No. 1 filter paper with 11µm pore size. The extract obtained was dried using a rotatory evaporator and refrigerated until required.

Experimental animals and treatments

Experimental animals

The Wistar rats used for these experiments were obtained from the Central Animal House, Department of Physiology, College of Health Sciences, Federal University of Technology, Akure. The animals were housed in the animal housed under standard laboratory conditions and had access to water and standard laboratory rat chow *ad libitum*. They were acclimatized for two weeks and, thereafter, treated as described below. The conduction of this study was in accordance with the International Ethical Norms on Animal Care and Used as contained in NIH publication/80-23, revised in 2010.

Animal treatments

The experiment consisted of 30 male rats aged 18 weeks with a weight of between 280-300 g each. They were divided into five groups (1, 2, 3, 4 and 5) of six rats per group. Group 1 served as control, and they were administered 10% DMSO. Dried extract, PCM and NAC were dissolved in 10% DMSO. Group 2 animals were administered methanol extract of *C. crepidioides* leaves (MECL) (300 mg/kg bwt/day). Group 3 was administered 250 mg/ kg bwt/day of PCM. These treatments lasted for two weeks. Group 4 was pretreated with MECL for one week and thereafter co-administered with 300 mg/kg bwt/day of MECL and 250 mg/ kg bwt/day of PCM for two weeks, while group 5 was pretreated with NAC for one week and thereafter was co-administered with 50 mg/kg bwt/day of N-acetylcysteine (NAC) and 250 mg/ kg bwt/day of PCM for two weeks. Pre-administration of MECL is a model that assumes that the regular consumption of *C. crepidioides* in diet before exposure to PCM toxicity may confer protection against subsequent PCM toxicity. The experiment aims toward the protective effect of regular intake of *C. crepidioides* on PCM-induced nephrotoxicity. NAC was used in order to compare its effect on PCM toxicity with that of MECL since NAC has been used for a long time as an antidote to PCM toxicity due to its ability to supply cysteine for the replenishment of GSH that is used for NAPQI detoxification. It also protects against oxidative stress through its electron-donating properties (Waring, 2012).

All administration was carried out by oral gavage. The animals maintained their diets for this period. The rats were fasted for 12 hours, and treatments were withdrawn from them 24 hours prior to sacrifice.

Table 1. Experimental Grouping. PCM (paracetamol); MECL (methanol extract of *C. crepidioides* leaves), NAC (N-acetylcysteine)

Tabela 1. Eksperimentalne skupine: PCM (paracetamol); MECL (metanolni ekstrakt listov *C. crepidioides*), NAC (N-acetilcistein)

Group	Treatment
Group 1	10% DMSO (Control) for two weeks
Group 2	300 mg MECL /kg bwt/day for two weeks
Group 3	250 mg PCM / kg bwt/day for 2 weeks
Group 4	Pre-administration with 300 mg MECL /Kg bwt/day for one week before co-administration with 300 mg MECL /kg bwt/day and 250 mg PCM/ kg bwt/day for 2 weeks
Group 5	Pre-administration with 50 mg NAC /Kg bwt/day for one week before co-administration with 50 mg NAC /kg bwt/ day and 250 mg PCM / kg bwt/day for 2 weeks

Collection of Blood Samples and Preparation of Rat Post Mitochondria Fractions of Kidney

Animals were killed by decapitation, and blood was taken into plain tubes by cardiac puncture. Blood samples were centrifuged for 10 minutes at 3000g to obtain the serum. After sacrifice, the kidney was immediately removed and rinsed in a cold solution of 1.15% KCl (Sigma-Aldrich). The post-mitochondria fraction was obtained by homogenizing 1.4 g of tissue in 4.2 ml of sodium phosphate buffer pH 7.4. The homogenate was centrifuged in a cold centrifuge for 10 minutes at 10,000 x g. The filtrate was used for enzyme assay.

Biochemical Analysis (Evaluation of Organ Damage and Antioxidant Status)

Kidney damage was determined by the evaluation of serum urea and creatinine using the Randox kit, oxidative stress was evaluated by the determination of the malondialdehyde level and antioxidant status was evaluated by determination of SOD activity, GST activity, catalase activity and GSH content.

Determination of Malondialdehyde level

The breakdown product of LPO is a thiobarbituric acid reactive substance (TBARS). The level of LPO was assessed by the reaction of malondialdehyde with TBARS, by the method of Buege and Aust (1978). 3ml containing 1g of the test sample and 9 ml of stock reagent were mixed in a cooked test tube and heated for 15 minutes in a boiling water bath. After cooling at room temperature, the precipitate was removed by centrifugation at 1000 x g for 10 minutes, and the absorbance of the supernatant was measured using a spectrophotometer at 532nm against a blank containing all the reagents except the test sample.

The MDA concentration in moles /g tissue (converted to µg/g tissue) was calculated from the absorbance using an extinction coefficient of 1.56 x 105 M-1 cm-1 according to the method of Adam-Vizi and Seregi (1982).

Determination of SOD activity

SOD activity was determined by the method of Misra and Fridovich (1972). This measures the ability of SOD to inhibit the auto-oxidation of adrenaline to adrenochrome by superoxide anion.

To 0.2 ml of the tissue, homogenates were added to 2.5 ml of 0.05 carbonate buffer (pH 10.2). The reaction was started by the addition of 0.3 ml of freshly prepared 0.3 mM epinephrine to the mixture, which was quickly mixed by inversion. The blank was prepared by replacing the tissue with 0.2ml of water. The increase in absorbance at 480nm was monitored every 30 seconds for 150 seconds. The absorbance of the sample was measured at 450 nm using a spectrophotometer. The % inhibition was determined from the equation below.

$$\% \text{ inhibition} = \frac{\text{increase in absorbance of substrate}}{\text{increase in absorbance of the blank}} \times 100$$

One unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the oxidation of adrenaline during 1 minute

Determination of GST activity

The activity of GST was estimated by the method of Habig et al. (1974). The method involves the conjugation of the substrate (1-chloro-2,4-dinitrobenzene) with reduced glutathione by GST. Absorption read at 340 nm is a measure of GST activity. 30 µl of reduced glutathione (0.1 M) was added to 150 µl of CDNB (20 mM), and 2.79 ml of 0.1 M phosphate buffer (pH 6.5), 30 µl of serum was then added. The blank was prepared as above with 2.82 ml of 0.1 M phosphate buffer without the serum. The absorbance was read against the blank at 340 nm every 60 seconds for 3 minutes. The temperature was maintained at approximately 31°C. GST activity was calculated in µ mole/min/mg protein using the extinction coefficient of CDNB (9.6 mm^{-1} cm^{-1}).

Determination of GSH level

GSH content was determined by the method of Beutler et al. (1963). The method involves the reaction of 5,5 – dithiobis – (2-nitrobenzoic acid) (Ellman's reagent) with sulfhydryl compounds such as GSH to produce a stable yellow colour. An aliquot of homogenate was deproteinized by the addition of an equal volume of 4 % sulphursalicyclic acid. This was centrifuged at 4,000 rpm for 10 minutes. 0.5 ml of the supernatant obtained was added to 4.5 ml of Ellman's reagent, and the absorbance read at 412 nm. The blank was prepared by the addition of 0.5 ml of 4 % sulphursalicyclic acid to 4.5 ml of Ellman's reagent. The concentration of reduced glutathione (GSH) is proportional to the absorbance at 412 nm. Concentrations of GSH were determined from the standard curve (plot of absorbance against standard concentrations of GSH).

Determination of Catalase activity

Catalase activity was determined according to the method of Sinha (1971). This method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H_2O_2, with the formation of perchromic acid as an unstable intermediate. The chromate acetate then produced is measured colourimetrically at 570-610nm. The reaction mixture contained 4 ml of H_2O_2 solution (800 µmoles), 5 ml of phosphate buffer, and pH 7.0 in a 10 ml flat bottom. The reaction was started with the addition of 1 ml of diluted sample. The reaction was stopped at different periods of time at an interval of 60 seconds by the addition of 2ml of dichromate acetic acid mixture to a portion (1ml) of the reacting mixture and thereafter heated for 10 minutes. The remaining H_2O_2 in the portion was determined by measuring chromic acetate colourimetrically and extrapolating the value on the standard curve. To each of the varying concentrations of standard H_2O_2 was added 2ml of dichromate/acetate. The mixtures were heated to produce a stable green due to the formation of chromic acetate. After cooling at room temperature, the volume of the reaction mixture was made up to 3ml and the optical density was measured with a spectrophotometer at 570nm. The concentrations of varying concentrations of standard H_2O_2 were plotted against absorbance.

The monomolecular velocity constant K for the decomposition of H_2O_2 by catalase was determined by using the equation for a first-order reaction.

$$K = \frac{1}{t} \log So/S$$

Where So = initial concentration of H_2O_2 and S = concentration of H_2O_2 at 1min intervals. The values of K were plotted against time in minutes, and the velocity constant of catalase $K_{(O)}$ at 0 minutes was determined by extrapolation.

The catalase content of enzyme preparation was expressed in terms of catalase feiahigkeit or "Kat f" (which is equivalent to micromole of H_2O_2 consumed per min mg protein) according to Von Euler and Josephson (1927):

$$\text{Kat f} = \frac{\text{Ko}}{\textit{Mg protein/ml}}$$

Statistical Analysis

The results were expressed as Mean ± SEM. The sample sizes were small; thus, a normality test was carried out on the data using a statistical technique that was done by the application of Sapiro-Wilk test using IBM SPSS statistic version 26. $P < 0.05$ was considered significant. The p values obtained were greater than 0.05, indicating that the data do not show evidence of non-normality, hence the use of a parametric test for comparison of means. Analysis of data was done by one-way analysis of variance (ANOVA) and LSD post hoc test by Fischer. Differences among groups were considered to be significant at a P-value of< 0.05. All analyses were carried out using GraphPad Prism version 8.0 (GraphPad® Inc, CA, USA).

Results

As shown in Table 2, the serum urea and creatinine concentrations for the group administered 250 mg/ kg but/day PCM were not significantly different from the control group and the other test groups. It also shows that the Kidney MDA level was significantly higher ($p<0.05$) in the group administered 250 mg PCM with respect to the control group, while in the MECL and NAC co-administered group, the MDA level was not significantly ($p<0.05$) different from the control group.

Table 3 shows that kidney GST activity was significantly higher ($p<0.05$) in the group administered 250 mg/ kg bwt/day PCM compared to the control group and was significantly ameliorated with the co-administration of MECL and NAC.

Table 3 also indicated that the activity of SOD was significantly ($p<0.05$) lowered in the 250 mg/ kg bwt/day PCM group in relation to the control group. However, co-administration of PCM with MECL and with NAC significantly increased ($p<0.05$) the SOD activity when compared with the 250 mg/ kg bwt/day PCM group. The trend observed for catalase activity was similar to that of SOD activity, although catalase activity was not significantly ($p<0.05$) different from the control group in MECL co-administered groups.

The GSH level shown in Table 3 indicates a significant ($p<0.05$) reduction of GSH level in the 250 mg/ kg bwt/day PCM administered group in relation to the control but co-administration of PCM with the extract significantly ($p<0.05$) increase the GSH levels in relation to the PCM group. Co-administration of NAC also significantly ($p<0.05$) increased the GSH levels in relation to the PCM group, and no significant increase was observed between the NAC co-administered group and the control.

Table 2. Effects of MECL on serum urea, creatinine and MDA concentration in PCM-induced nephrotoxicity. PCM (paracetamol); MECL (methanol extract of *C. crepidioides* leaves), NAC (N-acetylcysteine)

Tabela 2. Učinki MECL na serumsko urejo, kreatinin in koncentracijo MDA pri z PCM-inducirani nefrotoksičnosti. PCM (paracetamol); MECL (metanolni ekstrakt listov *C. crepidioides*), NAC (N-acetilcistein)

Group	Urea concentration (mmol/l)	Creatinine concentration (µmol/l)	MDA concentration µg/g tissue
G1 (Control)	6.805 ± 0.205	96.192 ± 3.372	298.898±6.8845
G2 (MECL)	3.824 ± 0.199	80.326 ± 2.425	180.492±12.6135$^{*\#}$
G3 (PCM 250)	6.910 ± 0.136	98.475±3.068	391.583±18.350*
G4 (PCM + MECL)	5.225 ± 0.127	90.543±4.032	277.611±13.381$^{\#}$
G5 (PCM + NAC)	4.342 ± 0.188	87.425 ± 4. 25	310.957±10.725$^{\#}$

*p<0.05 shows that the group is significantly different from the control group
$^{\#}$p<0.05 shows that the group is significantly different from the PCM group

Table 3. Effects of MECL on kidney GSH levels, GST, SOD and catalase activities in PCM-induced nephrotoxicity. PCM (paracetamol); MECL (methanol extract of *C. crepidioides* leaves), NAC (N-acetylcysteine)

Tabela 3. Učinki MECL na koncentracijo GSH ter GST, SOD in katalazno aktivnost v ledvicah pri PCM-inducirani nefrotoksičnosti. PCM (paracetamol); MECL (metanolni ekstrakt listov *C. crepidioides*), NAC (N-acetilcistein)

Groups	GST activity (µ mole/min/mg protein)	SOD activity (µmole/min/mg protein)	Catalase activity (Katf)	GSH levels (µg/ml)
G1 (Control)	0.125±0.0031	7.532±0.270	0.0721±0.0021	0.009±0.0008
G2 (MECL)	0.092±0.0043$^{*\#}$	8.186±0.270$^{*\#}$	0.0952±0.0023$^{*\#}$	0.014±0.0009$^{*\#}$
G3 (PCM 250)	0.170±0.0013*	2.058±0.122*	0.0246±0.0012*	0.003±0.0008*
G4 (PCM + MECL)	0.118±0.0056$^{\#}$	3.412±0.083$^{*\#}$	0.0634±0.0082$^{\#}$	0.006±0.0004$^{*\#}$
G5 (PCM + NAC)	0.109±0.024$^{\#}$	4.453±0.064$^{*\#}$	0.0534±0.0043$^{*\#}$	0.008±0.0005$^{\#}$

*p<0.05 shows that the group is different significantly from the control group
$^{\#}$p<0.05 shows that the group is different significantly from the PCM group

Discussion

GSTs are toxicologically important enzymes because they catalyze the conjugation of N-acetyl-p-benzoquinone imine (NAPQI) with glutathione resulting in the detoxification of NAPQI (Arakawa et al. 2013). The significant increase in the activity of GST in the 250mg/kg bwt administered PCM group suggests that an overdose of PCM may result in the overactivation of GST. Hernandez et al. (2018) have also shown that exposure to foreign compounds can increase the activity of GST in adaptation to chemical stress. Bello et al. (2019) reported in one of their *in vitro* studies that MECL possesses antioxidant and anti-inflammatory activity. Thus, the decreased activity of the GST observed in MECL co-administered group may be due to the high level of antioxidants in MECL, resulting in reduced chemical stress and, thus, reduced oxidative stress on the cells of the kidney.

Kidney GSH level was depleted in the 250mg/kgbwt administered PCM group. This finding is consistent with those of Das et al. (2010), Demirbag et al. (2010) and Ahmad et al. (2012), in which a decrease in renal GSH concentration was observed in PCM-induced nephrotoxicity. GSH helps in the detoxification of PCM thus, prevent PCM-induced nephrotoxicity (Orji et al., 2020). Therefore, in GSH depletion, which may occur during chronic administration or when high doses are administered, detoxification of NAPQI is reduced, and excess NAPQI that is retained in the kidney may result in renal injury. The increase in GSH level in kidneys by MECL administration may therefore prevent PCM-induced nephrotoxicity. A similar study also showed that *C. crepidioides* increased the GSH level during rifampicin toxicity (Omoregie et al. 2015).

SOD is a key cellular antioxidant responsible for eliminating superoxide radicals (Younus, 2018). This study showed that 250 mg/kg/bwt PCM decreased renal SOD activity, ameliorated by MECL treatment. Demirbag et al. (2010) and Orji et al. (2020) also reported a decrease in rats' renal SOD levels after acute PCM overdose, while *C. crepidioides* were shown to strongly scavenge superoxide anion ($O_2^{\bullet -}$) and hydroxyl radicals ($HO^{\bullet}$) (Bello et al. 2019). Catalase prevents oxidative stress and hydroxyl radicals ($HO^{\bullet}$) formation by dismutating hydrogen peroxide (H_2O_2) to give oxygen and water (Nandi et al. 2019). Administration of PCM reduced catalase activity which was attenuated by MECL. Orji et al. (2020) also observed that paracetamol treatment significantly reduced the activity of catalase, while Oboh et al. (2021) showed that *C. crepidioides* increased catalase activity in the *Drosophila melanogaster* model of Alzheimer's disease. The reduced activity of SOD and catalase in 250 mg/kg/bwt PCM administered group is an indication of the reduced ability of the animals in this group to scavenge the reactive oxygen species, $O_2^{\bullet -}$, H_2O_2 and the $HO^{\bullet}$ which may eventually result in oxidative stress (Lenzen et al. 2022) in these animals. The increase in the activities of these antioxidant enzymes by administration of *C. crepidioides* may, however, scavenge the reactive oxygen species and prevent oxidative stress.

The results from this study suggest that PCM elevated renal MDA levels. This may be caused by the decrease in GSH level, catalase and SOD activities. Tripathi et al. (2019) and Canayakin et al. (2016) also suggested lipid peroxidation as a mechanism for PCM-induced oxidative stress and tissue damage. Decreased MDA levels observed in the MECL-treated group indicated that MECL may reduce oxidative stress in the kidneys of these animals. Although lipid peroxidation may be involved in the pathogenesis of PCM-induced nephrotoxicity and injury, Demirbag et al. (2010), no significant difference was observed in creatinine and urea levels in all groups. This indicates that although the increase in the level of lipid peroxidation by PCM might result in oxidative stress in the kidney, this might not be enough to cause serious damage to the kidney since serum urea and creatinine are nephrotoxicity markers (Salazar, 2014).

Similarly, co-administration of NAC with PCM attenuates the toxicity of PCM by significantly increasing SOD, Catalase activity and GSH levels. In addition, a significant decrease in GST activity and MDA levels in the kidney occurred in this group. MDA level and GST activity were also significantly decreased in *C. crcrepidioides*-only administered group relative to the control and relatively to PCM-only administered group, while GSH level, catalase and SOD activities were significantly increased in this group relative to the control and relatively to PCM-only, administered group.

In conclusion, the use of PCM is still unrestricted, as it can be obtained without a doctor's prescription. This study showed that PCM overdose caused oxidative stress in the rat kidney by increasing the MDA level and decreasing the activities of SOD, catalase and GSH level. This may eventually lead to nephrotoxicity and renal damage. Thus the non-restriction of PCM, which makes it easy for an overdose to be taken, poses a health risk. Hence, the need to protect against PCM-induced nephrotoxicity is in high demand. In this study, MECL increased the activities of SOD, catalase and GSH levels; thus, it is promising in protecting against oxidative stress and nephrotoxicity that may be induced by PCM overdose in the kidney.

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ABS

Original Research Article

Different susceptibility of two *Botrytis cinerea* strains to supercritical CO_2 plant extracts

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Abstract

Botrytis cinerea is an airborne plant pathogen with a necrotrophic lifestyle. As a generalist, *B. cinerea* has no host specificity and infects over 500 plant species. There are many studies about the phenotypic and genotypic diversity of *B. cinerea* strains worldwide. Two different morphological strains of *B. cinerea* were previously isolated also in Slovenia from buckwheat. The morphological diversity of *B. cinerea* is also reflected in different susceptibility to plant extracts. We tested the susceptibility of two *B. cinerea* strains derived from buckwheat grain to eleven extracts of plant species *Humulus lupulus, Nepeta cataria, Taraxacum officinale, Achillea millefolium, Calendula officinalis, Chamomilla recutita, Helichrysum arenarium, Hypericum perforatum, Juniperus communis, Sambucus nigra* and *Crataegus* sp. obtained by supercritical fluid extraction using CO_2 (SFE-CO_2). The resistance profiles showed that strain II of *B. cinerea* was generally susceptible to the action of these SFE-CO_2 extracts, whereas strain I was more resistant. The concentration-dependent antifungal activity of the chamomile extract and sandy everlasting indicates their possible use as a fungicide for both strains of *B. cinerea*.

Keywords

antifungal activity, *Chamomilla recutita*, fungal pathogen, *Helichrysum arenarium*, phytochemicals

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Različna občutljivost dveh sevov glive *Botrytis cinerea* za rastlinske izvlečke, pripravljene z ekstrakcijo s superkritičnim ogljikovim dioksidom

Izvleček

Botrytis cinerea je rastlinski patogen, ki se prenaša po zraku in ima nekrotrofni način življenja. Kot generalist nima specifičnega gostitelja in lahko okuži več kot 500 rastlinskih vrst. Obstaja veliko študij o fenotipski in genotipski raznolikosti sevov *B. cinerea* iz različnih regij sveta. Predhodno sta bila v Sloveniji iz ajde izolirana dva različna morfološka seva, ki kažeta različno občutljivost za rastlinske izvlečke. V raziskavi smo testirali občutljivost obeh sevov *B. cinerea* za enajst rastlinskih izvlečkov iz vrst *Humulus lupulus, Nepeta cataria, Taraxacum officinale, Achillea millefolium, Calendula officinalis, Chamomilla recutita, Helichrysum arenarium, Hypericum perforatum, Juniperus communis, Sambucus nigra* in *Crataegus* sp., pridobljnih z ekstrakcijo s superkitičnim ogljikovim dioksidom (SFE-CO_2). Iz rezulatov vidimo, da je bil sev II na splošno bolj občutljiv za delovanje izvlečkov SFE-CO_2, medtem ko je bil sev I bolj odporen. Koncentracijsko odvisna protiglivna aktivnost izvlečkov kamilice in smilja kaže, da sta rastlinska izvlečka lahko uporabna kot fungicida proti obema sevoma *B. cinerea*.

Ključne besede

protiglivna aktivnost, *Chamomilla recutita*, glivni pathogen, *Helichrysum arenarium*, rastlinske spojine

Introduction

Botrytis spp. are fungal pathogens that can cause pre- and postharvest disease in a wide variety of economically important crops. As a generalist, *B. cinerea* does not exhibit host specificity and infects over 500 plant species (Cheung et al., 2020). It causes grey mould disease in a wide range of crops, including vegetables, ornamentals, fruits, and cereals. The disease primarily affects flowers and buds, but infections can also occur on fruits, leaves, and stems. Grey mould rot is characterized by light brown to soft brown spots or blotches covered with a dusty mould that can cause seedlings, young shoots, and leaves to wilt and collapse and buds, flowers, and fruits to become blotchy and rotten. It is most destructive to mature or senescent tissues of dicotyledonous hosts, but it usually invades these tissues at a much earlier stage of plant development and remains dormant for a considerable period of time before rapidly decaying the tissues when the environment is favourable and the host physiology changes (Williamson et al., 2007).

There are numerous studies on the phenotypic and genotypic diversity of *B. cinerea* isolates from different regions of the world (Martinez et al., 2008; Isenegger et al., 2008; Kuzmanovska et al., 2012; Asadollahi et al., 2013; Kumari et al., 2014; Plesken et al., 2021). Two different morphological isolates of *B. cinerea* were also previously isolated from buckwheat in Slovenia (Kovačec et al., 2016). The genetic diversity and phenotypic characteristics of the different isolates may be the reason why *B. cinerea* can infect a large number of potential plant hosts (Corwin et al., 2016). As a result, it can counteract a wide range of plant defence chemicals. Control of diseases caused by *B. cinerea* has been largely dependent on the use of synthetic fungicides. Therefore, *B. cinerea* is a pathogen at high risk for developing resistance to fungicides (Hahn, 2014). High frequencies of isolates resistant to one or more chemically unrelated fungicides have been reported in *B. cinerea* populations from many crops (Zhao et al., 2010; Weber, 2011; De Miccolis Angelini et al., 2014; Rupp et al., 2017; Saito and Xiao, 2018). Therefore, due to the fungicide-resistant phenotypes in the *B. cinerea* population among crop hosts, an alternative control method, such as biological control of the fungus, is a better choice and must be integrated into existing rot management programmes to control this emerging disease (Ons et al., 2020). In addition, stricter regulations on the use of chemicals and the modern view of harmful residues have led to a renewed focus on natural products such as plant extracts and essential oils and their use as antifungal agents. Šernaite et al. (2020) reported that cinnamon extract proved to be the most effective agent against apple grey mould. Behshti et al. (2020) confirmed the antifungal activity of the essential oils of anise, fennel, chamomile, and majaroam applied to fruits of grape both *in vitro* and *in vivo* after harvest. Among them, anise oil completely inhibited the growth of *B. cinerea* in vitro. Supercritical fluid extraction with CO_2 (SFE-CO_2) has gained attention as an environmentally friendly extraction method because CO_2 is a non-toxic, inert, and available solvent, and the extracts obtained are chemically more complex or different compared to conventional extracts (Fornari et al., 2012).

The aim of this study was to determine whether the morphological diversity of *B. cinerea* isolates from buckwheat is also reflected in the differential susceptibility to plant extracts. Although there is a report on the morphological and biochemical diversity of *B. cinerea* isolates from buckwheat (Kovačec et al. 2016), there are no reports on their pathogenic diversity. Given the different growth rates and morphology, different susceptibility of the two fungal strains to plant extracts is also expected.

Materials and Methods

Plant extracts

Supercritical CO_2 extracts of 11 plant species were obtained from IME Insol (Slovenia), using flowers but also whole herbs and fruits of wild-grown plants from the region of Bihač, Bosnia and Herzegovina (Table 1). The extracts were prepared with a BBES 2.0 extraction system (Waters, Milford, MA, USA) under 200-300 bar and 40-50 °C and analyzed for their content using a GC-MS system (GCMS-QP2010 Ultra, Shimadzu, Japan) as described in Schoss et al. (2022).

Fungal growth inhibition assay

The antifungal activities of the SFE-CO_2 extracts were tested against *B. cinerea* previously isolated from buckwheat grains (Kovačec et al., 2016) and deposited in the fungal bank of the Plant Physiology Laboratory (Biotechnical Faculty, University of Ljubljana, Slovenia). First, *B. cinerea* colonies were divided into two morphologically distinct groups based on mycelial growth rate and sclerotia formation. Subsequently, the fungal genomic DNA of each group was isolated using GenElute® Plant Genomic DNA Miniprep Kit (Sigma, USA) according to the manufacturer's instructions. DNA amplification was performed by PCR (Minicycler PTC 150, MJ Research) using Taq DNA polymerase and the primer pair ITS1F/ITS4 (Kovačec et al., 2016). The reaction mixtures and PCR conditions were the same as described in Likar and Regvar (2013). Purification and sequencing of PCR products were performed by Macrogen (The Netherlands). To identify the strains, the sequences were subjected to BLAST searches within the NCBI database (https://www.ncbi.nlm.nih.gov/).

The inhibitory effect of SFE-CO_2 extracts on the radial growth of *B. cinerea* mycelia was tested according to the

Table 1. The list of plants and plant parts used for the preparation of SFE-CO_2 extracts.

Tabela 1. Seznam rastlin in njihovih delov, uporabljenih za pripravo izvlečkov SFE-CO_2.

Plant	Latin name	Family	Plant part
Yarrow	*Achillea millefolium*	*Asteraceae*	flowering herb
Common marigold	*Calendula officinalis*	*Asteraceae*	flower
Sandy everlasting	*Helichrysum arenarium*	*Asteraceae*	flower
German chamomile	*Chamomilla recutita*	*Asteraceae*	flower
Common hops	*Humulus lupulus*	*Cannabaceae*	flower
Dandelion	*Taraxacum officinale*	*Cichoriaceae*	flower
Common juniper	*Juniperus communis*	*Cupressaceae*	fruit
St. John's wort	*Hypericum perforatum*	*Hypericaceae*	flowering herb
Catnip	*Nepeta cataria*	*Lamiaceae*	herb
Hawthorn	*Crataegus* sp.	*Rosaceae*	flower
Black elderberry	*Sambucus nigra*	*Sambucaceae*	flower

method described in our previous study (Anžlovar and Dolenc Koce, 2014). First, a 10% extract was prepared by mixing 0.1 g of each SFE-CO_2 extract with 1 mL of 70% ethanol (Merck, Germany) and stirring the extract on a vortex (IKA, USA) until it dissolved. Elderberry and dandelion extracts were dissolved in 100% acetone (Merck, Germany).

A volume of 50 µL of the 10% SFE-CO_2 extracts was spread with a Drigalski spatula in a Petri dish (2r = 90 mm; Golias, Slovenia) containing 2% (w/v) potato dextrose agar (Biolife, Italy). Disks of *B. cinerea* mycelia (2r = 5 mm) were cut from the margins of 7-day-old fungal cultures and aseptically inoculated by placing them in the centre of a fresh plate containing the extract. Control samples with 70% ethanol or 100% acetone and without extracts were prepared at the same time. fungal colonies were incubated for seven days at room temperature (23±2 °C) in the dark. Mycelial growth was assessed on the 7th day after the inoculation. The plates were photographed with a digital camera (EOS 1000D, Canon, Tokyo, Japan), and the area (cm^2) of fungal colonies was measured using the image analysis software ImageJ (Schneider et al., 2012). Inhibition of fungal growth was expressed as a percentage of growth reduction and calculated according to the formula of Anžlovar et al. (2020):

Inhibition (%) = $(AC - AT) / AC \times 100$,

where *AC* is the area of mycelial growth of control colonies, and *AT* is the area of mycelial growth of treated colonies. Three replicates (N = 3) were performed for the controls and each treatment with SFE-CO_2 extract.

In addition, the two most active extracts (chamomile and sandy everlasting) were tested for antifungal activity at concentrations of 50%, 25%, 12.5%, 6.25%, and 3.13%.

Statistical analysis

The data were statistically analyzed to calculate mean values and standard errors, and the treatments were compared with a t-test (MS Excel). The level of significance was set at a P-value < 0.05.

Results and Discussion

Differences between morphological types of *B. cinerea* isolated from buckwheat have been observed previously (Kovačec et al., 2016). *Botrytis cinerea* strain I formed circular, white, and cotton-like colonies with filiform margins, whereas strain II had lobed margins, and the colonies were compact with black sclerotia (Figure 1). The growth of the two strains was also significantly different ($p < 0.0001$); the mean size of fungal colonies after seven days of growth was 47.98 ± 1.67 cm^2 for *B. cinerea* strain I and 12.33 ± 3.00 cm^2 for *B. cinerea* strain II (N = 12). The same type of differences in growth between *B. cinerea* strains has also been reported previously (Martinez et al., 2003; Kovačec et al., 2016).

The morphological diversity of the two *B. cinerea* strains was also reflected in different resistance profiles to the SFE-CO_2 extracts used in this study. Strain II was generally sensitive to these SFE-CO_2 extracts, whereas

Figure 1. Mycelium of *Botrytis cinerea* strain I (left) and strain II (right) on PDA 7 days after inoculation.

Slika 1. Micelij glive *Botrytis cinerea* sev I (levo) in sev II (desno) na krompirjevem agarju 7 dni po inokulaciji.

strain I was more resistant (Table 2). The growth of strain I was most strongly inhibited by extracts of chamomile and sandy everlasting. Extracts of hops, juniper and yarrow also significantly inhibited strain I growth, but their inhibition was much lower and close to control values. In contrast, the growth of strain II was significantly inhibited by almost all extracts (chamomile, hops, marigold, black elderberry, catnip, hawthorn), except for St. John's wort extract, which had a growth-promoting effect on it (Table 2). The yarrow extract showed markedly different strain-specific inhibition of *Botrytis* growth, with strain II being completely inhibited, whereas strain I was inhibited by less than 10% (Table 2). A similar but less pronounced pattern was also observed with SFE-CO_2 extracts of common juniper, common hops, common marigold, and catnip. An even more pronounced strain-specific pattern of *Botrytis* growth was observed with SFE-CO_2 extracts of black elderberry, dandelion, and hawthorn, where the growth of strain I was promoted by 1-6 %, while the growth of strain II was inhibited by 40-80% (Table 2).

The different resistance profiles of the two *B. cinerea* strains were the expected result considering the different morphological types and growth rates of the two strains. Kovačec (2016) reported that these two *B. cinerea* strains differed by orders of magnitude in their cellulase and polyphenol oxidase activities, indicating major differences in their biology. Because *B. cinerea* can be necrotrophic, cellulase activity is essential for the degradation of plant cell walls to obtain nutrients (Boddy, 2016) and is, therefore, important for the pathogenicity of the fungus. Anand et al. (2008) reported that virulent isolates of *Alternaria alternata* had higher production of cellulolytic enzymes compared to avirulent isolates. Kumari et al. (2014) found that higher concentrations of oxalic acid and higher activity of lytic enzymes were associated with the pathogenicity of *B. cinerea* isolates. On the other hand, the resistance profiles of the two strains are not correlated with the production of extracellular enzymes, as strain II, which was more susceptible to SFE-CO_2 extracts, has much higher cellulase and polyphenol oxidase activity than strain I (Kovačec 2016). Saito (2018) reported that of 200 *B. cinerea* isolates obtained from mandarin fruit, five fungicide-resistant phenotypes with triple resistance to azoxystrobin, pyrimethanil, and thiabendazole were detected. Five percent of phenotypes were resistant to one fungicide class, 23.5% to two fungicide classes, and 62% to three fungicide classes.

Since the strongest inhibition of the two *B. cinerea* strains was obtained with the extracts of chamomile and sandy everlasting, the antifungal activities were further evaluated using the 16-fold concentration range of these extracts, from 50% to 3.125%. In general, higher extract concentrations resulted in greater inhibition of fungal growth of the two *B. cinerea* isolates. Growth of the sensitive strain II and the resistant strain I was completely inhibited by the 50- and 25-per cent concentrations of

Table 2. Antifungal activity of 10% SFE-CO_2 plant extracts on mycelial growth of two strains of *Botrytis cinerea*. Data present means ± standard error (N=3). Statistically significant differences between control and extract treatment are marked with an asterisk (*).

Tabela 2. Protiglivna aktivnost 10% SFE-CO_2 rastlinskih izvlečkov na rast micelija *Botrytis cinerea* I in II. Podatki so povprečja ± standardne napake (N = 3). Zvezdica (*) prikazuje statistično značilno razliko (P < 0,05) med izvlečkom in kontrolo.

	Growth inhibition (%)	
SFE-CO_2 extract	***B. cinerea* strain I**	***B. cinerea* strain II**
German chamomile	95.77 ± 7.32*	100.00± 0.00*
Sandy everlasting	59.09 ± 4.10*	49.81 ± 8.36
Common hops	37.91 ± 10.56*	76.87 ± 14.47*
Common juniper	13.10 ± 3.47*	67.01 ± 7.85
Yarrow	7.37 ± 3.60*	100.00 ± 0.00*
Common marigold	3.98 ± 2.81	69.57 ± 6.59*
Black elderberry	-5.79 ± 5.60	81.13 ± 3.68*
Catnip	6.65 ± 5.28	87.57 ± 2.19*
St. John's wort	8.38 ± 4.03	-11.86 ± 3.47*
Dandelion	-1.61 ± 5.46	44.76 ± 12.83
Hawthorn	-5.40 ± 1.2	79.43 ± 5.14*

chamomile SFE-CO_2 extract, and both showed similar dose-dependent inhibition of fungal growth (Fig. 2). The sandy everlasting SFE-CO_2 extract had different effects on the growth of the two isolates: while the growth of strain I was significantly dose-dependent inhibited, the growth of strain II was strongly inhibited even by the lowest, 3.125% concentration of sandy everlasting extract (Fig. 2). The composition of the sandy everlasting SFE-CO_2 extract was chemically poorly identified and not comparable to what was previously reported for its essential oil (Schoss et al., 2022). The major constituent was γ-curcumin, which may be responsible for the antifungal activity of the sandy everlasting extract, as it was not found in other SFE-CO_2 plant extracts we studied (Schoss et al., 2022).

The chamomile flower SFE-CO_2 extract completely inhibited the growth of both strains (Fig. 2). The major components of the chamomile extract are α-bisabolol oxide A and α-bisabolol oxide B (Schoss et al., 2022). Bisabolol and its oxidized metabolites contribute to the therapeutic properties of chamomile. α-bisabolol has a Generally Regarded as Safe status (GRAS) and has been found to have antibacterial, antifungal, insecticidal, anti-inflammatory, and anti-ulcer activity (Avonto et al., 2013). While α-bisabolol is found in a wide variety of plants, bisabolol oxides are less common natural products, but all these compounds are of potential pharmacological importance (Avonto et al., 2013). The second major constituent of SFE-CO_2 extract from chamomile flower is tonghausu (Schoss et al., 2022), which is known as an antifeedant compound in the Chinese vegetable tonghao and other plants of the Asteraceae tribe Anthemideae (Chen et al., 2004). Polygodial, which exhibits antifungal activity against normal and resistant isolates of *B. cinerea*, also shows insect antifeedant activity (Carrasco et al., 2017). Similarly, tonghausu, which has antifeedant and antifungal activity, could be the reason for the wider antifungal activity of chamomile extract, as it exhibits inhibitory activity against both strains of *B. cinerea*. The strong antifungal activity of the chamomile extract could also be due to the α-bisabolol oxide (Lucca et al., 2011), as these were not detected in the other SFE-CO_2 extracts tested.

The two *B. cinerea* strains were resistant to St. John's wort extract (Table 2), whose main components are caryophyllene oxide, heneicosane, phytol, and caryophyllene (Schoss et al., 2022). St. John's wort is a chemically and pharmacologically well-studied medicinal plant. Extracts from the aerial parts are used internally to treat depression and externally to cure skin disorders (Tocci et al., 2018). The xanthone-rich extracts from the roots are being studied for their marked antifungal activity against human pathogens, including *Candida* species (Zubricka et al., 2015), and their activity against planktonic cells and biofilm of *Malassezia furfur* (Simonetti et al., 2016). Crockett et al. (2011) isolated three xanthones from St. John's wort root extract and tested them for growth inhibition of plant pathogenic fungi of the genera *Colletotrichum*, *Botrytis*, *Fusarium*, and

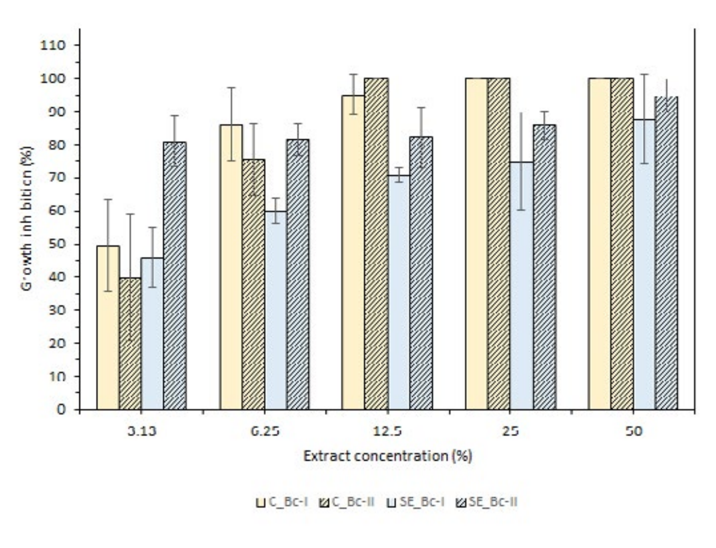

Figure 2. Concentration-dependent antifungal activity of chamomile and sandy everlasting SFE-CO_2 extracts. Data present means ± standard error (N = 3). Legend: C, chamomile; SE, sandy everlasting; Bc-I, *B. cinerea* strain I; Bc-II, *B. cinerea* strain II

Slika 1. Koncentracijsko odvisna protiglivna aktivnost SFE-CO_2 izvlečkov kamilice in smilja. Podatki so povprečja ± standardne napake (N = 3). Legenda: C, kamilica; SE, smilj; Bc-I, *B. cinerea* sev I; Bc-II, *B. cinerea* sev II

Phomopsis. Xanthone 1 was identified as a novel inhibitor of the plant pathogenic fungi *Phomopsis obscurans* and *P. viticola*, but no inhibition of *Colletotrichum*, *Botrytis*, and *Fusarium* species was observed. Since the SFE-CO_2 St. John's wort extract in this study was prepared from a flowering herb, the xanthone-rich roots would be a better choice for testing the antifungal activity of St. John's wort.

Control of diseases caused by *B. cinerea* depends largely on the use of fungicides. However, *B. cinerea* is a pathogen at high risk of developing resistance to fungicides (Hahn, 2014). The objective of this study was to test the resistance of two *B. cinerea* strains derived from buckwheat grain to eleven SFE-CO_2 plant extracts. The resistance profiles showed that strain II of *B. cinerea* was generally susceptible to the action of these SFE-CO_2 extracts, while strain I was more resistant. The extracts of chamomile and sandy everlasting have the potential to be effective as fungicides for both strains.

Author Contributions

Conceptualization, SA; methodology, SA; software, JDK; validation, SA, JDK; formal analysis, SA, JDK; investigation, SA..; data curation, SA, JDK; writing—original draft preparation, SA..; writing—review and editing, JDK; funding acquisition, JDK. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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ABS

Original Research Article

The external glutamic acid application results in significant effects on some characteristics of sunflower (*Helianthus annuus* L.)

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Abstract

The present study was conducted to determine the effects of glutamic acid treatments at different doses on determined characteristics of some sunflower varieties. Glutamic acid treatments were practiced as foliar sprays at four different concentrations (0, 100, 200 and 300 mg l^{-1}) to three different sunflower varieties (Sakha, Amar and Ishaqi-1). Field experiments were conducted in randomized blocks in a split-plot experimental design with three replications under Anbar-Iraq conditions in 2021. The highest average leaf area index was 5.82 cm^2. The 100 mg l^{-1} glutamic acid treatments of the variety Aqmar yielded the highest chlorophyll content (40.51%). For the variety Ishaqi-1, the 100 mg l^{-1} glutamic acid treatment resulted in the highest protein content (15.51%). However, the 200 mg l^{-1} glutamic acid treatments of the variety Ishaqi-1 resulted in an increase in empty seeds rate (75.29%) and oil content (44.09%). In general, medium external glutamic acid doses (100 mg l^{-1} and 200 mg l^{-1}) increase the chlorophyll content and protein content, and oil content, respectively. But, the empty seed rate also increased with 200 mg l^{-1} application.

Keywords

Sunflower, Amino Acids, Glutamic Acid, Oil Content, Protein Content, Leaf Area Index, Chlorophyll Content, Empty Seed Rate

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Aplikacija glutaminske kisline pomembno vpliva na nekatere lastnosti sončnice *Helianthus annuus*

Izvleček

V pričujoči študiji smo ugotavljali učinke tretiranja z glutaminsko kislino v različnih odmerkih na določene lastnosti nekaterih sort sončnic. Tretiranje z glutaminsko kislino je bilo izvedeno kot listno škropljenje v štirih različnih koncentracijah (0, 100, 200 in 300 mg l^{-1}) na treh različnih sortah sončnic (Sakha, Amar in Ishaqi-1). Poljski poskusi so bili izvedeni v randomiziranih blokih v poskusni zasnovi z razdeljenimi ploskvami s tremi ponovitvami na območju Anbar-Irak (leto 2021). Najvišje povprečje indeksa listne površine je bilo 5,82 cm^2. Po tretiranju z 100 mg l^{-1} glutaminske kisline pri sorti Aqmar smo določili najvišjo vsebnost klorofila (40,51 %). Pri sorti Ishaqi-1 smo po obdelavi z 100 mg l^{-1} glutaminske kisline določili najvišjo vsebnost beljakovin (15,51 %). Vendar se je pri obdelavi z 200 mg l^{-1} glutaminske kisline pri sorti Ishaqi-1 povečala stopnja praznih semen (75,29 %) in vsebnost olja (44,09 %). Na splošno lahko zaključimo, da srednji odmerki glutaminske kisline (100 mg l^{-1} in 200 mg l^{-1}) povečajo vsebnost klorofila in beljakovin ter vsebnost olja. Pri uporabi 200 mg l^{-1} pa se poveča tudi delež praznih semen.

Ključne besede

Sončnica, aminokisline, glutaminska kislina, vsebnost olja, vsebnost beljakovin, indeks listne površine, vsebnost klorofila, delež praznih semen

Introduction

Sunflower (*Helianthus annuus* L.), belonging to *Asteraceae* (*Compositae*) family, is one of the most important oil crops in the world and ranks third after soybean and rapeseed in the amount of oil produced at the global level. Sunflower originated from Mexico in Central America and then moved to European countries, especially to Spain (Al-Fahaadi, 2012).

The importance of the sunflower crop comes from the fact that its seeds contain a high percentage of oil that reaches nearly 50% of seed mass in some of the improved varieties, therefore providing the largest amount of oil per unit of cultivated area, in addition to good taste characteristics of the oil (Khalaf and Rahman, 2015). Since seed oil of this crop is characterized by its good taste, it is widely used in the manufacture of high nutritional oils, manufacture of butter, bread and biscuit products. It is also used in the manufacture of soap and gums. According to results of the study of Vasudha and Sarla (2021), sunflower seed cake contains 37.10% crude protein and 250.56 kcal energy per 100g, therefore, it is also a good sources of fodder for farm animals. Gul and Coban (2020) recorded that the oil contents of ten varieties in two different locations varied from 36.47 to 50.29%. Radić et al. (2009) reported that oil contents and protein contents varied between 31.34-48.90% and 15.07-26.74% for two genotypes and two locations, respectively expressed depend on result of their study with tow genotypes in two locations.

Recently, the importance of this oil crop has increased as a result of the shortage in the produced quantity of oils in the world. In the Middle East, sunflower is the third most important oil seed crop after soybean and palm oil (Al-Amery et al., 2011). Currently, it ranks second in terms of production after soybean and first in oil production in Iraq (Al-Rawi and Kavanagh, 1998). Sunflower is the first oil crop in Iraq (Ali Atiyah and Hasson Kadhim, 2019). Iraq is ranked fifth after Morocco, Syria, Egypt, and Tunisia in terms of the area planted with this crop in the Middle East (Nasralla et al., 2014). The productivity of this crop in Iraq is still below the required level due to the failure to follow the correct scientific methods and practices in sunflower cultivation.

Breeding and introduction of new varieties of sunflower in Iraq are needed to test them in terms of biological and agronomic characteristics and quality in Iraq and anywhere exposed to these new varieties. In the view of selecting suitable varieties for specific areas, scientific experiments were performed among others by Hamza et al. (2011), Nasralla et al. (2014), Al-Waeli et al. (2018), Abdel-Hafeez et al. (2019), Aljubouri et al. (2019), Al-Ramadan et al. (2021) in a similar temperate climate to Anbar. The difference in characteristics of the sunflower genotypes mostly comes from the difference in the physiological processes of these genotypes after flowering (de la Vega and Hall, 2002). Several studies have been conducted to compare different genotypes of sunflower, most of which indicated that the characteristics of sunflower could differ between varieties and according to climatic conditions, where they are grown (Kaya et al., 2007; Ayad et al., 2010; Dutta et al., 2011; Kaleem et al., 2011; Sarwar et al., 2013).

Amino acids, especially glutamic acid and glycine, enter the formation of the chlorophyll molecule and their use increased the rate of photosynthesis in plants. Amino acids and their activities in different plant growth stages increase the ability of the cell to absorb water and dissolved nutrients from the growth medium and thus increase vegetative growth. Amino acids increase protein synthesis and are involved in several plant functions, enhance metabolism and the rate of carbon uptake and increase total dry matter (Sharma-Natu and Ghildiyal, 2005; Dromantiene et al., 2013).

Glutamic acid is one of the amino acids that make up the proteins of all organisms and one of the most abundant ones in nature. Since organisms have intrinsic pathways for their biosynthesis, it is not considered essential (Forde and Lea, 2007). Glutamic acid belongs to the group of negatively-charged polar amino acids. It was discovered in 1866 by a German chemist Rittershausen while studying hydrolyzed wheat gluten, hence it was named "glutamic" acid. After the discovery, its presence was determined in a large part of living organisms, which is why, it is believed to have essential functions for life. It has a central role in the balance between carbon and nitrogen within the plant, as well as the synthesis of important proteins. It enters the synthesis of chlorophyll, which leads to an increase in the rate of photosynthesis. Glutamic acid is an α-amino acid that has a central carbon atom, the α-carbon, to which four other groups are attached: a carboxyl group, an amino group, a hydrogen atom and a substituent group (side chain or R group). The R-group of glutamic acid gives the molecule a second carboxyl group (-COOH) and its structure -CH2-CH2-COOH (-CH2-CH2-COO-in its ionized form), so the sum of the carbon atoms of the molecule is five. Glutamic acid releases special signals for growth. It plays an important role in balancing nutrients through plant tissues and increases the consumption and absorption of elements in addition to making up carbohydrates and proteins in the plant and improving physiological characteristics (Forde and Lea, 2007). The amino acid “glutamic acid”, which is in the free state in the form of L-Glutamic, activates the biosynthesis of (proline) L-Proline, which is one of the most important amino acids that help plants to resist most stress conditions such as salinity, cold and high temperatures, as well as drought (Farid et al., 2020).

Previous studies have proven that foliar sprays were effective in increasing some biological and agricultural characteristics.Alak (2016) conducted field experiments on the sunflower (*Helianthus annuus* L.) cultivar Luleo for two seasons to examine the phenotypic characteristics, yield and growth parameters under different irrigation treatments (40%, 50%, and 60%) and foliar proline (derived from glutamic acid) sprays at different concentrations (0, 30, 60 and 90 mg l^{-1}). Amino acid concentrations had significant effects on most of the studied traits. In both seasons, the greatest percentage of fertility (70.20% – 81.45%) was obtained from 60 mg l^{-1} amino acid concentration.

Majid and Al-Bahadli (2016) conducted field experiments to study the response of the sunflower (*Helianthus annuus* L.) cultivar Flame to sprays of amino acid proline derived from glutamic acid at different concentrations (0, 50, and 100 mg l^{-1}) and to different irrigation durations. Results revealed that amino acid concentration × irrigation duration interactions had significant effects on most of the studied traits. The highest average leaf area (0.41 m^2) was obtained from 100 mg l^{-1} proline concentration. They noted that seed oil contents also increased with increasing amino acid concentrations. Dahi et al. (2015) conducted field experiments in Baghdad during the spring season of 2013 to study the effects of different water stress levels (20%, 50% and 80%), different proline concentrations (0, 150 and 300 mg l^{-1}) and salicylic acid concentrations (0, 200, and 400 mg l^{-1}) on yield and growth parameters of Shmoos sunflower variety. The results of the study showed that 200 mg l^{-1} salicylic acid concentration was the most effective and yielded an average leaf area of 0.3494 m^2, and a total chlorophyll content of 0.802%. Mahdi et al. (2009) indicated in a study of two sunflower cultivars (Aqmar and Brodeferic) that the Aqmar cultivar was significantly superior in oil content, as it recorded the highest mean for oil content (47.68%), while the cultivar Brodeferic recorded the lowest mean value (45.18%).

The present experimental study was conducted to determine the effects of foliar glutamic acid treatments externally at different concentrations on various biological and agronomical traits of different sunflower varieties in Anbar district of Iraq, which are characterized by high temperatures throughout the growing season.

Materials and Methods

The present study was carried out in a private field in the city of Hit, in Anbar Governorate, western Iraq during the spring season in 2021. Effects of foliar sprays of glutamic acid at different concentrations (0, 100, 200, 300 mg l^{-1}) on yield and growth parameters of three sunflower cultivars (Sakha, Aqmar, Ishaqi-1) were investigated.

The experiments were conducted in randomized blocks in a split-plot experimental design with three replications. Glutamic acid concentrations coded Glu-0, Glu-100, Glu-200 and Glu-300 (0, 100, 200, 300 mg l^{-1}, respectively) were applied in the sub-plots and sunflower cultivars were placed into the main plots. Experimental plots were 3.50 m long and 2.80 m wide (9.8 m^2). Sowing was performed on 3/08/2021 at 0.70 m row spacing and 0.25 m on-row plant spacing. Weed control was done by hoeing and weeding. Pre-sowing period, diammonium phosphate (DAP, 18-46-0 % NPK) fertilizer was applied by calculating of 200 kg ha^{-1}. When plants reached 25-30 cm height 150 kg ha^{-1} urea (48% N) applied as a second batch of nitrogen fertilizer. During the growing season, five times irrigation was performed at 70-100 mm in each time when plants needed by the basin irrigation system. Glutamic acid foliar sprays were performed in two growth stages of plants: the first one at the beginning of plant growth seedling stage and the second one at the beginning of the head formation stage.

Measured characteristics were leaf area index, chlorophyll content, empty seed rate, oil content, and protein content. Leaf area index was calculated with the use of the following equation: Leaf area index = Average leaf area of per plant / the area that a plant occupies on the ground. Leaf chlorophyll content (%) was determined by using A SPAD meter (SPAD 502) by performing five readings on middle leaves. To determine the empty seed rate (%), about 50 g of seeds were taken from each plot, then the number of empty and filled seeds were counted. And, empty seed rate was calculated as: number of empty seeds / total number of seeds × 100. To determine oil content, seeds were ground and placed into a beaker, then supplemented with ethanol and placed into hexane-supplemented Soxhlet extraction device at 37°C for 12 hours (Chapman and Pratt, 1961). The following equation was used to calculate the oil content of the samples: Percentage of oil = Weight of oil extracted from seeds / weight of sample seeds × 100. To determine protein content, initially, sample total nitrogen (N) content was determined with the use of micro-kjeldahl method. The resulting N content was then multiplied by a coefficient of 25.6 to get protein content of the samples. And, used the formula: protein content = Total N × 25.6.

ANOVA, Variance and Standard Error analyses were used to determine the significance of differences and comparing means of treatments by using MSTAT-C software package (Freed et al.,1989).

Results and Discussion

Leaf Area Index (cm^2)

There were significant differences in leaf area values of the sunflower varieties (Table 1). The highest value (5.82 cm^2) was obtained from the variety Aqmar and the lowest value (5.06 cm^2) was obtained from the variety Sakha. In terms of leaf area index values of concentration × variety interactions, the highest value (6.26 cm^2) was obtained from Glu-100 (100 mg l^{-1}) treatments of the variety Aqmar and the lowest value (4.57 cm^2) was obtained from Glu-0 (control) treatments of the variety Sakha.

For leaf area index values at different glutamic acid concentrations, the highest value (5.78 cm^2) was obtained from Glu-300 (300 mg l^{-1}) and the lowest value (5.00 cm^2) was obtained from the Glu-0 (Control) treatments.

Present findings revealed that there were significant differences in leaf area index values of the sunflower varieties, probably because of the genetic structures of the varieties. Adeem (2015) also reported different leaf areas and thus leaf area index values for different sunflower varieties. Abdel Latef and Tran (2016) reported that amino acid treatments had significant effects on the growth parameters of maize plants, increased the production of soluble proteins, total free amino acids and proline, thus improving plant growth through enhancing photosynthetic activity and antioxidant mechanisms. There were significant differences in leaf area index values obtained

Table 1. Leaf area index values (cm²) of the sunflower cultivars exposed to external glutamic acid treatments

Tabela 1. Indeks listne površine (cm²) pri različnih sortah sončnic podvrženih tretiranju z glutaminsko kislino.

Leaf area index (cm²)					
Varieties	Glutamic Acid Concentrations				Mean Var.
	Glu-0	Glu-100	Glu-200	Glu-300	
Sakha	4.57i	4.76h	5.25f	5.64cd	**5.06c**
Aqmar	5.51de	6.26a	5.82c	5.70c	**5.82a**
Ishaki-1	4.93gh	4.98g	5.42ef	6.01b	**5.34b**
Mean Glu Con.	**5.00d**	**5.33c**	**5.50b**	**5.78a**	

SE± = 0.085
C. V. = 9.435

at different glutamic acid concentrations. Adeem (2015) also reported increasing leaf area index values with amino acid treatments. Consistent with the present findings, El-Hawary and Nashed (2019) reported significant effects of ascorbic and salicylic acid treatments on growth parameters of maize plants.

Chlorophyll Content (%)

In terms of chlorophyll contents of the varieties, the highest value (40.51%) was obtained from the variety Aqmar and the lowest value (39.03%) was obtained from the variety Sakha, with no significant difference between the variety Sakha and Ishaqi-1 (Table 2). There were significant differences in chlorophyll contents for the interactions. The highest value (41.50%) was obtained from Glu-100 (100 mg l^{-1}) treatments of the variety Aqmar and the lowest value (37.73%) was obtained from Glu-0 (control) treatments of the variety Sakha. Differences in chlorophyll contents of glutamic acid concentrations were not found to be significant. The highest value (40.38%) was obtained from Glu-100 (100 mg l^{-1}) treatments and the lowest value (38.53%) was obtained from Glu-0 (control) treatments.

Statistical analyses revealed that there were significant differences in chlorophyll contents of the sunflower varieties, since amino acid sprays reduced the harmful effects of stress factors and increased photosynthetic pigments (Sadak and Mostafa, 2015). Consistent with the present findings, Yan et al. (2011) indicated that proline treatments improved the defense mechanisms of horticultural crops against environmental stress, then promoted photosynthesis and enzyme activity. There were also significant differences in chlorophyll contents obtained at different glutamic acid concentrations, because amino acids worked as a growth regulator to maintain cell cytoplasm and build chlorophyll and carbohydrates (Al-Qaisi, 2016). Consistent with the present findings on chlorophyll contents, Hayat et al. (2012) found that amino acids acted on osmotic regulation of cells and protection of chromosomal systems under changing environmental conditions.

Table 2. Chlorophyll contents (%) of the sunflower cultivars exposed to external glutamic acid treatments.

Tabela 2. Vsebnost klorofila II (%) pri različnih sortah sončnic podvrženih tretiranju z glutaminsko kislino.

Chlorophyll Content (%)					
Varieties	Glutamic Acid Concentrations				Mean Var.
	Glu-0	Glu-100	Glu-200	Glu-300	
Sakha	37.73f	39.57cde	38.83e	39.97bcd	**39.03b**
Aqmar	39.23de	41.50a	40.57abc	40.73ab	**40.51a**
Ishaki-1	38.63ef	40.07bcd	40.43abc	39.17de	**39.58b**
Mean Glu Con.	**38.53b**	**40.38a**	**39.94a**	**39.96a**	

SE± = 0.194
C. V. = 2.935

Percentage of Empty Seeds (%)

Table 3 shows that there were no significant differences in the percentage of empty seeds of the varieties. The highest value (75.29%) was obtained from the variety Ishaqi-1 and the lowest value (74.52%) was obtained from the variety Aqmar. Interactions had significant effects on percentage of empty seeds. The greatest value (80.26%) was obtained from Glu-200 (200 mg l^{-1}) treatments of the variety Sakha and the lowest value (66.10%) was obtained Glu-0 (Control) treatments of the same variety. Significant differences were also seen in percentage of empty seeds of different glutamic acid concentrations. The greatest value (77.86%) was obtained from the Glu-200 (200 mg l^{-1}) treatments and the lowest value (62.24%) was obtained from the Glu-0 (Control) treatments. There were no significant differences between Glu-200 and Glu-300 treatments.

In the present study, statistical analyses revealed that there were no significant differences in percentage of empty seeds of the sunflower varieties, but there were significant differences in percentage of empty seeds obtained at different glutamic acid concentrations. The reason may be due to the role of amino acid in reducing the negative impact of stress, especially the water stress to which the plant was exposed throughout different growth stages. Taylor et al. (2002) reported positive effects of amino acid treatments on vegetative growth parameters through increasing carbon metabolism, dry matter accumulation, thus increasing the pollinated seeds in the head. Mohamed and Khalil (1992) reported positive effects of arginine treatments on yields components of some winter plants. Present findings on percentage of empty seeds are also consistent with the findings of Alak (2016) indicating the role of proline in improving yield and yield components of sunflower under water stress conditions.

Oil Content (%)

Table 4 shows that there were significant differences in the oil contents of the sunflower varieties. The highest value (44.09%) was obtained from the variety Sakha and

Table 3. Percentage of empty seeds (%) of the sunflower cultivars exposed to external glutamic acid treatments.

Tabela 3. Delež praznih semen (%) pri različnih sortah sončnic podvrženih tretiranju z glutaminsko kislino.

Percentage of Empty Seeds (%)					
Varieties	**Glutamic Acid Concentrations**				**Mean Var.**
	Glu-0	**Glu-100**	**Glu-200**	**Glu-300**	
Sakha	66.10h	75.56d	80.26a	78.05bc	**74.99a**
Aqmar	72.08f	73.99e	74.48de	77.51c	**74.52a**
Ishaki-1	69.52g	75.34d	78.85b	77.45c	**75.29a**
Mean Glu Con.	**69.24c**	**74.96b**	**77.86a**	**77.67a**	

SE± = 0.678
C. V. = 5.432

Table 4. Oil contents (%) of the sunflower cultivars exposed to external glutamic acid treatments.

Tabela 4. Vsebnost olja (%) pri različnih sortah sončnic podvrženih tretiranju z glutaminsko kislino.

Oil Content (%)					
Varieties	**Glutamic Acid Concentrations**				**Mean Var.**
	Glu-0	**Glu-100**	**Glu-200**	**Glu-300**	
Sakha	41.96d	45.55b	44.60bc	44.26bc	**44.09a**
Aqmar	39.75e	42.25d	47.49a	45.62b	**43.78ab**
Ishaki-1	38.23e	43.64cd	48.05a	43.00cd	**43.23b**
Mean Glu Con.	**39.98c**	**43.81b**	**46.71a**	**44.29b**	

SE± = 0.494
C. V. = 6.788

the lowest value (43.23%) was obtained from the variety Ishaqi-1. The differences between the variety Aqmar and Ishaqi-1 were not found to be significant. Interactions had also significant effects on oil contents. The greatest value (48.05%) was obtained from Glu-200 (200 mg l^{-1}) treatments of the variety Ishaqi-1 and the lowest value (38.23%) was obtained from Glu-0 (Control) treatments of the same variety. There were significant differences in oil contents of different glutamic acid concentrations. The greatest value (46.71%) was obtained from the Glu-200 (200 mg l^{-1}) treatments and the lowest value (39.98%) was obtained from the Glu-0 (control) treatments. There were no significant differences between Glu-100 and Glu-300 treatments.

Statistical analysis revealed that there were no significant differences in oil contents of the sunflower varieties, but there were significant differences in oil contents obtained at different glutamic acid concentrations. As compared to the control treatment, glutamic acid treatments generally increased oil contents, due to the role of amino acids in protecting the plant from oxidation resulting from plant exposure to environmental stresses such as high temperature and lack of water, consequently preserve oils from oxidation (Al–Fhadoya, 2016). This result is consistent with what Majid and Al-Bahadli (2016) mentioned in their study of the effect of proline in reducing moisture stress and prolonging irrigation periods for sunflower crop, where it was found that increasing proline from zero to 100 mg l^{-1} led to an increase in oil content from 28.33% to 32%. Present findings on oil contents are also consistent with the results of Hamed (2016) indicating significant effects of salicylic acid treatments on oil content of safflower plants.

Protein Content (%)

For protein contents of the varieties, the highest value (15.51%) was obtained from the variety Ishaqi-1 and the lowest value (14.63%) was obtained from the variety Aqmar. There was no significant difference between the variety Sakha and Aqmar and between the variety Sakha and Ishaqi-1. Interactions had significant effects on protein contents (Table 5). The greatest value (18.22%) was obtained from Glu-100 (100 mg l^{-1}) treatments of the variety Ishaqi-1 and the lowest value (13.31%) was obtained from Glu-0 (control) treatments of the variety Aqmar. There were significant differences in oil contents of different glutamic acid concentrations. The greatest value (16.51%) was obtained from the Glu-100 (100 mg l^{-1}) treatments and the lowest value (13.57%) was obtained from the Glu-0 (control) treatments. There was no significant difference between Glu-0 and Glu-200 treatments and between Glu-100 and Glu-300 treatments.

Statistical analyses revealed that there were significant differences in protein contents of the sunflower varieties, probably because of the differences in genetic structures of the varieties (Alsubaihi et al., 2020). There were also significant differences in protein contents obtained at different glutamic acid concentrations. Glutamic acid treatments increased protein contents since foliar application with amino acids can stimulate growth by increasing the activity of the antioxidant enzymes, thus it prevents protein loss and enhances the photosynthetic pigment (Paul and Nair, 2015). Al-Seedi and Al-Badry (2015) indicated that increasing acid concentrations encouraged the absorption of the basic elements for protein production (sulfur, phosphorous,

Table 5. Protein content (%) of the sunflower cultivars exposed to external glutamic acid treatments.

Tabela 5. Vsebnost proteinov (%) pri različnih sortah sončnic podvrženih tretiranju z glutaminsko kislino.

Protein Content (%)					
Varieties	**Glutamic Acid Concentrations**				**Mean Var.**
	Glu-0	**Glu-100**	**Glu-200**	**Glu-300**	
Sakha	13.63d	15.73bc	14.23cd	17.28ab	**15.22ab**
Aqmar	13.31d	15.59c	13.95d	15.68bc	**14.63b**
Ishaki-1	13.77d	18.22a	14.39cd	15.68bc	**15.51a**
Mean Glu Con.	**13.57b**	**16.51a**	**14.19b**	**16.21a**	

SE± = 0.276
C. V. = 10.933

and nitrogen) and the activity of some enzymes to make a protein. Raskin (1992) and Webber and Bledsoe (2002) also indicated that acid treatments promoted the production of proteins and some other compounds effective in inhibition of the activity of proteolytic enzymes such as proteases and peptidases.

Conclusions

The present study indicated that there were significant differences in leaf area index, chlorophyll content, empty seed rate, oil content, and protein content of the sunflower varieties.

According to the results of the study, the highest leaf area index was measured as 5.82 cm^2. The 100 mg l^{-1} glutamic acid treatments of the variety Aqmar yielded the highest chlorophyll content (40.51%). The 100 mg l^{-1} glutamic acid treatments of the variety Ishaqi-1 had the highest protein content (15.51%).

There were no significant differences in oil contents of the sunflower varieties, but there were significant differences in oil contents obtained at different glutamic acid concentrations. As compared to the control treatment, glutamic acid treatments generally increased oil contents. Statistical analyses revealed that there were significant differences in protein contents of the sunflower varieties, and, in general, glutamic acid treatments increased protein contents.

As a conclusion, the external glutamic acid application could have positive effects on the quality of sunflower.

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ABS

Review Article

A review of methodology for grassland restoration with practical examples

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Abstract

Currently, the majority of high nature value Slovenian grasslands have an unfavourable conservation status. Based on the available data from habitat type mappings, the surface of high nature value grasslands (6210(*) – semi-natural dry grasslands and scrubland facies on calcareous substrates, 6410 – *Molinia* meadows, and 6510 – lowland hay meadows) at Natura 2000 sites is decreasing. The existing agri-environment measures have been only partly effective in promoting grassland biodiversity. The main threats to biodiversity are driven by various anthropogenic activities, which result in a continuous change in landscape identity, habitat fragmentation, and ecosystem degradation. Therefore, biodiversity restoration became an urgent step in the conservation of high nature value grasslands. Multiple pathways may lead to the ecological restoration of grasslands with an altered, uncharacteristic floristic composition, or grasslands with an unfavourable conservation status. In this paper, we present an overview of the methods used in the restoration of grasslands from different parts of Europe. In an adapted form, these methods could also be used for the restoration of Slovenian grasslands. Grasslands may be left to spontaneous succession, which is mainly suitable for small-scale areas located in the proximity of grasslands with well-preserved biodiversity. However, to re-establish plant communities with specialist species, a more proactive approach is usually required, such as sowing of regional or commercial seed preservation mixtures, transfer of mature plant material, or topsoil transfer from donor sites with appropriate botanical composition. Grassland restoration methods should be carefully thought-out and carried out before the habitat or species is endangered. We conclude that optimally chosen post-restoration management may have an impact that is comparable to or even greater than the impact of a suitable restoration method. Nevertheless, the maintenance of well-preserved grasslands is still much more cost-effective than the restoration of degraded grasslands.

Keywords

grassland restoration, management, methodology, Natura 2000 grassland habitat types, agri-environment measures

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Pregled metodologije obnove travišč s primeri iz prakse

Izvleček

Večina slovenskih travišč z visoko naravno vrednostjo ima trenutno neugoden ohranitveni status. Glede na razpoložljive podatke s kartiranj habitatnih tipov se površine travinja z visoko naravno vrednostjo (6210(*) – polnaravna suha travišča in grmiščne faze, 6410 – travniki s prevladujočo modro stožko in 6510 – nižinski gojeni travniki) na območjih Natura 2000 zmanjšujejo. Predlagani ukrepi v preteklih kmetijsko-okoljskih programskih obdobjih so bili le delno učinkoviti pri ohranjanju biotske raznovrstnosti travišč. Glavno grožnjo biotski raznovrstnosti predstavljajo različne antropogene aktivnosti, ki vodijo v stalno spreminjanje krajine, razdrobljenost habitatov in degradacijo ekosistemov. Obnova biotske raznovrstnosti je zato postala nujen ukrep za ohranjanje travišč z visoko naravno vrednostjo. Do ekološke obnove travišč s spremenjeno, neznačilno floristično sestavo, ali pa travišč v neugodnem naravovarstvenem stanju, vodi več poti. V tem prispevku predstavljamo pregled metod, uporabljenih pri obnovi travišč iz različnih predelov Evrope, ki bi jih lahko ustrezno prilagojene uporabili tudi za obnovo slovenskih travišč. Travnike lahko prepustimo spontani sukcesiji, ki je primerna predvsem za manjše degradirane površine v bližini travišč z dobro ohranjeno biotsko raznovrstnostjo. Praviloma pa vnovična vzpostavitev rastlinskih združb z značilnimi vrstami zahteva bolj proaktiven pristop, kot je setev regionalnih ali komercialnih ohranjevalnih semenskih mešanic, prenos dozorelega rastlinskega materiala ali prenos vrhnje plasti tal z donorske površine z ustrezno vrstno sestavo. Metode obnove travinja je potrebno natančno pretehtati in z obnovo vrstne pestrosti začeti še preden je habitat ali določena vrsta ogrožena. Ugotavljamo, da ima lahko optimalen način upravljanja travinja po obnovi primerljiv ali celo večji učinek glede na tistega, ki ga lahko dosežemo z ustrezno metodo obnove. Kljub vsemu pa še vedno velja, da je vzdrževanje dobro ohranjenih travišč z ustreznim načinom rabe cenejše od obnove degradiranih travišč.

Ključne besede

obnova travišč, upravljanje, metodologija, Natura 2000 travniški habitatni tipi, kmetijsko-okoljski ukrepi

Introduction

Grasslands are areas dominated by naturally occurring grasses and other herbaceous species, used mainly for grazing by livestock and wildlife (Allen et al. 2011). They comprise approximately 40.5% of the Earth's surface (excluding Greenland and Antarctica), making them one of the world's largest ecosystems (Suttie et al. 2005).

In general, grasslands can be divided into two types: primary (natural) and secondary (semi-natural). The first type is represented by sites that are unfavourable for the establishment of trees, while the second type is of anthropogenic origin, sustained by regular mowing and/or livestock grazing. Despite their anthropogenic origin, secondary grasslands represent some of the most species-rich and also some of the most vulnerable habitats in Europe since they have been extensively managed for several hundred years (Török et al. 2020). In contrast to intensively managed systems with high productivity and thus high input requirements per unit area, extensively managed systems use small amounts of labour and capital per unit area and primarily rely on natural soil fertility, water availability, and climate (Ashton et al. 2012). Therefore, extensively managed grasslands have higher biodiversity value (Lesschen et al. 2014). The most species-rich secondary grasslands are dry and semi-dry grasslands. They are found on shallow to deep soils and are predominantly formed on calcareous or volcanic bedrocks from lowlands to mountains. Calcareous grassland types are especially species-rich. However, they are increasingly threatened by woody encroachment (Elias et al. 2018).

Grasslands may also be divided according to their age, namely into temporary and permanent grasslands. According to the European Commission, permanent grasslands are defined as "land used to grow grasses or other herbaceous forage naturally (self-seeded) or through cultivation (sown), and that is not included in the crop rotation of the holding for five years or longer" (Commission Regulation (EC) No 796/2004). Grasslands that are less than five years of age (including in a crop rotation) are therefore defined as temporary grasslands (Reheul et al. 2007).

Natural and semi-natural grasslands are usually defined by climatic, pedological, or topological factors. However, in natural grasslands, plant species richness is further increased by fires and grazing, which also prevent litter accumulation and thus limit woody encroachment in addition to climate and extreme habitat conditions (Kuzemko et al. 2016, Török et al. 2020). Both natural and semi-natural grasslands depend on microclimatic, soil, and bedrock gradients (Sutcliffe et al. 2016). Among these, the main drivers of species composition are soil depth, soil texture, and soil pH. The relationship between pH and plant diversity of a certain region is generally hump-shaped, as the highest plant species richness is found under neutral or slightly basic conditions (Palpurina et al. 2017). A hump-shaped relationship can also be found between plant species richness and primary productivity (Fraser et al. 2015). The productivity of grassland communities is mostly driven by the availability of water and nutrients, which affects the biodiversity of these communities. The amount of moisture and its seasonal variation influences the productivity of natural grasslands, whereas the productivity of semi-natural grasslands is controlled by the fertility gradient (Török et al. 2020). Limiting nutrients play an important role in shaping the richness-productivity pattern (Palpurina et al. 2017) since various species are adapted to nitrogen (N) and phosphorus (P) limitation (Roeling et al. 2018). Generally, grasslands rich in N, P, and potassium (K) have low biodiversity (Merunková and Chytrý 2012). Although fire is not as crucial for the formation of grassland plant communities as climate, its contribution to this process in terms of seasonality, intensity, and return rate is still significant (Ewing and Engle 1988, Biondini et al. 1989). Moreover, areas that have recently undergone fires are usually richer in nutrients, attracting large ungulates that may further change species composition (Milchunas et al. 1988, Hartnett et al. 1996).

Natural and semi-natural extensively managed grasslands provide many ecosystem services that cannot be provided by other land uses. Their ecosystem services are of higher value compared to those of sown and intensively managed grasslands (Wick et al. 2016). For example, natural and semi-natural extensively managed grasslands harbour rich and unique flora and fauna, they produce biomass, serve as forage for herbivores, provide natural medicines, present habitat for pollinators and birds, ensure resources for water infiltration, flood reduction, purification, and storage, they prevent erosion, balance local climate, and play an important role in nutrient cycling and nutrient retention (Wick et al. 2016, Török et al. 2020). They also act as carbon dioxide (CO_2) sinks and thus have a great carbon (C) sequestration potential (Ammann et al. 2007) since they contain up to 12% of the soil organic C global pool (Schlesinger 1997). Converting grasslands into arable land leads to a decline in soil C due to C loss by tillage and lower C input from litter (Jones and Donnelly 2004). Grasslands also offer a variety of intangible aesthetic, cultural, and recreational services (Török et al. 2017) and also have a good potential for biogas and biofuel production (Heinsoo et al. 2010).

Despite their ecological, cultural, and agricultural importance, grasslands are threatened globally due to numerous anthropogenic factors and climate change. Their degradation results in a reduced provision of ecosystem goods and services (Wick et al. 2016). The most common reasons for grassland degradation include, on the one hand, their fragmentation and isolation caused by the intensification of agriculture and increased development of the secondary and tertiary economic sector, especially in lowlands, along with the abandonment of extensive management by mowing or grazing in less accessible areas, resulting in overgrowth and encroachment by trees and shrubs. Other frequently mentioned causes include the absence of naturally occurring fires, biological invasions caused by introductions of non-native species, overgrazing, eutrophication, and the already mentioned climate change (Wick et al. 2016, Török et al. 2020).

In Slovenia, permanent grasslands cover 58% of the total agricultural land (Factsheet on the 2014-2022 Rural Development Programme for Slovenia 2022). About 20% of the Slovenian Natura 2000 network is represented by utilised agricultural land, among which extensive meadows are the most important (Prioritised action framework (PAF) for Natura 2000 in Slovenia 2019). The latest report by the European Environment Agency on the state of nature in the European Union from the year 2020 claims that 50% of the assessments for grasslands in Slovenia under the Habitats Directive are showing a bad conservation status (U2) and about 22% are showing an inadequate (U1) conservation status. Therefore, altogether, no less than 72% of Slovenian grassland areas have an unfavourable conservation status (EEA 2020). Among grassland areas, semi-natural dry grasslands and scrubland facies on calcareous substrates (Natura 2000 habitat type 6210(*)) are particularly affected. The main reason for such a negative trend is their dependency on particular sustainable management measures (EEA 2020). Kaligarič et al. (2019) studied four grassland-specific agri-environment measures (AEM) in Slovenia in the period from 2007 to 2013 and found that these failed to preserve high nature value (HNV) grasslands in Slovenia since AEM did not reach most of the HNV grasslands in Slovenia and since this limited interest in the AEM targeted any permanent grassland, irrespective of its conservation value. As a result, many grasslands included in the AEM were not HNV grasslands. The authors concluded that this was due to the lack of pre-selection criteria for grasslands and the lack of monitoring of the efficiency of the measures, and due to low interest of farmers in the subsidies (Kaligarič et al. 2019). Ivajnšič et al. (2019) also reported negligible integration of agricultural holdings in the AEM in Slovenia. Unfortunately, the AEM in the period from 2014 to 2020 retained more or less the same concept as in the previous period (Kaligarič et al. 2019). Therefore, Kaligarič et al. (2019) suggested that future schemes should be prepared on a completely different basis, wherein HNV grasslands should be prioritised, and monitoring of biodiversity should be the most important requirement. The proposed Common Agricultural Policy (CAP) for the period from 2021 to 2027 was said to be more flexible and effective (Lovec et al. 2020). However, Lovec et al. (2020) argue that the actual long-term impact was not thoroughly considered in the new CAP. Therefore, no significant improvements in biodiversity conservation can be expected (Lovec et al. 2020). Šumrada et al. (2021) conducted a study where they explored the potential of a payment-by-results approach as an alternative to management-based schemes (MBS) in Slovenia. MBSs are schemes that provide payments for farming practices, which are believed to secure certain services instead of being tied to their actual provision (Burton and Schwarz 2013). Alternatively, result-based schemes (RBS) remunerate farmers for ecological results, demonstrated by certain indicators (e.g., presence of certain plant species, breeding success of farmland birds, etc.) (Herzon et al. 2018). Šumrada et al. (2021, 2022) found that most farmers and experts were in favour of the introduction of such RBSs for grassland conservation. Farmers knew the selected plant indicators and preferred monitoring of their presence over the current system (Šumrada et al. 2021). However, for the successful conservation of HNV grasslands, institutional capacity is also needed to implement RBSs on a larger scale (Šumrada et al. 2021). In addition, regardless of their many advantages in comparison to MBSs (Pe'er et al. 2022), RBSs do not seem to be better suited than the current schemes in addressing the specific needs of small and (semi-)subsistence farmers (Davidova 2011) and in the cases where land ownership is highly fragmented (Hartvigsen 2014). For this reason, older farmers and those who manage semi-subsistent and small farms are mostly not in favour of RBSs (Šumrada et al. 2022). The findings of Šumrada et al. (2021) indicate that there is an institutional gap in the understanding of agroecology and of the importance of integration of biodiversity policy, which needs to be bridged to be able to enforce changes into the current system and achieve better conservation outcomes. Pe'er et al. (2022) argue that a combination of both result-based and action-oriented payments might be optimal.

According to the various ecosystem services that grasslands provide, it is vital to prevent their further degradation in the future. In this review article, we present an overview of the techniques for grassland restoration used in experiments carried out in various European countries (but not in Slovenia), evaluate their practical use in the past, and form recommendations for their future application. All findings and recommendations are drawn from these studies.

Grassland restoration in general

The best way to maintain the biodiversity of grasslands is to prevent both their abandonment and overexploitation by using extensive traditional agricultural practices, mainly grazing and/or mowing. These two practices have been shown to exert many positive effects on grassland biodiversity and are often recommended as tools for both the restoration and maintenance of grasslands (Galvánek and Lepš 2008, Török et al. 2016). Among these, wild herbivore grazing on open areas (by wild horses and cattle) and low-intensity grazing by herded livestock (local breeds) are often suggested (Török et al. 2016, Tóth et al. 2018).

To retain grassland biodiversity in (near) optimum conditions, not only one single type but a whole scheme of traditional management practices is required (Babai and Molnár 2014). In addition, mosaic management should also be taken into account to ensure the highest biodiversity (i.e., temporally and spatially dynamic combination of abandoned and mown grassland patches) (Török et al. 2017). Nonetheless, it is often the case that traditional practices are not possible or economically sustainable. For this reason, conservation authorities and site managers are looking for alternative practices, such as prescribed burning during the dormant season. Prescribed burning with long fire-return periods could be a suitable and economically feasible way of eliminating accumulated litter and sustaining grassland biodiversity (Valkó et al. 2014). However, in some cases, soil biota may be negatively affected by fires (McLeod and Gates 1998, Vasconcelos et al. 2017, Zaitsev et al. 2017). Annual prescribed fires were found to be inappropriate for maintaining the desired species richness and structure, whereas periodic prescribed fires every two to six years may have a positive impact even in terms of soil biological properties (Valkó et al. 2014).

For partly degraded grasslands, a decrease in management intensity is often sufficient for their recovery, while for fully degraded grasslands that have been converted into other land uses, spontaneous succession or technical reclamation is required for a successful restoration (Prach and Hobbs 2008). Spontaneous succession has been reported to be a promising restoration tool in several different Central European grassland habitats, especially where the proportion of target grassland communities is high (Albert et al. 2014, Prach et al. 2015). However, for large-scale restoration projects to be successful, well-preserved donor grasslands acting as spontaneous sources of propagules must be available nearby (Török et al. 2020). Regarding technical reclamation, the most common methods that have been successfully used in some extensive grassland restoration projects (Lengyel et al. 2012, Prach et al. 2015) are sowing of regional seed mixtures and transfer of plant material (Török et al. 2011).

The main goal of many restoration actions is not to directly increase biodiversity on chosen plots per se, but rather to (re-)establish and maintain the presence of characteristic indicator species of target grassland communities, with an additional aim of reducing cover and impact of non-target weeds (Lepš et al. 2007).

Restoration of species-rich grasslands can be time-consuming, and additionally, it usually requires plot preparation (Kiehl et al. 2010, Krautzer et al. 2011) and appropriate post-restoration management (Kiehl et al. 2010, Török et al. 2011). Many sources claim that seeding on bare soil improves the chances of vegetation establishment (Kiehl et al. 2010, Krautzer et al. 2011, Török et al. 2011). Soil preparation is mostly done by ploughing or disking, followed by raking for seedbed preparation. After sowing, the covering of seeds is carried out by raking or ring rolling (Török et al. 2011). If an existing sward is present, it should be cut to the height of 3-5 cm, when necessary, and then opened (Krautzer et al. 2011). Grassland restoration should first be carefully thought-out and then carried out according to the plan shown in Fig. 1.

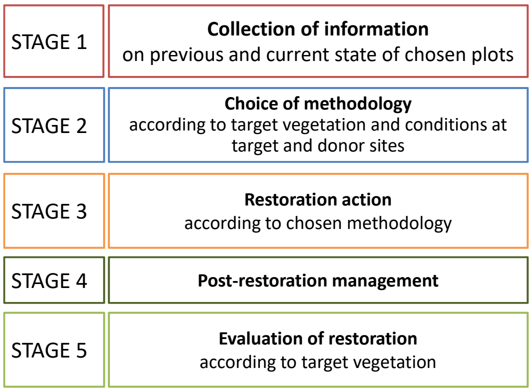

Figure 1. The stages of grassland restoration.

Slika 1. Zaporedje korakov pri obnovi travnikov.

Most commonly used methods in grassland restoration with practical examples

When restoring degraded permanent grasslands, we can choose between different methods. Each of them has its advantages and disadvantages, which are described below and summarised in Tab. 1.

Decrease in management intensity

Grassland management (especially traditional) is a crucial tool in the maintenance of semi-natural grasslands (Bischoff et al. 2009, Milberg et al. 2017, Goret et al. 2021). Cessation of management intensity (extensification) on previously intensively used grasslands has recently been discussed as a useful grassland restoration tool in many conservation policies and attempts. It might, theoretically,

Table 1. A summary of grassland restoration techniques used in different European countries.

Tabela 1. Povzetek tehnik obnove travnikov, ki so bile uporabljene v različnih evropskih državah.

Grassland restoration technique			Suitable for	Advantages	Limitations	Reference / An example of use
A decrease in management intensity			degraded grasslands of various habitat types (mesophilic, calcareous, and acidic grasslands)	reduction and prevention of further nutrient accumulation in the soil; general improvement of soil properties; an increase of taxonomical and functional biodiversity; increased landscape heterogeneity	drastic decrease in management intensity can lead to abandonment and biodiversity loss, causing overgrowth of weeds, forbs, and shrub encroachment; positive impacts of fertilisation reduction are not noticeable immediately; might negatively impact grass coverage in mesic grasslands and pastures	Marriott et al. 2004, Milberg et al. 2017, Mayel et al. 2021, Resch et al. 2021
Spontaneous succession			ruined grasslands; small-scale areas located in the proximity of well-preserved grasslands (mesic *Arrhenatherion* grasslands, dry *Festuco-Brometea* grasslands)	natural process; no action is needed	requires a high proportion of target grassland communities; high probability for an increased presence of weeds; long-term method; most suitable for recently degraded grasslands	Bossuyt and Honay 2008, Fagan et al. 2008, Lencová and Prach 2011, Knappová et al. 2012, Albert et al. 2014, Prach et al. 2014, Sojneková and Chytrý 2015, Prach et al. 2021a
Sowing of regional seed mixtures	low-diversity seed mixtures	manual collection	degraded grasslands; ruined grasslands; plots that are at risk of erosion; larger plots (mesic *Arrhenatherion* grasslands, dry *Festuco-Brometea* grasslands)	quick results	requires well-preserved donor grasslands; manual collection is time-consuming, requires good seed recognition; proper harvesting time	Lepš et al. 2007, Kiehl et al. 2010, Hofmann et al. 2020
		vacuum harvesting				
		combine harvesting				
	high-diversity seed mixtures	manual collection	ruined grasslands; smaller plots (mesic *Arrhenatherion* grasslands, dry *Festuco-Brometea* grasslands)	greater restoration success; a useful first step towards revegetation	requires well-preserved donor grasslands; limited availability; larger collecting effort; manual collection is time-consuming, requires good seed recognition; proper harvesting time	Jongepierová et al. 2007, Lepš et al. 2007, Kiehl et al. 2010, Török et al. 2011, Mitchley et al. 2012, Haslgrübler et al. 2013, Prach et al. 2013, 2014, Baasch et al. 2016, Hofmann et al. 2020, Prach et al. 2021a
		vacuum harvesting				
		combine harvesting				

Grassland restoration technique			Suitable for	Advantages	Limitations	Reference / An example of use
Transfer of plant material	hay threshing	plant clipping or green hay	ruined grasslands; all types of habitats	greater restoration success; genetic diversity preserved; low-cost; applicable on a larger scale	requires well-preserved donor grasslands; lower seed content	Kiehl et al. 2006, Donath et al. 2007, Edwards et al. 2007, Galvánek and Janák 2008, Kiehl et al. 2010, Rydgren et al. 2010, Krautzer et al. 2011, Albert et al. 2019, Hofmann et al. 2020
		dry hay/ 'hay sensu stricto'				
	seed stripping			does not require vegetation cutting	requires well-preserved donor grasslands	
	brush harvester (most common)		ruined grasslands; tall meadows (*Molinion, Cnidion*)	successful and widely used restoration method; does not require vegetation cutting; greater restoration success	requires well-preserved donor grasslands; seeds of shorter species underrepresented; specific equipment	
Topsoil transfer			ruined grasslands	greater restoration success	destructive for donor site; laborious; costly	Kiehl and Pfidenhauer 2007, Török et al. 2011
Mechanical disturbance, applied prior to restoration techniques			variously managed temperate grasslands; severely degraded grasslands; wet meadows; *Nardus* grasslands	increased soil seed bank potential and seedling recruitment	low specificity and selectivity; might improve the development of weeds	Hofmann and Isselstein 2004, Donath et al. 2007, Edwards et al. 2007, Mitchell et al. 2009, Krautzer et al. 2011, Klaus et al. 2018
Planting of entire plant individuals			degraded grasslands; individual/specific species; for stimulating succession to later stages	better establishment of endangered species	laborious; costly	Török et al. 2011
Cessation of fertilisation			grasslands degraded by over-fertilisation (mesic semi-natural grasslands containing *Molinio-Arrhenatherea* and *Festuco-Brometea* species; *Nardus* grasslands)	low-cost; no active measurements needed	unpredictable species shift; depends on abiotic parameters (climate); highly dependent on initial state; time-consuming	Marriott et al. 2004, Hejcman et al. 2007, Královec et al. 2009, Korzeniak 2016, Van Daele et al. 2017, Prach et al. 2021b

increase biodiversity on chosen plots by reducing work intensity, but at the same time increasing work effectiveness on larger grassland areas (Marriott et al. 2004, Milberg et al. 2017). However, results are context-dependent and differ according to the methodology used and the initial state at chosen sites. A study on the impact of grassland extensification by Marriott et al. (2004) showed that management cessation had a moderately negative impact on the diversity of calcareous and mesic grasslands, wherein changes were primarily visible in the long term. On the other hand, management abandonment had a significant negative impact primarily on mesic grasslands, which were showing signs of succession to forest vegetation in a 13-year period and were overgrown by shrubs and forest species in 30 years. Acidic grasslands were overgrown by non-palatable grasses (such as *Molinia caerulea* and *Deschampsia flexuosa*) and ericoid shrubs, whereas calcareous grasslands were overgrown by *Brachypodium pinnatum* (Marriott et al. 2004). Milberg et al. (2017) found that cessation of mowing intensity (from annually to once every three years) had a weak impact on diversity of wet grasslands, whereas the diversity of dry grasslands decreased significantly. Significant biodiversity loss was noticeable one decade into the experiment, more specifically after 11-14 years, which is somehow similar to the results of the study by Marriott et al. (2004). Rare mowing has a particularly negative impact on short-growing species, which can be impaired by higher and/or woody species that out-compete them and benefit from sporadic management. A combination of annual mowing of grasses and forbs in swards and occasional (once every few years) mowing of shrubby, overgrown parts of vegetation might be a solution that would combine the benefits of the aforementioned policy of reduced, but more efficient work, and conservation of grassland habitats (Milberg et al. 2017). The benefits of grassland management are discussed in more detail in the chapter Post-restoration management with grazing and mowing.

Spontaneous succession

Spontaneous secondary succession is a natural process of changes in species' structure and interaction dynamics in an already established ecosystem following natural or anthropogenic disturbance events. Particularly in the case of grassland restoration actions, secondary succession often occurs following the abandonment of previously used arable land. It is the most natural way of grassland restoration, relying on naturally (spontaneously) occurring processes in an ecosystem (Török et al. 2011). It is strongly dependent on the local availability of seeds (propagules) of the target grassland species and their efficient dispersal by different vectors, deriving from natural vegetation near the restoration target sites (Hölzel and Otte 2003, Donath et al. 2007, Kardol et al. 2008, Kiehl et al. 2010, Krautzer et al. 2011, Török et al. 2011, Hofmann et al. 2020). Natural succession of ex-arable fields usually begins with the establishment of annual and perennial ruderal plants, followed by perennial grasses and forbs – species of later successional stages, which are better adapted to specific grassland conditions (such as resource limitation). However, the transition to more complex plant communities during later stages of ecological succession rarely occurs. The structure of these plant communities stagnates with an increased presence of weeds, ruderals, and grasses, due to the low germination potential of grassland species' seeds in the seed banks and their limited dispersal potential (Kleijn 2003, Lawson et al. 2004). As stable ecosystems, grasslands do not typically rely on soil seed banks since characteristic grassland species do not produce long-lived or numerous seeds (Bossuyt and Honnay 2008). They rather rely on the dispersal of seeds (Edwards et al. 2007, Bossuyt and Honnay 2008). A study by Knappová et al. (2012) reported on the ability of dry grassland species (*Festuco-Brometea*) to colonise grasslands in their proximity as an alternative to restoration actions of a particular grassland type. While about 68% of the species present showed dispersal potential, some of the 'grassland specialists', such as *Helianthemum nummularium* subsp. *grandiflorum*, *Carex humilis*, *Anthericum ramosum*, *Filipendula vulgaris*, *Melampyrum nemorosum*, *Gymnadenia conopsea*, and *Campanula glomerata*, did not colonise proximal area. Overall, the authors defined the distance of 0.5 kilometres as the limit for successful propagule spreading, pointing out that in addition to distance and plot area, biotic and abiotic factors of the selected grasslands also need to be considered when discussing this topic (Knappová et al. 2012).

A review study by Bossuyt and Honnay (2008) showed that grassland soil seed banks were amongst the ones with the highest diversity, richness, and evenness, in comparison to those of forests, marshes, and heathlands. However, their generally low seed density and common absence of target species' seeds (especially in calcareous grasslands) made them an unreliable source for restoration. Exceptionally, seed banks might be useful at sites that were recently degraded (up to 5 years ago) (Bossuyt and Honnay 2008). Intensive use of agricultural fields and increased soil fertility negatively impact grassland soil seed banks (Edwards et al. 2007), favouring the development of weeds as remnants of previous successional stages (Török et al. 2011), along with invasive and non-target species that are commonly found at earlier stages of succession (Bossuyt and Honnay 2008). Another common issue with spontaneous succession is landscape fragmentation, resulting in the isolation of grassland plots and the absence of suitable propagule sources (Kleijn 2003, Kiehl et al. 2010, Lawson et al. 2004, Török et al. 2011), along with their dispersal limitation (Donath et al. 2007). All these issues generally cause delayed restoration (Kleijn 2003, Török et al. 2011). In addition, natural regeneration is often limited by inadequate germination conditions found on plots where vegetation is already established (mainly regarding light availability and soil). These differ between species of early and later successional stages. Species of early successional stages are usually taller and are consequently better competitors for light. Furthermore, they have a better capability for vegetative reproduction and thus out-compete species of later successional stages (Kleijn 2003).

Proactive measures of grassland restoration are usually needed since restoration by spontaneous succession is mainly suitable for small-scale areas located in the proximity of well-preserved grasslands, which serve as an adequate source of propagules. It is a good restoration method where no urgent results are expected due to slow and often unpredictable development of vegetation (Török et al. 2011). For example, Fagan et al. (2008) showed that they preferred the regeneration of dry grasslands by natural succession, combined with moderate management, defined by local agro-environmental schemes, rather than regeneration by sowing seed mixtures. Another study favouring natural succession over sowing of seed mixtures as a form of dry grassland restoration was conducted by Lencová and Prach (2011). They claim that if target sites are in the proximity of adequate propagule sources, properly managed, and not (extremely) loaded with nutrients, no sowing is required unless urgent results for grassland production are needed.

Sowing of seed mixtures

Many grassland restoration attempts rely on different methods of plant material introduction (Hofmann et al. 2020). One of the most commonly used methods is seed addition by sowing seed mixtures (Török et al. 2011, Hofmann et al. 2020). Seed mixture composition depends on numerous factors, such as target vegetation, conditions at the target site, and seed availability (Török et al. 2011).

Török et al. (2011) mention two main types of seed mixtures used in restoration: (1) low-diversity (LD) seed mixtures, usually consisting of seeds of 2-8 species, mostly dominant grasses and/or forbs of the target vegetation, and (2) high-diversity (HD) seed mixtures, which contain seeds of 10 or more target species. LD seed mixtures are often applied in restoration experiments that aim for quicker results – for instance, on plots that are at high risk of erosion. On larger plots, where HD seed mixtures might not be quantitatively sufficient due to their limited availability and greater collection effort, mixtures may be combined and/or applied in patches. Seeds can be either commercially sourced or locally harvested. Commercial seed mixtures are acceptable if they contain seeds of target species from local populations. It is, however, highly suggested to use seeds of plants grown or harvested locally, as this way the chances of successful restoration are higher. Non-native ecotypes in commercial seed mixtures namely have lower genetic and ecological compatibility with local conditions (Kiehl et al. 2010, Török et al. 2011). Additionally, foreign ecotypes may hybridise with local ecotypes, causing genetic biodiversity loss and reduced fitness in new hybrid populations (Kiehl et al. 2010). Seed collection, as opposed to the application of commercially sourced propagules, may help in the reintroduction of certain rare species, whose seeds are not readily available (Kiehl et al. 2010). Seeds can be collected manually or by using specific equipment, such as vacuum or combine harvesters (Edwards et al. 2007), which require special attention in the choice of proper harvesting time (Krautzer et al. 2011). An exception is vacuum harvesting, which can be easily carried out with machines used for leaf vacuuming and blowing even after seed shedding. Due to its complexity, this method should be carried out primarily at locations where other methods are not readily applicable (Kiehl et al. 2010). Application of different seed densities results in varying success rates of grassland restoration. Generally, the application of a larger number of seeds results in greater restoration success. However, it could also cause greater resource competition among sown species, which might result in decreased diversity of target species (Lepš et al. 2007, Török et al. 2011). As a potentially good alternative to spontaneous succession, Török et al. (2011) recommend sowing densities from 4000 to 13000 seeds/m^2 on areas of up to a few hundred square meters, whereas larger areas (measured in hectares) might require between 20 and 45 kg of seeds per hectare (for more details, see Török et al. 2011). Sowing of regional seed mixtures has been repeatedly proven as a successful restoration method for dry grasslands (Lepš et al. 2007, Jongepierová et al. 2007, Mitchley et al. 2012, Johanidesová et al. 2014, Prach et al. 2021a). Jongepierová et al. (2007) reported that sowing of regional seed mixtures is a very good method for the restoration of *Bromion* grasslands, indicating the use of a specific methodology in the form of strip sowing. Regional seed mixtures may be expensive and not readily accessible. In this case, strips of regional seed mixtures could be applied inside fields designated for natural regeneration, which should theoretically be colonised by previously sown target species. Mitchley et al. (2012) studied vegetation at the same plots in 2009, following the first part of the survey by Jongepierová et al. (2007), further confirming the positive effects of sowing regional seed mixtures, with the highest success achieved by sowing regional grasses, which covered around 50% of the studied plots. All experimental plots showed divergence towards ancient grasslands in the 10-year period, but donor and recipient sites still showed significant differences in species composition, suggesting that the 10-year period might not be sufficient to achieve the intended results and prove the importance of time dimension in grassland restoration experiments. Regarding different types of seed mixtures, Lepš et al. (2007) confirmed the positive impact of sowing both HD and LD seed mixtures on grassland vegetation succession. Both LD and HD seed mixtures were proven as suitable, even though HD seed mixtures were generally more successful and had higher productivity. An additional benefit that they provided was a higher probability of compensation in the case of species establishment failure, expressing the ‘insurance effect’ on diversity. Grasses were generally more successful than forbs. However, non-sown control plots subjected to natural succession had higher species richness and diversity, which did not result from dispersal from sown plots. Namely, sown species never achieved dominance on control plots, even though they spread relatively successfully over short distances into adjacent plots. Sowing of species is therefore defined as better suited for species introduction and not necessarily for biodiversity increase. An ideal combination might be planned to sow seed mixtures in strips, with some space left for natural colonisation between the strips. When seed mixtures are used in restoration actions, the species composition of both applied mixtures and recipient sites should be taken into consideration, focusing on the colonisation potential and competitiveness of present species. The presence of highly competitive species in mixtures might negatively impact the development and survival of other species, which were either previously sown or initially present at the site. Due to the competitiveness or invasiveness of various plant species (particularly weeds), the presence of those species in plant communities that are located near the restored grasslands should also be taken into account. By doing that, we might prevent their spreading into newly sown sites, where they might cause negative shifts in target species composition. Johanidesová et al. (2014) additionally reported that restoration by seed addition could be a successful first step towards revegetation and that its continuation in the form of natural succession depends on the vicinity of appropriate grasslands that would serve as a natural source of propagules. The latter is important due to limitations in the sowing of some grassland species’ seeds for many reasons, either biological or technical. If natural spreading does not fulfil expectations in the set time frame, repeated sowing of selected target species may be required. Prach et al. (2014) studied the differences between restoration attempts of two grassland types of the *Arrhenatherion* and *Bromion* alliances in the White Carpathian Mountains, pointing out that spontaneous succession and use of commercially prepared mixtures led to the establishment of mesic vegetation, whereas local mixtures favoured dry grasslands. A study by Prach et al. (2013) on the same area gave the same results, favouring the use of local seed mixtures. 98% of sown species grew successfully, along with unsown species that spread from nearby reference ancient grasslands.

Transfer of plant material

Other commonly used seed collection techniques include threshing, seed stripping, and the use of a brush harvester. Brush harvesting is a generally successful and widely used restoration method that does not require vegetation cutting. It is overall better suited for tall meadows since seeds of shorter species are often underrepresented in the harvested material. Shortgrass meadows could therefore benefit from collecting plant material by cutting or even raking, followed by transfer of raked material, which would additionally help transfer propagules of bryophytes and lichens. Hay threshing and brush harvesting are more expensive methods due to the use of specific equipment (Kiehl et al. 2010).

Restoration of species-rich grasslands usually requires transplantation of plant material from donor sites since the collection of adequate HD seed mixtures demands a lot of time and resources (Török et al. 2011). The application of plant material containing seeds is a cost-effective method that is applicable on a large scale and in a wide range of habitats. In addition, this method might be beneficial due to the introduction of an entire gene pool of the donor community, which might also include some rare or endangered species that cannot be sown. Therefore, the genetic diversity of locally-adapted ecotypes is preserved, along with the provision of microsites required for seed germination. Like other restoration methods, it is highly context-dependent, and it requires the proper selection of quality donor plant material applied at corresponding, ecologically compatible sites. Although the seed content of the transferred plant material is an important variable in the evaluation of restoration success, colonisation ability is also very important (Hölzel and Otte 2003).

When plant material with propagules is collected by mowing (cutting), it can be applied either as fresh or dry hay (Kiehl et al. 2010, Krautzer et al. 2011). Application of fresh hay is known as ‘plant clippings’ or ‘green hay’, whereas dry hay is known as ‘hay *sensu stricto*’ (Kiehl et al. 2010). Application of fresh plant material increases the chances of species establishment (Kiehl et al. 2010, Török et al. 2011), while dry hay requires additional manipulation of the material and therefore increases the costs of the process (Krautzer et al. 2011), with a limited restoration potential due to lower seed content. Low-productivity grasslands, such as calcareous grasslands, typically require around 300-600 g/m^2 of freshly cut hay with a 2:1 to 3:1 donor-to-recipient plot ratio, whereas other habitat types, such as fens, require a 1:1 to 2:1 plot ratio (Kiehl et al. 2010). Immediate transfer of freshly cut plant material with ripe seeds is known as hay strewing (Edwards et al. 2007). Plant material can be applied in a 5-15 cm layer, which is advised for mesotrophic to eutrophic grasslands (Kiehl et al. 2010). Thicker layers are not recommended since they can suppress the colonisation of target species (Kiehl et al. 2010, Török et al. 2011). Transfer of plant material containing seeds is best combined with prior preparation of recipient sites with various soil disturbance methods, which are chosen according to the grassland type and its nutrient status. The importance of mechanical disturbance for grassland restoration based on seed bank potential was studied by Klaus et al. (2018). Results showed that mechanical disturbance could potentially increase the diversity of severely degraded grasslands. The sole impact of seed banks on partly developed grasslands is, however, questionable. The similarity between seed banks and vegetation stands did not increase with disturbance frequency. Thus, other measures of propagule introduction, such as sowing, were required (Klaus et al. 2018). Apart from the quality of applied seed mixtures, another crucial factor is the availability of microsites that would provide adequate conditions for seedling germination and development, preventing seed desiccation. This can be achieved by providing an additional mulch layer that also prevents soil erosion (Kiehl et al. 2010).

Diaspore transfer was proved as a successful method in the restoration of wet meadows of the *Molinion* and *Cnidion* alliances (Hölzel and Otte 2003, Donath et al. 2007). Previous disturbance of existing grass swards in the form of rotavating before the application of seed-containing plant material was proved beneficial for the establishment of target vegetation of wet grasslands, although the authors noted that competitive relationships within the stand (with a specific focus on grasses) severely dictated the dynamics of restoration. They also noted that heavier disturbance (such as ploughing of existing sward) might be needed to expand niches for introduced species (Donath et al. 2007). Both studies reported low establishment rates for sedges (*Carex* spp.) (Hölzel and Otte 2003, Donath et al. 2007). This can be explained by their specific phenology and ecology. Namely, their seeds ripen early in the vegetation season and are absent at the time of seed collection in late summer or autumn. They also have specific germination requirements, such as prolonged incubation in warm and moist conditions, and due to their successful vegetative reproduction, they are better suited for other methods of propagule introduction, such as turf transfer (Donath et al. 2007). Donath et al. (2007) do not recommend simultaneous sowing of grasses when applying seed mixtures due to the potential of grasses to out-compete target vegetation and their ability to successfully colonise sites of stands in the proximity, even though simultaneously sown grasses do not disturb the development of newly introduced vegetation (Donath et al. 2006, 2007). Already established grass stands presented a bigger obstacle to the recruitment of new vegetation (Donath et al. 2007).

Green hay transfer was proved by Kiehl et al. (2006) to be a successful method of restoration of ancient grasslands, although it is also not suitable for the reintroduction of sedges (*Carex* spp.). Albert et al. (2019) found that green hay transfer was the most efficient restoration method for *Bromion erecti* grasslands, followed by triple brush harvesting and single brush harvesting. Donor sites were located in the Protected Landscape Area and Biosphere Reserve of the White Carpathian Mountains in the Czech Republic. Thirty-five species were recorded at the recipient site, on which green hay consisting of 112 species collected at donor plots was applied, whereas triple harvesting showed a similar success rate with 33 newly established species. The success of this method may be due to the fact that plants were transferred as specimens, providing a possibility for the seeds to ripen on parent plants, whereas harvesting methods require effort to remove fruits and seeds from plants. Harvesting proved to be more successful when repeated during the season. However, all of the tested options had a relatively low success rate, with minimal differences between green hay and triple harvesting. Albert et al. (2019) noted that, due to the higher practicality of seed mixtures collected by harvesting, which can be stored and transferred, brush harvesting has a significant potential for restoration, whereas hay transfer needs to be applied as soon as possible after mowing and is usually limited to smaller areas. Hofmann et al. (2020) tested different methods of grassland restoration on various vegetation types (*Festuco-Brometea*, *Nardo-Callunetea*, *Sedo-Scleranthetea*, and *Trifolio-Geranietea*). Their results confirmed the efficiency of hay transfer. However, they noted the importance of choosing the right time for the application of this method, which should be before the seeds are ripe, and should be repeated if necessary.

Edwards et al. (2007) studied the influence of preparatory disturbance practices (power harrowing and turf stripping) on the efficiency of two commonly used grassland restoration methods (brush harvesting and green hay strewing) applied to lowland hay meadows and chalk grasslands as two target vegetation types. Both methods of seed addition proved successful. Hay strewing was more efficient for seed collection of low-growing plant species due to the greater height at which the brush harvester collected seeds. In addition, it sampled a wider phenological range of different plants, a trait specific to mesic chalk grasslands. Early cutting might favour perennial grasses, whereas later hay cut includes a wider range of different forbs, of which many have a conservation value. This confirms the importance of phenology knowledge in choosing the adequate restoration method. Disturbance generally had a positive impact on grassland establishment, except for power harrowing in chalk grasslands. They did, however, benefit from turf stripping, even though the positive impact might have been related to a decrease in nutrients in the soil caused by the given disturbance. This study confirmed the importance of background knowledge and context dependence. The choice should be in line with phenology, and management techniques should be considered.

Other methods of grassland restoration

Other, not commonly used methods of grassland restoration include topsoil transfer, turf transplantation, and community translocation directly from donor sites to recipient plots (Török et al. 2011). Potential advantages of chosen methods include the transfer of diaspores with their associated soil fauna and microbiota, which could increase the chances of vegetation establishment in its original form and dynamics (as close as possible) (Török et al. 2011). Kiehl and Pfidenhauer (2007) confirmed a positive impact of topsoil removal on the re-establishment of dry *Bromus* grasslands by an increased cover of various target species, such as *Thymus praecox*, *Hippocrepis comosa*, and *Dorycnium germanicum*, following the experiment. However, transplantation methods are still not widely used due to their destructive nature, the high effort that they require, and the costs they cause (Török et al. 2011). This was also noted by the authors of the original research (Kiehl and Pfidenhauer 2007). In addition to sowing or hay transfer, planting of entire plant individuals or their belowground parts is sometimes carried out in restored areas. Due to higher costs, it is only recommended in specific cases, for example, for a better establishment of endangered species or for stimulating succession to later stages (Török et al. 2011). Other potential issues in soil transfer are eutrophication, caused by increased nutrient mineralisation in the applied soil, and ruderalisation, which require additional management measures afterwards (Kiehl et al. 2010).

As an option for the restoration of grasslands dominated by *N. stricta*, Mitchell et al. (2009) suggested the creation of gaps in turf for other plant seedlings by rotavation. Nonetheless, regular cutting and chopping of biomass using a flail or rotary mower is not advised since it may cause changes in species composition and dominance of some grasses (Krahulec et al. 2007, Galvánek and Janák 2008). Too regular burning is also not recommended, as it can promote the spreading of invasive species (Bensettiti et al. 2005).

For long-term maintenance of *Nardus* grasslands in Serbia, Dajić Stevanović et al. (2008) recommend practices such as juniper burning or roller chopping, or the introduction of horse grazing to control the spreading of some undesired low-quality grasses, and mechanical clearance of woody species, along with the reintroduction of cattle and sheep grazing.

Parolo et al. (2011) defined a few management practices necessary for the preservation of *Nardus* grasslands. They suggested mechanical removal of woody pioneer species to prevent encroachment at pasture edges, establishing electric paddocks for more intensive grazing at the periphery of pastures, and using electric fences on lower pastures that are grazed twice to ensure the concentration of cows at pasture edges. It is advisable to distribute animals along the peripheral parts of pastures so that nitrophilous species have less potential for spreading (Bensettiti et al.

2005). Moreover, Parolo et al. (2011) recommended turf stripping in the centre and fertilisation at the periphery of pastures, and combining cattle with small herbivores, such as sheep and goats, for a more efficient limitation of woody encroachment following restoration. However, fertilisation in these habitats is usually forbidden or at least very restricted, as it can lead to eutrophication (Galvánek and Janák 2008). Some countries encourage liming for the improvement of *Nardus* grasslands, as higher calcium (Ca) levels may positively affect species diversity (Common et al. 1991). Nevertheless, a thorough assessment is required before application since liming, like P, has a long-term effect on species composition (Hejcman et al. 2007). Turf stripping is otherwise mainly used in the case of eutrophication of the upper soil horizons. This technique removes nutrients from the upper soil layers, and thus restoration of such oligotrophic habitats is possible (Galvánek and Janák 2008).

Sometimes it is necessary to apply several restoration techniques at once. However, this correspondingly also requires more effort. Since grasslands that are to be restored are often abandoned and, therefore, densely overgrown, a technique that is often used is the removal of trees and shrubs by hand or with machinery. If a habitat is not densely overgrown, cutting and chopping of biomass with a flail or rotary mower may be used. When, however, trees and shrubs are too lush, a cultivator has to be used. Another possible, but costly method is also manual cutting with a brush cutter (Galvánek and Janák 2008). All these methods for scrub cutting are effective, but only if regular management is assured afterwards (Bensettiti et al. 2005), as scrub encroachment could turn out to be even more intense after cutting (Galvánek and Janák 2008).

Restoration of completely destroyed habitats is very expensive, yet feasible. There were some attempts of restoration of severely damaged habitats in Belgium by turf transplantation using sod-cutting techniques and by utilisation of hay or mulch from species-rich donor grasslands (Galvánek and Janák 2008). The turf should be placed on open land in a chessboard layout. Such an arrangement prompts seed dispersal and recruitment, and thus enables faster rehabilitation of disturbed habitats. In addition, turf also prevents soil erosion (Stanová et al. 2007). If farmers are not interested in maintaining these habitats, or when these habitats are threatened by various economic activities, land acquisition is of great importance in order to ensure their proper management. In any case, no matter which restoration measure is used, regular active management of these habitats is still much more cost-effective than their restoration (Galvánek and Janák 2008).

Timeline of grassland restoration

When designing restoration studies, it is important to take into account the conditions and area of both donor and recipient grassland plots, along with the phenology of the community and target grassland species (Edwards et al. 2007, Török et al. 2011). Seed collection should take place when most of the seeds in the donor plant community are ripe (Edwards et al. 2007, Török et al. 2011). The most appropriate times for seed collection in different habitat types are shown in Tab. 2. Nevertheless, it should be noted that these data were collected in different parts of Europe and, therefore, cannot be directly applied to the conditions in Slovenia.

For example, to maximise the yield of grass seeds, mowing of European dry grasslands should be carried out in June, whereas mesic grasslands should be mown later during the vegetation season (between June and July) (Török et al. 2011). To promote better biomass decomposition at the recipient sites in *Nardus* grasslands, propagule collection for restoration purposes in these grasslands should be carried out as early as May (Van Daele et al. 2017), in the period from June to July (Rūsiņa et al. 2017), in mid-July (Galvánek and Janák 2008, Kurtogullari et al. 2020), or by the end of July at the latest (Háková et al. 2004). Actions taken later in the vegetation season are generally more suitable for *Nardus* grasslands and pastures at higher altitudes (Kurtogullari et al. 2020). Wet grasslands are usually mown in late summer, only in August (Török et al. 2011). Generally, earlier cuts of grasslands favour grasses, whilst later or repeated cuts favour forbs (Krautzer et al. 2011, Haslgrübler et al. 2013). Late propagule collection can severely decrease the chances of successful grassland restoration. To avoid this, it is recommended to carry out multiple collections throughout the vegetation season (Török et al. 2011). The area ratio between recipient and donor site size varies between 1:2 and 1:10, and mostly depends on the state of vegetation at donor sites (Edwards et al. 2007, Török et al. 2011).

Table 2. An approximate timeline of plant material collection (primarily by mowing), which is used for the restoration of different European grassland habitat types. Differently coloured cells in the following table indicate theoretically defined optimal timing for mowing at different grassland habitat types. Individual restoration actions should, however, consider climate conditions and the state of donor grassland vegetation at a given time. Following works by Háková et al. (2004), Galvánek and Janák (2008), Krautzer et al. (2011),Török et al. (2011), Haslgrübler et al. (2013), Rūsiņa et al. (2017), Van Daele et al. (2017), Kurtogullari et al. (2020).

Tabela 2. Časovni okvir nabiranja rastlinskega materiala (predvsem s košnjo) za obnovo različnih evropskih habitatnih tipov travišč. Različno obarvana polja v spodnji preglednici označujejo teoretično optimalen čas za košnjo in nabiranje rastlinskega materiala v različnih travniških habitatnih tipih. Kljub temu pa je pri akcijah obnavljanja travnikov vedno potrebno upoštevati klimatske razmere in stanje vegetacije območja izbranih donorskih površin. Prirejeno po Háková in sod. (2004), Galvánek in Janák (2008), Krautzer in sod. (2011), Török in sod. (2011), Haslgrübler in sod. (2013), Rūsiņa in sod. (2017), Van Daele in sod. (2017), Kurtogullari in sod. (2020).

	January	February	March	April	May	June	July	August	September	October	November	December
Dry calcareous grasslands (habitat type 6210(*))						▨						
Mesic grasslands (habitat types 6510, 6520)						▨	▨					
Oligotrophic *Nardus* grasslands (habitat type 6230)					Dependent on area	▨	▨					
Wet grasslands (habitat type 6410)								▨				

Choice of donor sites for grassland restoration

When choosing adequate donor sites for grassland restoration, one should opt for donor sites that fulfil specific criteria. Primarily, we should choose donor sites that are biogeographically and phytocoenologically suitable for recipient sites, with representative target plant community structure and low presence of neophytes. Some databases for grassland sites in Germany also cite the need for adequate management status and naturalness of the site (not sown with commercial seeds), without incoming/planned changes in land use (Krautzer et al. 2011). Additionally, donor sites must be compatible with recipient plots regarding their nutrient status, hydrology, and substrate, with special attention paid to differences in water and nutrient status of dry, nutrient-poor grasslands (*Bromion*), mesic and mesotrophic grasslands (*Arrhenatherion*), and wet grasslands (*Molinion* and *Deschampsion*) (Krautzer et al. 2011).

Soil fertility as a crucial factor in grassland restoration

On land formerly used for cropland, the establishment of target grassland species can be limited by increased nutrient levels in topsoil, which stimulate the growth of highly competitive annuals and weeds (Kardol et al. 2008). The removal of the topsoil layer might be applied to lower nutrient concentrations (Hölzel and Otte 2003, Kardol et al. 2008, Kiehl et al. 2010, Török et al. 2011) and to remove propagules of weeds (Hölzel and Otte 2003, Kiehl et al. 2010, Török et al. 2011). To prevent soil erosion, the removal of 25-50 cm of soil is recommended, whereas soil removal on a larger scale is not advised. Soils of ex-arable fields are often saturated with inorganic N, which stimulates the development of non-target species in the initial phases of vegetation succession. To prevent overgrowth by non-target species (namely weeds), a decrease in soil fertility is required and carried out by different actions: topsoil removal, offtake optimisation, or the currently popular C addition (for more details, see Török et al. 2011).

Two different studies, one from Switzerland and one from the Czech Republic, reported a long-term impact of fertilisation on *Nardus* grasslands. In the latter study, the impact of fertilisation was evident even 37 years after the last nutrient application, especially on behalf of P and Ca (Hegg et al. 1992, Hejcman et al. 2007). Therefore, due to their preference for oligotrophic soils, restoration of extensive *Nardus* grasslands where fertilisers were applied in the past is difficult (Dähler 1992, Hejcman et al. 2007). Van Daele et al. (2017) claim that to restore species-rich *Nardus* grasslands, it is crucial to reduce bioavailable

P below 10 mg/kg or to select sites with bioavailable P contents below this threshold. Similarly, Korzeniak (2016) found that, especially for mesic *Nardus* grasslands in the lower montane zone, nutrient levels should be kept low to successfully control the expansion of nitrophilous species. For the restoration of species-rich *Nardus* grasslands, Van Daele et al. (2017) suggest inoculation of soil with a native soil community since native soil communities are known to promote restoration management (Middleton and Bever 2012, Wubs et al. 2016). Another criterion for selecting suitable sites for restoration could be a pH of approximately 4.5 to lift seed limitation. Last but not least, they recommend mowing in May, as this measure could reduce the competitive disadvantage of the slower-germinating *Nardus* species. They emphasised that to increase the success of restoration management, knowledge about establishment limitation should be considered (Van Daele et al. 2017).

According to Schelfhout et al. (2017), a prerequisite for successful restoration is that the requirements regarding the abiotic conditions are met. These authors conducted a study where they tried to restore *Nardus* grasslands on formerly intensively managed agricultural land. They discovered that traditional mowing and grazing did not change community composition in such a way that it would resemble *Nardus* grasslands. They concluded that when threshold values for abiotic conditions are exceeded, abiotic restoration should be performed before biotic restoration. Therefore, it is crucial to perform measurements of important initial soil characteristics (e.g., pH, nutrients, etc.) before restoration. For abiotic restoration, the authors suggest P-mining or topsoil removal. Nevertheless, topsoil removal is a costly procedure from both the perspectives of time and money. Topsoil removal is, therefore, more feasible in larger restoration projects with more funding. Restoration projects with less funding should focus on sites that were previously managed less intensively (Schelfhout et al. 2017).

Post-restoration management with grazing and mowing

The most commonly applied methods in post-restoration management, used in the studies analysed, are listed in Tab. 3. It is very important to use an appropriate type of post-restoration management since optimally chosen post-restoration management may have an impact that is comparable to or even greater than the impact of a suitable restoration method (Paolinelli Reis et al. 2022).

Grasslands in Europe are semi-natural ecosystems that greatly depend on how they are managed (Butaye et al. 2005). This fact confirms the importance of disturbance in grasslands (Edwards et al. 2007), primarily mowing and/or grazing in restored grasslands, once basic vegetation is established (Butaye et al. 2005, Kiehl et al. 2010, Török et al. 2011). Disturbance in the form of grazing and mowing enhances the colonisation of target species by creating better germination and establishment conditions at microsites by reducing the cover of highly competitive species (Edwards et al. 2007). Cutting taller plants is especially effective, as it stimulates the establishment of sown forbs (Lawson et al. 2004) and improves plot diversity (Török et al. 2011). One of the many positive impacts that grazing and mowing as forms of management also have on grasslands is the reduction of accumulated aboveground plant (litter) biomass since they open space and offer new niches for new plants to emerge and establish in the community (Török et al. 2011). This is a specificity of mesic grasslands due to their higher biomass production in comparison to xeric grasslands, which do not benefit from grazing and/or mowing as much (Hayes and Holl 2003). Huhta et al. (2001) indicated that different mowing timeline has a different impact on grasslands: late mowing as a form of grassland upkeep and litter removal, whereas early mowing might shift species structure due to disturbed seed production.

Turtureanu et al. (2014) claim that the highest plant species richness in semi-natural grasslands is provided by mowing. However, grazing and minor disturbance by animals may also be important for a higher diversity of plant species in semi-natural grasslands (Enyedi et al. 2008). Mowing may affect species composition since, for example, early mowing facilitates early-flowering species. As opposed to mowing, grazing usually inhibits graminoids and promotes the development and replenishment of forb species. However, grazing has a positive impact on the development and germination of many important grassland species, primarily by providing open soil surfaces and niches by livestock trampling while grazing (Török et al. 2020). Nevertheless, grazing animals may exert a large force on the soil surface due to their heavy weight and, at the same time, relatively small hoof area (Bilotta et al. 2007). As a result, trampling may reduce both biodiversity and vegetation cover at grazed sites (Matches 1992). Thus, the load imposed on the soil by grazing animals should always be taken into account. The amount of pressure exerted on the soil differs according to the species and age of grazing animals (Bilotta et al. 2007). Furthermore, there are differences regarding grazing preference according to the type of grazing livestock. Cattle and horses feed on taller grasses, whereas sheep prefer short grasses and forbs (Tóth et al. 2018). Moreover, browsers, such as goats, can reduce shrub encroachment into grasslands (Elias et al. 2018). On *Nardus* grasslands, late onset of grazing, badly organised grazing, or too low grazing intensity might lead to the spreading of small shrubs, such as *Vaccinium myrtillus* or *V. uliginosum*, which results in a lower pasturing value of these habitats (Bensettiti et al. 2005).

Table 3. A list of post-restoration grassland management techniques used in the studies reviewed.

Tabela 3. Seznam tehnik vzdrževanja travnikov po njihovi obnovi, ki so bile uporabljene v pregledanih raziskavah.

Grassland management technique	Suitable for	Advantages	Limitations	Reference / An example of use
Grazing	mesic and semi-dry semi-natural grasslands, e.g., habitat types 6210(*) and 6230*; not optimal for hay meadows and xeric grasslands	relatively frequent, but low-intensity disturbance; when carried out adequately (in line with species phenology), it can prevent shrub overgrowth and encroachment; enhancement of target species colonisation; removal of accumulated biomass and litter; grazing animals might serve as vectors for propagule transfer; decreases fire hazard; provision of open areas (niches) for the development of grassland species by animal trampling; increase and maintenance of microhabitat heterogeneity	selective (might cause an overgrowth of woody and thorny species, not palatable for certain types of livestock – primarily cows, so grazing by goats is recommended in this case); favours grasses over forbs due to their biology; dependent on species phenology; not suitable for xeric grasslands	Halada et al. 2001, Muller 2002, Hayes and Holl 2003, Pykälä 2004, Edwards et al. 2007, Galvánek and Lepš 2008, Török et al. 2011, Turtureanu et al. 2014, Török et al. 2016, Bonari et al. 2017, Tóth et al. 2018, Silva et al. 2019, Köhler et al. 2020, Kurtogullari et al. 2020, Mrázková-Štýbnarová et al. 2020, Török et al. 2020, Tölgyesi et al. 2022, Zarzycki et al. 2022
Mowing	various semi-natural grasslands, including habitat types 6210(*), 6230*, 6410, 6510, 6520	generally more accessible than grazing; early mowing facilitates the development of early-flowering species; enhancement of target species colonisation; improvement of the development of sown forbs by increasing competitiveness of higher plants; elimination of accumulated biomass and litter	beneficial only if carried out at an appropriate time in the season (when most diagnostic grassland forb species are ripe) and not too frequently (regime dependent on grassland type); when carried out with machinery, it is a non-selective and high-intensity disturbance single event (compared to grazing) – hand mowing has more advantages that are similar to grazing, but it is costly and time-consuming; might cause vegetation homogenisation; heavy machinery threatens invertebrate diversity	Halada et al. 2001, Muller 2002, Lawson et al. 2004, Edwards et al. 2007, Enyedi et al. 2008, Galvánek and Lepš 2008, Královec et al. 2009, Halada et al. 2011, Török et al. 2011, Valkó et al. 2012, Török et al. 2016, Bonari et al. 2017, Milberg et al. 2017, Van Daele et al. 2017, Tälle et al. 2018, Török et al. 2020, Zarzycki et al. 2022
Prescribed burning (with long fire-return periods)	various types of grasslands	elimination of accumulated biomass and litter	might cause drastic species shift; might increase chances of biological invasions	Bensettiti et al. 2005, Galvánek and Janák 2008, Valkó et al. 2014
Mulching	nutrient-poor grasslands	prevents overgrowth by woody species; promotes the development of target species found at nutrient-poor sites		Moog et al. 2002

Grassland management technique	Suitable for	Advantages	Limitations	Reference / An example of use
Cessation of fertilisation	suitable for oligotrophic grasslands (such as *Nardus* grasslands); previously used arable fields	retains naturally occurring nutrient status of oligotrophic habitats	revegetation might be time-consuming	Critchley et al. 2007, Galvánek and Janák 2008, Královec et al. 2009, Korzeniak 2016, Van Daele et al. 2017, Kurtogullari et al. 2020
Mechanical elimination of woody vegetation	*Nardus* grasslands	prevention of overgrowth at pasture edges	if not followed by regular management, it might cause worse shrub overgrowth compared to initial conditions	Galvánek and Janák 2008, Parolo et al. 2011
Mixed management	various semi-natural (including restored) grassland types	favours plant and butterfly diversity; takes into consideration the fact that different habitats require different approaches and that there is no ultimate solution that could be applied to all habitats	time- and resource-challenging	Bonari et al. 2017

Grazing is a more selective form of management that favours rosette and prostrate plant life forms, so its abandonment favours the development of taller plant stands. Pykälä (2004) also found that grazing improves the diversity of different plant functional types and life forms in mesic grasslands more than in wet or arid habitats. The latter are additionally limited by natural limiting factors, such as drought or flooding. In this study, grazing particularly benefitted annual, biennial, and perennial plants, whereas geophytes had a negative response to grazing. Cattle grazing might increase the coexistence of different species with different life strategies and functional types, if given species are not under selective pressure by cows as a food source. Apart from species' traits and habitat type, impacts of this specific disturbance are also geographically defined and, therefore, context-dependent. Török et al. (2011) noted that grazing could potentially have more positive impacts on grasslands in comparison to mowing since grazing animals can serve as vectors for propagule transfer. Furthermore, through their selective grazing, they can form more heterogeneous landscapes with different microclimatic and microstructural conditions. However, selective grazing can negatively affect the diversity and establishment of target species since grazers (especially cows) usually avoid woody and thorny species. Consequently, these can overgrow the target area. Such common field weeds should be avoided in forage production and may be suppressed with occasional clean cuts. Otherwise, goat grazing is also recommended in areas under pressure by shrub vegetation (Török et al. 2011). This was confirmed by Köhler et al. (2020), whose study showed that grazing by goats (browsing) had a positive effect on orchid-rich dry calcareous grasslands in Germany at a Natura 2000 site classified as habitat type 6210(*), due to the presence of *Gymnadenia conopsea*, *Orchis purpurea*, *O. militaris*, *Ophrys sphegodes*, *O. apifera*, and *O. insectifera*. Browsing should be conducted in early spring in accordance with the phenology of orchids and the onset of shrub development, when young shrub plants are still palatable. The results of this study are promising. However, they should be further researched since this study was conducted on a relatively small scale and with orchid cover varying throughout the eight-year study. Grazing and trampling probably enhance light availability in pastures, promoting the annual recruitment of orchids (Köhler et al. 2020). The positive effects of grazing are also reflected in epizoochory (Tölgyesi et al. 2022).

Mowing is a more accessible form of management. However, it does not offer the same probability of possible improvement in grassland and landscape diversity as grazing does, often causing homogenisation of vegetation and negatively impacting invertebrate diversity in grasslands, particularly when carried out by heavy machinery. When possible, mowing by hand is recommended, although this method itself is not very useful at a larger scale (Török et al. 2011).

Muller (2002) compared different management practices between different Natura 2000 grassland habitat types and denoted mowing without fertilisation as an adequate form of management for dry grasslands (6210(*))

since fertilisation led to structurally- and diversity-degraded forms of habitats dominated by grasses. On the other hand, abandonment led to overgrowth by shrubs. Low-intensity mowing without fertilisation is also recommended for *Molinia* grasslands (6410), which are very sensitive to eutrophication. Grazing without fertilisation turned out to be the best method for species-rich *Nardus* grasslands (6230*) since grazing prevents regressive succession to the previous form (heathlands), whereas fertilisation would disturb the oligotrophic nature of this habitat. Hay meadows (Natura 2000 habitat types 6510 and 6520) require extensive management with low fertilisation and late cutting. Moog et al. (2002) noted that for *Arrhenatherum elatius* and *Bromus erectus* grasslands, the best management regimes for grassland conservation include regular grazing, mowing, and mulching since these practices promote the development of species that are typical of nutrient-poor habitats. On the other hand, irregular mulching was proved to have a very similar impact as natural succession. Succession favoured the development of seedlings of woody species, such as *Fraxinus excelsior*, *Acer platanoides*, *A. pseudoplatanus*, and species of nutrient-rich habitats. Therefore, to prevent grassland degradation, it is highly discouraged to cease their management. Královec et al. (2009) reported on the possibility of natural regeneration of agriculturally used fields following cessation of fertilisation and regular management in the form of multiple cuttings per year. However, this experiment was relatively small-scale and was probably highly dependent on the colonisation of target species in the proximity and regular management rather than on the seed bank itself. A positive impact of post-restoration management on dry grasslands, evident from the suppression of ruderal perennial vegetation, was experimentally proved by Kiehl and Pfidenhauer (2007), particularly in the later phases of experiments.

The importance of regular management of grassland habitat types 6210(*), 6410, and 6510 was confirmed by Milberg et al. (2017), whose results showed that in comparison to annual mowing, mowing every three years had a negative impact on plant diversity after 11-14 years from cessation of management. Cessation of management had the highest impact on short plants, so the authors recommended extensive management (for example, mowing once every two years) on taller grasslands, whereas short-grown swards are suited for annual mowing. Even though irregular mowing might not be the ideal solution for the conservation of all grassland plants, it is still more reasonable and sustainable regarding diversity in comparison to abandonment, with additional benefits for pollinators.

One of the largest attempts at grassland restoration was carried out as part of the project LIFE04 NAT/HU/119 titled ‘Grassland restoration and marsh protection in Egyek-Pusztakócs’ in the Hortobágy National Park in the Great Hungarian Plain (Valkó et al. 2021). Following restoration actions in the form of seed mixture sowing, the authors studied the importance of post-restoration management and the impact of seed bank on grassland development. Their results showed that cessation of management had a negative impact on both seed bank and vegetation stands, favouring weed development. Furthermore, seed bank itself had limited potential in grassland restoration since it mainly consisted of weed species and not target species. The similarity between vegetation stands and seed bank was low (Valkó et al. 2021).

When trying to reduce *N. stricta* cover in the case of *N. stricta* dominance on *Nardus* grasslands, it is generally better to choose cattle than sheep, as sheep usually avoid *N. stricta* (Grant et al. 1996), and plant species diversity increases under cattle grazing (Armstrong et al. 1997). Similarly, in a study in the Hrubý Jeseník Mountains in the Czech Republic, overall species richness increased on a previously long-term unmanaged pasture after six years of revived cattle grazing. In addition, rare and endangered species became more dominant. The authors concluded that this was probably the result of cattle trampling, which formed small open habitats that enabled the germination and survival of new species (Mrázková-Štýbnarová et al. 2020). However, according to Hejcman et al. (2008), sheep grazing proved to be a suitable management practice for degraded meadows in the Giant Mts. in the Czech Republic, as it reduced the extent of undesirable species typical of long-term abandoned swards. Crawley (1983) even promotes mixed stocking with sheep and cattle (or goats), as one species improves the environment for the other, and thus facilitation is enabled. Accordingly, Holland et al. (2008) observed that the combined use of cattle and sheep was more effective in creating structural change on *N. stricta* grasslands compared to when sheep alone were used. Next, Kurtogullari et al. (2020) believe that pastures at lower altitudes should have a lower grazing intensity. Velev and Apostolova (2008) also found in their study in Bulgaria that the abundance of *N. stricta* was lower at lower grazing intensity. This could be achieved by moving cattle to higher altitudes earlier during the season. At higher altitudes, cattle should first be sent and enclosed in areas where *N. stricta* is dominant in order to reduce its cover as long as it is still palatable (Kurtogullari et al. 2020).

According to Bedia and Busqué (2013), the most suitable management practice for maintaining species-rich *Nardus* grasslands is grazing by large herbivores, as it reduces not only *N. stricta* dominance through defoliation, but also shrub encroachment through trampling and fertilisation (Hartley and Mitchell 2005). In general, low-intensity management by mowing, grazing, or a combination of both is crucial to retain *Nardus* grasslands (Krahulec et al. 2001, Dullinger et al. 2003). A combination is especially beneficial, as it enables the occurrence of a greater number of different species (Galvánek and Janák 2008). Most of all, a spatially variable disturbance regime is needed (Dullinger et al. 2003). Parolo et al. (2011) showed that heterogeneous grazing management had a positive impact on plant diversity in the alpine species-rich *Nardus* pastures in Italia. Likewise, Zarzycki et al. (2022) claim that to preserve the biodiversity of mountain grasslands, it is vital to preserve a mosaic spatial structure and retain extensive management practices with various forms of human impact, such as mowing and grazing. On the contrary, Vassiliev et al. (2011) suggested the formation of sheep pens for a more spatially uniform grassland management within the mountain in less accessible sites with abandoned pastures in the Western Balkan Mts. in Bulgaria. However, they also advocated extensive management with a zonation regime (Vassiliev et al. 2011). Yearly rotational grazing is also recommended to limit woody encroachment in remote areas on the one hand and also overgrazing near populated areas on the other hand (Başnou et al. 2009). In a study by Lüth et al. (2011), the *Sieversio montanae-Nardetum strictae* grasslands were most species-rich in the case of mowing, slight fertilisation, and grazing in autumn. Thus, they suggested that traditional hay management is the most appropriate practice (Lüth et al. 2011). Some use of manure or leaving cut grass on the ground every now and then is advisable when the only management practice on *Nardus* grasslands is mowing (Háková et al. 2004), as regular removal of biomass might gradually lead to oligotrophisation of these grasslands, especially on very poor soils, which reflects in a lower number of species (Krahulec et al. 1996, Halada et al. 2001). However, several soil parameters have to be considered first to prevent eutrophication (Galvánek and Janák 2008). In the case when *Nardus* grasslands are only mown, it is also highly recommended to introduce artificial disturbance to ensure space for those plant species that are less competitive (Háková et al. 2004). Fischer and Wipf (2002) advised to continue with traditional mowing of subalpine meadows and to switch back to mowing in the case of recently grazed meadows, as grazing negatively affected plant species richness in their study. However, this traditional management practice presents high costs and is, therefore, often no longer feasible (Galvánek and Janák 2008). Considering this, traditional mowing may also be alternated with sheep grazing. In fact, the alternation between mowing and grazing is advisable not only from the perspective of lowering the costs, but also to suppress the spreading of invasive species, as utilisation of only one restoration technique is often not enough to curb the expansion of invasive species (Pecháčková and Krahulec 1995, Krahulec et al. 2001). Pecháčková and Krahulec (1995) reported that by using multiple restoration techniques at once, it is possible to restore species-rich grasslands in three to five years. Nonetheless, Halada et al. (2001) found that the vitality of invasive species was also suppressed by applying regular mowing only. Along with the position of grasslands along the altitudinal gradient, management practices should also always take into consideration the sensitivity of grasslands to isolation and fragmentation (Reitalu et al. 2012, Janišová et al. 2014). Korzeniak (2016) stated that the above-mentioned suggestions are necessary especially for thermophilous grasslands with *Nardus* in the lower montane zone.

Evaluation of grassland restoration techniques

Evaluation of grassland restoration techniques and their comparison is a very demanding task since every restoration action has specific initial conditions at both target and donor sites (Török et al. 2011). Every restoration action depends on interspecies' interactions on the field, particularly competition (Hölzel and Otte 2003), and therefore requires individual approaches (Török et al. 2011). Additionally, technical details differ for each restoration action. Different methods are generally harder to compare. Therefore, only similar actions should be compared (e.g., sowing of seeds and addition of plant material). In general, any restoration planning effort should start with the collection of reliable information regarding the previous and current state of chosen plots. The choice of methodology should be carefully considered and planned according to the target vegetation and conditions at both target and donor sites, which should be ecologically similar (Török et al. 2011). Sengl et al. (2017) noted that commonly used variables, such as similarity and dissimilarity indices, biodiversity indices, number and coverage of chosen functional plant groups, and species number, are applicable only if sites are similar or located nearby, and not when they are distant and/or degraded. For instance, sites with high diversity could be degraded by invasive species and weeds, and thus their ecosystem services could be disrupted. Therefore, Sengl et al. (2017) recommend the evaluation of grassland naturalness, more specifically, the use of ecological indicator values, which explain the state of grasslands through the presence of specific plant species, level of invasiveness, grassland functional diversity, and resilience of grasslands to disturbance and degradation.

The evaluation of success is highly context-dependent. According to Prach et al. (2021a), varying abiotic and biotic factors at chosen sites affect restoration success and are also influenced by the chosen restoration method. Furthermore, soil and landscape characteristics are also highly important. Their experiment showed that the use of regional seed mixtures benefitted the establishment of dry grasslands, whereas methods like natural succession and commercial seed mixtures favoured the establishment of mesic grassland species (Prach et al. 2021a). They also noted that researchers should evaluate measures of post-restoration management, which would primarily help in the establishment of characteristic target species. In addition, restoration must also be thought out from the technical and economic points of view. When possible, the desired restoration actions should be tested. Restoration progress should be monitored and sampled using an appropriate methodology, which should be able to show the differences and results of revegetation attempts. In addition, these should be documented for future reference (Török et al. 2011). The survival of newly established plant populations is often limited by ecosystem services, such as seed production and dispersal, but also by other factors, such as lack of suitable pollinators, limited genetic diversity in sexual mating, vegetative reproduction by dominant species, herbivory, and inadequately planned management (Albert et al. 2021). Albert et al. (2021) found that donor sites provide more ecosystem services in comparison to recipient sites. These show signs of pressure by previous arable use and dominance of ruderals and weeds, supporting mostly herbivory, whereas the presence of late-flowering meadow generalists supports pollination and pollinator diversity on donor grasslands.

Conclusions

Today, grasslands are under threat all over the world, mainly due to human activity and climate change, which is why their ability to provide ecosystem services is rapidly decreasing.

The agri-environment measures for the preservation of high nature value grasslands in Slovenia are not successful for various reasons. Therefore, they should be reconsidered and based on a different basis.

Given the many benefits that grasslands provide, it is vital to prevent their further decline. The best way to maintain grassland biodiversity is to ensure traditional extensive agricultural practices, such as mowing and/or grazing. For this reason, these two practices are also often recommended for grassland restoration. In order to successfully maintain grassland biodiversity, not only one type of appropriate practice but rather the introduction of a wider scheme of traditional management practices is necessary. In addition, mosaic management should also be taken into account.

In the case of partly degraded grasslands, a reduction in management intensity is usually sufficient for their successful restoration. On the other hand, for entirely degraded grasslands, spontaneous succession or technical reclamation (for example, sowing of regional seed mixtures or transfer of plant material) is necessary for their recovery. In any case, the proximity of well-preserved donor grasslands, which serve as a natural source of reproductive units (propagules) for degraded grasslands, is key to the success of larger grassland restoration projects.

Restoration of species-rich grasslands can be a very time-consuming process and usually requires a certain amount of soil preparation on the land that will be subject to restoration, as well as further management after restoration. It was found that optimally chosen post-restoration management may have an impact that is comparable to or even greater than the impact of a suitable restoration method.

When designing restoration studies, it is always necessary to take into account the conditions and area of donor and recipient grassland plots, as well as the phenology of the community and target grassland species.

When selecting donor plots for grassland restoration, attention should be paid to the fact that donor plots should be compatible with recipient plots in terms of nutrient status, hydrological conditions, and substrate. In addition, donor plots must also demonstrate a biogeographically and phytocoenologically representative plant community composition, with as little presence of neophytes as possible.

In any case, active maintenance of well-preserved grasslands that have not yet been degraded is more cost-effective than the restoration of grasslands that have already been degraded.

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ABS

Review Article

Sekundarni metaboliti v navadni konoplji (*Cannabis sativa* L.) in njihova vloga pri obrambi rastline pred škodljivci in patogeni

Luka Biteznik [1,*], Marina Dermastia [2], Stanislav Trdan [1]

Izvleček

Navadna konoplja (*Cannabis sativa L.*) je razširjena rastlina z zapleteno fitokemijo in bogatim sekundarnim metabolizmom. Sekundarni metaboliti so snovi, katerih vloga je interakcija rastline z okoljem. Njihova sinteza je pogojena s prisotnostjo stresa in je energetsko zelo potratna. Rastline varujejo pred škodljivci in patogeni ter abiotskimi dejavniki. V konoplji so v največji meri prisotni kanabinodi, terpenodi in flavonoidi, skoncentrirani v socvetjih ženskih rastlin. Njihova ekološka vloga in delovanje ni povsem raziskano. Kanabinoidi Δ-9-*trans*-tetrahidrokanabinol (Δ^9-THC), kanabidiolna kislina (CBDA) in drugi naj bi bili vpleteni v antioksidativne procese in obrambni sistem rastline, zaradi prostorsko izolirane sinteze in citotoksičnih stranskih produktov. Ob prisotnosti stresa pride do povečanja njihove vsebnosti in spremembe kemijskega profila. Stres aktivira stresne signalne molekule – jasmonsko kislino in njene derivate, ki vplivajo na sproščanje terpenodiov, ter poveča izražanje genov v fenilpropanoidni poti, ki je ključna za sintezo flavonoidov. Sekundarni metaboliti delujejo sinergično in v povezavi z mikrobiomom – endofitnimi bakterijami in glivami – negativno vplivajo na škodljive organizme ter varujejo rastlino.

Ključne besede

Cannabis sativa L, flavonoidi, kanabinoidi, navadna konoplja, terpenoidi, patogeni, škodljivci

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Secondary metabolites of hemp (*Cannabis sativa* L.) and their role in defence against pests and pathogens

Abstract

Hemp (*Cannabis sativa* L.) is a globally distributed plant with a complex phytochemistry. Secondary metabolism is extremely rich. Secondary metabolites are substances whose role is the interaction of the plant with the environment. Their synthesis depends on the presence of stress and is very energy-consuming. They protect plants from pests and pathogens, as well as from abiotic factors. Cannabinoids, terpenoids and flavonoids are the predominant substances concentrated in the inflorescences of female plants. Their ecological role and function are not yet fully understood. The cannabinoids Δ-9-*trans*-tetrahydrocannabinol (Δ9-THC), cannabidiolic acid (CBDA), and others probably act as antioxidants, and based on their spatially isolated synthesis and cytotoxic by-products, they are thought to be involved in the plant's defense system. Under stress, their content increases and the chemical profile changes. Stress activates stress signalling molecules - jasmonic acid and its derivatives, which affect the release of terpenoids, and increases the expression of genes in the phenylpropanoid pathway, which is crucial for the synthesis of flavonoids. Secondary metabolites act synergistically and in conjunction with the microbiome - endophytic bacteria and fungi - have a negative effect on harmful organisms and protect the plant.

Keywords

cannabinoids, *Cannabis sativa* L, hemp, flavonoids, pests, pathogens, terpenoids

Uvod

Navadna konoplja (*Cannabis sativa* L.) je zelo razširjena rastlina na naravnih rastiščih, ki ji ustrezajo. Izvirala naj bi iz območij centralne in jugovzhodne Azije, od koder se je razširila po vsem svetu (Schultes in sod. 1974). Konoplja je vsestransko uporabna v tekstilni industriji in gradbeništvu zaradi hitre rasti in visoke vsebnosti celuloze ter lesnih vlaken. Ker pa vsebuje veliko bioaktivnih snovi – rastlinskih sekundarnih metabolitov z biološkim učinkom v človeškem telesu, je že tisočletja del vzhodnjaških kultur in je vse bolj cenjena tudi v farmacevtski industriji in medicini (Andre in sod. 2016). Pripravke iz konoplje danes uporabljamo za lajšanje kroničnih in nevropatskih bolečin ter bolezenskih znakov, ki se pojavljajo pri obolelosti z rakom, multiplo sklerozo, Parkinsonovo, Crohnovo boleznijo, številnimi psihičnimi motnjami in drugimi boleznimi (Hill 2015). Pripravki so se izkazali za učinkovite tudi pri zmanjševanju slabosti in stimulaciji apetita v primeru obolelosti s HIV-om (Woolridge in sod. 2005).

Sekundarni metaboliti rastlinam omogočajo interakcije z okoljem in s tem pomagajo pri njihovem preživetju (Yazaki 2004). Vključeni so v obrambne mehanizme pred biotičnimi in abiotičnimi dejavniki, kot je UV-B sevanje, ter tudi v komuniciranje med samimi rastlinami (Schafer in Wink 2009). V splošnem velja, da so rastline z višjo vsebnostjo sekundarnih metabolitov odpornejše, vendar je proizvodnja teh snovi energetsko zelo potratna. To vpliva na rast in razvoj rastline, zato je povečana sinteza pogojena s prisotnostjo stresa. Rastline so razvile kompleksne obrambne sisteme, v katerih biotični ali abiotični stresni dejavniki sočasno vplivajo na številne signalne in odzivne poti. Obrambni sistemi omogočajo rastlini odkrivanje patogena in tudi sprožitev tarčne obrambe (Zaynab in sod. 2018).

Kljub številnim raziskavam sekundarnih metabolitov konoplje in njihovih učinkov na ljudi, je podatkov o vlogi teh snovi v biotičnem stresu in dejanskem ekološkem pomenu dokazanega protimikrobnega delovanju sekundarnih metabolitov iz konoplje zelo malo. V tem pregledu smo te skope informacije zbrali.

Obrambni sistem konoplje

Obrambni sistem konoplje vključuje fizično in kemijsko obrambo. Fizično rastlinske dele ščitijo trihomi; med njimi je največ žleznih. Žlezne votline žleznih trihomov (slika 1) so občutljive na dotik in se zlahka poškodujejo ter na listno površino izločijo sekundarne metabolite – kanabinoide in terpenoide. Te spojine oksidirajo in polimerizirajo v smolo, ki mehansko in kemijsko prepreči napad škodljivca (Potter 2009). Bogat terpenski profil in intenzivne arome delujejo repelentno na številne škodljivce (Pate 1994).

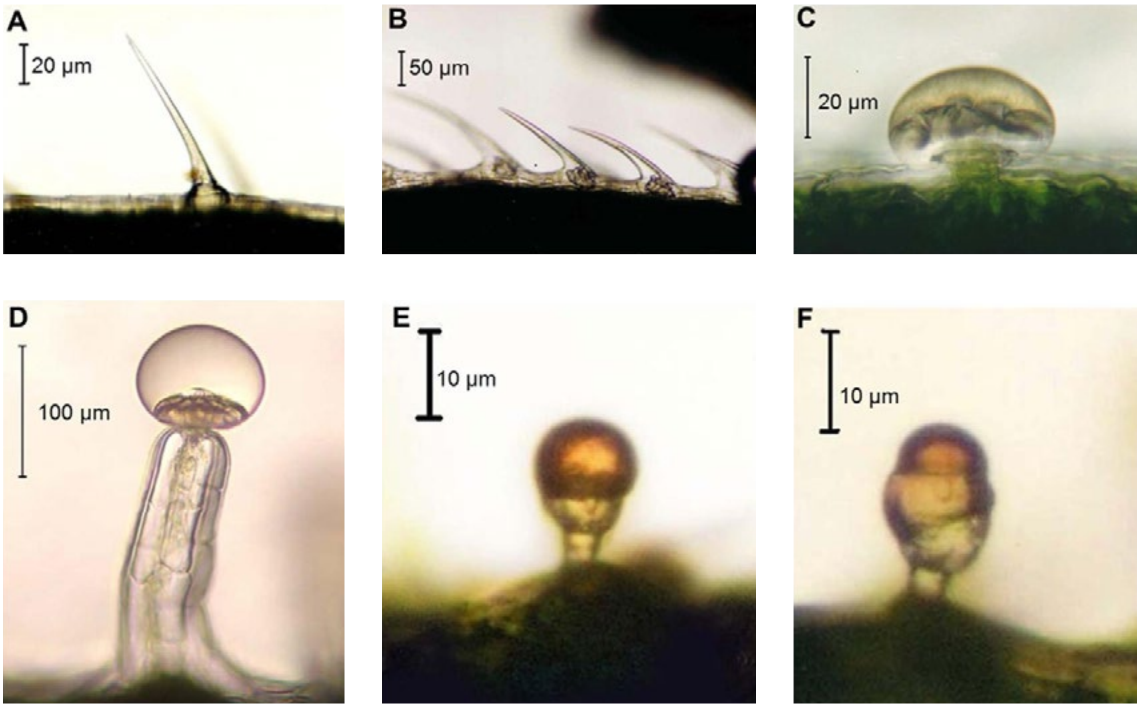
V kombinaciji s sekundarnimi metaboliti, kot dodatna mehanska prepreka škodljivcem delujejo na listni površini prisotni posebni trihomi – cistolitni laski (slika 1 B) (Glattstein in Gorski 2001). Cistolitni laski so mikroskopsko majhni, močno silificirani kristali kalcijevega karbonata, ki ovirajo mobilnost manjšim herbivorom, večjim pa poškodujejo ustni aparat (Levin 1973).

Fitokemija konoplje je zelo zapletena in zajema več kot 480 spojin (ElSohly in Slade 2005) iz različnih kemičnih skupin. Nekatere spojine so primarni metaboliti, vendar je večina sekundarnih. Med slednjimi so najpogostejši kanabinoidi, terpenoidi, flavonoidi, stilbenoidi, lignani in alkaloidi. Njihova koncentracija v rastlinskem tkivu je pogojena z delom rastline, starostjo rastline, sorto, okoljskimi dejavniki in agrotehničnimi ukrepi (Keller in sod. 2001).

Prevladujoči sekundarni metaboliti v navadni konoplji in njihova interakcija z okoljem

Kanabioidi

Kanabinoidi so skupina terpenofenolnih spojin, sestavljeni iz 21 ogljikovih atomov in so v rastlini prisotni v obliki karboksilne kisline. V rodu *Cannabis* so najpogosteje zastopana skupina sekundarnih metabolitov (Głodowska 2016), saj jih več kot 100 različnih. Med njimi je najbolj raziskan Δ-9-*trans*-tetrahidrokanabinol (Δ^9-THC). Pri sesalcih



Slika 1. Vrste žleznih in nežleznih trihomov na rastlinah konoplje. (A) Enocelični nežlezni trihom; (B) cistolitni laski; (C) »glavasti sedeči« (angl. capitate-sessile) trihom (D) »pecljati glavasti« (angl. capitate-stalked) trihom; (E) »preprost čebulni« (angl. simple bulbous) trihom; (F) »kompleksen čebulasti« (angl. complex bulbous) trihom (Andre in sod. 2016).

Figure 1. Types of glandular and non-glandular trichomes on cannabis plants. (A) Unicellular non-glandular trichome; (B) cystolitic hairs; (C) “capitate-sessile” trichome (D) “capitate-stalked” trichome; (E) “simple bulbous” trichome; (F) “complex bulbous” trichome (Andre et al. 2016).

Δ^9-THC ob zaužitju povzroča psihoaktivne učinke, zato je v večini držav po svetu prepovedan (Whiting in sod. 2015). Najpogosteje prisotni kanabinoidi oziroma fitokanabinoidi v rastlini so tetrahidrokanabinolna kislina (THCA), kanabidiolna kislina (CBDA), kanabigerolna kislina (CBGA), kanabinolna kislina (CBNA), kanabikromeska kislina (CBCA) in kanabikiklolna kislina (CBLA) (Jin in sod. 2020). Vsi kanabinoidi imajo pomembno vlogo pri obrambi rastline pred škodljivci, patogeni in abiotičnimi dejavniki (McPartland in sod. 2000; Pate 1994).

V rastlini so kanabinoidi nakopičeni predvsem v žleznih votlinah trihomov (slika 1. C-F) na ženskih socvetjih (Whiting in sod. 2015; Tanney in sod. 2021). Vsebnost kanabinoidov v socvetjih konoplje je odvisna od genetske osnove rastline, mineralne prehrane, osvetlitve, relativne zračne vlage in časa vzorčenja (Keller 2001). Njihova vsebnost in razmerja se močno spreminjajo glede na rastlinsko tkivo in se razlikujejo tudi na podlagi geografskega porekla rastline (Hemphill in sod. 1980). V neoprašenih ženskih socvetjih dosegajo vsebnosti do 21 % suhe mase socvetja, medtem ko je vsebnost v listih lahko le 0,3 % (Richins in sod. 2018). Rastline iz severnih območji imajo višje vsebnosti Δ^9-THC in CBD, kar lahko pojasnimo z ostrejšimi podnebnimi razmerami (Leizer in sod. 2000). Poleg tega je vsebnost Δ^9-THC v primerjavi s CBD višja pri rastlinah, ki rastejo bližje ekvatorju, zato naj bi Δ^9-THC med drugim rastlino ščitil pred UV žarki. Δ^9-THC je viskozna hidrofobna in nizko hlapna snov, ki v povezavi z drugimi kanabinoidi in terpeni tvori voskom podobne snovi. Te imajo številne funkcije, med katerimi je tudi omejitev transpiracije in izgube vode v suhih rastnih razmerah (Pate 1994).

Okoljski dejavniki povzročajo v rastlini oksidativen stres, ki ga uravnavajo antioksidanti in njihovi encimi. Pri varovanju celic pred reaktivnimi kisikovimi spojinami so najpomembnejši tokoferoli, fenoli, superoksid dismutaza, askorbat peroksidaza in katalaza. Reaktivne kisikove spojine vplivajo na biokemijo kanabinoidov, zato predvidevamo, da so kanabinoidi vpleteni v antioksidativne procese. Prosti radikali in hidroksilirani intermediati sodelujejo pri oksidaciji nevtralne in kislinske oblike Δ^9-THC v nevtralno in kislinsko obliko CBN ali Δ^8-THC (Flores-Sanchez in Verpoorte 2008).

Specifično mesto skladiščenja kanabinoidov v žleznih trihomih na površini rastline kaže še na drugo vlogo v rastlini, kar so potrdili Sirikantaramas in sodelavci leta 2005. Stranski produkt THCA-sintaze in oksidacije CBGA je vodikov peroksid, ki nastaja v velikih količinah. THCA in vodikov peroksid sta za rastlinske celice toksična, zato se THCA-sintaza izloča iz sekretornih celic v žlezne votline. Prostorsko izolirana biosinteza prepreči poškodbo celic. V žleznih trihomih poteka sinteza tudi drugih kanabinoidov, kot na primer izomer CBDA in CBCA, kar bi lahko pomenilo vključenost kanabinoidov v obrambnem mehanizmu rastline (Taura in sod. 1996).

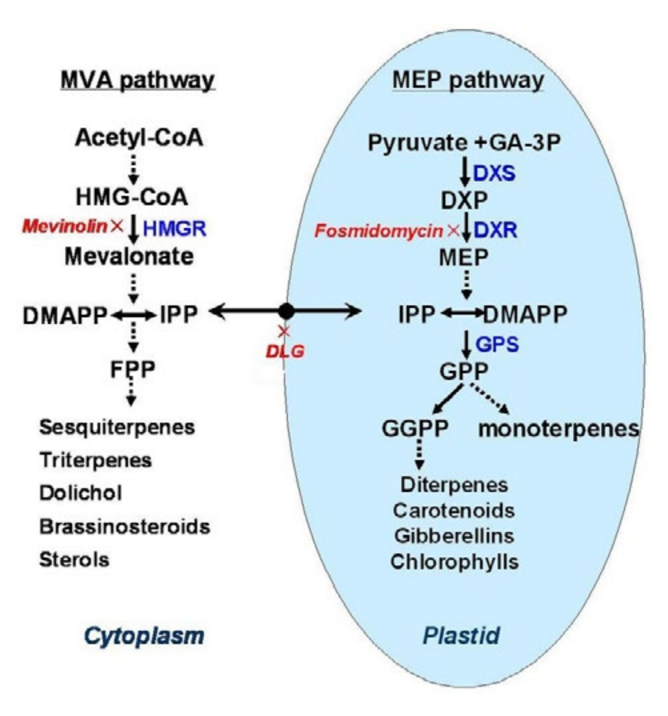
Terpenoidi

Terpenoidi so spojine, ki vključujejo terpene in njihove oksigenirane derivate. Terpeni imajo skupaj z drugimi sekundarnimi metaboliti pomembno vlogo v obrambnem sistemu rastline pred škodljivci in patogeni (Gershenzon in Dudareva 2007). Terpenoide nekaterih rastlinskih vrst danes uporabljamo kot insekticide, med najbolj poznanimi so azadirahtin in piretrini. Konoplja sintetizira več kot 140 terpenoidov, med katerimi so v največji meri zastopani terpeni (McPartland in Sheikh 2018). Od prisotnosti terpenov je odvisna aroma in intenzivnost vonja rastline. Vsebnost terpenov v rastlinskem tkivu je odvisna od genetske osnove, okoljskih dejavnikov in razvojne stopnje rastline (Brenneisen 2007).

Ob poškodbi rastlinskega tkiva in sprožitvi sinteze rastlinske signalne molekule v primeru prisotnosti stresnih dejavnikov – jasmonske kisline in njenih derivatov (Ruan in sod. 2019), se iz rastline začno sproščati hlapne spojine. To so večinoma terpenoidi in nekatere snovi nastale po šikimski sintezni poti. Za povečano tvorbo terpenoidov sta odgovorni dve sintezni poti – pot mevalonske kisline in pot metil-eritriol-4-fosfata (slika 2) (Bartram in sod. 2006).

Izražanje genov za pot mevalonske kisline in pot metil-eritriol-4-fosfata je zelo nadzorovano, saj obe poti predstavljata pomemben člen v biosintezi izoprenoidov, osnovnih gradbenih enot terpenoidov (Vranová in sod. 2013). Izoprenoidi geranil difosfat (GPP), fernesil difosfat (FPP) in geranilgeranil difosfat (GGPP) so začetne molekule tudi za številne sekundarne metabolite, vključno s kanabinoidi (Bergman in sod. 2019).

Terpene klasificiramo na podlagi števila ponavljajočih se izoprenskih enot, sestavljenih iz petih ogljikovih atomov. V socvetjih, koreninah in listih rastlin konoplje je največ monoterpenov in seskviterpenov. Prevladujoči hlapni terpeni v ženskih socvetjih so monoterpeni, med katerimi so najpogostejši D-limonen, β-mircen, α- in β-pinen, terpinolen ter linalol (Fischedick in sod. 2010). Hlapne terpene lahko z uporabo parne destilacije skoncentriramo



Slika 2. Pot mevalonske kisline (MVA) in pot metil-eritriol-4-fosfata (MEP). 3-hidroksi-3-metilglutaril koencim A reduktaza (HMGR), dimetilalil difosfat (DMAPP), izopentenil difosfat (IPP), fernesil difosfat (FPP), D, L-gliceraldehid (DLG), gliceraldehid 3-fosfat (GA-3P), 1-deoksi-D-ksiluloza 5-fosfat (DXP), 1-deoksi-D-ksiluloza 5-fosfat sintaza (DXS), 1-deoksi-D-ksiluloza 5-fosfat reduktoizomeraza (DXR), 2-C-metil-d-eritritol-4-fosfat MEP, geranilgeranil difosfat (GGPP), geranil difosfat (GPP) in geranilgeranil difosfat sintaza (GGPPS) (Yang in sod. 2012).

Figure 2. The mevalonic acid (MVA) pathway and the methyl-erythritol-4-phosphate (MEP) pathway. 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), dimethylallyl diphosphate (DMAPP), isopentenyl diphosphate (IPP), farnesyl diphosphate (FPP), D, L-glyceraldehyde (DLG), glyceraldehyde 3-phosphate (GA-3P), 1-deoxy-D-xylulose 5-phosphate (DXP), 1-deoxy-D-xylulose 5-phosphate synthase (DXS), 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR), 2-C-methyl-d-erythritol-4-phosphate MEP, geranylgeranyl diphosphate (GGPP), geranyl diphosphate (GPP), and geranylgeranyl diphosphate synthase (GGPPS) (Yang et al. 2012).

v eterično olje (Beek in Joulain 2017). Izmed vseh monoterpenov, pinen, mircen in limonen predstavljajo približno 85 % vseh hlapnih terpenov, ki jih zaznamo v bližini rastline, vendar skupno prispevajo manj kot 10 % k celokupni vsebnosti eteričnega olja v rastlinskem tkivu konoplje (Hood in sod. 1973). Konoplja z visoko vsebnostjo Δ9-THC ima tri do šest krat višjo vsebnost limonena in pinena v primerjavi z industrijsko konopljo (Mediavilla in Steinemann 1997).

Flavonoidi

V konoplji so določili že več kot 20 flavonoidov, od katerih večina pripada flavon- (apigenin in luteolin) in flavonol- (kaempferol in kvartecin) aglikonom in glikozidom (Flores-Sanchez in Verpoorte 2008). Biološka funkcija flavonoidov je povezana z njihovo potencialno citotoksičnostjo in sposobnostjo interakcije z encimi. Nekateri delujejo kot stabilizatorji prostih radikalov, na primer reaktivnih kisikovih spojin, lahko pa tudi kot kelatorji kovin, pri čemer v Fentonovi reakciji nastajajo reaktivne kisikove spojine (Williams in sod. 2004). Sposobnost keliranja kovin daje OH skupina na tretji poziciji flavonoidnega skeleta (Verdan in sod. 2011).

Posledica abiotičnega stresa je velikokrat povečana sinteza flavonoidov. Flavon luteolin ima na primer varovalno vlogo pred UV-B sevanjem (Ferreyra in sod. 2012). Posebni flavonoidi, ki jih najdemo v rastlinah konoplje so kanaflavini A, B in C. Kanaflavin A so določili tudi v *Mimulus bigelovii*, rastlini iz družine *Phrymaceae*, medtem ko sta preostala dva znana izključno v navadni konoplji (Bautista in sod. 2021).

Sinteza kanaflavinov je tako kot pri večini sekundarnih metabolitov pogojena s stresnimi razmerami, kar so potrdili v raziskavi leta 2020. Z večanjem nadmorske višine se povečuje vsebnost kanaflavina A, B in C (Giupponi in sod. 2020). Sinteza flavonoidov se poveča tudi pri biotskem stresu, saj sprožena obramba pred patogenom poveča izražanje genov v fenilpropanoidni poti. Ob napadu patogena se zmanjša sinteza antocianov in poveča izražanje genov, odgovornih za sintezo flavonov in izoflavonov. Predvidevajo, da zaradi nasprotno delujočega uravnavanja sinteze, izoflavoni prevzamejo vlogo antocianov in delujejo kot antioksidativne in protimikrobne spojine (Samac in Graham 2007).

Pomembni škodljivci in patogeni navadne konoplje

Leta 1976 sta Small in Cronquist izoblikovala uradno nomenklaturo za konopljo in jo uvrstila v družino konopljevk (Cannabaceae). Razvrstitev v podvrste temelji na vsebnosti sekundarnega metabolita Δ^9-THC, vendar so med podvrstami tudi znatne razlike v prisotnosti drugih sekundarnih metabolitov. Poznavanje lastnosti gostiteljske rastline in njene kemijske sestave je ključnega pomena za uspešno prepoznavanje patogenov in škodljivcev. Kanabinoid Δ^9-THC in terpenoida humulen ter kariofilen imajo insekticidno delovanje, medtem ko imajo metil-ketoni repelentno delovanje na listne herbivore (Mediavilla in Steinemann 1997).

Variabilnost vsebnosti sekundarnih metabolitov je zelo velika, zato je zaradi sočasne evolucije patogenov in škodljivcev ter gostiteljske rastline prišlo do speciacije. Škodljivi organizmi napadajo le izbrane rastline (McPartland in sod. 2000). Speciacija škodljivih organizmov je prisotna med podvrstami konoplje, vendar ni dokazov, s katerimi mehanizmi se ti organizmi izogibajo rastlinski obrambi.

McPartland (1992) je identificiral številne patogene, ki okužujejo konopljo. Skupno naj bi bila konoplja gostiteljska rastlina okrog 100 različnim patogenom in številnim škodljivcem, kot so insekti in pajkovci. Novejše raziskave se osredotočajo na patogene na posušenih ženskih socvetjih (Thompson in sod. 2017).

Škodljivci konoplje

Navadna pršica (*Tetranychus urticae*) in karminasta pršica (*Tetranychus cinnabarinus*), iz skupine pršic prelk sta izraziti termofilni vrsti ter zato pogosta škodljivca pri gojenju konoplje v zavarovanih prostorih (McPartland in sod. 2000). Optimalne razmere za razvoj škodljivcev so v času cvetenja rastlin konoplje, zato takrat povzročajo največ škode. Prehranjujejo se na spodnji strani listov, prebadajo posamezne celice in povzročajo izgubljanje klorofila ter zmanjšanje neto fotosinteze rastline. Poškodbe opazimo kot bele ali rumene pike na zgornji strani listov (Park in Lee 2002). Problematična je tudi pršica *Aculops cannabicola*. Je monofag, ki napada liste, listne peclje, meristeme in socvetja ženskih in moških rastlin. Povzroča zvijanje in nekroze listov, ter zmanjšuje produkcijo smolnih snovi pri konoplji (Petanović in sod. 2007). Konoplja je priljubljena gostiteljska rastlina tudi ušem. Večinoma jo napadajo siva breskova uš (*Myzlis persicae*), konopljina uš (*Phorodon cannabis*) in črna fižolova uš (*Aphrid fabae*). Prehranjujejo se s floemskim sokom, le redke sesajo ksilem. Zadržujejo se na spodnji strani listov in povzročajo venenje in rumenenje ter so prenašalci številnih gliv, bakterij in virusov (Kennedy in sod. 1959; Prado in Tjallingii 1994). Izločajo eksudat, medeno roso, s katero se prehranjujejo mravlje in glive sajavosti, ki povzročajo sekundarne poškodbe na rastlinah (McPartland in sod. 2000). Uši na konoplji so toplo in vlago ljube, previsoke temperature povzročajo pritlikavost osebkov, zmanjšujejo plodnost in skrajšujejo njihovo življenje. So pogostejši škodljivec pri pridelavi na prostem (Chi in Su 2006). V nadzorovanih prostorih in rastlinjakih je zelo pogost škodljivec rastlinjakov ščitkar (*Trialeurodes vaporariorum*). Bolezenska znamenja napada in poškodbe so podobne ušem, rastline izgubljajo vigor, listi venijo in rumenijo. Ob močnejšem napadu rastline propadejo (McPartland in sod. 2000). Rastlinjakov ščitkar ravno tako izloča medeno roso, s katero privabi glive sajavosti, ki posledično zmanjšujejo neto fotosintezo rastline (Hall 1982; Byrne in Miller 1990). Optimalne razmere za razmnoževanje so pri 24 °C in visoki relativni zračni vlagi, ko samice ležejo sto in več jajčec na spodnjo stran listov (Gamarra in sod. 2020). Poleg rastlinjakovega ščitkarja sta zelo problematična še tobakov resar (*Thrips tabaci*) in cvetlični resar (*Frankliniella occidentalis*). Cvetlični resar je škodljivec zunaj rastočih rastlin, medtem ko se tobakov resar pojavlja pri pridelavi rastlin konoplje v zavarovanih prostorih (McCune in sod. 2021). Resarji sesajo celični sok na spodnji strani listov, zato so prve poškodbe težko vidne. Ob močnejšem napadu na listih najdemo njihove izločke, vidne kot majhne črne pike. Izleže se do osem generacij letno, vendar je število močno odvisno od temperature (Van Rijn in sod. 1995, McPartland in sod. 2000).

Patogeni konoplje

Najpogostejši patogen, ki napada rastline konoplje je siva plesen, ki jo povzroča gliva *Botrytis cinerea*. Njen razvoj pospešuje visoka relativna zračna vlaga, zato na obdelovalnih površinah v deževnih mesecih povzroča velike izpade pridelka (Van der Werf in sod. 1995). Do okužbe koreninskega sistema pride v vseh razvojnih stopnjah rastline, socvetja pa gliva kolonizira ob poznem cvetenju ali po spravilu pridelka socvetij. Okužba lahko izbruhne v sušilnicah ali med skladiščenjem in s tem zmanjšuje kakovost in uporabnost socvetij (Punja in sod. 2019). Pogosti patogen rastočih rastlin je pepelovka, ki jo povzročata glivi *Podosphaera macularis* (prej *Sphaerotheca macularis*) in *Leveillula taurica*. Napadata mlade rastline na katerih se bolezenska znamenja pokažejo nekaj tednov po okužbi. Okužba je omejena na liste in se širi v suhih rastnih razmerah, ob nizki stopnji osvetlitve in slabem kroženju zraka. Konidiji uspešno kalijo pri relativni zračni vlagi 0 – 100 %. Ob močnem napadu je okužba za rastlino usodna (Nour 1958; McPartland in sod. 2000).

Vpliv sekundarnih metabolitov konoplje na škodljivce in patogene

Prevladujoči terpeni v eteričnem olju konoplje – α-pinen, β-mircen, ocimen, terpinolen in *trans*-kariofilen delujejo insekticidno na navadno pršico (*T. urticae*) in uš *Aulacorthum solani* (Górski in sod. 2016). Pri rastlinah, ki so bile izpostavljene napadu navadne pršice se je povišala vsebnost vseh analiziranih terpenov, razen α-pinena. Vsebnost terpenov se je povišala tudi v socvetjih rastlin, ki so bile pred cvetenjem obdelane z akaricidom, kar naj bi nakazovalo pomembnost interakcije s škodljivcem (Kostanda in Khatib 2021). Čeprav je v konoplji veliko terpena β-kariofilena, njegovega insekticidnega delovanja na konopljo še niso proučili. Kljub temu sklepajo, da naj bi deloval podobno (Kostanda in Khatib 2021) kot proti komarju ščitarju (*Aedes aegypti*), žitnemu kutarju (*Rhyzopertha dominica*), riževemu žužku (*Sitophilus oryzae*) in riževemu mokarju (*Tribolium castaneum*) (Satyal in Setzer 2014). Za enkrat tudi ni znano, če na konopljo deluje kot atraktant moških osebkov navadne pršice in posledično večjo dovzetnosti konoplje na napad pršice, prisoten seskviterpen alkohol farnesol (Nuutinen 2018), kot je to pri rastlini iz iste družine – hmelju (Regev in Cone 1975).

Po izpostavitvi konoplje navadni pršici se je v listih in socvetjih povečala vsebnost kanabinoidov CBG za 29 %, CBC za 41 % in Δ^9-THC za 52 %, medtem ko je bila vsebnost CBCA višja pri kontrolnih rastlinah, ki niso bile izpostavljene škodljivcu.

Zaradi sinteze hlapnih snovi z insekticidnim delovanjem, ima konoplja dobre sosedske odnose z drugimi rastlinskimi vrstami. Rastline konoplje v bližini bombažnih polj naj bi zmanjševale pojavnost škodljivca listov bombaža *Alabama argillacea*, kot vmesni posevek v pridelavi zelenjadnic zmanjšuje prisotnost kapusovega belina (*Pieris brassicae*), rastline krompirja ščiti pred koloradskim hroščem (*Leptinotarsa decemlineata*) in zmanjšuje pojavnost poškodb *Delia coarctata* na posevkih pšenice (McPartland 2000).

Že leta 1952 so se začele raziskave o potencialnem protimikrobnem delovanju bioaktivnih snovi konoplje. Danes vemo, da na kanabinoidno biosintezno pot v konoplji dokazano vpliva prisotnost bakterijskih in glivnih patogenov. Številni kanabinoidi delujejo celo na odporne seve bakterij (Gorelick in Bernstein 2017).

Eterično olje konoplje deluje močno protimikrobno proti Gram-pozitivnima bakterijama *Bacillus cereus* in *Staphylococcus aureus*; Gram-negativnim bakterijama *Pseudomonas aeruginosa* in *Escherichia coli* ter glivam *Candida albicans* in *Aspergillus niger* (Satyal in sod. 2012). Nissen in sodelavci so leta 2010 izvedli raziskavo protimikrobnega delovanja konopljinega ekstrakta v razmerah *in vitro*. Izmed treh izbranih sort konoplje se je za najučinkovitejšega proti Gram-pozitivnima bakterijama *Clostridium* spp. in *Enterococcus* spp. ter Gram-negativnima bakterijama *Pseudomonas* spp. in *Pectobacterium* spp., ter kvasovkam pokazal ekstrakt sorte Futura. Razlog za širok spekter delovanja ekstrakta te sorte je najverjetneje višja vsebnost terpena terpinolena.

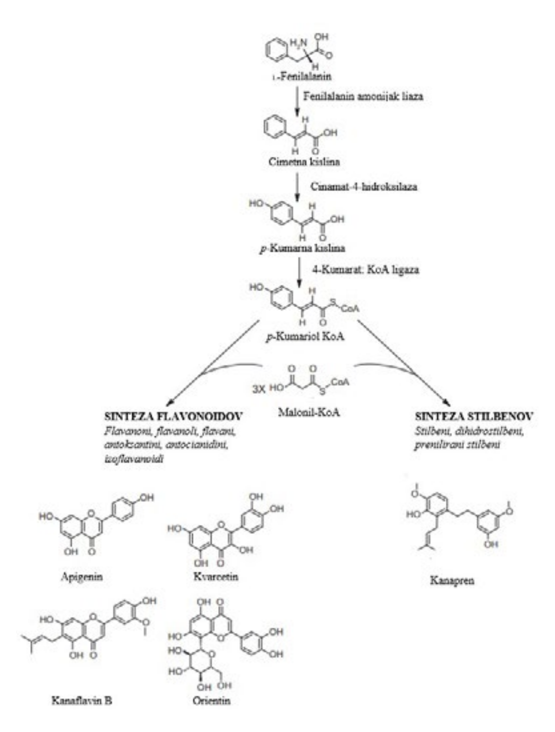
Nekateri terpeni imajo v povezavi s kanabinoidi sinergistični učinek, zato poleg lastnega repelentnega delovanja na škodljivce, povečajo tudi insekticidno delovanje kanabinoidov (Russo 2011).

Konopljini sekundarnim metaboliti, tako terpenoidi kot kanabinoidi, imajo tudi protiglivne lastnosti. Izolata THC in CBD preprečita kalitev glive *Phomopsis ganjae* (McPartland 1984). Kanabinoida CBC (Turner in Elsohly 1981) in CBG (Elsohly in sod. 1982) ter terpenoidi in fenoli linalol, citronelol, geraniol, eugenol (Kurita in sod. 1981) limonen, evkaliptol, β-myrcene, α- in β-pinen delujejo tako proti bakterijam kot proti glivam (De Groot 1972). Ekstrakt listov konoplje inhibira rast in razvoj glive *Aspergillus niger* in s tem prepreči nastanek črne plesni, ki je pogosto prisotna bolezen na sadju in zelenjavi (Wasim in sod. 1995). V celičnih kulturah konoplje, suspenzija micelija dveh najpogostejših patogenih gliv, *Pythium aphanidermantum* in *B. cinerea*, pospeši biosintezne poti in poviša vsebnosti triptofana, fumarata, aspartata in glutamina (Flores-Sanchez in sod. 2009).

Ob okužbi z glivo *B. cinerea* se poveča izražanje genov vpletenih v signalno pot salicilne kisline, jasmonske kisline in etilena (Balthazar in sod. 2020). V večini primerov rastlinski obrambni mehanizem proti nekrotrofnim organizmom deluje po signalni poti jasmonska kislina/etilen (Thomma in sod. 2001). Ob okužbi mladih listov konoplje z glivo *B. cinerea*, se poveča izražanje encima fenilalanin amonijak liaze (slika 3.), označevalca v signalni poti jasmonska kislina/etilen, ki katalizira začetno reakcijo biosinteze fenilpropanoidov. Fenilpropanoidi so začetniki za kompleksne sekundarne metabolite, kot so na primer pigmenti ali protimikrobni fitoaleksini (Balthazar in sod. 2020). Jasmonska kislina je pomembna za sintezo flavonoidov in alkaloidov (Hayat in sod. 2010) ter vpliva na vsebnost terpenoidov in kanabinoidov v rastlinah konoplje (Salari in Mansori in sod. 2013). Salicilna kislina je povezana s sistemsko pridobljeno odpornostjo, v kateri se povečuje tudi sinteza sekundarnih metabolitov (Hayat in sod. 2010), vendar njena vloga v obrambi konoplje pred *B. cinerea* ni povsem jasna (AbuQamar in sod. 2006). Eksogena salicilna kislina povečuje ekspresijo gena za THC-sintazo, kar potrjuje njeno vlogo v stimulaciji biosinteznih poti sekundarnih metabolitov (Jalali in sod. 2019). Predvidevajo, da določene sorte konoplje, okužene s *B. cinerea* lahko pridobijo sistemsko odpornost (Balthazar in sod. 2020).

Vse bolj je jasen pomen mikrobioma in z njim tudi endofitnih bakterij in endofitnih gliv pri rastlinah, vključno s konopljo (Kusari in sod. 2013; Kusari in sod. 2014; Taghinasab in Jabaji 2020). Predvidevajo, da so endofiti antagonisti številnih patogenov, med njimi konopljini endofiti tudi antagonisti glivnih rodov *Trichothecium* in *Botrytis* (Kusari in sod. 2013; Kusari in sod. 2014). Ob prisotnosti endofitov se poveča izražanje genov vpletenih v biosintezni poti mevalonske kisline in metil-eritriol-4-fosfata (slika 2), kar naj bi bilo ključno pri zvišanju odpornosti rastline proti stresu (Mishra in sod. 2018). Endofitne bakterije pogosto izločajo sekundarne metabolite, ki so podobni gostiteljski rastlini, kar še dodatno poveča tolerantnost konoplje proti stresnim dejavnikom (Kusari in sod. 2013). *Bacillus megaterium* B4, *Brevibacillus borstelensis* B8, *Bacillus* sp. B11, in *Bacillus* sp. B3 povzročajo motnje komunikacijskih signalov (angl. 'quorum quenching') med celicami bakterij, kar prepreči razvoj odpornosti proti gostiteljskim in/ali endofitnim sekundarnim metabolitom (Taghinasab in Jabaji 2020).

V celičnih kulturah navadne konoplje, tobaka BY-2 in ameriške koruzne sovke (*Spodoptera frugiperda*) delujejo kanabinoidi citotoksično in inducirajo celično apoptozo (Sirikantaramas in sod. 2005).



Slika 3. Biosintezna pot fenilpropanoidov (Thomas in ElSohly, 2016).

Figure 3. Biosynthetic pathway of phenylpropanoids (Thomas and ElSohly, 2016).

Sklepi

Sekundarni metabolizem konoplje je kompleksen sistem, v katerem je še veliko neznank. Številne snovi med seboj sodelujejo in imajo sinergičen učinek pri obrambi rastline pred škodljivci in patogeni. Čeprav ekološka vloga konopljinih kanabinoidov, terpenoidov in flavonoidov še ni povsem razkrita, je jasno, da kanabinoidi in flavonoidi varujejo rastline pred abiotičnim stresom v največji meri pred UV sevanjem, medtem ko kanabinoidi in terpenoidi delujejo repelentno, insekticino in protimikrobno.

Več študij potrjuje insekticidno in antimikrobno delovanje kanabinoidov THC in CBD. Povečana sinteza THC in CBD se v rastlinah konoplje pojavlja ob napadu in po napadu škodljivca. THC in CBD s številnimi terpeni učinkujeta sinergično.

Ko rastlino napade škodljivec ali jo okuži patogen, se aktivirajo signalne poti, ki delujejo na encim fenilalanin amonijak liaza ali na poti mevalonske kisline in metil-eritriol-4-fosfata. Fenilalanin amonijak liaza katalizira začetno reakcijo fenilpropanoidov, ki so ključni za sintezo fenolnih snovi, medtem ko sta sintezni poti mevalonske kisline in metil-eritriol-4-fosfata odgovorni za sintezno kanabinoidov in terpenoidov. Glive in bakterije dokazano sprožijo kanabinoidne in terpenske biosintezne poti. To potrjuje vlogo kanabinoidov in terpenov v interakciji rastline z biotskimi dejavniki.

Na rastlinski površini in v rastlini so prisotni številni glivni in bakterijski endofiti, ki sodelujejo pri rastlinski obrambi. V največji meri so prisotni *Chaetomium globosum*, *Eupenicillium rubidurum* in *Penicillium sumatrense* (Kusari in sod. 2014). Delujejo neposredno antagonistično s patogenovimi sekundarnimi metaboliti ali posredno z izločanjem sekundarnih metabolitov, toksičnih za patogena. Endofiti lahko spodbujajo rastlinski sekundarni metabolizem ali celo omejujejo razvoj škodljivega organizma v prid rastlini.

Zaradi pomanjkanja raziskav ekološkega pomena sekundarnih metabolitov pri navadni konoplji, ta pomen ni povsem jasen. Kljub temu vemo, da imajo številne snovi v konoplji varovalno vlogo pred škodljivimi organizmi in da je stres osnova za povečano sintezo sekundarnih metabolitov. Z načrtovanim in pravilnim izvajanjem stresa in ohranjanjem zdravega rastlinskega mikrobioma lahko s tehnologijo pridelave povečamo tvorbo obrambnih snovi in s tem izboljšamo odpornost konoplje proti patogenom in škodljivcem.

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