ČESKÁ ZEMĚDĚLSKÁ UNIVERZITA V PRAZE
Fakulta agrobiologie, potravinových a přírodních zdrojů
Katedra agroenvironmentální chemie a výživy rostlin

Vliv výživy na tvorbu biomasy a obsah terapeuticky využitelných sekundárních metabolitů rostlin konopí (*Cannabis sativa* L.)

....

doktorská disertační práce

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Poděkování

Rád bych touto cestou poděkoval především svému školiteli prof. Ing. Pavlu Tlustošovi, CSc., dr. h. c., který si vždy udělal čas, když bylo potřeba a zajistil patřičné finanční zdroje pro mé důstojné bytí, ne jenom pouhé přežití.

Velké „díky dík“ dále pak patří též všem kolegům z interkatedrového vědecko-výzkumného týmu léčebného konopí a katedry Agroenvironmentální chemie a výživy rostlin, fakulty Agrobiologie, potravinových a přírodních zdrojů, České zemědělské univerzity v Praze za věcné rady a přátelské prostředí.

V neposlední řadě bych chtěl poděkovat za důvěru a trpělivost mé snoubence, rodině a přátelům.

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1. ÚVOD

V posledních letech zaznamenal výzkum léčivého konopí velký rozmach v biomedicínském a farmaceutickém odvětví. Kontroverze spojené s psychoaktivními účinky, a vůbec s touto léčbou v rámci právních a sociálních důsledků na základě užívání, nestandardizovaná aplikace a dávkování, nepříznivé zdravotní následky způsobené intoxikací a terapeutickými indikacemi na základě omezených klinických údajů, představují určité komplikace.

Přes všechny tyto obtíže se aplikovatelnost a dostupnost léčebného konopí neustále usnadňuje, rozšiřuje a vyvíjí, o čemž svědčí i rostoucí počet zemí, které dnes umožňují jeho použití pro konkrétní terapeutické indikace a rekreační užívání. Počet sledovaných účinných látek i nadále roste a je studován jejich účinek na řadu různých onemocnění.

V evropských zemích se ale dosud většina odrůd konopí pěstuje pro průmyslové účely. Z tohoto důvodu je technologie pěstování technického konopí poměrně dobře prozkoumána, zatímco o klíčových faktorech ovlivňujících pěstování konopí pro lékařské účely je známo jen málo. Účinné látky v rostlinách konopí se nazývají fytokanabinoidy. Kromě kanabinoidních sloučenin obsahují rostliny konopí mimo jiné terpeny a flavonoidy. Biosyntéza fytokanabinoidů je poměrně dobře prozkoumána, ale mnohem méně byly studovány specifické faktory prostředí, které ovlivňují jejich obsah a spektrum. Pěstování ve sklenících nebo vnitřních prostorách s automatizovaným osvětlením, ventilací, zavlažováním a komplexními systémy výživy rostlin se stává stále propracovanějším a jeví se jako nejúčinnější metoda pro homogenní produkci léčebného konopí.

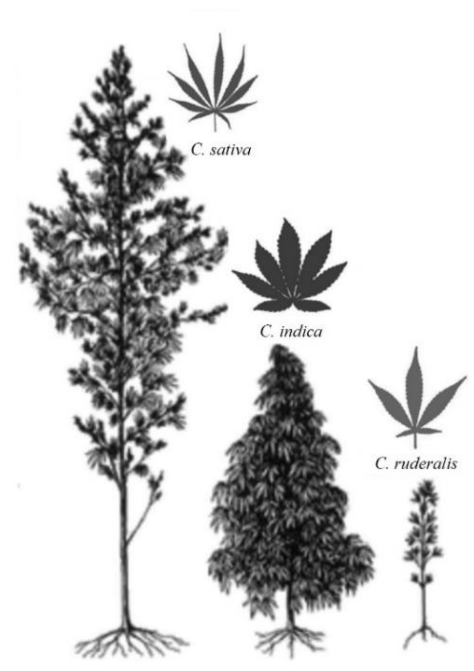
2. LITERÁRNÍ PŘEHLED

2.1. Taxonomie konopí

Debata o správné botanické klasifikaci konopí je stále otevřená, protože jeho genetická plasticita ztěžuje katalogizaci.

2.1.1. Historie

Linnaeus (1753) popsal konopí (*Cannabis*) jako rod s jediným druhem *Cannabis sativa*. Na základě srovnávacích analýz psychoaktivních účinků, velikosti listů, tvaru a struktury indických a evropských odrůd klasifikoval o 33 let později de Lamarck (1786) indické kultivary jako další samostatný druh, *Cannabis indica*. Na začátku 20. století pak ale ruský botanik Janischevsky (1924) zjistil, že místní rostliny mají odlišné vlastnosti od *C. sativa* i *C. indica*, přesto stále patří do taxonu konopí. Tyto malé, divoce rostoucí, samonakvétací rostliny byly tedy klasifikovány jako třetí samostatný druh s názvem *Cannabis ruderalis*. Rozdíly v jednotlivých druzích rodu *Cannabis* lze vidět na Obrázku 1.

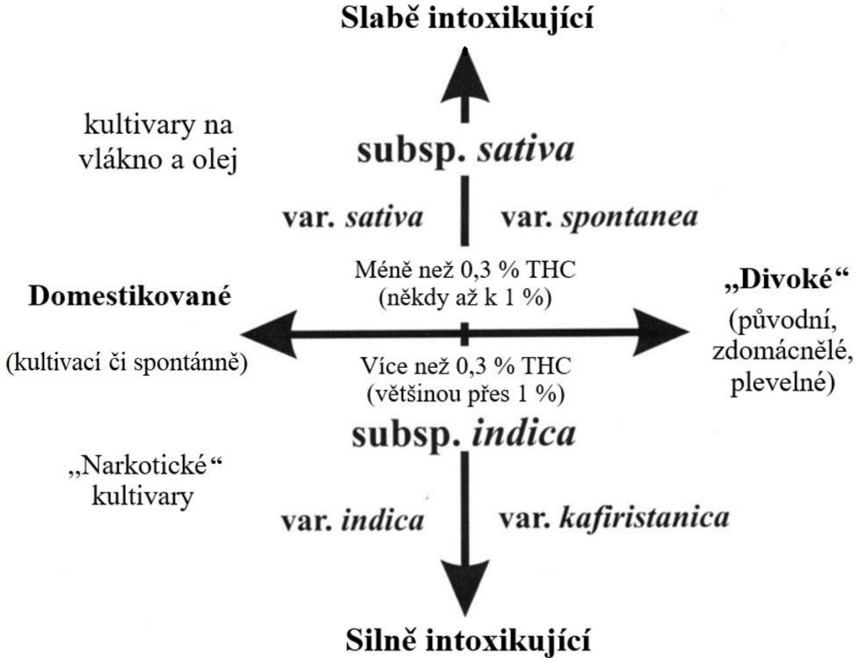


Obrázek 1: Druhy konopí (Hartsel et al. 2016).

2.1.2. Současná nomenklatura

Koncem 20. století Small a Cronquist (1976) využili dvoufázový přístup kombinující morfologické a chemické vlastnosti ke kategorizaci rodu *Cannabis* na následující čtyři skupiny:

1. *C. sativa* L. subsp. *sativa* var. *sativa*,
2. *C. sativa* L. subsp. *sativa* var. *spontanea* Vavilov,
3. *C. sativa* L. subsp. *indica* Small & Cronquist var. *indica* (Lam) Wehmer,
4. *C. sativa* L. subsp. *indica* Small & Cronquist var. *kafiristanica* (Vavilov) Small & Cronquist (Obrázek 2).



Obrázek 2: Chemotypy konopí (Small & Cronquist 1976).

Hillig (2005) ve své genomické studii taxonomické klasifikace konopí na základě analýz odlišných genotypů různého zeměpisného původu dospěl k závěru, že žádné z předchozích rozdělení dostatečně nedefinovalo diference mezi geny *sativa* a *indica*, a proto se přiklání k vícedruhové klasifikaci (*C. sativa*, *C. indica* a *C. ruderalis*).

Small (2015) nedávno navrhl dvě možná taxonomická uspořádání. První je v souladu s dřívějším rozdělením (Small & Cronquist 1976) a Mezinárodním kódem nomenklatury řas, hub a rostlin (McNeill et al. 2012). Druhé, pro domestikované konopí, se řídí pokyny pro Mezinárodní kód nomenklatury kulturních rostlin (Brickell et al. 2009):

1. Nenarkotické rostliny, domestikované pro vlákna a/nebo olejnatá semena v západní Asii a Evropě. Nízký obsah Δ^9-tetrahydrokanabinolu (THC) and vysoký obsah kanabidiolu (CBD) (Hillig a Mahlberg (2004) *C. sativa* „biotyp technického konopí“).

2. Nenarkotické rostliny, domestikované pro vlákna a/nebo olejnatá semena ve východní Asii, především v Číně. Nízký až střední obsah THC a vysoký obsah CBD (Hillig a Mahlberg (2004) *C. indica* „biotyp technického konopí“).

3. Psychoaktivní rostliny, domestikované v jižní a střední Asii. Vysoký obsah THC a žádný až nízký obsah CBD (Hillig a Mahlberg (2004) *C. indica* „drogový biotyp s úzkými listy“).

4. Psychoaktivní rostliny, domestikované v jižní Asii (Afghánistán a sousední státy). Střední až vysoký obsah THC a CBD (Hillig a Mahlberg (2004) *C. indica* „drogový biotyp s širokými listy“).

Kromě výše zmíněných byly navrženy také dvě hybridní třídy:

(5.) Nenarkotické rostliny, hybridní kultivary mezi skupinami 1 a 2.

(6.) Psychoaktivní rostliny, hybridní kultivary mezi skupinami 3 a 4.

Hillig a Mahlberg (2004) analyzovali obsah kanabinoidů v různých rostlinách konopí a na základě geografického původu, morfologických rysů a předpokládaného účelu kultivace je přiřadili k vnitrodruhovým taxonům (biotypům):

1. *C. sativa* „biotyp technického konopí“ - bylo analyzováno 62 rostlin, percentuální obsah kanabinoidů v sušině byl stanoven v rozmezí 0,1-11,5 % pro THC a 0,0-13,6 % pro CBD.

2. *C. indica* „biotyp technického konopí“ - bylo analyzováno 45 rostlin, percentuální obsah kanabinoidů v sušině byl stanoven 0,1-9,3 % pro THC a 0,0-8,5 % pro CBD.

3. *C. indica* „drogový biotyp s úzkými listy“ - bylo analyzováno 68 rostlin, percentuální obsah kanabinoidů v sušině byl stanoven v rozmezí 1,4-12,4 % pro THC a 0,0-0,1 % pro CBD.

4. *C. indica* „drogový biotyp s širokými listy“ - bylo analyzováno 40 rostlin, percentuální obsah kanabinoidů v sušině byl stanoven 0,1-14,7 % pro THC a 0,0-11,0 % pro CBD.

Všechny druhy konopí se úspěšně kříží a produkují plodné hybridy (Beutler & Marderosian 1978). Bylo též zjištěno, že rostliny *indica* a *sativa* se liší v terpenových a kanabinoidních profilech. Tyto chemotaxonomické markery jsou tedy slibným nástrojem

pro screening daných hybridů (Hillig 2004; Hillig & Mahlberg 2004; Fischedick et al. 2010; Elzinga et al. 2015).

Zhang et al. (2018) doporučují, aby konopí bylo uznáno jako monotypický druh *C. sativa* L. se třemi poddruhy subsp. *sativa*, subsp. *indica* a subsp. *ruderalis*. Jejich návrh je podložen studií zaměřenou na sekvenování DNA těchto rostlin. Také McPartland (2018) se na základě analýzy konopného DNA ve své práci přiklání k rozdělení konopí na poddruhové úrovni a uznává nomenklaturu *C. sativa* subsp. *sativa* a *C. sativa* subsp. *indica*.

2.2. Sekundární metabolity konopí

V tomto odvětví již dávno uplynula doba, kdy se při experimentech využívaly neznámé kmeny konopí s nedefinovaným obsahem alespoň dvou hlavních kanabinoidů, THC a CBD. Nyní jsou nastaveny pěstitelské postupy s cílem optimalizovat a standardizovat šlechtění konopných kmenů se specifickým složením majoritních i minoritních kanabinoidů a dalších důležitých fytochemikálií, zejména terpenů a flavonoidů. Ačkoliv se většina stávající vědecké literatury o konopí zaměřuje stále především na dva již zmíněné hlavní kanabinoidy, minoritní kanabinoidy, terpeny a flavonoidy jsou obecně ignorovány. Důkazy však naznačují, že tyto složky, zejména kanabinoidy a terpeny, hrají významnou roli při vzájemném ovlivňování a synergickém působení. Tento jev bývá souhrnně označován jako „doprovodný efekt konopí“ (Russo 2011, 2018; Koltai & Namdar 2020). Dále budou přiblíženy pouze dvě skupiny konopných fytochemikálií, a to fytokanabinoidy a terpenické sloučeniny.

2.2.1. Fytokanabinoidy

Současný výzkum kanabinoidů stojí na řadě významných objevů profesora Raphaela Mechoulama a profesora Yechiela Gaoniho. Ti v 60. letech identifikovali psychoaktivní složku *C. sativa*, Δ^9-tetrahydrokanabinol, popsali její chemickou strukturu (Gaoni & Mechoulam 1964; Mechoulam & Gaoni 1967) a syntetizovali ji (Mechoulam et al. 1967). V 90. letech byly v savčích tkáních identifikovány ligandy endogenních kanabinoidních receptorů, nazývané endokanabinoidy. Nejznámějšími zástupci jsou anandamid (Devane et al. 1992) a 2-arachidonoylglycerol (Mechoulam et al. 1995). Endokanabinoidy jsou odvozeny od arachidonové kyseliny, jako potenciální zdroj této mastné kyseliny slouží membránové lipidy (Giuffrida et al. 2001).

Z tohoto důvodu jsou kanabinoidní látky z konopí často označovány jako fytokanabinoidy, aby se odlišily od výše zmíněných endokanabinoidů.

2.2.1.1. Biosyntéza

Fytokanabinoidy lze rozdělit do dvou skupin, a to na neutrální kanabinoidy a kanabinoidní kyseliny. Diverzifikace je založena na tom, kolik karboxylových skupin má daná molekula. Během skladování a při zvýšených teplotách může ale dojít k neenzymatické dekarboxylaci (Kimura & Okamoto 1970; Shoyama et al. 1970).

Fytokanabinoidy, prenylované polyketidy smíšeného biosyntetického původu, jsou syntetizovány z prekurzorů mastných kyselin a izoprenoidů. Všechny fytokanabinoidní struktury obsahují monoterpenovou jednotku připojenou k fenolickému kruhu se substituentem v podobě alkylu na C3 (Dewick 2002). Alkylový postranní řetězec může mít různou délku od jednoho do pěti atomů uhlíku (Obrázek 3), ale *n*-pentyl je nejhojnější (Elsohly & Slade 2005). Fytokanabinoidy obsahující postranní řetězec v podobě *n*-propylu se označují jako kanabivariny. Tetrahydrokanabivarin (THCV), analog THC s postranním řetězcem *n*-propylu, se často vyskytuje u *C. indica* (Hillig & Mahlberg 2004).

Obrázek 3: Chemická struktura fytokanabinoidů.

Výchozími sloučeninami pro syntézu aromatických kruhů, včetně alkylu na třetím uhlíku (Hanuš et al. 2016), jsou tři molekuly malonyl-CoA a jedna molekula hexanoyl-CoA odvozená od kyseliny hexanové (kapronové) (Dewick 2002). Hexanoyl-CoA působí jako primer pro enzymy, polyketidové syntázy typu III, známé také jako tetraketid syntázy (TKS). V dalším kroku jsou vyžadovány enzymy, cyklázy olivetolové kyseliny (OAC), katalyzující C2-C7 intramolekulární aldolovou kondenzaci s retencí karboxylové skupiny za vzniku olivetolové kyseliny (Taura et al. 2009; Gagne et al. 2012). V průběhu těchto biosyntetických kroků vznikají též vedlejší produkty jako 4-hydroxy-6-pentylpyran-2-on (PDAL), 4-hydroxy-6-(2-oxoheptyl)pyran-2-on (HTAL) a olivetol. Olivetolová kyselina je alkylována monoterpenovou jednotkou, geranylpyrofosfátem, za účasti geranylpyrofosfát: olivetolát geranyltransferázy (GOT), a tím vzniká kanabigerolová kyselina (CBGA) (Obrázek 4) (Fellermeier & Zenk 1998). V menší míře je syntetizován také (*Z*)-izomer

kanabigerolové kyseliny, kanabinerolová kyselina (CBNRA) (na Obr. 4 z důvodu přehlednosti nezmíněna), a to v případě, kdy je nerylpyrofosfát využit enzymem GOT namísto geranylpyrofosfátu (Taura et al. 1995a). Existují tři kyseliny, které lze vytvořit z CBGA a CBNRA.

První z nich, tetrahydrokanabinolová kyselina (THCA) je syntetizována z CBGA nebo CBNRA za formování heterocyklického kruhu pomocí enzymu THCA syntázy (Taura et al. 1995b). Nízká substrátová specificita THCA syntázy pro CBNRA ve srovnání s CBGA však naznačuje, že THCA je převážně syntetizována z CBGA. Průběh této reakce je obdobný jako u jiných reakcí katalyzovaných monoterpenickými cyklázami (Obrázek 5). Většina cykláz vyžaduje pro svou aktivitu dvojmocné ionty, jako jsou Mg^{2+} nebo Mn^{2+}, ale u THCA syntázy tomu tak není (Taura 2009). Přítomnost karboxylové skupiny u substrátu je pro danou reakci zásadní, protože THCA syntáza nerozpozná jako substráty neutrální kanabinoidy, jako je kanabigerol (CBG) (Taura et al. 2007a).

Struktura druhé, kanabidiolové kyseliny (CBDA) je výsledkem pericyklické reakce zahrnující ztrátu protonu (Obrázek 6) (Dewick 2002). Modifikace je katalyzována intramolekulární oxidoreduktázou, CBDA syntázou, která selektivně upřednostňuje tvorbu CBDA z CBGA před jeho (*Z*)-izomerem, CBNRA (Taura et al. 1996). Byly zkoumány účinky různých kovových iontů (Mg^{2+}, Mn^{2+}, Zn^{2+}, Ca^{2+}, Co^{2+} a Cu^{2+}) na aktivitu daného enzymu, ale tyto nezměnily rychlost katalýzy. Naproti tomu iont Hg^{2+} při koncentraci 2 mM zcela inhiboval aktivitu enzymu a chelatační činidlo, ethylendiamintetraoctová kyselina (EDTA), v koncentracích do 5 mM vykazovalo nízký pozitivní účinek na aktivitu enzymu. Nezdá se tedy, že by CBDA syntáza vyžadovala kovové ionty pro oxidativní cyklizaci CBGA (Taura et al. 1996). CBDA syntáza a THCA syntáza katalyzují tvorbu jednotlivých optických izomerů o čistotě vyšší než 95 % (Taura et al. 2007b).

Obrázek 4: Biosyntéza fytokanabinoidů 1/2. TKS - tetraketid syntáza; PDAL - 4-hydroxy-6-pentylpyran-2-on; OAC - cykláza olivetolové kyseliny; HTAL - 4-hydroxy-6-(2-oxoheptyl)pyran-2-on; GOT - geranylpyrofosfát: olivetolát geranyltransferáza

Obrázek 5: Syntéza Δ^9-THCA. CBGA - kanabigerolová kyselina; Δ^9-THCA - tetrahydrokanabinolová kyselina

Třetí, kanabichromenová kyselina (CBCA) je syntetizována z CBGA pomocí oxidace a cyklizace CBCA syntázou (Obrázek 7). CBCA je syntetizována jako enantiomerní směs v poměru 5:1, pravděpodobně kvůli částečnému uvolňování meziproduktů z aktivního místa CBCA syntázy před dokončením reakce (Morimoto et al. 1997). Testy kovových iontů Mg^{2+}, Zn^{2+}, Ca^{2+} a Cu^{2+} ukázaly, že žádný ze zmíněných nestimuluje aktivitu enzymu. Naopak, kupříkladu iont Hg^{2+} úplně inhiboval danou reakci již v koncentraci 1 mM a EDTA mírně zvyšovala aktivitu enzymu. Vše tedy naznačuje, že reakce CBCA syntázy nevyžaduje kovové ionty (Morimoto et al. 1998).

Obrázek 6: Syntéza CBDA. CBGA - kanabigerolová kyselina; CBDA - kanabidiolová kyselina

Croteau (1987) zjistil, že terpenické cyklázy vyžadují pro svoji funkci bivalentní kationty. Tyto kovové ionty jsou schopné neutralizovat negativní náboj na difosfátových skupinách terpenických molekul, a dále ionizují allyldifosfátový substrát. Protože ale CBGA difosfátovou skupinu neobsahuje, lze očekávat, že CBCA syntáza, CBDA syntáza a THCA syntáza tento požadavek na bivalentní kationty nemají. Z kanabinoidů přítomných v *C. sativa* lze většinu klasifikovat jako Δ^9-THC, CBD, kanabichromen (CBC), CBG, kanabinol (CBN), kanabicyklol (CBL), kanabielsoin (CBE) a kanabitriol (CBT) (Turner et al. 1980; Razdan 1986; Ross & ElSohly 1995). THCA, CBDA a CBCA bývají také někdy nazývány

jako primární fytokanabinoidy, protože další fytokanabinoidy jsou generovány z těchto tří prekurzorů převážně neenzymatickými degradačními cestami.

Obrázek 7: Syntéza CBCA. CBGA - kanabigerolová kyselina; CBCA - kanabichromenová kyselina

Primární fytokanabinoidy lze buď dekarboxylovat na neutrální formu (Obrázek 8), nebo konvertovat na CBE, CBN, CBT, Δ^8-tetrahydrokanabinol (Δ^8-THC) či CBL vystavením světlu, teplu a kyslíku (Obrázek 9). CBD může podstoupit fotooxidaci nebo pyrolýzu za vzniku CBE. Δ^9-THC se při vystavení teplu převede na termodynamicky stabilnější Δ^8-THC, nebo může v přítomnosti kyslíku oxidovat na CBT nebo CBN (Elsohly & Slade 2005). Přítomnost CBT a CBN spolu s vysokými hladinami dekarboxylovaných fytokanabinoidů jsou chemickými indikátory dlouhého skladování za špatných podmínek (Shoyama et al. 1970). Rychlost degradace primárních fytokanabinoidů se zvyšuje spolu s teplotou, vyššími počátečními koncentracemi primárních fytokanabinoidů a při zvětšení povrchu květů (nadrcením), a tím tedy i větší povrchovou expozicí vzduchu (Milay et al. 2020). CBC v přítomnosti světla degraduje na kanabinoidy typu CBL (Elsohly & Slade 2005).

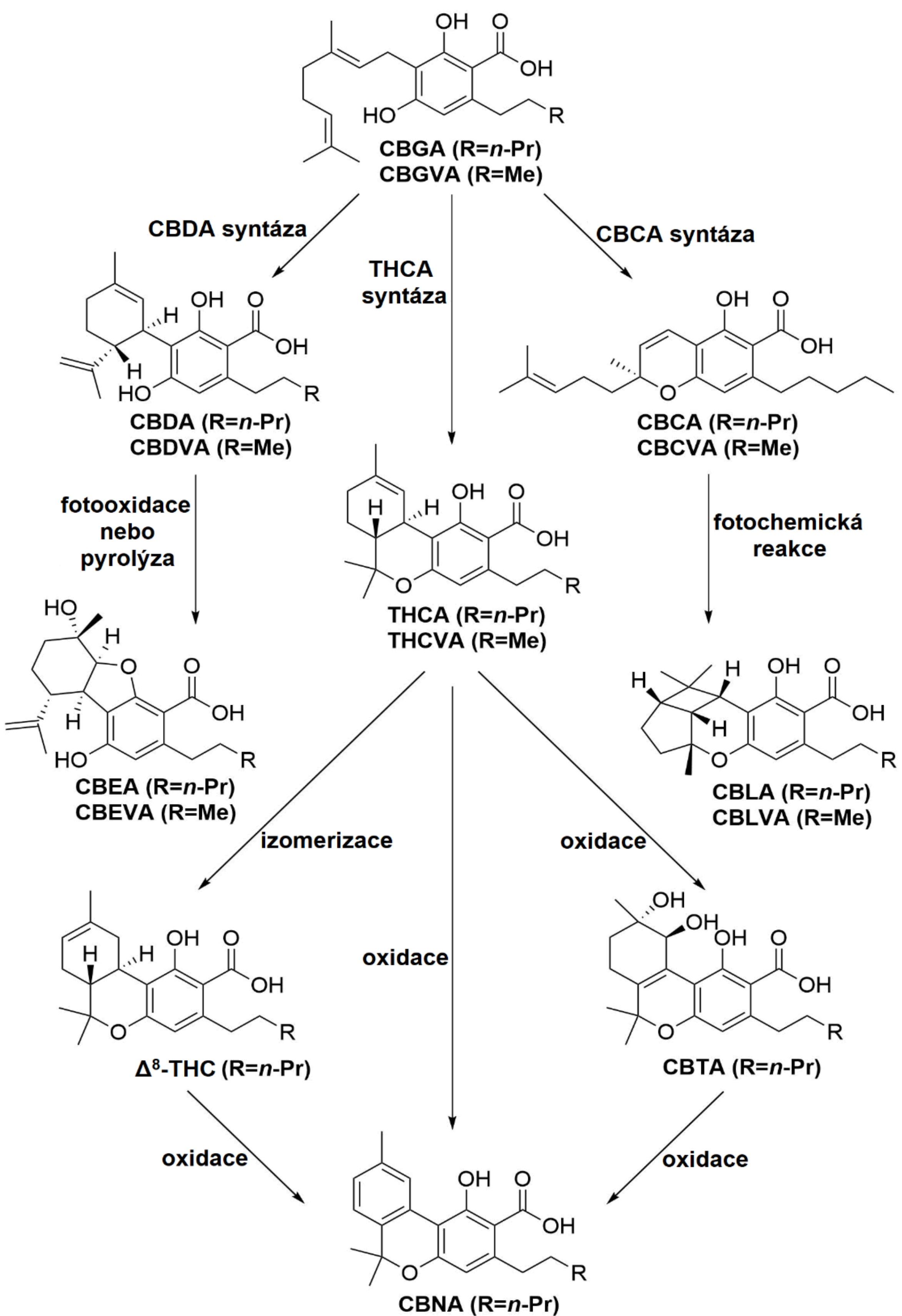
Kanabivariny jsou generovány stejnými biosyntetickými cestami z kyseliny kanabigerovarinové (CBGVA), homologního prekurzoru CBGA (Obrázek 9) (Shoyama et al. 1984). Kanabinoidní profil v konopí prochází rychlými změnami v raných fázích růstu (Potter 2014). CBDA a THCA syntáza mají velmi podobné katalytické rychlosti (k_{cat} = 0,19 s^{-1} a 0,20 s^{-1}) a afinitu (K_M = 134 µM a 137 µM) pro CBGA (Taura et al. 1995b; Taura et al. 1996). Ale CBCA syntáza vykazuje nižší Michaelisovu konstantu (K_M = 23 µM) a vyšší katalytickou rychlost (k_{cat} = 0,04 s^{-1}). V raných fázích kultivace, kde je CBGA stále přítomna v nízkých koncentracích, převládá tedy syntéza CBCA (Morimoto et al. 1998). Jak se však v průběhu času zvyšuje koncentrace CBGA, zvyšuje se účinnost biosyntézy THCA a CBDA, a tyto sloučeniny brzy převáží nad koncentrací CBCA. V pozdějších fázích růstu se syntéza

CBGA zpomaluje a její relativní podíl v kanabinoidním profilu rostliny se postupně snižuje (Potter 2014).

Obrázek 8: Dekarboxylace primárních fytokanabinoidů. Δ^9-THCA - tetrahydrokanabinolová kyselina; Δ^9-THC - tetrahydrokanabinol; CBDA - kanabidiolová kyselina; CBD - kanabidiol; CBCA - kanabichromenová kyselina; CBC - kanabichromen

2.2.1.2. Vliv výživy na tvorbu fytokanabinoidů

V oblasti výživy léčebného konopí chybí současně stále v literatuře podstatné experimentální údaje (Caplan et al. 2017a). Tudíž většina těchto informací je stále vyvozována na základě pěstování konopí technického. Technické konopí bylo však selektivně vyšlechtěno k produkci vlákna a pěstování na poli, a proto bude mít pravděpodobně mírně odlišné potřeby živin než konopí pěstované pro léčebné účely za kontrolovaných podmínek (Hillig & Mahlberg 2004; van Bakel et al. 2011; Amaducci et al. 2015).



Obrázek 9: Biosyntéza fytokanabinoidů 2/2. CBGA - kanabigerolová kyselina; CBGVA - kanabigerovarinová kyselina; CBDA - kanabidiolová kyselina; CBDVA - kanabidivarinová kyselina; CBCA - kanabichromenová kyselina; CBCVA - kanabichromevarinová kyselina; THCA - tetrahydrokanabinolová kyselina; THCVA - tetrahydrokanabivarinová kyselina; CBEA - kanabielsoinová kyselina; CBEVA - kanabielsovarinová kyselina; CBLA - kanabicyklolová kyselina; CBLVA - kanabicyklolvarinová kyselina; CBTA - kanabitriolová kyselina; CBNA - kanabinolová kyselina

Přijatelné formy jednotlivých základních živin rozdělují Barker a Pilbeam (2015) do dvou skupin podle potřeb rostlin, a to na makroživiny: dusík (NO_3^-, NH_4^+), fosfor ($H_2PO_4^-$, HPO_4^{2-}), draslík (K^+), vápník (Ca^{2+}), síra (SO_4^{2-}), hořčík (Mg^{2+}) a mikroživiny: železo (Fe^{2+}, Fe^{3+}), chlor (Cl^-), mangan (Mn^{2+}), zinek (Zn^{2+}), měď (Cu^+, Cu^{2+}), bór (H_3BO_3, $H_2BO_3^-$), molybden (MoO_4^{2-}) a nikl (Ni^{2+}).

2.2.1.2.1. Vliv makroživin na tvorbu fytokanabinoidů

2.2.1.2.1.1. Dusík, fosfor a draslík (NPK)

Předpokládá se, že obsah dusíku ve vegetativních částech technického konopí pozitivně koreluje s obsahem THC (Haney & Kutscheid 1973). Starší listy tedy obsahují méně THC než listy mladší, protože obsahují méně dusíku (Bócsa et al. 1997). Optimální hladiny přístupného dusíku v půdě pro pěstování technického konopí se pohybují v rozmezí 50-200 kg/ha (Vera et al. 2004; Aubin et al. 2015). Tato doporučení však nejsou plně aplikovatelná při hydroponickém pěstování léčebného konopí, kde by při kultivaci za kontrolovaných podmínek mělo být zajištěno 190-400 mg N/l. Tato hodnota byla taktéž uvedena při organickém pěstování rajčat ve sklenících (Zhai et al. 2009; Surrage et al. 2010). Experimentálně bylo nedávno dokázáno, že pro rostliny léčebného konopí je z hlediska morfofyziologických funkcí ve vegetativní fázi optimální dávka 160 mg N/l (Saloner & Bernstein 2020). Tato koncentrace se taktéž jevila jako optimální kompromis mezi morfofyziologickým stavem rostlin a výtěžkem sekundárních metabolitů ve fázi generativní (Saloner & Bernstein 2021). Jako ideální poměr amonný (NH_4^+)/nitrátový (NO_3^-) dusík bylo při hladině 200 mg N/l stanoveno 10-30 % NH_4^+. Vyšší poměr zvyšoval potenciál vážného až smrtelného poškození toxicitou NH_4^+ (Saloner & Bernstein 2022b).

Růst technického konopí a jeho obsah THC pozitivně korelují s obsahem přístupného P v půdě (Coffman & Gentner 1977), ale naopak obsah CBD v listové tkáni je s půdně dostupným P v souvztažnosti negativní. Technické konopí pěstované na půdách zbavených P vykazovalo zvýšený obsah CBD (Coffman & Gentner 1975). Zvýšení fosforu také neprokázalo žádný pozitivní vliv na koncentrace THC, CBD, CBN či CBG v květech rostlin léčebného konopí (Bernstein et al. 2019b). Produkce rostlinné biomasy, rychlost fotosyntézy, stomatální vodivost a rychlost transpirace ve vegetativní fázi rostlin léčebného konopí excelovaly při hladině 30 mg P/l (Shiponi & Bernstein 2021b). Tato koncentrace taktéž postačovala k produkci 80% maximálního výnosu v generativní fázi rostlin. Ale

přísun fosforu vyšší než 5 mg P/l snížil finální obsah THCA a CBDA v květech až o 25 % (Shiponi & Bernstein 2021a).

Dle Saloner et al. (2019) se růstová odezva rostlin léčebného konopí na různé koncentrace draslíku suplementované během vegetativní fáze liší v závislosti na genotypech. Dávka 15 mg K/l byla nedostačující pro optimální růst a funkci, a vyvolala symptomy deficitu ve všech genotypech. Naopak 240 mg K/l se již ukázalo jako dávka nadměrná a škodlivá. Byla prokázána kompetice o příjem mezi K, Ca a Mg, ale žádný účinek na příjem N a P s výjimkou draslíkového deficitu. Rychlost fotosyntézy rostlin se zvyšovala s dávkou K až do maxima, kterého bylo dosaženo při dávce 100 mg K/l. Ačkoli biomasa listů, stonků a kořenů se zvyšovala až do koncentrace 175 mg K/l. Optimální dávka K v růstové fázi se tedy pohybuje mezi 100-175 mg K/l v závislosti na genotypu léčebného konopí. V návazném pokusu, již během fáze květu a následné sklizně rostlin, výsledky ukázaly, že vývoj a funkce rostlin, které obdržely nízké koncentrace draslíku (15 mg K/l), byly narušeny. Tyto rostliny trpěly chlorózou a byl u nich snížen výnos květenství. Rostliny, které dostávaly vyšší dávky draslíku až do 175 mg K/l, vykazovaly optimální fyziologické funkce a vysoký výnos. Naopak ale koncentrace většiny kanabinoidů a terpenoidů klesaly se zvyšováním dávek draslíku. Koncentrace 60 mg K/l se tedy jeví jako doporučená aplikační dávka pro udržení optimálních fyziologických funkcí rostlin v kombinaci se stále vysokým výtěžkem sekundárních metabolitů (Saloner & Bernstein 2022a).

Podle Hanus a Dostalova (1994) mohou různé kombinace vybraných makroelementů (N, P, K) ve výživě technického konopí významně ovlivnit typ daných kanabinoidů i jejich individuální obsahy. Caplan et al. (2017a, 2017b) se experimentálně zabývali přímo touto problematikou organické výživy ve vztahu k léčebnému konopí. Koncentrace 389 mg N/l se jim jevila jako optimální pro maximální výnos během růstové fáze. Poměr základních makroelementů (N, P a K) ve vegetativním období byl 4:1,3:1,7. Po provedení přepočtů dle poměru byly získány hodnoty 126 mg P/l a 165 mg K/l. V generativní fázi bylo stanoveno optimální množství dusíku 212-261 mg/l. Při koncentraci dusíku 283 mg/l poskytovaly rostliny maximální výtěžek květů a biomasy, ale výsledná koncentrace kanabinoidů v sušeném produktu byla nižší. Poměr N, P a K v generativním období byl stanoven na 2:0,87:3,32. Při koncentraci 283 mg N/l by tedy mělo být následně doplněno 123 mg P/l a 470 mg K/l. Výsledky předložené Bernstein et al. (2019b) ukazují, že zvýšení koncentrace NPK v živném roztoku zvýšilo hladiny CBG v květech o 71 % a snížilo hladiny CBN v

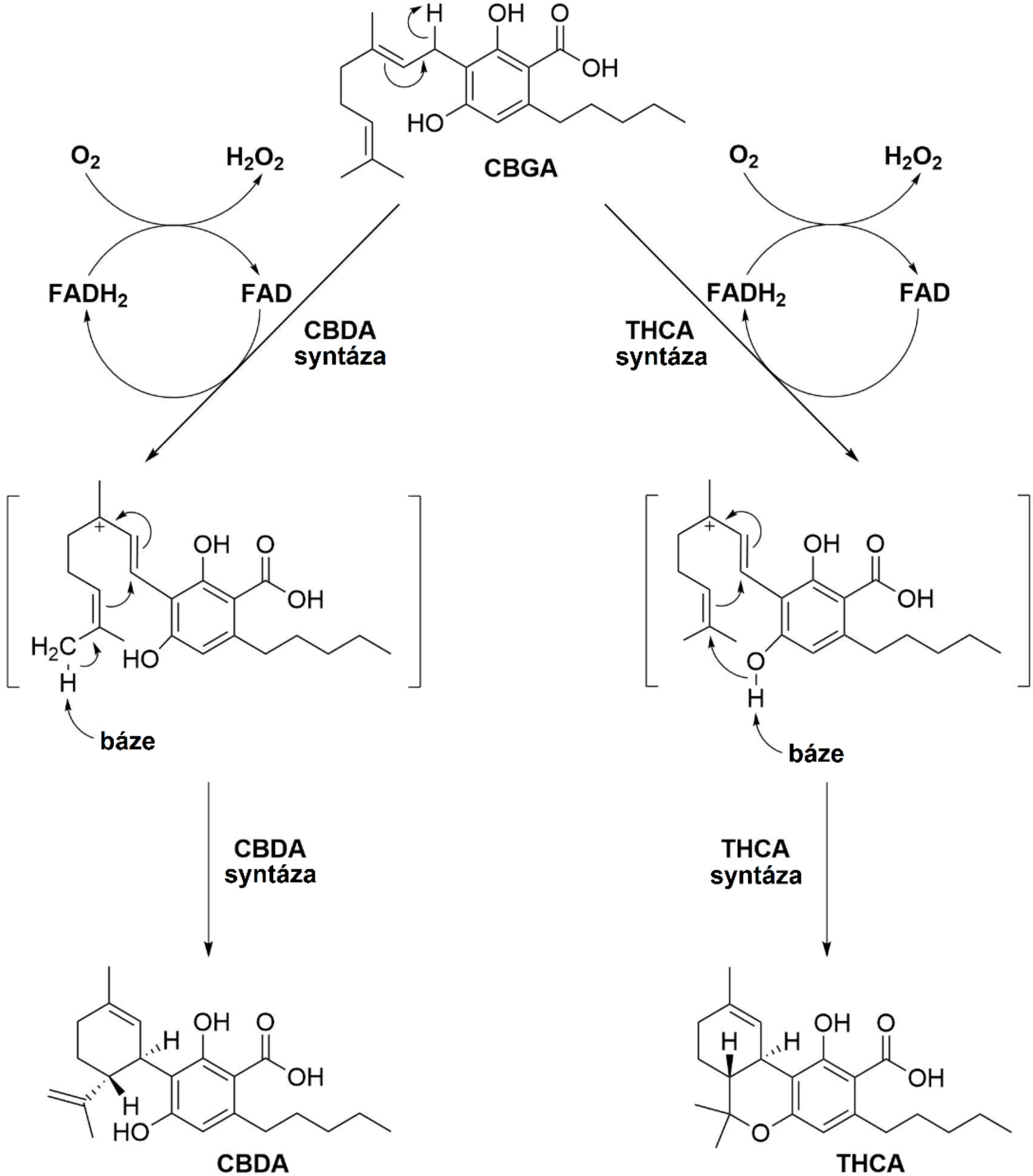
květech o 38 % ve srovnání s kontrolní výživovou variantou. Koncentrace rozpuštěných živin v kontrolní variantě byla následující: 65 ppm N (poměr 1:2 NH_4^+/NO_3^-), 17 ppm P a 90 ppm K. Mikronutrienty byly dodávány jako EDTA cheláty v koncentraci 0,4 ppm Fe, 0,2 ppm Mn a 0,06 ppm Zn.

2.2.1.2.1.2. Ostatní makroživiny

Hořčík je v půdě relativně mobilní a jeho koncentrace v rostlinách, zejména v listech, je vysoká, protože je součástí chlorofylu. Negativní korelace mezi tímto kovem a mědí vyplývá ze skutečnosti, že poloměry jejich iontů jsou podobné, a tím pádem soutěží o stejná vazebná místa. Obsah Δ^9-THC a CBD v listech technického konopí klesá s rostoucí koncentrací Mg v půdě. Pozitivní korelace hořčíku s Δ^8-THC byly vysloveny s hypotézou, že tato živina může být kofaktorem v enzymu odpovědném za produkci daného kanabinoidu. Obsah Δ^9-THC v listech technického konopí pozitivně koreluje s poměrem přístupného Ca/Mg v půdě, ale CBD negativně koreluje s přístupnými poměry Ca/Zn a Mg/Cu (Coffman & Gentner 1975; Pate 1994; Radosavljevic-Stevanovic et al. 2014).

2.2.1.2.2. Vliv mikroživin na tvorbu fytokanabinoidů

Obdobné výsledky byly pozorovány u požadavků na mikroživiny. Bylo zjištěno, že koncentraci CBN a Δ^9-THC v rostlině lze ovlivnit množstvím manganu, zatímco obsah CBD je závislý na koncentraci železa (Radosavljevic-Stevanovic et al. 2014). THCA syntáza je enzym obsahující flavinadenindinukleotidovou (FAD) prostetickou skupinu. Jak již bylo zmíněno, katalyzuje oxidační cyklizaci CBGA na THCA. Tato reakce vyžaduje molekulární kyslík pro reoxidaci $FADH_2$ na FAD, tím pádem tedy produkuje toxické množství peroxidu vodíku, a to v molárním poměru 1:1 k vznikající THCA (Shoyama et al. 2012). CBDA syntáza je také enzym obsahující FAD kofaktor. Rozdíl mezi reakcemi THCA syntázy a CBDA syntázy spočívá v kroku přenosu protonů, jak ukazuje Obrázek 10. Dále je redukovaný $FADH_2$ též reaktivován za vytvoření peroxidu vodíku (Taura et al. 2007b).



Obrázek 10: Reakční mechanismus THCA syntázy a CBDA syntázy. CBGA - kanabigerolová kyselina; CBDA - kanabidiolová kyselina; THCA - tetrahydrokanabinolová kyselina; FAD - flavinadenindinukleotid

Odhaduje se, že zhruba 1 % kyslíku u rostlin je využito pro tvorbu reaktivních forem kyslíku s různou subcelulární lokalizací, přičemž nejvýznamnější podíl připadá na peroxid vodíku. U rostlin byl tedy vyvinut velmi sofistikovaný systém tvorby, detoxikace a signalizace H_2O_2, který v rostlinném organismu zastává celou řadu rozličných funkcí.

Akumulace peroxidu vodíku vede k oxidačnímu poškození buněk a následně až k programované buněčné smrti (Quan et al. 2008).

Vzhledem k tomu, že je tedy peroxid vodíku toxický, je dále přeměněn na vodu a kyslík katalázami, které obsahují mangan nebo hemové železo. Na základě pozitivní korelace manganu, THC a CBN můžeme předpokládat, že kataláza, která rozkládá peroxid vodíku získaný syntézou THCA, obsahuje ve svém katalytickém centru mangan (Mn) v oxidačním čísle III. Během rozkladu peroxidu vodíku na vodu a kyslík se Mn s oxidačním číslem III redukuje na Mn s oxidačním číslem II, který se znovu oxiduje z Mn (II) na Mn (III) další reakcí s peroxidem dle následujících rovnic:

$$2Mn^{3+} + H_2O_2 \leftrightarrow 2Mn^{2+} + O_2 + 2H^+$$

$$2Mn^{2+} + H_2O_2 + 2H^+ \leftrightarrow 2H_2O + 2Mn^{3+}.$$

Obě reakce jsou energeticky výhodné ($\Delta G < 0$). Korelace mezi manganem a CBN je taktéž pozitivní, jelikož CBN je primárním produktem degradace THC (Wu et al. 2004).

Negativní korelaci železa (Fe) a chromu (Cr) s CBD lze vysvětlit následovně. Kataláza odpovědná za přeměnu peroxidu vodíku, pocházejícího z reakce CBDA syntázy, obsahuje hemové železo. Peroxid vodíku je po vstupu do hemové kavity silně stericky bráněn. V této pozici probíhá první fáze katalýzy. Přenos protonu z jednoho atomu kyslíku molekuly peroxidu vodíku na druhý atom kyslíku prodlužuje a polarizuje vazbu O–O, která se nakonec heterolyticky rozpadá. První atom kyslíku molekuly peroxidu vodíku je koordinován s hemovým centrem. Při této koordinaci se uvolňuje voda a vytvoří se $O{=}Fe^{(IV)}$–Enzym (+) hemový radikál. Radikál následně rychle zaniká v dalším elektronovém přenosu. Molekula se zbavuje radikálového elektronu, a díky tomu tedy zůstává porfyrinový kruh nezměněný. Během druhé fáze, v obdobné reakci dvou elektronových přenosů, reaguje $O{=}Fe^{(IV)}$–Enzym (+) s druhou molekulou peroxidu vodíku za vzniku původní molekuly $Fe^{(III)}$–Enzym, další vody a molu molekulárního kyslíku (Boon et al. 2007; Vlasits et al. 2010).

Předpokládaný mechanismus reakce:

$$H_2O_2 + Fe^{(III)}\text{–Enzym} \leftrightarrow H_2O + O{=}Fe^{(IV)}\text{–Enzym } (+)$$

$$H_2O_2 + O{=}Fe^{(IV)}\text{–Enzym } (+) \leftrightarrow H_2O + Fe^{(III)}\text{–Enzym} + O_2$$

Fe–Enzym představuje centrum hemového železa připojeného ke zbytku enzymu

Tranzitní stav (O=Fe$^{(IV)}$–Enzym (+)) je energeticky nestabilní, lze tedy konstatovat, že tyto reakce jsou nevýhodné (Boon et al. 2007; Vlasits et al. 2010). Přestože chrom není důležitý pro růst rostlin, jeho negativní korelace s CBD je vysvětlena vzájemně pozitivní korelací Fe a Cr, protože se vyskytují spolu v přírodě jako komplexní oxid (Radosavljevic-Stevanovic et al. 2014). Pozitivní korelace železa s Δ^8-THC byly zaznamenány s hypotézou, že tato živina může být kofaktorem v enzymu odpovědném za jeho produkci (Pate 1994; Radosavljevic-Stevanovic et al. 2014).

Bernstein et al. (2019a) popisují translokaci jednotlivých makro a mikroelementů ve vztahu ke stáří jednotlivých rostlinných částí a také distribuci kanabinoidů v rostlinách léčebného konopí. Závěr experimentu ohledně rozložení kanabinoidů je ve shodě s tvrzením Hemphill et al. (1980), kteří dělali obdobný pokus na technickém konopí, a to že obsah kanabinoidů v listech postupně klesá od vrcholu rostliny směrem dolů. Jejich nejvyšší koncentrace lze též nalézt v květech a květových listech. Koncentrace nalezené ve vějířovitých listech dosahují asi 1/10 obsahu kanabinoidů nalezené v květech. Distribuce minerálních živin mezi rostlinnými orgány vykazuje typický příjem a translokaci v rostlině. Nižší koncentrace N, P, K a vyšší koncentrace Ca ve vějířovitých listech ve srovnání s květy podporují fyziologické zjištění, že vějířovité listy jsou starší.

2.2.1.2.3. Hodnota pH

Doporučený optimální rozsah hodnot pH živného roztoku je mezi 5,5-6,5. Hodnota pH je důležitá, protože ovlivňuje dostupnost a vstřebávání živin potřebných pro růst rostlin. V hydroponické kultuře je doporučené rozmezí pH mezi 5,5-6,0 a maximální absorpce živin je při pH 5,8 (Velazquez et al. 2013). Při pěstování v substrátu se doporučuje rozmezí pH 5,8-7,2 a maximální absorpce základních živin je obvykle při pH 6,5. Když pH klesne pod tento rozsah, velká část makroživin začíná být méně dostupná a časem se mohou projevit jejich nedostatky. Když naopak hodnota pH stoupne nad tento rozsah, časem projeví nedostatek některých mikroživin (Caplan et al. 2017a). Tito autoři také zmiňují potřebu dalšího výzkumu k potvrzení optimálního rozmezí pH pro více konopných odrůd.

2.2.1.2.4. Vliv rostlinných biostimulantů na tvorbu fytokanabinoidů

Rostlinný biostimulant je jakákoli látka, druh mikroorganismu nebo jejich směs aplikovaná na rostliny za účelem zvýšení tolerance vůči abiotickému stresu, nutriční

efektivity či kvalitativní charakteristiky plodiny, bez ohledu na obsah živin. Bylo navrženo sedm hlavních kategorií biostimulantů: huminové a fulvové kyseliny, proteinové hydrolyzáty, mořské řasy a botanické extrakty, chitosan a biopolymery, prospěšné bakterie, prospěšné houby a minerály (du Jardin 2015).

2.2.1.2.4.1. Vliv huminových a fulvových kyselin na tvorbu fytokanabinoidů

Huminové látky jsou přirozenými složkami půdní organické hmoty. Jedná se o směs heterogenních sloučenin původně klasifikovaných podle jejich molekulových hmotností a rozpustnosti na huminy, huminové kyseliny a fulvokyseliny (du Jardin 2015).

Suplementace huminové kyseliny měla na konopí pozitivní vliv v případě výšky rostlin, obsahu chlorofylu a účinnosti fotosyntézy, zejména bezprostředně po období vodního stresu (Da Cunha Leme Filho et al. 2020).

Podle současné literatury je účinek na kanabinoidy spíše negativní. Bernstein et al. (2019b) uvedli, že výživové doplňky, jako jsou huminové kyseliny, výrazně snižují prostorovou variabilitu kanabinoidů na příč celou rostlinou. Tato zvýšená uniformita je na úkor obsahu THC a CBD, který byl v nejvyšších částech rostlin snížen o 37, respektive 39 %. Pokles THC je spojen s dalším trendem zvyšování CBN. To bylo pravděpodobně způsobeno zrychlenou degradací kanabinoidů v částech rostlin s jejich vysokou koncentrací.

2.2.2. Terpenické sloučeniny

Terpeny a terpenoidy jsou hlavními složkami rostlinných pryskyřic a éterických olejů mnoha léčivých bylin, včetně konopí. Z chemického hlediska se tyto dvě skupiny ale trochu liší. Terpeny se skládají pouze z uhlovodíků, kdežto u terpenoidů je na daném uhlovodíkovému skeletu navázána ještě funkční skupina či jiný chemický prvek. Tyto termíny se však v literatuře často zaměňují. Terpenické sloučeniny představují jednu z nejpočetnějších a strukturně nejrozmanitějších skupin přírodních látek. Lze je rozdělit podle počtu stavebních jednotek izoprenu v molekule na monoterpeny, seskviterpeny, diterpeny, sesterterpeny, triterpeny, tetraterpeny a polyterpeny. Spolu se steroly tvoří rozsáhlou skupinu izoprenoidů (Ludwiczuk et al. 2017).

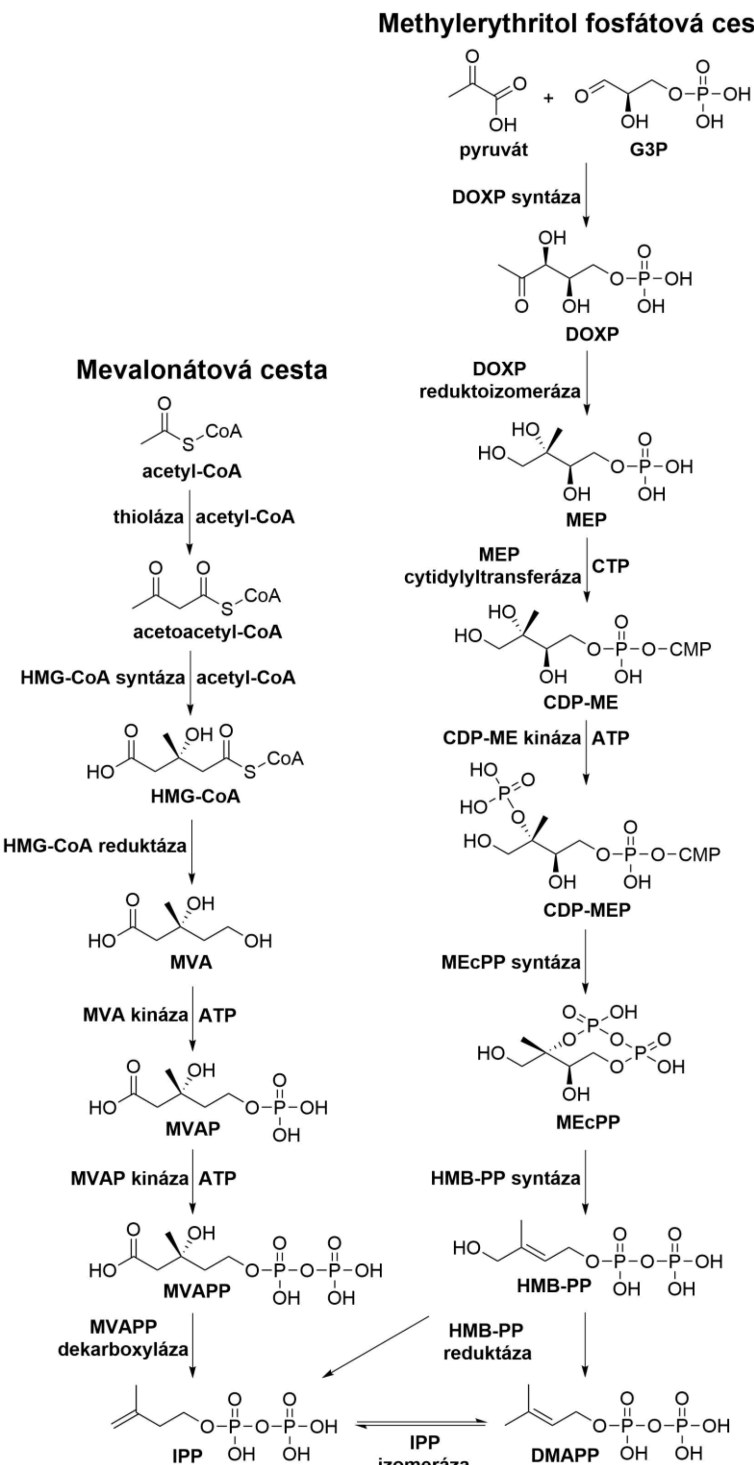
V minulosti byly terpenické sloučeniny v knihách popisovány jako produkty detoxikace, avšak začátkem sedmdesátých let bylo několik terpenů demonstrováno jako toxiny, repelenty či atraktanty. Toto vedlo k závěru, že mají zásadní funkci v

antagonistických a mutualistických vztazích mezi organismy. V přírodě hrají důležitou roli při řízení růstu, obraně, komunikaci či vábení opylovačů mnohých druhů rostlin (Langenheim 1994). Terpenické sloučeniny jsou taktéž zodpovědné za rozdílnou vůni u různých genotypů konopí. Pravděpodobně tedy následně přispěly k záměrné selekci narkotických genotypů při domestikaci (Small 2015). Obsah a distribuce terpenických sloučenin v rostlině se liší dle procesu získávání, podmínek prostředí nebo zralosti rostliny (Meier & Mediavilla 1998). Mono a seskviterpeny byly detekovány v květech, kořenech a listech konopí, přičemž hlavním místem produkce jsou sekreční žlázové chloupky (trichomy). Monoterpeny obecně dominují těkavému terpenickému profilu (3,1-28,3 mg/g sušiny květu) s hlavními konkrétními zástupci jako jsou D-limonen, β-myrcen, α-pinen, β-pinen, terpinolen a linalool. Seskviterpeny, zejména β-karyofylen a α-humulen, se ale v konopí také vyskytují ve velké míře (0,5-10,1 mg/g sušiny květu). Při pěstování konopí za standardizovaných podmínek byla zjištěna významná pozitivní korelace mezi úrovní terpenů a kanabinoidů (Fischedick et al. 2010). To lze vysvětlit skutečností, že monoterpeny a seskviterpeny jsou syntetizovány ve stejných žlázových trichomech jako kanabinoidy (Meier & Mediavilla 1998).

2.2.2.1. Biosyntéza

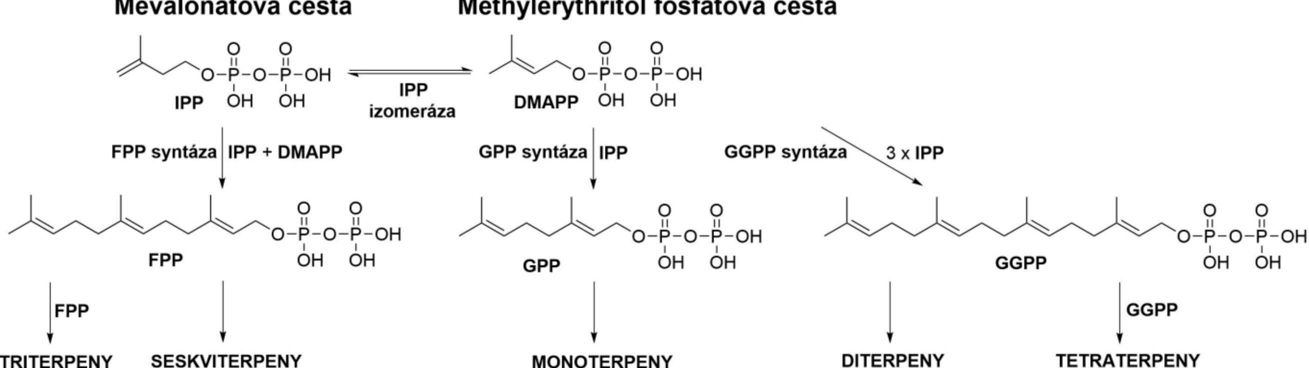
K syntéze rostlinných terpenů přispívají ve svých raných krocích dvě biosyntetické dráhy. První, dráha cytosolické mevalonové kyseliny (MVA), která je zapojena do biosyntézy seskviterpenů a triterpenů. Druhá, plastidově lokalizovaná methylerythritol fosfátová (MEP) dráha, účastnící se syntézy monoterpenů, diterpenů a tetraterpenů. Tyto dráhy regulují různé substráty (Bouvier et al. 2005). Základními stavebními kameny všech terpenických struktur jsou izopentenyldifosfát (IPP) a dimethylallyldifosfát (DMAPP). Obratlovci a bezobratlí živočichové syntetizují tyto prekurzory izoprenoidů z acetyl koenzymu A (acetyl-CoA) cestou mevalonové kyseliny, což je též kritická dráha pro syntézu cholesterolu a prekurzorů farnesylu a geranylgeranylu pro prenylaci proteinů. Tato cesta je rovněž důležitým zdrojem IPP a DMAPP v cytoplazmě rostlinných buněk (McGravey & Croteau 1995). Zde jsou v prvním kroku konjugovány 3 jednotky acetyl-CoA za vzniku 3-hydroxy-3-methylglutaryl koenzymu A (HMG-CoA), a ten je dále přeměněn na mevalonovou kyselinu pomocí HMG-CoA reduktázy (Obrázek 11). Statiny, včetně mevinolinu, mevistatinu, pravastatinu a simvastatinu, mohou blokovat tento krok limitující

rychlost biosyntézy (Luthra et al. 1999). Přestože statiny blokují produkci fytosterolů v rostlinách, nezabraňují produkci terpenů v plastidech (Chang et al. 2013).



Obrázek 11: Biosyntéza terpenů 1/2. CDP-MEP - 4-difosfocytidyl-2-C-methyl-D-erythritol-2-fosfát; ATP - adenosintrifosfát; CTP - cytidintrifosfát; HMB-PP - (*E*)-4-hydroxy-3-methyl-but-2-enylpyrofosfát; MEcPP - 2-C-methyl-D-erythritol 2,4-cyklodifosfát; MVAPP - mevalonát-5-pyrofosfát; HMG-CoA - 3-hydroxy-3-methylglutaryl koenzym A; IPP - izopentenyldifosfát; DMAPP - dimethylallyldifosfát; G3P - D-glyceraldehyd-3-fosfátu; DOXP - 1-deoxy-D-xylóza-5-fosfát

Počátečním krokem plastidické methylerythritol fosfátové cesty je konverze pyrohroznové kyseliny (pyruvátu) a D-glyceraldehyd-3-fosfátu (G3P) na 1-deoxy-D-xylóza-5-fosfát (DOXP) pomocí DOXP syntázy. DOXP je poté pomocí DOXP reduktoizomerázy přeměněn na 2-C-methyl-D-erythritol-4-fosfát (MEP). Tento krok je možné inhibovat antibiotikem fosmidomycin (Rohmer 1999; Wanke et al. 2001; Dubey et al. 2003). Koncovým bodem obou zmíněných biosyntetických drah (MVA a MEP cesta) je IPP, který se poté izomerizuje na DMAPP pomocí izomerázy IPP v cytosolu (Obrázek 11). V cytosolu jsou poté dvě molekuly IPP a jedna molekula DMAPP kondenzovány za vzniku farnesyldifosfátu (FPP), farnesyldifosfát syntázou. FPP slouží jako prekurzor seskviterpenů. Dvě molekuly FPP mohou být dále kondenzovány skvalen syntázou (SQS) na endoplazmatickém retikulu za vzniku skvalenu, prekurzoru triterpenů a sterolů. V plastidu je jedna molekula IPP a jedna molekula DMAPP kondenzována za vzniku geranylpyrofosfátu (GPP), GPP syntázou (Obrázek 12) (Kempinski et al. 2015). GPP je bezprostředním prekurzorem monoterpenů a také stavebním kamenem biosyntézy kanabinoidů (Fellermeier et al. 2001). Tyto lineární izoprenoiddifosforečnany jsou substráty pro monoterpensyntázy a seskviterpensyntázy, které následně diverzifikují tyto prekurzory enzymatickými modifikacemi, jako jsou hydroxylace, dehydrogenace, acylace a glykosylace (Booth et al. 2017; Nagegowda & Gupta 2020).



Obrázek 12: Biosyntéza terpenů 2/2. IPP - izopentenyldifosfát; DMAPP - dimethylallyldifosfát; GPP - geranylpyrofosfát; GGPP - geranylgeranylpyrofosfát; FPP - farnesyldifosfát

2.2.3. Izolace fytokanabinoidů a terpenických sloučenin

2.2.3.1. Extrakce fytokanabinoidů

V literatuře lze rozlišovat dvě kategorie procesu extrakce konopných květů. Za prvé, konvenční metody, zahrnující maceraci rostlinného materiálu v organickém rozpouštědle. Extrakt se při nich koncentruje odstraňováním rozpouštědla. Fytokanabinoidy bývají při tomto procesu vystaveny kombinovaným účinkům tepla, světla, vzduchu a kyselého prostředí, a tím pádem mohou podléhat chemickým modifikacím a degradaci. Jedna z nevýhod těchto konvenčních technik souvisí hlavně s termolabilitou extrahovaných složek. Efektivitu tohoto typu extrakce lze kupříkladu zlepšit procesním zapojením ultrazvuku nebo mikrovlnného ošetření (Fairbairn & Liebmann 1973; Ramirez et al. 2019; Nahar et al. 2021).

Za druhé, inovativní metody, jako je superkritická fluidní extrakce, což je proces separace jedné složky od ostatních použitím superkritické látky jako extrakčního rozpouštědla. Extrahuje se obvykle z pevné matrice, ale může to být i z kapaliny. Nejpoužívanější superkritickou kapalinou je oxid uhličitý, a to navzdory své neúčinnosti při rozpouštění polárních sloučenin. Pro zlepšení rozpustnosti polárních látek je někdy modifikován přídavkem malého množství jiného rozpouštědla (ethanolu či methanolu), které by mělo být zcela mísitelné se superkritickým CO_2. Extrakční podmínky pro CO_2 jsou nad kritickou teplotu 31,1 °C a nad kritický tlak 73,8 barů (7,38 MPa). Přidáním modifikátoru se mohou tyto hodnoty ale měnit. Tekutina v superkritickém stavu je velmi mobilní. Rozpustností se přibližuje kapalným rozpouštědlům, zatímco penetrace do pevné matrice je usnadněna transportními vlastnostmi blížícími se plynu. Vysoce stlačený plyn pak v superkritickém stavu vykazuje mimořádné vlastnosti, s rostoucí hustotou vrůstá totiž schopnost rozpouštět dané látky. Zatímco zvyšování tlaku zvyšuje hustotu, zvyšování teploty hustotu obvykle snižuje. Tyto parametry lze tedy pro dosažení optimálních výsledků různě kombinovat (Sihvonen et al. 1999; Ramirez et al. 2019; Lancaster 2020).

Nejdůležitějšími parametry, které je třeba při extrakci vzít v úvahu jsou teplota, tlak, předběžná úprava bylin a povaha rozpouštědla. Extrakci lze považovat za úspěšnou, když je dosaženo optimální rozpustnosti aktivních sloučenin ve vhodném rozpouštědle. Za tímto cílem je nutné rozbít buněčné struktury rostlin natolik, aby účinné látky byly dobře přístupné a mohly tak interagovat s rozpouštědlem (Ramirez et al. 2019). Následuje několik příkladů extrakčních metod.

2.2.3.1.1. Dynamická macerace

Před samotnou dynamickou macerací je naváženo přesné množství konopných květů, a poté je ke vzorku přidáno extrakční rozpouštědlo. Následuje magnetické míchaní po určitou dobu při laboratorní teplotě. Roztok je potom přefiltrován přes filtrační papír. Zbytek je extrahován stejným postupem ještě dvakrát, vždy se shodným extrakčním poměrem (hmotnost sušiny/objem rozpouštědla). Filtráty ze všech tří extrakcí jsou slity dohromady. Před nástřikem na kolonu vysokoúčinné kapalinové chromatografie (HPLC) jsou ještě extrakty zfiltrovány pomocí PTFE filtru do HPLC vialek (Brighenti et al. 2017; Pellati et al. 2018; Žampachová et al. 2021).

2.2.3.1.2. Ultrazvukem asistovaná extrakce

Na začátek je znovu naváženo určité množství konopných květů a přilito dané množství extrakčního rozpouštědla. Vzorek se dále po určitý čas nechá v laboratorní ultrazvukové lázni při 40 °C. Roztok je poté přefiltrován přes filtrační papír do odměrné baňky. Postup se opakuje ještě dvakrát. Filtráty ze všech tří extrakcí se poté spojí. Před nástřikem do systému HPLC jsou extrakty ještě filtrovány pomocí PTFE filtru do HPLC vialek (Porto et al. 2014; Brighenti et al. 2017; Agarwal et al. 2018).

2.2.3.1.3. Extrakce mikrovlnným zářením

Odvážené množství vzorku je v prvním kroku extrahováno rozpouštědlem do skleněné nádoby při 60 °C za nepřetržitého magnetického míchání. Extrakce podporovaná mikrovlnným ohřevem s fokusovaným polem probíhá v uzavřeném systému nádob. Po skončení extrakce je vzorek přefiltrován do odměrné baňky. Druhý a třetí krok extrakce probíhá obdobně. Filtráty ze tří extrakčních kroků jsou následně spojeny. Před samotnou analytickou koncovkou ve formě HPLC jsou extrakty ještě zfiltrovány pomocí PTFE filtru do HPLC vialek (Brighenti et al. 2017; De Vita et al. 2020).

2.2.3.1.4. Extrakce fytokanabinoidů superkritickým CO_2

Extrakce se provádí superkritickým CO_2 s 20% přídavkem ethanolu jako spolurozpouštědla. Extrakční nádoba je naplněna konopnými květy rozemletými na prach a smíchanými se stejným množstvím matrice. Průtok CO_2 se zpravidla udržuje na průměrné úrovni 2,5 l/min a celý proces se provádí při 100 barech a 35 °C po dobu 5 minut statické

extrakce. Následované 15 minutami dynamické extrakce. Ke sběru extraktu je použit ethanol. Extrakt se poté vysuší a rozpustí v ethanolu. Před nástřikem do systému HPLC je extrakt zředěn a zfiltrován pomocí PTFE filtru do HPLC vialek (Omar et al. 2013; Brighenti et al. 2017).

2.2.3.2. Izolace terpenických sloučenin

V průběhu času bylo vyvinuto několik technik izolace terpenů a terpenoidů. Silice (esenciální či éterické oleje) bývají tradičně z trichomů konopí izolovány pomocí hydrodestilace. Obdobně se ale již také využívá konvenční extrakce rozpouštědlem či metod inovativních (Isidore et al. 2021). Je potvrzeno, že úprava biomasy má významný vliv na výtěžek a složení esenciálních olejů. Sušení a skladování rostlin vede k vyšší ztrátě nejvíce těkavých složek, monoterpenů. K dalšímu snížení výtěžku silic dochází při zvýšení dané teploty sušení (McGraw et al. 1999; Kwaśnica et al. 2020; Wanas et al. 2020).

2.2.3.2.1. Destilace vodní parou a hydrodestilace

Parní destilace a hydrodestilace patří mezi nejoblíbenější metody používané k izolaci silic z rostlinných zdrojů. Tyto metody jsou užívány k oddělení látek, které těkají s vodní párou při nižší teplotě, než je jejich bod varu. Vodní pára proniká biomasou a strhává těkavé sloučeniny. Rozpouštědlo a rozpuštěné látky poté kondenzují, což způsobuje jejich oddělení, přičemž horní fází kapaliny jsou éterické oleje. Rozdíl v těchto dvou metodách spočívá v tom, že při destilaci vodní parou je pára přímo přiváděna do rostlinného materiálu, zatímco při hydrodestilaci je rostlinný materiál zpočátku nasáknut vodou, a následně zahříván na teplotu varu (Chemat & Boutekedjiret 2015). Ukazuje se, že typ destilace ovlivňuje selektivitu vůči určité skupině terpenů. Fiorini et al. (2019) porovnávali chemické složení silice získané pomocí destilace vodní parou a hydrodestilace a všimli si, že při parní destilaci se získá větší obsah monoterpenů (54 %) ve srovnání se seskviterpeny (44,2 %). Naopak hydrodestilace vedla k většímu zastoupení seskviterpenů (48,5 %) oproti monoterpenům (43,9 %). Karyofylen však zůstal nejvíce zastoupeným seskviterpenem v silicích bez ohledu na destilační typ. Hydrodestilace byla díky vyššímu procentu extrahovaných bioaktivních sloučenin vyhodnocena jako účinnější. Je to pravděpodobně proto, že u parní destilace je vyvinut nižší tlak a pára nepronika rovnoměrně rostlinným materiálem. Proto je též zapotřebí více času na extrakci složek s vyšším bodem varu. Byly rovněž testovány různé teploty u obou typů destilací květů konopí. Optimální teploty jsou 110 °C pro hydrodestilaci a 130 °C

pro destilaci vodní parou. Rozemletí rostlinného materiálu vedlo k dalšímu zvýšení obsahu monoterpenů, ale snižovalo seskviterpenovou frakci (Naz et al. 2017; Zheljazkov et al. 2020).

2.2.3.2.2. Konvenční extrakce rozpouštědlem

Konopí se extrahuje dobře jak polárním (Bakro et al. 2020), tak nepolárním rozpouštědlem (Krill et al. 2020). Nicméně Namdar et al. (2018) zjistili, že směs polárních a nepolárních rozpouštědel, konkrétně hexan/ethanol (7:3), vede k nejúčinnější extrakci terpenů a současně kanabinoidů z květenství ve srovnání s čistým hexanem a ethanolem. Případné využití polárních rozpouštědel vede ke zvýšení výtěžku kanabinoidů.

Z kořenů a stonkové kůry konopí byly extrahovány triterpeny. Friedelin a epifriedelin byly izolovány z kořenů konopí po ethanolové extrakci (Slatkin et al. 1971), ale malé množství různých triterpenů bylo též identifikováno při extrakci na Soxhletově extraktoru stonkové kůry acetonem (Gutiérrez et al. 2006).

2.2.3.2.3. Superkritická fluidní extrakce

Superkritický CO_2 je dobrým rozpouštědlem pro extrakci těkavých sloučenin, jako jsou terpeny, z *Cannabis sativa* L. (Darani & Mozafari 2010). Extrakce superkritickým CO_2 byla porovnána s hydrodestilací a prokázalo se, že superkritická fluidní extrakce dosahuje lepších extrakčních výtěžků silic než hydrodestilace. Dalšími výhodami jsou možná frakcionace, přímé získávání silic a nižší spotřeba energie ve srovnání s hydrodestilací (Naz et al. 2017). Da Porto et al. (2014) studovali vliv extrakčního tlaku na výtěžek a složení konopného esenciálního oleje. Při 40 °C vedlo zvýšení extrakčního tlaku ze 100 barů na 140 barů ke snížení výtěžku z 0,67 % hmotnosti sušiny na 0,34 %. Dále bylo též zjištěno, že terpenové spektrum v extraktech získaných při tlaku 100 barů je blíže původní terpenické skladbě v konopných květech.

2.2.3.2.4. Ultrazvuková extrakce

Bylo prokázáno, že ultrazvuková amplituda a složení rozpouštědla (poměr cyklohexanu a izopropanolu) má významný vliv na obsah terpenů, zatímco zvyšování doby působení nemá ve studovaném rozsahu signifikantní vliv. Dále byly navrženy optimální provozní podmínky extrakce květů konopí pro tuto metodu, rozpouštědlo

izopropanol/cyklohexan (1:1), čas působení 5 min s 80% amplitudou a v cyklech 3 s^{-1}. Ultrazvukem asistovaná extrakce se také provádí v minutách ve srovnání s hodinami práce v případě destilace (Omar et al. 2013; Palmieri et al. 2020).

2.2.3.2.5. Mikrovlnná extrakce

Při extrakci mikrovlnným zářením je pozorován obdobný výtěžek silic jako při hydrodestilaci, ale za kratší čas. Tyto dvě metody se však liší svým terpenickým profilem, podíl seskviterpenů je v případě mikrovlnné extrakce upřednostňován před monoterpeny. Vysoká energie dodávaná mikrovlnami pravděpodobně způsobuje ztrátu těkavějších sloučenin (Fiorini et al. 2020; Gunjević et al. 2021).

2.2.4. Identifikace a kvantifikace fytokanabinoidů a terpenických sloučenin

2.2.4.1. Identifikace a kvantifikace fytokanabinoidů

Kanabinoidní sloučeniny je v této době možné stanovit různými chromatografickými technikami s odlišnými detekčními metodami.

2.2.4.1.1. Tenkovrstvá chromatografie

Chromatografie na tenké vrstvě (TLC) je vhodná metoda pro rychlý screening různých druhů vzorků. Lze ji snadno provést při rutinních testech a na trhu je již k dispozici několik komerčních sad přímo pro testování kanabinoidů (Ramirez et al. 2019). Fischedick et al. (2009) vyvinuli jednoduchou a rychlou metodu vysokoúčinné TLC (HPTLC) pro kvantifikaci Δ^9-THC a kvalitativní screening hlavních neutrálních kanabinoidů nalezených v kultivarech konopí.

2.2.4.1.2. Plynová chromatografie

Plynová chromatografie (GC) je jednou z nejpopulárnějších technik pro analýzu biologických matric a rostlinných extraktů. Tato metoda pracuje na principu, kdy se daný vzorek dávkuje do proudu nosného plynu (mobilní fáze) a je dále unášen kolonou. Aby mohl být vzorek transportován, musí se ihned taktéž přeměnit na plyn. V koloně se pak látky separují na základě odlišné schopnosti interagovat se stacionární fází (Jennings et al. 1997). Použití vysokých teplot ale vede ke ztrátě karboxylové skupiny z kyselých forem kanabinoidů. Při této metodě tudíž není možné rozlišovat kyselinu od neutrálních forem,

pokud není provedena předchozí derivatizace. Je však třeba vzít též v úvahu, že výtěžky derivatizace nemusí být kvantitativní (Hazekamp et al. 2005).

2.2.4.1.3. Vysokoúčinná kapalinová chromatografie

V rostlinných extraktech konopí, které nebyly podrobeny tepelnému ošetření, bývá většinový podíl kanabinoidů ve formě kyselin. Výhodou technik založených na kapalinové chromatografii (LC) je schopnost analyzovat při laboratorní teplotě, a tím pádem zabránit případné tepelné degradaci a dekarboxylaci kanabinoidních sloučenin ve vzorku (Hazekamp et al. 2005). V dnešní době je pro tyto analýzy běžně využívaná kapalinová chromatografie s obrácenými (reverzními) fázemi, tedy s nepolární (C18) stacionární fází a polární mobilní fází (Ramirez et al. 2019).

De Backer et al. (2009) aplikovali HPLC s detektorem s diodovým polem (DAD) za účelem identifikace a kvantifikace neutrálních a kyselých forem kanabinoidů z *C. sativa*. Byla použita nepolární kolona C18 v kombinaci s gradientovou elucí methanol/voda od poměru 68:32 do 95:5 s přídavkem 50 mM mravenčanu amonného a při pH 5,19. Určili mez detekce (LOD) a mez stanovitelnosti (LOQ) pro THCA, Δ^9-THC, CBDA, CBD, CBGA, CBG a CBN. Metoda byla plně validována dle Mezinárodní organizace pro normalizaci, ISO 17025 (Rodima et al. 2005).

Aizpurua-Olaizola et al. (2014) použili HPLC s tandemovou hmotnostní spektrometrií (HPLC-MS/MS) pro analýzu konopných extraktů. Kvantifikovali 6 kanabinoidů (THCA, Δ^9-THC, CBD, THCV, CBG a CBN) a dalších 7 identifikovali pomocí kvadrupólového detektoru doby letu (Q-TOF) a chemické ionizace za atmosférického tlaku (APCI) při pozitivní ionizaci.

Relativně nedávno byly též testovány kolony naplněné částicemi silikagelu o průměru pod 2 µm. Tato metoda byla nazvána jako ultra vysoce účinná (UHPLC) či ultra účinná kapalinová chromatografie (UPLC). Heo et al. (2016) ji při simultánní analýze kanabinoidů v doplňcích stravy kombinovali s detektorem ultrafialového (UV) záření a detektory MS/MS. Metoda se ukázala jako citlivá a reprodukovatelná. Umožňovala identifikaci a kvantifikaci rostlinných a syntetických kanabinoidů přítomných v tabletách, kapslích, práščích a cukrovinkách s validací dle mezinárodních pokynů.

2.2.4.2. Identifikace a kvantifikace terpenických sloučenin

Kanabinoidní sloučeniny a jejich metabolity lze analyzovat jak pomocí kapalinové, tak i plynové chromatografie. Terpeny se ale kvůli vysoké volatilitě analyzují hlavně prostřednictvím plynové chromatografie (GC) (Leghissa et al. 2018).

2.2.4.2.1. Plynová chromatografie

Terpeny jsou snadno ionizovány, ale nejběžnější analytické metody neberou v úvahu izomerní povahu a různé aromatické vlastnosti těchto sloučenin, což vede k méně než komplexní charakterizaci (Leghissa et al. 2018). K překonání tohoto nedostatku by mohly být použity chirální GC kolony (se stacionární fází na bázi cyklodextrinu). Dalším problémem v analýze terpenů jsou strukturní podobnosti těchto složek, které vedou k podobným až identickým fragmentům (Booth et al. 2017).

Primární volbou kombinace této separační techniky s detektorem, založenou na nízkých pořizovacích nákladech a jednoduchosti, je plynová chromatografie s plamenovým ionizačním detektorem (GC-FID). Využívá se k semikvantitativní analýze, čímž se zjistí relativní zastoupení daných složek v aromaprofilu (Leghissa et al. 2018). Hazekamp a Fischedick (2012) použili ke kvantifikaci 20 terpenických složek vzorku pouze jeden terpenový standard, γ-terpinen, kvůli velmi malým rozdílům mezi FID faktory odezvy monoterpenů a seskviterpenů.

Kvalitativní povaha terpenů je obvykle zkoumána pomocí jednoduchých kvadrupólových MS detektorů porovnáním vypočtených retenčních indexů složek a hmotnostních spekter daných látek s databázemi. Snadná ionizace umožňuje detekovat sloučeniny s vysokou spolehlivostí. Strukturální podobnosti monoterpenů a seskviterpenů však vedou k podobným a nerozeznatelným MS spektrům, což způsobuje problémy při jejich identifikaci. K překonání těchto nevýhod se pro jednoznačné přiřazení velmi doporučuje kombinovat FID a MS spektra (Micalizzi et al. 2021).

2.2.5. Potencionální terapeutické využití

Výzkum konopí se v posledních letech rozvinul (Grotenhermen & Muller-Vahl 2012; Felson et al. 2019). Přijatelnost, využití a následné předepisování léčebného konopí se nadále rozšiřuje, jak ukazuje i rostoucí počet zemí, které umožňují jeho použití pro specifické terapeutické indikace (Shelef et al. 2011; Troutt & DiDonato 2015; Balneaves & Alraja

2019). Doposud ale převládá upřednostňování konopí s majoritním zastoupením kanabinoidu CBD, tedy rostlin chemického fenotypu III (Marinotti & Sarill 2020). V současnosti je stále tedy bohužel nedostatek základních výzkumných informací o regulaci biosyntézy THCA a ostatních sekundárních metabolitů u chemického fenotypu léčebného konopí s majoritním zastoupením kanabinoidu THCA, chemotypu I, a jeho farmaceutickém potenciálu. K tomuto také přispívá přetrvávající právní omezení ve většině zemí (Aguilar et al. 2018). Počet zkoumaných fytokanabinoidů se ale stále zvyšuje a jejich účinky na různé nemoci, jako je chronická bolest (Lynch & Campbell 2011; Portenoy et al. 2012), nevolnost a zvracení (Duran et al. 2010), spasticita (Pooyania et al. 2010; Corey-Bloom et al. 2012), psychózy, motorické a nemotorické symptomy Parkinsonovy choroby (Lotan et al. 2014), deprese (Selvarajah et al. 2010), úzkost a poruchy spánku (Russo et al. 2007; Bonn-Miller et al. 2014; Babson et al. 2017), glaukom (Järvinen et al. 2002), zánětlivé onemocnění střev (Ravikoff Allegretti et al. 2013) a v neposlední řadě léčba různých druhů rakoviny (Abrams & Guzman 2015; Heider et al. 2022), jsou studovány.

2.2.5.1. Antikarcinogenní působení

Mnoho vědecko-výzkumných týmů se v současnosti snaží najít nové aktivní sloučeniny s cytostatickými a cytotoxickými účinky. Až donedávna se fytokanabinoidy používaly převážně k léčbě nechutenství, nevolnosti a zvracení u pacientů s rakovinou podstupujících chemoterapii. V současné době, ale přibývá experimentálních důkazů *in vitro* a na zvířecích modelech podporujících protirakovinnou aktivitu jednotlivých kanabinoidů prostřednictvím modulace klíčových buněčných signálních drah zapojených do kontroly proliferace a přežití rakovinných buněk, inhibice angiogeneze a redukce metastáz u různých typů nádorů (Guzmán 2003; Velasco et al. 2016). Fytokanabinoidy taktéž vykazují specifickou cytotoxicitu vůči nádorovým buňkám a současně chrání zdravou tkáň před apoptózou (Bogdanović et al. 2017). Některé studie již tyto pozitivní efekty prokázaly u různých typů rakoviny včetně rakoviny prsu (Sarnataro et al. 2006), slinivky (Fogli et al. 2006), prostaty (De Petrocellis et al. 2013) a střev (Javid et al. 2016; Velasco et al. 2016). Nicméně jejich využití v onkologii je zatím omezené, protože klinické důkazy stále chybí. Výzkum brzdí variabilita a nedostatečná standardizace designu studií, lékové formy a farmakodynamiky (Abrams 2019; Turgeman & Bar-Sela 2019). Úspěch takové léčby bude taktéž záviset na dávce, jedinci, stadiu nádoru a mnoha dalších okolnostech a faktorech (Urruticoechea et al. 2010; Lafaye et al. 2017).

3. HYPOTÉZY A CÍLE PRÁCE

3.1. Hypotézy práce

Z literatury vyplývá, že intenzita biosyntézy kanabinoidních látek se v různých fázích vývoje konopného květenství diferencuje. Na základě dosavadních informací lze předpokládat, že koncentrace a poměr živin a biostimulantů v živném roztoku hraje zásadní roli a následně tedy ovlivní obsah sekundárních metabolitů, tvorbu květenství a výnos nadzemní biomasy rostlin léčivého konopí. Připravené konopné extrakty budou vykazovat potencionálně využitelné terapeutické účinky.

3.2. Cíle práce

Tato práce si klade za cíl vyhodnotit intenzitu biosyntézy kanabinoidních látek během vývoje konopného květenství. Porovnat vliv různých druhů výživy v nezávislých hydroponických pěstebních cyklech na tkáňový ionom, tvorbu biomasy, obsah sekundárních metabolitů a potencionální antikarcinogenní účinky rostlin konopí.

4. PUBLIKOVANÉ PRÁCE

Doktorská disertační práce předkládaná formou svázaných vědeckých článků vznikla na základě níže uvedených čtyř publikovaných prací v časopisech databáze Web of Knowledge s Impact Factor indexem a jedné práce, která je aktuálně „Under Review“. Ostatní publikace, uveřejněné mimo rozsah práce, jsou uvedené v kapitole 8, Publikované práce mimo rozsah disertace, na konci této práce.

4.1. The overview of existing knowledge on medical cannabis plants growing

Autoři: Malík M, Velechovský J, Tlustoš, P

Rok publikace: 2021

Časopis: Plant, Soil and Environment **67**: 425-442

https://doi.org/10.17221/96/2021-PSE

The overview of existing knowledge on medical cannabis plants growing

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Citation: Malík M., Velechovský J., Tlustoš P. (2021): The overview of existing knowledge on medical cannabis plants growing. Plant Soil Environ., 67: 425–442.

Abstract: The use of cannabis for medicinal purposes dates back well before the era of modern medicine, but in recent years research into the use of medical cannabis in the medical and pharmaceutical sciences has grown significantly. In European countries, most cannabis plants have been and still are grown for industrial purposes. For this reason, hemp cultivation technology is relatively well researched, while little is known about the key factors affecting cannabis cultivation for medical purposes. The active substances of cannabis plant targeted by this review are called phytocannabinoids. The biosynthesis of phytocannabinoids is relatively well understood, but the specific environmental factors that influence the type and number of phytocannabinoids have been much less studied. Indoor or greenhouse cultivation, which uses automated lighting, ventilation, irrigation systems and complex plant nutrition has become much more sophisticated and appears to be the most effective method for producing medical cannabis. There are many different cultivation systems for cannabis plants, but one of the essential elements of the process is an optimal plant nutrition and selection of fertilisers to achieve it. This review summarises the existing knowledge about phytocannabinoid biosynthesis and the conditions suitable for growing plants as sources of medical cannabis. This review also attempts to delineate how nutrient type and bioavailability influences the synthesis and accumulation of specific phytocannabinoids based on contemporary knowledge of the topic.

Keywords: *Cannabis sativa* L.; tetrahydrocannabinol; cannabidiol; chemical profile; growing conditions

Cannabis is one of the earliest of domesticated crops. According to Chinese historical records and archaeological findings, its cultivation and utilisation can be traced back to 3 000 to 4 000 years BCE (Yu 1987, Jiang et al. 2006). The first use of cannabis for therapeutic purposes, directly evidenced by the finding of the stable cannabis compound, Δ^6-tetrahydrocannabinol (Δ^6-THC), has been dated to around 400 CE in a carbonised material discovered in a tomb at Beit Shemesh near Jerusalem (Zlas et al. 1993). Recent years have seen a boom in research on medical cannabis in the biomedical and pharmaceutical sectors. The applicability and acceptability of medical cannabis is expanding, as seen by the growing number of countries that allow its use for specific therapeutic indications (Shelef et al. 2011, Troutt and Didonato 2015, Balneaves and Alraja 2019). The number of active phytocannabinoids under investigation continues to increase and their effects on a variety of diseases such as chronic pain (Lynch and Campbell 2011, Portenoy et al. 2012, Wilsey et al. 2013), nausea and vomiting (Lane et al. 1991, Duran et al. 2010), spasticity (Pooyania et al. 2010, Corey-Bloom et al. 2012), depression (Wade et al. 2004, Selvarajah et al. 2010, Portenoy et al. 2012), glaucoma (Järvinen et al. 2002), inflammatory bowel disease (Ravikoff Allegretti et al. 2013), psychosis, motor and non-motor symptoms of Parkinson disease (Lotan et al. 2014), anxiety and sleep disorder (Russo et al. 2007, Bonn-Miller et al. 2014, Babson et al. 2017) are being studied (Doyle and Spence 1995, Järvinen et al. 2002, Lynch and Campbell 2011, Grotenhermen and

https://doi.org/10.17221/96/2021-PSE

Muller-Vahl 2012, Ravikoff Allegretti et al. 2013, Lotan et al. 2014). Nearly 150 different phytocannabinoid compounds are currently known (Hanuš et al. 2016).

TAXONOMY

History

The genetic plasticity of cannabis makes it difficult to catalog, and there is still a debate about its proper botanical classification. Linnaeus (1753) described *Cannabis sativa* as a single species. Based on comparative analyses of the psychoactive effects, leaf size, shape and structure of Indian and European varieties, Jean-Baptiste Lamarck (1786) classified the Indian cultivars as a separate species, *Cannabis indica*. At the beginning of the 20th century, the Russian botanist Janischevsky (1924) found that the local Russian plants possessed different characteristics from both *C. sativa* and *C. indica* yet still belonged to the cannabis taxon. These small, wild-growing, auto-flowering plants have been classified as a separate species named *Cannabis ruderalis* (Figure 1).

Current nomenclature

Small and Cronquist (1976) utilised a biphasic approach combining morphological and chemical characteristics to divide the *Cannabis* genus into the following four groups:

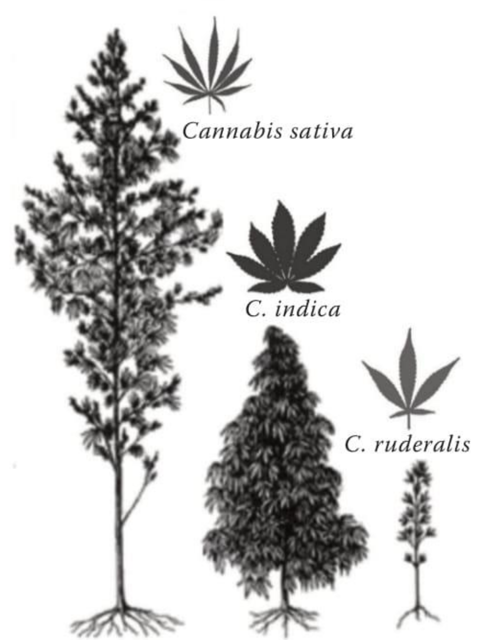

Figure 1. Species of cannabis (Hartsel et al. 2016)

1. *Cannabis sativa* L. subsp. *sativa* var. *sativa*,
2. *Cannabis sativa* L. subsp. *sativa* var. *spontanea* Vavilov,
3. *Cannabis sativa* L. subsp. *indica* Small & Cronquist var. *indica* (Lam) Wehmer,
4. *Cannabis sativa* L. subsp. *indica* Small & Cronquist var. *kafiristanica* (Vavilov) Small & Cronquist (Figure 2).

Hillig (2005) concluded from his genomic study of the classification of *C. sativa* that none of the previous taxonomic concepts sufficiently defined the *sativa* and *indica* genes. He analysed different genotypes from various geographical origins and was therefore inclined to a multi-species classification including *C. sativa*, *C. indica* and *C. ruderalis*. Small (2015) has recently proposed two possible cannabis taxonomic classifications. The first is consistent with an earlier division (Small and Cronquist 1976) and is in accordance with the International Code of Nomenclature for Algae, Fungi, and Plants (McNeill et al. 2012). The second, for domesticated cannabis, follows the guidelines of the International Code of Nomenclature for Cultivated Plants (Brickell et al. 2009):

Non-narcotic plants, domesticated for stem fiber and/or oilseeds in West Asia and Europe. Low Δ^9-tetrahydrocannabinol (THC) content and high cannabidiol (CBD) content (Hillig and Mahlberg (2004) *Cannabis sativa* "hemp biotype").

Non-narcotic plants, domesticated for stem fiber and/or oilseeds in East Asia, mainly China. From low to moderate THC content and high CBD content (Hillig and Mahlberg (2004) *Cannabis indica* "hemp biotype").

Psychoactive plants, domesticated in Southern and Central Asia. High THC content and low or absent CBD content (Hillig and Mahlberg (2004) *Cannabis indica* "narrow-leaflet drug (NLD) biotype").

Psychoactive plants, domesticated in Southern Asia (Afghanistan and neighboring countries). From moderate to high THC and CBD content (Hillig and Mahlberg (2004) *Cannabis indica* "wide-leaflet drug (WLD) biotype").

In addition, two hybrid classes have also been generated:

5. Non-narcotic plants, hybrid cultivars between two fiber (hemp) groups (1 and 2).
6. Psychoactive plants, hybrid cultivars between two narcotic groups (3 and 4).

Hillig and Mahlberg (2004) analysed the content of cannabinoids in various cannabis plants and based on geographical origins, morphological features and

https://doi.org/10.17221/96/2021-PSE

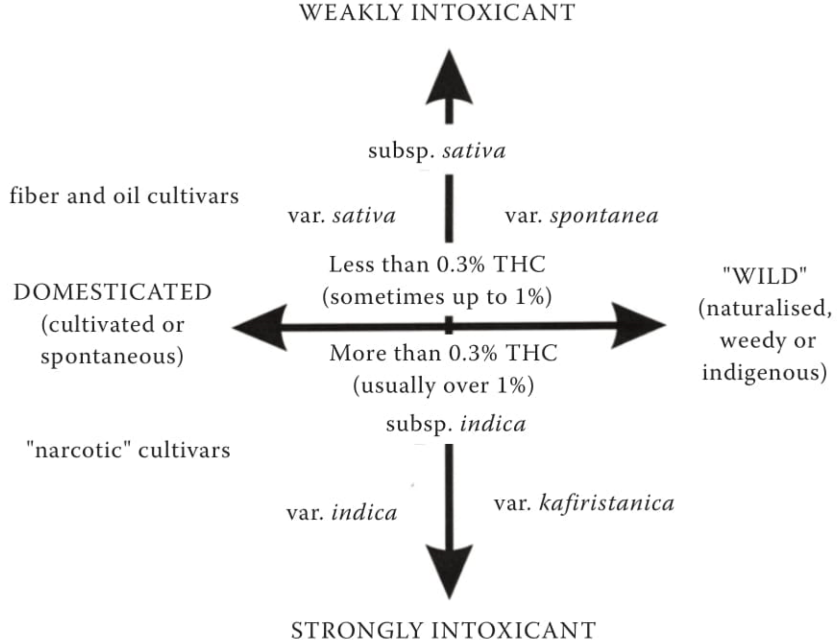

Figure 2. Cannabis chemotypes (Small and Cronquist 1976). THC – Δ^9-tetrahydrocannabinol

the supposed purpose of cultivation assigned them to the intraspecific taxa (biotypes):

Cannabis sativa "hemp biotype" – 62 plants were analysed, ranges of the dry-weight percentages of THC were measured 0.1–11.5% and CBD were measured 0.0–13.6%.

Cannabis indica "hemp biotype" – 45 plants were analysed, ranges of the dry-weight percentages of THC were measured 0.1–9.3% and CBD were measured 0.0–8.5%.

Cannabis indica "narrow-leaflet drug (NLD) biotype" – 68 plants were analysed, ranges of the dry-weight percentages of THC were measured 1.4–12.4% and CBD were measured 0.0–0.1%.

Cannabis indica "wide-leaflet drug (WLD) biotype" – 40 plants were analysed, ranges of the dry-weight percentages of THC were measured 0.1–14.7% and CBD were measured 0.0–11.0%.

All cannabis species successfully cross and produce fertile hybrids (Beutler and Marderosian 1978). *Indica* and *sativa* plants have also been found to differ in terpene and cannabinoid profiles. Thus, these chemotaxonomic markers are a promising tool for screening hybrids (Hillig 2004, Hillig and Mahlberg 2004, Fischedick et al. 2010, Elzinga et al. 2015). Zhang et al. (2018) are recommending that Cannabis should be recognised as a monotypic species typified by *Cannabis sativa* L., containing three subspecies: subsp. *sativa*, subsp. *indica*, and subsp. *ruderalis*. This proposal is based on their study focused on DNA sequence variations of cannabis plants. Also, McPartland (2018) in his work mentions that DNA barcode analysis supports the separation cannabis at a subspecies level and recognising the nomenclature of *C. sativa* subsp. *sativa* and *C. sativa* subsp. *indica*.

BIOSYNTHESIS OF CANNABINOIDS

History

Actual cannabinoid research is based on a number of major discoveries made by Professor Raphael Mechoulam and Professor Yechiel Gaoni. In the 1960's they identified the psychoactive component in *Cannabis sativa*, Δ^9-tetrahydrocannabinol, determined and described its chemical structure (Gaoni and Mechoulam 1964, Mechoulam and Gaoni 1967) and synthesised it (Mechoulam et al. 1967). Endogenous cannabinoid receptor ligands, called endocannabinoids, were identified in mammalian tissues in the 1990s. The best-known examples are anandamide (Devane et al. 1992) and 2-arachidonoylglycerol (Mechoulam et al. 1995). Endocannabinoids are derived from arachidonic acid, and membrane lipids serve as a potential source of this fatty acid (Giuffrida et al. 2001). For this reason, cannabinoids from cannabis are often referred to as phytocannabinoids to differentiate them from endocannabinoids.

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Biosynthesis of phytocannabinoids

Phytocannabinoids can be divided into two groups, neutral cannabinoids and cannabinoid acids. Diversification is based on how many carboxyl groups the molecule has, but non-enzymatic decarboxylation can occur during storage and especially at elevated temperatures when cannabis is smoked (Kimura and Okamoto 1970, Shoyama et al. 1970). Phytocannabinoids, prenylated polyketides of mixed biosynthetic origin, are synthesised from fatty acid precursors and isoprenoids. All phytocannabinoid structures contain a monoterpenic unit attached to the phenolic ring having the C3 alkylated carbon (Dewick 2002). The alkyl side chain can vary in length from one to five carbons (Figure 3) and n-pentyl is the most abundant (Elsohly and Slade 2005). Phytocannabinoids containing an n-propyl side chain are referred to as cannabivarins. Tetrahydrocannabivarin (THCV), the THC analogue with an n-propyl side chain, often occurs in *C. indica* (Hillig and Mahlberg 2004).

The starting materials for aromatic ring synthesis, including the alkyl on the third carbon (Hanuš et al. 2016), are three molecules of malonyl-CoA and one molecule of hexanoyl-CoA derived from hexanoic (caproic) acid (Dewick 2002). The hexanoyl-CoA acts as a primer for the type III polyketide synthase enzyme, also known as tetraketide synthase (TKS), which also requires the olivetolic acid cyclase enzyme (OAC) catalysing a C2–C7 intramolecular aldol condensation with carboxyl group retention to produce olivetolic acid (Taura et al. 2009, Gagne et al. 2012). These transformations can give rise to by-products such as 4-hydroxy-6-pentylpyran-2-one (PDAL), 4-hydroxy-6-(2-oxoheptyl)pyran-2-one (HTAL) and olivetol. Cannabigerolic acid (CBGA) is further derived from olivetolic acid after alkylation with a monoterpene unit, geranylpyrophosphate, with the participation of geranylpyrophosphate:olivetolate geranyltransferase (GOT) (Figure 4) (Fellermeier and Zenk 1998). Also, the (Z)-isomer of cannabigerolic acid, cannabinerolic acid (CBNRA), is synthesised to a small extent when neryl pyrophosphate is used by the GOT enzyme instead of geranyl pyrophosphate (Taura et al. 1995a). There are three acids that can be formed from CBGA and CBNRA.

Figure 3. Structure of cannabinoids

Tetrahydrocannabinolic acid (THCA) is produced during the formation of the heterocyclic ring by the THCA synthase enzyme, which can convert CBGA or CBNRA to THCA (Figure 5) (Taura et al. 1995b). However, the low THCA synthase specificity for CBNRA compared to CBGA suggested that THCA was predominantly synthesised from CBGA. The course of this reaction is similar to that of other reactions catalysed by monoterpenic cyclases. Most of the cyclases require divalent ions such as Mg^{2+} or Mn^{2+} for their activity, but this is not the case with THCA synthase (Taura 2009). The presence of a carboxyl group in the substrate molecule is essential for the reaction because THCA synthase does not recognize neutral phytocannabinoids such as cannabigerol (CBG) as substrates (Taura et al. 2007a).

The structure of cannabidiolic acid (CBDA) is the result of a pericyclic reaction involving loss of a proton (Figure 6) (Dewick 2002). The modification is catalysed by the intramolecular oxidoreductase, CBDA synthase, which selectively favours the formation of CBDA from CBGA over its (Z)-isomer, CBNRA (Taura et al. 1996). The effects of various metal ions (Mg^{2+}, Mn^{2+}, Zn^{2+}, Ca^{2+}, Co^{2+} and Cu^{2+}) on its activity were investigated, but they did not alter the rate of catalysis. In contrast, the Hg^{2+} ion completely inhibited enzyme activity at a concentration of 2 mmol, and the chelating agent, ethylenediaminetetraacetic acid (EDTA), at concentrations up to 5 mmol showed a low positive effect on enzyme activity. Thus, CBDA synthase does not appear to require metal ions for CBGA oxidocyclization (Taura et al. 1996). CBDA synthase and THCA synthase catalyse the formation of single optical isomers at a purity of greater than 95% (Taura et al. 2007b).

Cannabichromenic acid (CBCA) is derived from CBGA by oxidation and cyclisation by cannabichromenic acid synthase (CBCA synthase) (Figure 7). CBCA is synthesised as a 5:1 enantiomeric mixture, probably because of the partial release of intermediates from the CBCA synthase active site prior to completion of the reaction (Morimoto et al. 1997). Tests of the metal ions, Mg^{2+}, Zn^{2+}, Ca^{2+} and Cu^{2+}, showed that none of them stimulated enzyme activity. Hg^{2+}, however, completely inhibited the reaction at a concentration of 1 mmol. EDTA slightly increased enzyme activity suggesting that the CBCA synthase reaction does not require metal ions (Morimoto et al. 1998).

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Figure 4. Biosynthesis of phytocannabinoids 1/2. TKS – tetraketide synthase; PDAL – 4-hydroxy-6-pentylpyran-2-one; OAC – olivetolic acid cyclase enzyme; HTAL – 4-hydroxy-6-(2-oxoheptyl)pyran-2-one; GOT – geranylpyrophosphate: olivetolate geranyltransferase

Figure 5. Δ^9-THCA (tetrahydrocannabinolic acid) synthesis

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cannabigerolic acid → CBDA synthase → cannabidiolic acid

Figure 6. Cannabidiolic acid (CBDA) synthesis

Croteau (1987) discovered that all terpene cyclases require bivalent cations for their function because these metal ions are able to neutralise the negative charge on the diphosphate groups on the terpene molecules and ionise the allyl diphosphate substrate. Since CBGA does not contain a diphosphate group it is to be expected that CBCA synthase, CBDA synthase and THCA synthase have no requirement for bivalent cations. The most of the cannabinoids present in *C. sativa* can be categorised as Δ^9-tetrahydrocannabinol (Δ^9-THC), CBD, CBC, CBG, cannabinol (CBN), cannabicyclol (CBL), cannabielsoin (CBE) and cannabitriol (CBT) (Turner et al. 1980, Razdan 1986, Ross and Elsohly 1995). Δ^9-THCA, CBDA and CBCA are also sometimes called primary phytocannabinoids because other phytocannabinoids are generated from these three precursors predominantly by nonenzymatic degradative pathways.

Primary phytocannabinoids can either be decarboxylated to their neutral form (Figure 8) or converted to CBE, CBN, CBT, Δ^8-tetrahydrocannabinol (Δ^8-THC) or CBL *via* exposure to light, heat and oxygen (Figure 9). CBD can undergo photooxidation or pyrolysis to form CBE. Δ^9-THC is converted to the thermodynamically more stable Δ^8-THC when exposed to heat, or it may be degraded to CBT or CBN in the presence of oxygen (Elsohly and Slade 2005). The presence of CBT and CBN together with high levels of decarboxylated phytocannabinoids, are the chemical indicators of lengthy storage under poor conditions (Shoyama et al. 1970). The degradation rate of primary phytocannabinoids to these secondary phytocannabinoids increases with higher temperature, higher initial concentrations of primary phytocannabinoids, and with an increase in the inflorescence surface, and thus greater surface exposure to air (Milay et al. 2020). CBC in the presence of light converts to CBL-type phytocannabinoids (Elsohly and Slade 2005). Cannabivarins are generated by the same biosynthetic pathways from cannabigerovarinic acid (CBGVA), a homologous CBGA precursor (Shoyama et al. 1984). The cannabinoid profile in Cannabis undergoes rapid changes in the early stages of growth (Potter 2014). CBDA and THCA synthases have very similar catalytic rates (k_{cat} = 0.19/s and 0.20/s) and affinity (K_M = 134 μmol and 137 μmol) for cannabigerolic acid (Taura et al. 1995b, 1996). The CBCA synthase, however, shows a lower Michaelis constant (K_M = 23 μmol) as well as a higher catalytic rate (k_{cat} = 0.04/s). In the early stages of cultivation, where CBGA is still present at low concentrations, CBCA synthesis predominates (Morimoto et al. 1998). However, as the CBGA concentration increases over time, the efficacy of THCA and CBDA biosynthesis increases, and these molecules soon outweigh the CBCA concentration. At later stages of growth, CBGA synthesis slows and its relative proportion in the phytocannabinoid profile is gradually reduced (Potter 2014).

CULTIVATION

In European countries, most cannabis is grown for industrial purposes (Zuk-Golaszewska and Golaszewski

cannabigerolic acid → CBCA synthase → cannabichromenic acid

Figure 7. Cannabichromenic acid (CBCA) synthesis

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heat
decarboxylation

delta-9-tetrahydrocannabinolic acid delta-9-tetrahydrocannabinol

heat
decarboxylation

cannabidiolic acid cannabidiol

heat
decarboxylation

cannabichromenic acid cannabichromene

Figure 8. Decarboxylation of primary phytocannabinoids

2018) such as hemp fibers (Pickering et al. 2007), seeds as a source of oil (Mölleken and Theimer 1997, Kriese et al. 2004) and protein (Patel et al. 1994). For this reason, the procedures for hemp cultivation are well known, while the growth factors affecting cannabis cultivation for medical purposes are poorly understood (Zuk-Golaszewska and Golaszewski 2018).

Indoor or outdoor medical cannabis cultivation?

The conditions under which cannabis plants are grown for drug production is subject to more stringent protocols relating to the content and type of the active phytocannabinoids. Among the factors influencing the composition and yield of phytocannabinoids are the genotype of the plant, the growing conditions, maturity at harvest time, storage and handling (Potter 2014).

It is much more efficient to grow medical cannabis plants in a greenhouse where light, temperature and humidity can be controlled. Until recently, this method of cultivation was used mainly by illegal cannabis growers (Drugs 2009). Outdoor cultivation is less expensive, but the variability of the environment makes it almost impossible to obtain a high-potency, homogeneous product. Cannabis that is grown outdoors is also at greater risk from pests and plant diseases (Potter 2014). Cannabis entrepreneurs now use sophisticated indoor cultivation methods with automated control of lighting and photoperiod, temperature, ventilation and irrigation, and complex systems for providing nutrients. However, much of the information on indoor cannabis production is still obtained from anecdotal sources (Vanhove et al. 2011). Current data on the influence of photoperiod and even light spectrum allow indoor growers to regulate such aspects as leaf and shoot growth and time of vegetation cycle and thus achieve several growth cycles per year (Farag and Kayser 2015). Three to six harvests per year (six harvests per year is the maximum, and in this case, you have to skip the vegetative phase) can be attained by applying modern controlled growing practices (Leggett 2006).

Hydroponics *versus* soil

Indoor cannabis cultivation can be accomplished in several ways, but primarily either in soil or in soilless culture using hydroponic media. Hydroponic

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CBGA (*R* = n-Pr)
CBGVA (*R* = Me)

CBDA synthase
THCA synthase
CBCA synthase

CBDA (*R* = *n*-Pr)
CBDVA (*R* = Me)

THCA (*R* = *n*-Pr)
THCVA (*R* = Me)

CBCA (*R* = *n*-Pr)
CBCVA (*R* = Me)

photooxidation or pyrolysis

photochemical reaction

CBEA (*R* = *n*-Pr)
CBEVA (*R* = Me)

CBLA (*R* = *n*-Pr)
CBLVA (*R* = Me)

isomerisation
oxidation
oxidation

delta-8-THCA (*R* = *n*-Pr)

CBTA (*R* = *n*-Pr)

oxidation
oxidation

CBNA (*R* = *n*-Pr)

Figure 9. Biosynthesis of phytocannabinoids 2/2. CBGA – cannabigerolic acid; CBGVA – cannabigerovarinic acid; CBDA – cannabidiolic acid; CBDVA – cannabidivarinic acid; CBCA – cannabichromenic acid; CBCVA – cannabichromevarinic acid; THCA – tetrahydrocannabinolic acid; THCVA – tetrahydrocannabivarinic acid; CBEA – cannabielsoin acid; CBEVA – cannabielsovarinic acid acid; CBLA – cannabicyclolic acid; CBLVA – cannabicyclolvarinic acid; CBTA – cannabitriolic acid; CBNA – cannabinolic acid

cultivation has become increasingly popular among growers. A soilless media such as mineral wool, coconut fibers, perlite or expanded clay are used while nutrients provided by solutions are applied directly

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to the roots (Vanhove et al. 2011). The conventional type of cultivation is in soil with fertilisers applied through irrigation or by mixing with the soil substrates. Potter (2014) found that there was no increase in phytocannabinoid potency or in biomass under hydroponic conditions compared to standard soil cultivation, and the hydroponic system was more complicated and difficult to operate and maintain.

Vegetation cycle of cannabis

Cannabis is a short-day plant, naturally blooming in autumn, and the induction of flowering is regulated by specialised photoreceptor proteins called phytochromes. Therefore, the effect of photoperiod must be taken into an account in indoor cultivation (Halliday and Fankhauser 2003). The vegetative phase lasts from 2–4 weeks after rooting the clones or germinating the seeds (De Backer et al. 2012). The relative humidity in this phase should be from 70% to 80% with a temperature from 21 °C to 28 °C (Chandra et al. 2008). The generative phase is induced by shortening the photoperiod to 12 h light and 12 h dark. The first flowers should appear about one week after the reduction of the light period. The development of stems and leaves gradually slows down and stops after three weeks of this photoperiod, while the flowers continue to develop over the next 8 weeks (De Backer et al. 2012). The monitoring of 200 high THC cannabis varieties showed that the average flowering time with 12 h light period was 57 days, and 88% of the plants flowered between 7 and 9 weeks (Carpentier et al. 2012). The recommended temperatures are similar to the previous phase from 21 °C to 28 °C. However, humidity should be lowered to 40% over the generative phase to reduce the risk of fungal diseases (Vanhove et al. 2011, 2012).

Effect of CO_2 concentration

In order to prevent mold, a dry environment and constant air circulation should be ensured in indoor cannabis growing rooms, either from outdoor ventilation with filters or by indoor fans. It is also recommended to increase the concentration of CO_2 during the light phase of the day (cycle) to improve photosynthesis, plant growth and thus increase biomass yield (Kimball 1983, Wheeler et al. 1996, Chandra et al. 2008, 2011). Elevated CO_2 concentration can improve the assimilation of carbon, thereby accelerate plant growth and potentially improve productivity (Kimball 1983). There is a close correlation between plant yield and photosynthesis rate because more than 90% of plant dry matter is derived from photosynthetic CO_2 assimilation (Zelitch 1975). However, the improved level of plant photosynthesis and growth appear to be species- and variety-specific (Minorsky 2002).

Wang et al. (2008) investigated the effects of standard (370 ppm) and high (700 ppm) CO_2 concentrations on photosynthesis tolerance to acute heat stress (daily growth temperature was increased by 15 °C every day for 4 h) in cool-season and warm-season of C3 plants. High CO_2 concentration increased the cool-season and warm-season C3 plants tolerance of photosynthesis to acute heat stress. Hamilton et al. (2008) further elaborated the previous idea and concluded that the effects of growth temperature on photosynthetic thermotolerance between C3 and C4 plants are different and affected by the state of acclimatisation of the plants. A high concentration of CO_2 (700 ppm) increases the thermotolerance of C3 plants photosynthesis, except for C3 plants grown at the supra-optimal (5 °C above optimal) growth temperature, then increased CO_2 may provide no advantage or even reduce photosynthesis. On the other hand, increased CO_2 often reduces the photosynthetic thermotolerance of C4 plants at both optimal and supra-optimal growth temperatures.

Chandra et al. (2011) performed experiments directly on cannabis and showed that increasing CO_2 concentration from 390 ppm to 700 ppm increased the rate of photosynthesis in different varieties of *Cannabis sativa* by 38–48% and improved efficiency of water uptake.

Artificial light

To achieve optimal biomass and phytocannabinoid production, artificial lighting must meet certain parameters. These include light intensity in lumens per m^2 (lux units) and radiation intensity in watts per m^2 and the wavelength. Wavelength is particularly important because plants require different wavelengths of light during the growth. In the vegetative (roots and shoots) phase, the light should be 420–460 nm which corresponds to blue light, which promotes phototropism and growth hormone production in the plants. In the flowering phase, a red spectrum (600–680 nm) that is well absorbed by chlorophyll is best (Mahlberg and Hemphill 1983). For indoor cannabis cultivation, fluorescent T-5 lighting, metal-halide lamps (MH), high-pressure sodium lamps (HPS) for the growth and light-emitting diodes (LED),

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high-pressure sodium lamps (HPS) for the generative phase are most commonly used (Sweet 2016). These lamps differ in the composition of the inside gases, and they produce the light of different wavelengths.

The optimal intensity of illumination. The experiments of Potter and Duncombe (2012) showed positive relationship between the intensity of illumination and amount of biomass harvested. They determined three zones with elevated illumination energy 270, 400 and 600 W/m^2. Five plants of each variety were placed in each of the three zones at a density of 10 plants/m^2. In the growth rooms, daily average temperatures were maintained at 25 ± 2 °C. A constant supply of fresh air kept CO_2 concentration in the environment between 350 ppm and 390 ppm. Irradiance levels at the surface of the plant canopy were measured using a hand-held light meter determined the photosynthetically active radiation 80, 120 and 180 W/m^2 according to variants. Within plants growth, the lamps were kept at a constant distance from the cannabis canopy. The greatest harvest was achieved at 600 W/m^2 of the illumination intensity. Furthermore, the THC contents in the leaves and inflorescences of the mentioned variants were measured, but no significant increase in the concentration of THC was recorded with an increase of light intensity. Toonen et al. (2006) also reported that plants grown under 600 W lamps achieved higher yields than plants grown under 400 W lamps.

Decreasing tendency of plants to convert light energy into biomass with increasing levels of radiation is probably due to the fact, that plants have a limited ability to use light for photosynthesis. Under low light conditions, plants normally show an initial linear increase in the rate of photosynthesis and thus a tendency to convert light energy into biomass in response to increasing irradiation. However, under brighter conditions, the growth rate slows as chloroplasts become more and more saturated with light (Evans et al. 1993, Ögren and Evans 1993). This has also been proven on cannabis. The rate of increase in photosynthetic activity went down rapidly when irradiation levels rose above 100 W/m^2 of photosynthetically active radiation. Since 300 W/m^2 of photosynthetically active radiation, almost no increase in photosynthetic activity has been observed (Lydon et al. 1987).

HPS lamps *versus* LED. Magagnini et al. (2018) concluded that HPS-lit plants were higher and had a larger amount of dry matter than LED-lit plants. Conversely, plants under LED fixtures contained higher levels of CBD and THC than under the HPS. Namdar et al. (2019) also found out significant increase in concentration of CBGA in the inflorescences that flowered under LED illumination, with CBGA:THCA ratio of 1:2 as opposed to 1:16 when grown under HPS. Because of the high level of illumination, it was necessary to install a ventilation fan for cooling to the optimum temperature for photosynthesis of 25 °C to 30 °C (Bazzaz et al. 1975). A more efficient alternative is to use banks of LEDs that produce relatively little heat (Bessho and Shimizu 2012). LEDs do not consume much energy, do not require ballasts, and produce only a small amount of heat compared to high intensity discharge lamps. LEDs are compact, have long lives, very good wavelength specificity, relatively cool radiating surfaces, and linear photon output with electrical input current (Massa et al. 2008).

NUTRITION

In the area of plant nutrition for medical cannabis production, there is currently a lack of experimental data in the literature (Caplan et al. 2017a). It is known that the content of cannabinoids in leaves gradually decreases from top to bottom of the hemp plant (Hemphill et al. 1980) and from the literature about hemp cultivation can be deduced that nutrient application can affect the final cannabinoid content of the plants as well as their total yield. This suggests that nutrition could play a similar role for medical cannabis grown under controlled conditions. However, cannabis for hemp production has been selectively bred to produce fiber and is therefore likely to have slightly different nutrient needs than cannabis grown for medicinal purposes. The hemp crop is also grown in the field and not indoors (Hillig and Mahlberg 2004, Van Bakel et al. 2011, Amaducci et al. 2015).

Acceptable forms of individual essential nutrients are divided by Barker and Pilbeam (2015) into two groups according to plant needs, namely macronutrients: nitrogen (NO_3^-, NH_4^+), phosphorus ($H_2PO_4^-$, HPO_4^{2-}), potassium (K^+), calcium (Ca^{2+}), sulfur (SO_4^{2-}), magnesium (Mg^{2+}), and micronutrients: iron (Fe^{2+}, Fe^{3+}), chlorine (Cl^-), manganese (Mn^{2+}), zinc (Zn^{2+}), copper (Cu^+, Cu^{2+}), boron (H_3BO_3, $H_2BO_3^-$), molybdenum (MoO_4^{2-}) and nickel (Ni^{2+}).

Macronutrients

Nitrogen, phosphorus and potassium (NPK). It is assumed that the nitrogen content in the vegetative parts of

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the hemp plant positively correlates with the THC content (Haney and Kutscheid 1973). Thus, older leaves contain less THC than younger leaves because they contain less nitrogen. In contrast, high nitrogen levels in applied nitrogen fertilisers reduce the THC content of the hemp leaves (Bócsa et al. 1997). For example, good hemp production requires optimum soil nitrogen levels in the range of 50–200 kg/ha (Vera et al. 2004, Aubin et al. 2015), but these recommendations are not applicable for hydroponic or soil cultivation where studies on indoor cannabis cultivation indicated nitrogen fertilisation should be provided in the range of 190–400 mg N/L. This value has also been reported for nitrogen supplementation of organic, greenhouse-grown tomatoes (Zhai et al. 2009, Surrage et al. 2010).

Hemp growth and an increase in THC content were positively correlated with soil P content (Coffman and Gentner 1977). A negative relationship has been reported for CBD content in leaf tissue relative to available P. Hemp grown on soils depleted of P showed an increased CBD content (Coffman and Gentner 1975). Conversely, phosphorus enhancement did not show any positive effect on THC, CBD, CBN or CBG concentrations in buds from the top of the medical cannabis plants (Bernstein et al. 2019b).

Saloner et al. (2019) investigated response of medical cannabis to different potassium supply in vegetative growing phase. The results show that the response to nutrition is highly dependent on the genotype. Plants in this study were exposed to five different levels of K supply (15–240 ppm). Generally, both cultivars showed increased K concentration in all plant parts with increased K supply. Insufficient K dose for optimal growth and function was the lowest tested supply 15 ppm of K. Also, the highest dose proved excessive and damaging effect to development for one of the two tested genotypes. Similarities proven at both genotypes were in trends of accumulation and uptake. Results demonstrated competition between K and Ca with Mg uptake and no effect on P and N uptake except in the K deficiency range. Potassium supply showed only little effect on micronutrient accumulation in the plant shoot which was similar for both cultivars.

In contrast, no significant effect on hemp biomass and THC was observed in relation to different doses of N and K (Coffman and Gentner 1977). According to Hanuš and Dostálová (1994), various combinations of selected macroelements (N, P, K) in hemp culture can significantly affect the type of phytocannabinoids present and their individual contents. One of a few available sources of scientific literature dealing directly with this issue is the article by Caplan et al. (2017a, b), who reported a concentration of 389 mg N/L as optimal during the growth phase for maximum yield. The ratio of the basic macroelements (N, P, and K) in the vegetative period was 4:1.3:1.7. After making the calculations for P and K, we obtained values of 126 mg P/L and 165 mg K/L. In the generative phase, 212–261 mg N/L was the optimal amount. A nitrogen concentration of 283 mg N/L gave the maximum yield of inflorescence and biomass, but the concentrations of phytocannabinoids in the dried product was lower. The ratio of N, P, and K in the generative period was set at 2:0.87:3.32. Therefore, an initial concentration of 283 mg N/L, would require 123 mg P/L and 470 mg K/L. The plants tested were propagated from 17 day-old cuttings, which were fertilised with a solution of the indicated concentration for the following 21 days of vegetative growth. Another study has proved sensitivity of phytocannabinoids metabolism to mineral nutrition. The results presented by Bernstein et al. (2019b) show that increased treatment of inorganic NPK increased levels of CBG in flowers by 71% and decreased levels of CBN in flowers by 38% compared to a control treatment. Plants in the control variant were cultivated in potting mixture with fertigation. Concentration of dissolved nutrients in the control variant was as follows: 65 ppm N (1:2 ratio of NH_4^+/NO_3^-), 17 ppm P (40 ppm P_2O_5), 90 ppm K (108 ppm K_2O). Micronutrients were supplied chelated with EDTA at concentration of 0.4 ppm Fe, 0.2 ppm Mn, and 0.06 ppm Zn.

The rest of macronutrients. The magnesium cation content in soils is relatively mobile and its concentration in plants, especially in leaves, is high because it is a component of chlorophyll. The negative correlation between this metal and copper results from the fact that the radii of their ions are similar and both ions can compete for the same binding sites. The content of Δ^9-THC and CBD in hemp leaves decreases with increasing Mg concentration in the soil. The Δ^9-THC content in leaves is positively correlated with the ratio of accessible Ca/Mg in soil. CBD is negatively correlated with available Ca/Zn and Mg/Cu ratios. Positive correlations of magnesium with Δ^8-THC have been reported with the hypothesis that this nutrient may be cofactor in the enzyme responsible for its production (Coffman and Gentner 1975, Pate 1994, Radosavljevic-Stevanovic et al. 2014).

Micronutrients

Similar results have been seen for micronutrient requirements. Positive correlations of iron with

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Δ^8-THC have been reported with the hypothesis that this nutrient may be cofactor in the enzyme responsible for its production (Pate 1994, Radosavljevic-Stevanovic et al. 2014). CBD content in hemp plants is decreasing with increasing iron concentration (Radosavljevic-Stevanovic et al. 2014). The negative correlation of iron (Fe) and chromium (Cr) with CBD can be explained because the catalase responsible for the decomposition of hydrogen peroxide from the CBDA synthase reaction is a member of the class that contains four heme iron groups. Hydrogen peroxide is strongly sterically hindered upon entry into the heme cavity where the first step of catalysis takes place. Transferring a proton from an oxygen atom to a hydrogen peroxide molecule, and then to a second oxygen atom extends and polarises the O-O bond, which eventually decays heterolytically. The first oxygen atom of the hydrogen peroxide molecule is coordinated with a heme center, which releases water and creates an O=$Fe^{(IV)}$-$enzym^{(+)}$ heme radical. The radical then quickly breaks down by electron transfer, removing the radical electron from the porphyrin ring, which remains unchanged. During the second step, in a similar two-electron transmission reaction, the O=$Fe^{(IV)}$-$enzym^{(+)}$ reacts with a second molecule of hydrogen peroxide to form the parent molecule $Fe^{(III)}$ – enzym, water, and molecular oxygen (Boon et al. 2007, Vlasits et al. 2010).

Proposed reaction mechanism:

$$H_2O_2 + Fe^{(III)}\text{-enzyme} \leftrightarrow H_2O + O{=}Fe^{(IV)}\text{-enzyme}^{(+)}$$
$$H_2O_2 + O{=}Fe^{(IV)}\text{-enzyme}^{(+)} \leftrightarrow H_2O + Fe^{(III)}\text{-enzyme} + O_2$$

Fe-enzyme represents the center of heme iron attached to the rest of the enzyme.

The transition state, O=$Fe^{(IV)}$-$enzyme^{(+)}$ is energetically unstable, so these reactions are disadvantageous (Boon et al. 2007, Vlasits et al. 2010). Although chromium is not important for plant growth, its negative correlation with CBD is explained by the fact that Fe and Cr occur together in nature as a complex oxide (Radosavljevic-Stevanovic et al. 2014).

The concentration of CBN and Δ^9-THC in hemp plants can be influenced by the amount of manganese (Radosavljevic-Stevanovic et al. 2014). A positive correlation of manganese with CBN has been reported (Pate 1994, Radosavljevic-Stevanovic et al. 2014). THCA synthase, which catalyses the oxidative cyclisation of CBGA to THCA, contains a flavin adenine dinucleotide (FAD) prosthetic group that is reduced to $FADH_2$. Molecular oxygen is required to re-oxidise the $FADH_2$ to FAD, with the formation of hydrogen peroxide in a 1:1 molar ratio to the resulting THCA as a by-product of the reaction (Flores-Sanchez and Verpoorte 2008, Shoyama et al. 2012). CBDA synthase also contains FAD that is reduced to $FADH_2$ with release of H_2O_2, but the reaction differs from THCA synthase in the proton transfer step (Figure 10) (Taura et al. 2007a). It is estimated that about 1% of oxygen in plants is used to form reactive oxygen species in different subcellular locations with hydrogen peroxide being the most abundant. Hydrogen peroxide causes oxidative damage to cells that can lead to apoptosis (Quan et al. 2008), and plants have evolved efficient ways of eliminating toxic levels of H_2O_2. Catalase is a peroxidase enzyme found in all oxygen-using organisms that rapidly converts H_2O_2 to water and oxygen. There are three types of catalase and the non-heme form utilises manganese (Mn^{3+}) in its catalytic center that is reduced to Mn^{2+} during the decomposition of H_2O_2 to water and oxygen. Mn^{2+} can then react with more peroxide and be converted back to Mn^{3+} according to the following equations:

$$2Mn^{3+} + H_2O_2 \leftrightarrow 2Mn^{2+} + O_2 + 2H^+$$
$$2Mn^{2+} + H_2O_2 + 2H^+ \leftrightarrow 2H_2O + 2Mn^{3+}.$$

Both reactions are energetically advantageous ($\Delta G < 0$). The correlation between manganese and CBN is also positive since CBN is the primary THC degradation product (Wu et al. 2004).

Bernstein et al. (2019a) describes translocation of individual macro and microelements in relation to individual plant parts' age. The work also describes, inter alia, the distribution of cannabinoids in the plant. The research shows that the concentration of cannabinoids increases with the height of the plant and the highest concentration can be found in flowers and inflorescence leaves. The concentration found in fan leaves is about 1/10 the concentration found in flowers. The distribution of mineral nutrients between plant organs shows a typical uptake and translocation in the plant. Lower concentrations of N, P, K, and higher Ca in fan leaves compared to inflorescence supports physiological findings that the fan leaves are older than the inflorescence leaves.

pH value

Suggested optimal pH range of nutrient solution is between 5.5–6.5. pH is important because it affects the availability and absorption of nutrients needed

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Figure 10. Reaction mechanism of tetrahydrocannabinolic acid (THCA) synthase and cannabidiolic acid (CBDA) synthase. CBGA – cannabigerolic acid; FAD – flavin adenine dinucleotide

for plant growth. In hydroponic culture, the recommended pH range is 5.5–6.0 and the maximum absorption of nutrients is usually at pH 5.8 (Velazquez et al. 2013). In growing substrate, a pH range of 5.8–7.2 is recommended and the maximum absorption of essential nutrients is typically at pH 6.5. When the pH falls below this range, many macronutrients are less available and macronutrient deficiencies can be developed. When pH values rise above this range, many micronutrients will not be available for the plant uptake causing micronutrient deficiencies (Caplan et al. 2017a). These authors also mention the need for further research to confirm the optimal range of pH for multiple cannabis varieties.

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Plant biostimulants

A plant biostimulant is any substance, micro-organism strain or mixture of both applied to plants to increase tolerance to abiotic stress, nutritional efficiency or crop quality characteristics, regardless of its nutrient content. Seven main biostimulant categories were proposed: humic and fulvic acids, protein hydrolysates, seaweed and botanical extracts, chitosan and biopolymers, beneficial bacteria, beneficial fungi and beneficial minerals (Du Jardin 2015).

Humic and fulvic acids in cannabis nutrition. Humic substances are natural components of soil organic matter. It is a mixture of heterogeneous compounds originally classified according to their molecular weights and solubility into humins, humic acids and fulvic acids (Du Jardin 2015).

Humic acid supplementation had a positive effect on cannabis in the case of the height of cannabis plants, the chlorophyll content and the efficiency of photosynthesis, especially immediately after the period of water stress (Da Cunha Leme Filho et al. 2020).

According to the current literature, the effect on phytocannabinoids is rather negative. Bernstein et al. (2019b) mentioned that nutritional supplements such as humic acids significantly reduced spatial variability of cannabinoids throughout the plant parts. This increased uniformity came at the expenses of THC and CBD content which was reduced by 37% and 39% respectively in the top parts of plants. The decrease of THC has been associated with an additional trend of CBN increasing. This was probably due to the accelerated degradation of cannabinoids in the plant parts with their high concentration.

Other biostimulants in cannabis nutrition. Conant et al. (2017) demonstrated that microbial biostimulant Mammoth P™ promoted cannabis growth during the blooming phase. Lyu et al. (2019) hypothesised that future research will show that plant growth-promoting bacteria can affect the accumulation of phytocannabinoids, increase inflorescence yields, protect against plant pathogens by producing antimicrobial compounds and reduce the impact of abiotic stresses.

CONCLUSIONS AND FUTURE PERSPECTIVE

Based on the above information, it can be stated that quality of medical cannabis biomass, spectrum and concentration of phytocannabinoids can be influenced by cultivation conditions as well as nutrition during cultivation.

For the cultivation of medical cannabis, due to safety reasons, unpredictable environmental influences and required homogenity of harvest, indoor cultivation is definitely a better option because optimal growing conditions can be set and cannabis can be harvested from three to six times per year. Of the growing conditions, artificial light, the level of CO_2 concentration and the humidity of the surrounding environment influence the harvest quantity and quality the most. It is very important to choose the right combination of all mentioned conditions because they affect each other.

There is currently only a few experimental data on the medical cannabis nutrition, so most of this information is based on the hemp cultivation, which was bred for fiber production rather than inflorescence. However, it can be concluded from the current literature the concentration and spectrum of individual macronutrients, micronutrients and plant biostimulants in plant nutrition has a fundamental impact on biomass formation, spectrum and amount of medical cannabis cannabinoids.

In the future, the effects of nutrient ratios and availability can reasonably be expected to be one of the main factors influencing the content and type of cannabinoids in medical cannabis plants, separate from genotype and microclimate. These issues should be explored through further experiments, which will certainly be beneficial because of growing interest in the phytocannabinoids development in public and commercial spheres. Future technical research in this area should focus on possible new indoor medical cannabis cultivation techniques or the automation of existing cultivation technologies to facilitate work.

Acknowledgement. We thank for financial support from Faculty SGS project SV20-4-21140, and prof. Gary Bentley for improving the use of English in the manuscript.

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Received: February 23, 2021
Accepted: June 4, 2021
Published online: July 19, 2021

4.2. Application of Individual Digestate Forms for the Improvement of Hemp Production

Autoři: Velechovský J, Malík M, Kaplan L, Tlustoš, P

Rok publikace: 2021

Časopis: Agriculture **11**:1137

Article

Application of Individual Digestate Forms for the Improvement of Hemp Production

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Abstract: In a two-year vegetation field experiment, the fertilizing effects of by-products from the agricultural biogas plant—a solid phase of digestate (SPD) and a liquid phase of digestate (LPD)—were studied and compared with mineral fertilization (NPK) on the biomass yield, content and nutrient uptake by *Cannabis sativa* L. plants. Furthermore, the agrochemical properties of the soil were evaluated at the end of the experiment. In all variants of the experiment, a uniform nitrogen dose of 150 kg/ha was applied. The dose of other nutrients corresponded to the fertilizer used. The biggest fertilizing effect, and therefore the greatest hemp biomass yield and nutrient uptake, was demonstrated when combining SPD and LPD fertilization in one treatment. However, the differences were statically insignificant ($p \leq 0.05$). The applied amount appeared to be sufficient for the nutrition of hemp plants and was comparable to mineral fertilization. The distribution of nutrients between leaves and stems varied depending on the nutrient monitored. Analyses after the end of the experiment did not show different contents of accessible nutrients in the soil on the studied variants. The content of accessible risk elements in the soil was not affected by the application of the SPD and the LPD. The experiment showed that cannabis plants are able to achieve equivalent biomass yields (8.68 t/ha) using the combination of LPD and SPD by-products from a biogas plan compared to commercial mineral fertilizer (7.43 t/ha). Therefore, we can recommend a split application of LPD and SPD as a suitable alternative to mineral fertilization. Due to prolonged nutrient release from SPD, we can expect a smaller negative environmental impact than current fertilization practices.

Keywords: *Cannabis sativa* L.; fertilization; biogas plants; solid phase of digestate; liquid phase of digestate

Citation: Velechovský, J.; Malík, M.; Kaplan, L.; Tlustoš, P. Application of Individual Digestate Forms for the Improvement of Hemp Production. *Agriculture* **2021**, *11*, 1137. https://doi.org/10.3390/agriculture11111137

Academic Editor: Claudio Marzadori

Received: 8 October 2021
Accepted: 11 November 2021
Published: 13 November 2021

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1. Introduction

As a result of ever-increasing greenhouse gas emissions from burning fossil fuels, there has been a great boom in the development and use of biogas in recent years, especially as a source of environmentally friendly energy in the production of heat and electricity [1–3]. It is estimated that the consumption of biogas in Europe in the coming years will double, from 14.5 gigawatts in 2012 to 29.5 gigawatts in 2022 [4]. From the perspective of farmers, agricultural biogas plants also offer the possibility of stable extra income [5].

Biogas is produced by the anaerobic microbiological degradation of organic compounds, and it usually contains two major components: methane and carbon dioxide; it can also include other gases such as hydrogen sulfide and nitrogen (N_2) [6]. The positive aspect of anaerobic digestion is the fact that it reduces pathogens, kills viruses, fungi, bacteria of the genus *Listeria, Salmonella* and *Escherichia coli* and inactivates plant seeds [7–11]. The secondary product of this wet fermentation is digestate [12]. Hemp appears to be a suitable alternative crop for biogas production due to high biomass yields. It also has low adverse environmental impacts compared to other crops commonly used in Europe for biogas production (corn, sugar and beet) [13–15]. Considering soil ecology and sustainable soil use

in the Czech Republic, *Cannabis sativa* L. could work as a plant that alternates commonly grown plants used for biogas production. The aim is to reach a closed feedstock circle in which *Cannabis sativa* L. is grown due to using biogas station outputs as a fertilizer. In addition, soil conditions are improved by *Cannabis sativa* L. growth, and the consequently gained biomass might be used for further biogas production.

1.1. Composition of Digestate

Digestate is a heterogeneous liquid formed as a by-product of biogas production from organic matter with a significant proportion of undecomposed solid organic fraction (60–80% in dry matter). The dry matter of digestate is in the range of 7–12% and is comparable to slurry. It has a similar nitrogen content in fresh matter as manure (0.2–1%) but a higher pH value ranging from 7–8 [16–18]. The nutrient content in the dry mass (DM) of digestate is reported by Möller and Müller as follows: total N: 3.1–14%, P: 0.6–1.7% and K: 1.9–4.3% [19]. The application of digestate as an organic fertilizer to agricultural land is already considered as a standard method of its use [20,21]. The use of biogas residue as a substitute for mineral fertilizers has also been mentioned by other authors. Studies show that the use of digestate from agricultural biogas plants reduces the environmental risks that are generally associated with the use of mineral fertilizers and, at the same time, achieves comparable yield parameters for agricultural crops such as *Medicago sativa* L. and *Triticum aestivum* L. At the same time, the availability of nutrients depends on the input of raw materials, and it is not possible to say that, in general, we achieve better field crop yields by using by-products of anaerobic digestion [19,22–24].

The solid phase (SPD) and the liquid phase of digestate (LPD) are formed by the mechanical separation of the digestate in order to obtain two homogeneous materials. The composition of the SPD and the content of macronutrients and micronutrients is influenced by the composition of the input raw materials into the fermentation process and the retention time of the raw materials in the fermenter [25,26]. Slurry, silage corn, grass silage, cereals, sorghum and sugar beet pulp are produced as input materials [27]. The dry matter content in the SPD is in the range of 21–30% [16]. Möller and Müller present a content of nitrogen in the range of 2.2–3%, a content of phosphorus of 1.9% and a potassium content of 3.6% in the dry matter (DM) of the solid part of the digestate [19]. Due to the chemical composition and physical features, the applied SPD can positively affect biomass yield and soil structure [28,29].

Kolář et al. [26] refer to the LPD as a dilute solution containing a wide range of nutrients in a form acceptable to plants. The values given for the liquid part of the digestate (DM) are 7.7–9.2% for nitrogen, 0.4–0.7% for phosphorus and 3.9% for potassium [19]. The LPD appears to be a suitable raw material for application to arable land during vegetation, and its fertilizing and irrigation effects can be used [16,26]. Schievano et al. [30] characterize the LPD as an organic fertilizer that contains mineral nutrients along with organic matter. The dry matter of the LPD is in the range of 0.8–4%. Nitrogen is mainly present in mineral form, which means that it is easily accessible to plants. Its concentration is in the range of 0.15–0.30%, which is comparable to the potassium content. A study presented by Coelho et al. showed concentrations of plant essential nutrients as follows: N (6.6–24.1%, average 11.7%), P (0.81–3.28 % DW, average 1.74%) and K (0.81–17.35 % DW, average 6.15%). Because the proportions of N–P–K are variable in each digestate, it is necessary to provide an analysis of the specific digestates before actual application on the field [31]. Other nutrients are present in significantly lower concentrations [32].

1.2. Hemp (Cannabis sativa L.) Plants

Hemp (*Cannabis sativa* L.) comes from western Asia and is one of the earliest domesticated agricultural crops. According to Chinese historical records and archaeological findings, its cultivation for fiber and seeds dates back to the period of approximately 3000–4000 years BCE [33,34]. Over the centuries, hemp fibers have been used to manufacture

fabrics, sails, ropes and paper, while hemp seeds have been used as protein-rich food and feed [35].

According to European regulations, industrial hemp may contain no more than 0.3% of tetrahydrocannabinol (THC). In several European countries (e.g., the Netherlands and Belgium), a maximum THC content of 0.2% is allowed. In the European Union, only hemp cultivars approved by the European Commission—i.e., industrial hemp cultivars with a THC content below 0.2%—are permitted for industrial hemp cultivation [36–38].

Hemp biomass has been used for energy purposes for centuries. However, the energetic use of hemp has traditionally been limited to the use of oil pressed from hemp seeds; e.g., for lighting purposes. To date, the commercial use of industrial hemp biomass for energy purposes has been proposed in many countries. Hemp can be used to produce heat and energy by directly burning biomass from whole plants, or it can be converted to biomass-bound energy into liquid or gaseous transport biofuels such as bioethanol and biogas [39–41].

According to van der Werf et al. [42], the maximum yield of industrial hemp stems in field cultivation can be reached at a plant density of 90 plants/m^2. Amaducci et al. [43] reached an average yield of 14 tons/ha in a three-year field hemp cultivation experiment. The fertilization of hemp plants with nitrogen at a rate of 150 kg N/ha ensures optimal plant height, higher seed yield, higher stem strength [44] and overall high biomass production [44–47]. Nitrogen uptake is, according to Landi [48], greatest in the early stages of growth. An adequate nitrogen supply is ensured if the nitrogen content of the plant in dry matter is in the range of 5–6% [49]. The need for nitrogen by plants depends on the variety, as stated by Finnan and Burke [46]. Alaru et al. [50] compared the use of nitrogen by plants in variants where ammonium nitrate, waste sludge and beef manure were applied. The nitrogen dose for all variants was chosen identically, at 100 kg N/ha. Their results showed the suitability of using sludge as an organic fertilizer in the cultivation of cannabis for energy purposes. On the contrary, the application of beef manure failed to ensure the maximum yield of biomass. Malceva et al. [51] demonstrated the negative effects of increasing the nitrogen dose on the fiber content of the hemp stem. During a growing season with a higher level of precipitation, an application of 60 kg N/ha is recommended. According to the authors, this dose can be considered optimal. Regarding nitrogen cannabis nutrition, application rates vary from country to country. In Canada, a rate of 60–90 kg N/ha is used, while in EU countries, higher rates are used, ranging from 80–160 kg/ha and depending on soil properties and climatic conditions.

Phosphorus from the soil is taken up evenly by the plant, and its consumption is increased during the flowering and ripening period of the seeds [44]. Phosphorus uptake by cannabis plants ranges from 25–38 kg/ha, depending on the yield [45]. Ivanyi [49] states that a sufficient supply of phosphorus is at a content of 0.5–0.6% P in young fully developed leaves. The required amount of phosphorus to form one ton of dry plant matter is 1.7 kg. If the soil is rich in phosphorus, fertilization with this nutrient can be omitted [52].

Potassium is an important nutrient for the formation of cannabis stems and fibers. Interactions between nitrogen and phosphorus increase the quality of the fiber and the yield of hemp stalks. Potassium is mostly absorbed by cannabis plants during periods of intensive growth [48]. According to Barron et al. [47], potassium requirements for cannabis plants are high. They range from 75 to 100 K kg/ha, and in extreme cases up to 300 kg/ha. However, hemp is able to use potassium from the deeper layers of the soil profile. Cannabis concentrates most of the potassium in the stem, at up to 70–75% [46]. Iványi and Izsáki [53] state that the optimal potassium content in the plant is 2.7–3.0%.

According to Johnson [44] and Landi [48], calcium is also necessary for soils with a neutral pH due to its high consumption in the growth of the root system, stems and seeds. Landi [48] states that the need for calcium, together with nitrogen and potassium, is dominant for cannabis in terms of macronutrients. Depending on the yield, calcium intake is in the range of 151–227 kg/ha at yields of 8–10 tons/ha. In soils with a deficiency,

compensatory fertilization is necessary. Cannabis plants absorb calcium mainly at the end of the growing season.

Magnesium is involved in ensuring the good health of the plant [44]. Landi [48] indicates a magnesium uptake by cannabis plants in the range of 36–54 kg/ha, depending on the yield.

From micronutrients, hemp initially accumulates zinc and copper into the vegetative organs of the plant; later, it transports them to the generative organs, while iron, boron and manganese accumulate mainly in the vegetative organs.

For the field study of hemp growth, we hypothesized that the application of byproducts from a biogas station can sufficiently saturate plants with nutrients to achieve a comparable yield to hemp fertilized by mineral NPK fertilizers. The goal of our experiment was to verify our formulated hypotheses.

2. Materials and Methods

2.1. Vegetation Experiment Establishment

Cannabis plants were grown in a precise two-year vegetation field experiment. The experiment occurred on a demonstration and experimental site of the Faculty of Agrobiology, Food and Natural Resources of the Czech University of Life Sciences in Prague (GPS 50°7′40″ N and 14°22′33″ E). The land is located at an altitude of 286 m above sea level, with an average annual temperature of 9.1 °C and an average total annual precipitation of 495 mm [54]. The soil type is a partly degraded Chernozem—slightly agglomerated on loess and loess clays.

2.2. Description of the Used Hemp Variety

Cannabis sativa L. variety "Tiborszállási", native to Hungary, was used in a precise field experiment. It is a dioecious variety with a proportion of sex individuals in the stand of approximately 1:1. In the case of cultivation, in order to achieve the maximum biomass yield, the growing season is in the range of 105–110 days; in our case, the plants were harvested after 101 days for both years of the experiment. This variety is specific for its early ripening and provides a high yield of stems as well as green biomass. The THC content is declared to be below 0.2% [36].

2.3. Origin of the Digestate and Its Separation into Liquid and Solid Parts

Within the experiment, a digestate originated from the agricultural biogas station Krásná Hora nad Vltavou with an energy output of 526 kWh. Corn silage, grass silage and livestock manure from local stables were used as energy sources. On average, over 20 tons of silage and 44 tons of cattle manure served as daily input. The solid and liquid phases were obtained by the mechanical separation of the digestate on the principle of a centrifuge and a press. All used raw materials were taken in the required amount before starting the experiment directly from the mentioned biogas plant. Some of the raw materials, which were applied only during the experiment, were stored in closed containers in a cooling box at a constant temperature of 4 °C.

2.4. Layout of Individual Plots and Sowing of Plants

The area in which the experiment took place was divided into 12 separate sub-plots measuring 2.5 m × 5 m. Eighty-five grams of seed were sown on each plot, up to 12.5 cm distance per row at a depth of 3 cm. Thus, a total of 1050 g of seed was sown for the entire area of the experiment. The seed rate was calculated from the seeding amount value (70 kg seed/ha).

In the experiment, four variants were established. Each variant was realized in three repetitions arranged such that two identical fertilization variants were not adjacent.

2.5. Amount of Applied Fertilizers in Individual Variants

In the first (NPK) variant, a mineral fertilizer was used in which nitrogen, phosphorus and potassium were added to the soil. Nitrogen was supplied in the form of ammonium nitrate with limestone containing 27% nitrogen (50% NH_4^+, 50% NO_3^-). The amount of nitrogen determined for the field experiment was 150 kg/ha. The dose was chosen depending on the intention to use the cannabis (biomass yield), according to the authors Sausserde and Adamovičs [55], Vera, Malhi, Phelps, May and Johnson [45] and Finnan and Burke [46]. Phosphorus was supplied at a rate of 20 kg/ha in the form of triple superphosphate with a phosphorus content of 21% (48% P_2O_5). Potassium was supplied at a rate of 150 kg/ha in the form of a potassium salt (60% K_2O).

The second variant included a corresponding dose of the SPD converted to a nitrogen content corresponding to 150 kg N/ha, with respect to the first control variant. The analysis of the SPD itself revealed a dry matter of 21.71% and a nitrogen content of 2.49% in the dry matter. For the delivery of 150 kg N/ha, it was necessary to deliver 34.80 kg of SPD on a partial plot with an area of 12.5 m^2. The application of the SPD took place 14 days before sowing, with simultaneous incorporation to a depth of about 8 cm.

The third variant was fertilized with a divided dose of SPD and LPD in a ratio of N (1:1), such that the total dose corresponded to 150 kg N/ha. A corresponding dose of 17.4 kg/12.5 m^2 was separated into the soil two weeks before sowing. The LPD fertilization took place three times during the vegetation, at two-week intervals, with the first application taking place 32 days after sowing. Later application was impossible in practice due to the involvement of cannabis. The LPD at 6% dry mass contained 5.78% N in dry matter. For the purposes of the experiment, it was necessary to supply 27.01 kg of the LPD divided into three equal batches, weighing 9 kg per sub-plot. For each of the three applications, the LPD was applied using a can.

The fourth variant was fertilized only with the LPD, divided into four doses. In the fourth variant, it was necessary to supply a quantity of 54 kg of the LPD per sub-plot. The application was identical to the previous variant. The amount of nitrogen supplied in individual variants during the vegetation is shown in Table 1.

Table 1. The amount of nitrogen supplied in individual variants during vegetation.

	The Amount of N Supplied (kg/ha)				
Variant	**Basic Fertilization**	**1. Additional Fertilization**	**2. Additional Fertilization**	**3. Additional Fertilization**	**Total**
NPK	150	0	0	0	150
SPD	150	0	0	0	150
SPD + LPD	75	25	25	25	150
LPD	75	25	25	25	150

LPD fertilization in the third and fourth variants was performed using a watering can 3 times in an interval of 14 days during the phase of intensive cannabis growth. Prior to the actual application, a groove was formed, into which the LPD was applied and then covered with soil to prevent volatilization of the ammonium. The purpose of this method of application was to simulate a hose applicator commonly used in agricultural practice. During the vegetation, the plants were not treated against diseases or pests. The inter-row treatment against weeds was performed using a hand hoe as needed. The amount of individual biogas by-product for the delivery of 150 kg N/ha was as follows: SPD—27.8 t/ha, LPD—43.2 t/ha, combined dose of SPD + LPD—13.9 + 21.6 t/ha, respectively.

2.6. Harvesting and Plants Sampling, Soil Sampling

Cannabis plants were harvested by hand by plucking, including the root, from an area of 1 m^2, separately from each plot of all variants. After washing and drying the roots, the whole biomass from 1 m^2 was weighed, and the yield was subsequently evaluated.

Individual plant parts (root, stem and leaf) were separated from the harvested plant sample. These samples for analysis were then dried and homogenized. After the plants were harvested, soil samples were taken from individual plots. Samples were taken with a soil probe (20 punctures) to a soil profile depth of 20 cm. The samples were used to determine agrochemical properties. Analyses of all samples took place at the Department of Agroenvironmental Chemistry and Plant Nutrition, Czech University of Life Sciences Prague. All plant samples, samples of the SPD and the LPD, were dried at 35 °C and then homogenized in a 1 mm sieve grinder.

2.7. Determination of pH Value and Content of Soluble Salts in Soil Samples

From the chemical features, the pH value and the content of soluble salts in the aqueous extract of the sample and demineralized water were determined in a ratio of 1:10 (volume:weight). A 10 g sample of dried soil was weighed at 25 °C into plastic PE bottles with lids and poured into 100 mL of distilled water. The samples were shaken for 5 min and then stood still for 5 min. The measurement was performed with a calibrated pH meter and a calibrated conductometer marked HI 991,300 Hanna Instruments.

2.8. Determination of Individual Nitrogen Forms in Soil Samples

For the purpose of the analysis, 10 g of fresh homogenized soil (sieved through a mesh size of 5 mm) was weighed into 250 mL polyethylene bottles and filled with 100 mL of a 0.01 mol/L $CaCl_2$ solution. The solution was then shaken for two hours. The samples were then removed and filtered. The total contents of mineral nitrogen, ammonium and nitrate form were determined in fresh soil by the colorimetric method on the SKALAR SAN^{PLUS} SYSTEM analyzer (Breda, The Netherlands).

2.9. Determination of Acceptable Nutrients from Soil Samples According to Mehlich 3

The soil samples were dried at 35 °C and then sieved through a sieve with a mesh diameter of 2 mm. A 10 g sample soil was weighed into plastic PE bottles, which was extracted with 100 mL of Mehlich 3 reagent [56]. Shaking was performed for 10 min, and then the obtained solution was filtered. Individual extracts were analyzed for phosphate content by the colorimetric method with ICP OES. The extracts were also measured for potassium, magnesium and calcium using an atomic absorption spectrometer (ASS), type Varian Vista Pro (Mulgrave, Australia).

2.10. Determination of Nitrogen Content in Samples of Plant Material

The Kjeldahl method was used to determine the total nitrogen content of the plant material. Total nitrogen includes both organic and ammoniacal nitrogen. For the determination, 0.50 g of a dry homogenized sample of plant material was weighed and mineralized. Mineralization took place in glass cuvettes. To the sample in the cuvette, 2 g of catalyst (mixture of 100 g K_2SO_4, 1 g $CuSO_4$, 0.1 g Se) and 10 mL of concentrated sulfuric acid (H_2SO_4) were added. Decomposition was performed for 90 min at 420 °C. After mineralization, the samples were prepared for distillation by adding 20 mL of distilled water to the cuvette, which was placed in the Gerhardt Vapodest 30s (Königswinter, Germany). Then, distillation into H_3BO_3 took place, and the total nitrogen content in the sample was determined.

2.11. Determination of Macronutrients, Micronutrients and Hazardous Substances Using an Absorption Spectrometer

The decomposition of the samples was carried out in a microwave system in cuvettes, into which 0.5 g of dry plant material, SPD and LPD, ground to a fraction size of 1 mm, was weighed. Then, 8 mL of HNO_3 (65% p.a.) and 2 mL of H_2O_2 (30% H_2O p.a.) were added to the sample. The resulting mineralizate was evaporated after 20 min. Internal reference material (IRM) analysis was performed on each series of samples. The contents

of macroelements, microelements and hazardous elements were determined by ICP-OES (Varian Vista Pro, Mulgrave, Australia).

2.12. Statistical Evaluation

As part of the statistical evaluation, the average yields of fresh and dry biomass, nutrient content and nutrient intake of individual cannabis variants were statistically evaluated by the Statistica 12 program (Statsoft) by a test of homogeneity of variance and then by an analysis of variance ($p \leq 0.05$). More detailed differences between individual averages were evaluated by Tukey's HSD test ($p \leq 0.05$).

3. Results and Discussion

The SPD and the LPD used for fertilization were characterized by a pH value ranging from 8.3–8.6, which matches the approach of Makádi, Tomócsik and Orosz [16]. The LPD obtained on the pressure production separator contained, on average, 6.04% of dry matter, which was significantly more than stated by Kolář et al. [26]. This was probably due to the meshes in the sieve, which, due to their size, allowed the passage of small solid particles into the LPD. Tables 2 and 3 show the different contents of soluble salts. In the SPD, the content of dry matter was more than twice as much as in the LPD, which coincides with the approach of Makádi et al. [16].

Table 2. Specifications of applied SPD in dry mass.

Dry Matter (%)	pH (H_2O)	EC (mS/cm)	Total N (mg/kg)	P (mg/kg)	K (mg/kg)	Ca (mg/kg)	Mg (mg/kg)	S (mg/kg)
21.71 ± 0.261	8.6 ± 0.141	2.749 ± 0.072	24,900 ± 452	3127 ± 129	29,419 ± 632	40,358 ± 772	4364 ± 518	2793 ± 516
Fe (mg/kg)	**Zn (mg/kg)**	**Cu (mg/kg)**	**B (mg/kg)**	**Mn (mg/kg)**	**Pb (mg/kg)**	**Cd (mg/kg)**	**Cr (mg/kg)**	**As (mg/kg)**
296 ± 23.3	90 ± 9.33	5.65 ± 0.919	70.1 ± 19.5	144 ± 10.8	0.065 ± 0.001	0.085 ± 0.002	1.425 ± 0.13	0.07 ± 0.001

Table 3. Specifications of the LPD applied in dry mass.

Dry Matter (%)	pH (H_2O)	EC (mS/cm)	Total N (mg/kg)	P (mg/kg)	K (mg/kg)	Ca (mg/kg)	Mg (mg/kg)	S (mg/kg)
6.04 ± 0.127	8.35 ± 0.353	>4000 ± 0	57,800 ± 1265	12,912 ± 562	42,988 ± 1214	39,996 ± 2586	4268 ± 272	3228 ± 342
Fe (mg/kg)	**Zn (mg/kg)**	**Cu (mg/kg)**	**B (mg/kg)**	**Mn (mg/kg)**	**Pb (mg/kg)**	**Cd (mg/kg)**	**Cr (mg/kg)**	**As (mg/kg)**
267 ± 96	251 ± 68	6.1 ± 0.52	76.5 ± 12.5	189 ± 15.7	0.9 ± 0.02	0.09 ± 0.001	1.01 ± 0.02	3.79 ± 0.12

Furthermore, the LPD was characterized by an approximately twice greater content of total N compared to the SPD, even when taking into account errors according to the standard deviation. The total contents of other macronutrients were similar for both applied raw materials. Of the macronutrients, the greatest content was found for calcium in the applied SPD. The contents of macroelements and microelements in the SPD and the LPD coincide with the approaches of Makádi et al. [16], Kolář et al. [26] and Dubský et al. [29]. Analyses of the SPD and LPD confirmed the findings of Makadi et al. [28], in which these raw materials possess features suitable for plant nutrition. Of the micronutrients, the greatest content was found for iron and zinc. The lowest content in both materials analyzed found was for copper. Both components, SPD and the LPD, were characterized by a low content of hazardous substances. Therefore, neither the SPD nor the LPD posed a risk of soil contamination and transport of these substances to the plant parts of cannabis.

Tables 4 and 5 show the individual nutritional variants of the experiment, recalculated for the application of kg of a nutrient per hectare.

Table 4. Specifications of applied NPK in relation to the application of nutrients per hectare of soil.

Total N	P	K
(kg/ha)	(kg/ha)	(kg/ha)
150.0	20.0	150.0

Table 5. Specifications of the applied SPD, SPD + LPD and LPD in relation to the application of nutrients per hectare of soil.

	SPD	SPD + LPD	LPD
		(kg/ha)	
Total N	150.50	150.70	150.8
P	18.90	26.30	33.7
K	177.80	145.00	112.2
Ca	243.90	174.20	104.4
Mg	26.40	18.80	11.1
S	16.90	12.70	8.4
Fe	1.80	1.20	0.7
B	0.40	0.30	0.2
Mn	0.90	0.70	0.5

Table 6 presents the analysis of soils before sowing cannabis seeds according to nutritional variants, two weeks after fertilizer application.

Table 6. Soils before sowing.

Variant	Nitrate N (mg/kg)	Ammonia N (mg/kg)	Carbon (mg/kg)	Total N (mg/kg)	P (mg/kg)	K (mg/kg)	Ca (mg/kg)	Mg (mg/kg)	S (mg/kg)
NPK	21.25	29.96	287.79	57.50	534.56	502.78	8170.00	221.89	24.44
SPD	17.18	25.62	291.63	50.35	542.89	545.56	8233.33	234.44	25.56
SPD + LPD	9.31	17.83	303.57	39.62	548.89	543.33	8190.00	246.89	26.26
LPD	8.97	15.35	294.41	33.24	530.50	523.63	8443.75	234.75	25.23

The average dry mass yield of cannabis plants is shown in Figure 1. There were no significant differences between variants caused by the high non-uniformity of harvested plants in the field experiment. Similar results were reported by Tsachidou et al., who claim that applications of anaerobic digestion residues as a nitrogen source have shown the ability to maintain forage yields at a similar level as when using mineral NPK fertilizer. At the same time, the environmental risk associated with nitrogen leaching is reduced in this practice [23]. In both years, the average biomass yield was greatest in the variant fertilized by a divided dose of SPD and LPD (8.68 tons/ha), while the lowest dry mass yield was found in the variant where NPK was applied (7.45 tons/ha). The yield differences between the variants were statistically insignificant. The greatest yield in the variant with pre-sown-applied SPD and with fertilization with LPD was probably caused by the sufficient development of the root system in soil fertilized with a lower dose of SPD and regular fertilization with LPD during vegetation, which coincides with the findings of Alaru et al. [50] and Landi [48].

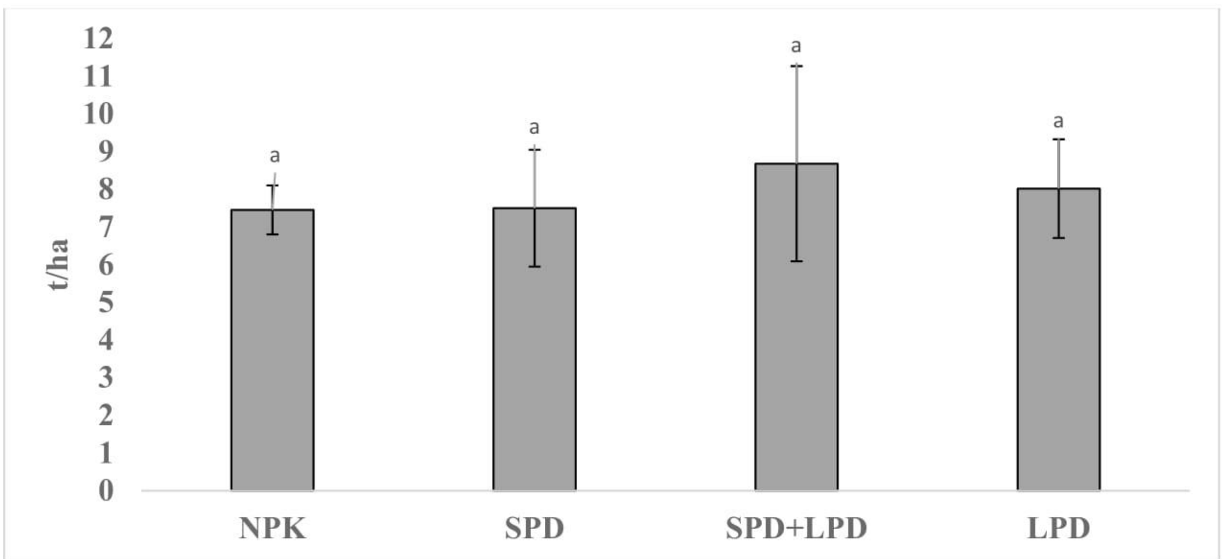

Figure 1. Average dry biomass yield of cannabis with individual variants.

The divided application of the LPD in the phase of intensive cannabis growth ensured a sufficient increase in biomass, which was reflected in the second greatest yield in the experiment. The results agree with the statements of Landi [48] and Barron et al. [47], who both state that the greatest nitrogen intake by cannabis plants is in the phase of their intensive growth. Nitrogen is also a key factor that influences the quantity and quality of cannabis production [57].

The contents of macronutrients, micronutrients and hazardous substances in dry matter in individual parts of cannabis plants are shown in Tables 7 and 8. The differences between the individual variants were not statistically significant for any of the analyzed plant parts. Overall, the greatest N content was found in cannabis leaves and decreased in the order of stem and root.

In the SPD + LPD variant, the greatest content of N (3.5%) was found in the leaves and stems (2.15%) of cannabis. The value found is slightly lower than that indicated by Iványi and Izsáki [53]. In contrast, the lowest nitrogen concentration in the leaves was found after the application of a divided dose of the LPD (3.2%) in the stems (1.95%) and roots (0.57%) of the NPK variant. The nitrogen concentration in the stems of all variants was lower than indicated by Hakala et al. [58], except for in the roots, in which it was relatively similar (0.61%).

Similar to N, other macronutrients were found in higher amounts in the leaves and in significantly lower amounts in the stems and roots, where they did not differ statistically. The greatest content in the leaves was found for calcium in the variant fertilized with NPK, which could have been caused by the release of a significant amount of calcium after a single application of water-soluble fertilizers. Our assumptions are also confirmed by the fact that the lowest calcium content was measured in cannabis leaves in the variant fertilized SPD, where, on the contrary, the share of available nutrients was clearly the lowest. In the case of other evaluated macronutrients, their content in leaves or in other parts of the plant did not significantly statistically differ between individual variants. Phosphorus and potassium contents in cannabis leaves were, on average, lower than 0.5–0.6% P and 2.7–3% K, which Ivanyi [49] had stated regarding young leaves.

This was probably due to the fact that, in our case, the leaves of the whole plant were analyzed, including old and dried leaves, which both contain significantly fewer nutrients.

When evaluating the content of micronutrients, the trend of their accumulation in individual parts of plants was far from unambiguous, as in the case of macronutrients (Table 8). Again, most of the elements accumulated in the highest concentrations in the leaves; only iron was found in the greatest concentrations in the roots, thus confirming its

limited mobility [59]. Despite its high accumulation in the roots, its content in the leaves was also the greatest of all monitored microelements. Lower contents were determined for boron and manganese and the lowest were found for zinc and copper. Overall, the contents of micronutrients in cannabis plants corresponded to the values reported by Ivanyi [49]. Differences in the contents of microelements in individual parts of plants were not statistically affected by the fertilizer used. The contents of molybdenum and risk elements were low in all plant parts, specifically below the detection limit of the analytical technique used.

Table 7. Macronutrient contents in individual parts of cannabis in dry biomass.

Variant	Root	Stem	Leaf
		Calcium (%)	
NPK	0.59 [a]	0.89 [a]	3.40 [a]
SPD	0.60 [a]	0.80 [a]	2.07 [a]
SPD + LPD	0.61 [a]	0.47 [a]	2.81 [a]
LPD	0.57 [a]	0.68 [a]	2.85 [a]
Variant		**Magnesium (%)**	
NPK	0.079 [a]	0.100 [a]	0.32 [a]
SPD	0.076 [a]	0.089 [a]	0.30 [a]
SPD + LPD	0.130 [b]	0.060 [a]	0.23 [a]
LPD	0.083 [a]	0.093 [a]	0.24 [a]
Variant		**Sulfur (%)**	
NPK	0.047 [a]	0.036 [a]	0.14 [a]
SPD	0.034 [a]	0.034 [a]	0.12 [a]
SPD + LPD	0.055 [a]	0.030 [a]	0.10 [a]
LPD	0.049 [a]	0.032 [a]	0.12 [a]
Variant	**Root**	**Stem**	**Leaf**
		Nitrogen (%)	
NPK	0.57 [a]	1.95 [a]	3.36 [a]
SPD	0.73 [a]	1.98 [a]	3.30 [a]
SPD + LPD	0.73 [a]	2.15 [a]	3.53 [a]
LPD	0.75 [a]	2.01 [a]	3.22 [a]
Variant		**Phosphorus (%)**	
NPK	0.11 [a]	0.11 [a]	0.24 [a]
SPD	0.096 [a]	0.12 [a]	0.25 [a]
SPD + LPD	0.14 [a]	0.089 [a]	0.21 [a]
LPD	0.11 [a]	0.11 [a]	0.21 [a]
Variant		**Potassium (%)**	
NPK	1.31 [a]	1.27 [a]	1.98 [a]
SPD	1.10 [a]	1.18 [a]	1.95 [a]
SPD + LPD	1.20 [a]	1.37 [a]	1.75 [a]
LPD	1.28 [a]	1.21 [a]	1.92 [a]

Different superscript letters indicate statistical significance.

Figure 2 shows the average macronutrient total uptake of cannabis plants in kg/ha. Consumption by plants was calculated on the basis of the yield of individual parts of dry biomass on the plot and the content of individual nutrients in these parts of cannabis plants. The calculated samples confirmed that cannabis plants have a high uptake capacity and that nutrient samples exceeded their application rates in all cases. For this ability, cannabis is also commonly used in soil phytoremediation [60,61]. Overall, the greatest average consumption, at a level of about 300 kg/ha, was determined to be for nitrogen, only slightly lower for potassium, and at a level of 200 kg/ha for calcium. LPD and SPD fertilization led to higher N and K uptake on all variants in comparison with the variant

fertilized with NPK. The greatest nitrogen uptake was found in plants in the variant with a divided dose of SPD and LPD. The high nitrogen uptake was probably caused by a sufficient supply of accessible nitrogen during the growing season. This effect can only be expected with the direct application of digestates immediately incorporated into the soil to minimize losses of ammonia N present in high portions in both components of the digestate [19]. Nitrogen uptake in the mentioned variants exceeds the values presented by Ivanyi and Izsaki [52], who both reported an average sampling over a four-year trial of 213 kg N/ha. On the contrary, the plants in the variant with the application of NPK achieved the lowest nitrogen uptake. In addition, the results of Sogn et al. showed that digestates are a promising alternative to NPK mineral fertilizers, although the levels of K and P in particular may fluctuate in these raw materials. When evaluating wheat yields using anaerobic digestion residues, comparable yields were achieved when using NPK fertilizer as a control [24].The greatest potassium uptake was again determined in the variant with the divided dose of SPD and LPD (345 kg/ha) and the lowest was in the variant with NPK (179 kg/ha). The observed values of potassium uptake by cannabis plants in the experiment are higher than indicated in Barron, Coutinho, English, Gergely, Lidouren and Haugaard-Nielsen [47]. The authors stated the range of potassium intake to be in the range of 75–300 kg/ha.

Table 8. Contents of micronutrients (mg/kg) in individual parts of cannabis in dry mass.

Variant	**Root**	**Stem**	**Leaf**
		Iron (ppm)	
NPK	182 [a]	28.84 [a]	94.61 [a]
SPD	280 [a]	50.72 [a]	77.38 [a]
SPD + LPD	236 [a]	33.90 [a]	67.52 [a]
LPD	125 [a]	49.07 [a]	73.01 [a]
Variant		**Manganese (ppm)**	
NPK	21.29 [a]	26.53 [a]	54.14 [a]
SPD	20.96 [a]	23.11 [a]	36.27 [a]
SPD + LPD	22.76 [a]	14.31 [a]	26.20 [a]
LPD	17.51 [a]	25.30 [a]	37.93 [a]
Variant		**Boron (ppm)**	
NPK	11.39 [a]	12.50 [a]	45.73 [a]
SPD	12.79 [a]	15.65 [a]	41.82 [a]
SPD + LPD	24.50 [a]	8.36 [a]	35.77 [a]
LPD	14.54 [a]	12.27 [a]	35.04 [a]
Variant	**Root**	**Stem**	**Leaf**
		Zinc (ppm)	
NPK	7.37 [a]	6.49 [a]	19.24 [a]
SPD	7.18 [a]	6.07 [a]	13.62 [a]
SPD + LPD	11.45 [a]	5.03 [a]	8.91 [a]
LPD	6.54 [a]	6.34 [a]	15.71 [a]
Variant		**Copper (ppm)**	
NPK	2.68 [a]	2.31 [a]	4.72 [a]
SPD	2.67 [a]	2.68 [a]	4.62 [a]
SPD + LPD	3.25 [b]	2.28 [a]	3.80 [a]
LPD	2.64 [a]	2.64 [a]	4.45 [a]

Different superscript letters indicate statistical significance.

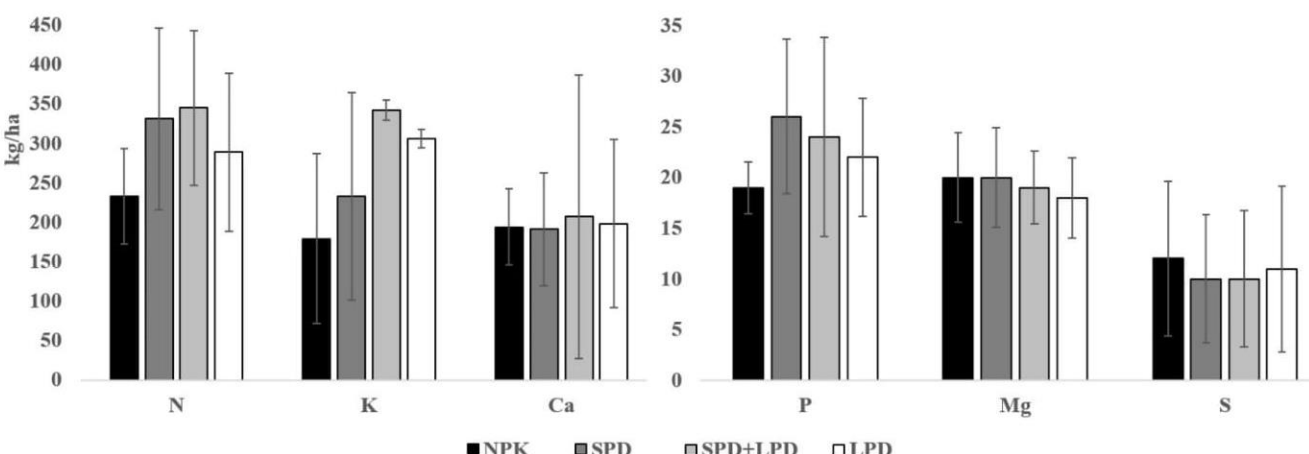

Figure 2. The average total uptake of cannabis macronutrients of individual experimental variants.

Calcium uptake by cannabis was high, not significantly affected by the fertilizer used and averaged at 191–207 kg/ha. These values correspond to the calcium samples given by Landi [48], at 150–227 kg Ca/ha.

The average phosphorus uptake ranged from 19–26 kg/ha and was higher for the variants fertilized with SPD and LPD than for the variant fertilized with NPK. These values match those of Landi [48], who reported P withdrawals by cannabis plants from 12 to 35 kg/ha. In addition, they are in line with the values stated by Ivanyi and Izsaki [52].

The average intakes of magnesium and sulfur were similar in all variants. The consumption of magnesium ranged from 18–20 kg/ha and that of sulfur ranged from 10–12 kg/ha.

Micronutrient uptake by plants was significantly reduced (Figure 3). The greatest consumption was found for iron; the consumption was lower for manganese and boron and lowest for zinc and copper. In the case of iron and boron, fertilization by SPD and LPD had a favorable effect. Especially in the variant with a divided dose of SPD and the LPD, cannabis plants took up most of these microelements. The lowest consumption by plants was for manganese and boron in the variant fertilized with LPD and NPK. For other nutrients, no significant differences were found between the individual variants. A higher uptake of iron and boron by plants on variants with SPD and LPD was probably due to their higher content in these materials and easier accessibility (Tables 2 and 3).

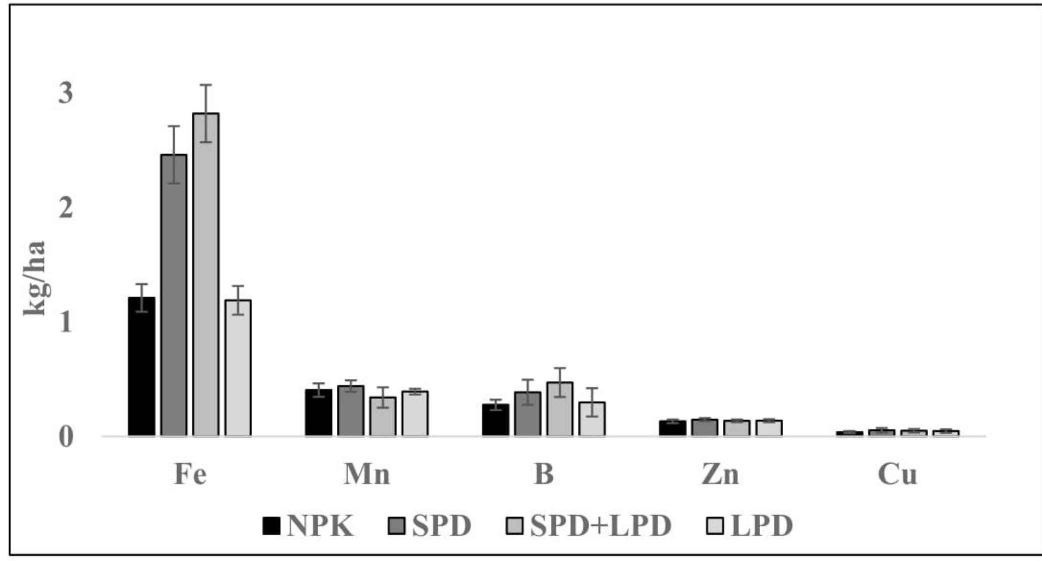

Figure 3. The average intakes of cannabis micronutrients of individual experimental variants (kg/ha).

After each harvest, the basic agrochemical features were determined (Table 9). The obtained values confirmed that the experiment was based on fertile soil; therefore, the application of NPK, SPD and LPD did not have a statistically significant effect on the evaluated parameters. The pH value determined in the aqueous extract was similar for all variants and corresponded to a pH of 8.7. The content of dissolved salts in the soil was the same in all variants. Slight differences were found in the content of individual forms of mineral nitrogen. The nitrogen supplied by the LPD oxidized more rapidly, and the ammonium N content was the lowest for these variants. The improvement of the soil structure led to the greatest overall consumption of N on the SPD + LPD variant, which was reflected in the lowest content of nitrate N after the cannabis harvest on this variant.

Table 9. Dry matter content, pH value, soluble salt value and mineral nitrogen content in soil samples after plant harvest.

Variant	Dry Matter (%)	pH (H_2O)	EC (mS/cm)	N (mg/kg)	
				NO_3^-	NH_4^+
NPK	90.8 ± 0.07	8.71 ± 0.00	0.114 ± 0.008	21.55 ± 0.49	18.35 ± 6.33
SPD	90.3 ± 0.14	8.74 ± 0.04	0.105 ± 0.001	21.12 ± 5.53	21.13 ± 6.32
SPD + LPD	89.7 ± 0.07	8.76 ± 0.08	0.110 ± 0.013	18.23 ± 2.60	15.54 ± 3.34
LPD	90.3 ± 0.02	8.73 ± 0.02	0.108 ± 0.003	23.09 ± 2.06	11.84 ± 5.04

The contents of accessible nutrients and risk elements in the soil after the cannabis harvest confirmed that the application of LPD and SPD did not significantly affect their accumulation in the topsoil layer (Table 10). The high pH value was confirmed by the high content of accessible calcium, which did not differ between the individual variants of the experiment. The contents of other cations were also high. The K content was not affected by the applied fertilizer, and the Mg content was slightly increased on the variants fertilized with the LPD and SPD, which could be caused by its supply in organic fertilizers. This trend was reflected on a smaller scale in the case of the evaluation of the available sulfur content in the soil.

Table 10. Macronutrient content in soil samples after harvest of plants in dry matter.

Variant	P (mg/kg)	K (mg/kg)	Ca (mg/kg)	Mg (mg/kg)	S (mg/kg)
NPK	614 ± 113	460 ± 59	7328 ± 1190	240 ± 27.6	29.0 ± 7.1
SPD	628 ± 122	468 ± 108	7637 ± 843	252 ± 26.2	30.5 ± 7.8
SPD + LPD	629 ± 114	479 ± 90	7367 ± 1163	253 ± 10.6	32.5 ± 9.2
LPD	613 ± 117	476 ± 66	7374 ± 1512	259 ± 35.4	34.5 ± 13.4

Similar to the contents of the macroelements, the accessible content of microelements depended mainly on their amount in the soil and was only slightly affected by the applied SPD and LPD (Table 11). Only in the case of Cu can we assume that its high affinity for organic matter meant a slight decrease in its accessible forms in the soil. In the case of the micronutrients, their content depended on the habitat when assessing the accessible proportion of risk elements, and the applied fertilization did not lead to any significant changes in their content.

Table 11. Microelements content in soil samples after harvest of plants in dry matter.

Sample	Fe (mg/kg)	Zn (mg/kg)	Cu (mg/kg)	B (mg/kg)	Mn (mg/kg)	Mo (mg/kg)
NPK	62.6 ± 19.1	16.4 ± 3.1	9.87 ± 1.59	21.9 ± 5.45	291 ± 78	<0.005
SPD	63.9 ± 16.1	16.9 ± 3.2	8.68 ± 0.40	20.6 ± 3.33	302 ± 83	<0.005
SPD + LPD	64.5 ± 15.1	17.2 ± 2.4	8.82 ± 0.31	21.5 ± 4.60	299 ± 74	<0.005
LPD	64.0 ± 21.9	17.0 ± 3.6	8.59 ± 0.27	21.4 ± 4.81	290 ± 77	<0.005

In conclusion, the data obtained from this experiment suggest that the by-products from anaerobic digestion can be used as an alternative to mineral NPK fertilizers. Comparable yield parameters were achieved by cannabis plants and were supported by a greater degree of nutrient accumulation in individual plant tissues. However, these materials are variable both in the composition of specific nutrients and in their accessibility to plants. This variability is due to differences of the input raw materials into the anaerobic digestion process, and this factor must be taken into account.

Author Contributions: Conceptualization, J.V., L.K. and P.T.; methodology, J.V., L.K. and P.T.; software, J.V., M.M.; validation J.V. and P.T.; formal analysis, J.V. and L.K.; investigation, J.V., L.K., M.M. and P.T.; resources, P.T.; data curation, J.V., M.M. and P.T.; writing—original draft preparation, J.V.; writing—review and editing, J.V.; visualization, J.V.; supervision, P.T.; project administration, P.T.; funding acquisition, P.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by European Regional Development Fund project NUTRISK, grant number [CZ.02.1.01/0.0/0.0/16_019/0000845]. The financial support is greatly appreciated.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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4.3. Amino Acid Supplementation as a Biostimulant in Medical Cannabis (*Cannabis sativa* L.) Plant Nutrition

Autoři: Malík M, Velechovský J, Praus L, Janatová A, Kahánková Z, Klouček P, Tlustoš, P

Rok publikace: 2022

Časopis: Frontiers in Plant Science **13**:868350

ORIGINAL RESEARCH
published: 31 March 2022
doi: 10.3389/fpls.2022.868350

Amino Acid Supplementation as a Biostimulant in Medical Cannabis (*Cannabis sativa* L.) Plant Nutrition

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There is growing evidence to support the involvement of nutrients and biostimulants in plant secondary metabolism. Therefore, this study evaluated the potential of amino acid-based supplements that can influence different hydroponic nutrient cycles (systems) to enhance the cannabinoid and terpene profiles of medical cannabis plants. The results demonstrate that amino acid biostimulation significantly affected ion levels in different plant tissues (the "ionome"), increasing nitrogen and sulfur content but reducing calcium and iron content in both nutrient cycles. A significantly higher accumulation of nitrogen and sulfur was observed during the recirculation cycle, but the calcium level was lower in the whole plant. Medical cannabis plants in the drain-to-waste cycle matured 4 weeks earlier, but at the expense of a 196% lower maximum tetrahydrocannabinolic acid yield from flowers and a significantly lower concentration of monoterpene compounds than in the recirculation cycle. The amino acid treatments reduced the cannabinolic acid content in flowers by 44% compared to control in both nutritional cycles and increased the monoterpene content (limonene) up to 81% in the recirculation cycle and up to 123% in the drain-to-waste cycle; β-myrcene content was increased up to 139% in the recirculation cycle and up to 167% in the drain-to-waste cycle. Our results suggest that amino acid biostimulant supplements may help standardize the content of secondary metabolites in medical cannabis. Further experiments are needed to identify the optimal nutrient dosage and method of administration for various cannabis chemotypes grown in different media.

Keywords: medical cannabis, phytocannabinoids, amino acids, *Cannabis sativa* L., terpenoids, biostimulant, hydroponics

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Plant Metabolism and Chemodiversity, a section of the journal Frontiers in Plant Science

Received: *02 February 2022*
Accepted: *08 March 2022*
Published: *31 March 2022*

Citation:
Malík M, Velechovský J, Praus L, Janatová A, Kahánková Z, Klouček P and Tlustoš P (2022) Amino Acid Supplementation as a Biostimulant in Medical Cannabis (Cannabis sativa L.) Plant Nutrition. Front. Plant Sci. 13:868350. doi: 10.3389/fpls.2022.868350

INTRODUCTION

Medical cannabis research has developed dramatically in recent years (Grotenhermen and Muller-Vahl, 2012). The use of these plants in healthcare and pharmaceutics places rigorous demands on the growing environment for optimal production of the desired active compounds (Potter, 2014). For these reasons, and because it is now legal, many growers have opted to use indoor facilities as a more efficient way to grow medical cannabis, a method used mainly by illegal growers until recently (Drugs and Crime, 2009). Consequently, indoor cultivation has become

more sophisticated with automated lighting, ventilation, and irrigation systems being commonly in use. It can be implemented in several ways, but always comes down to two basic methods—cultivation in soil substrates or hydroponically. The nutrients are dissolved in the irrigation water, or already fertilized soil substrates can be used. Hydroponics is currently one of the fastest developing methods in the horticultural industry (Vanhove et al., 2011) and cannabis growers have already started to use it extensively. In hydroponic cultivation the nutrients are supplied in the form of an aqueous solution directly in contact with the plant's root system. Thanks to the possibility of year-round growth in a controlled environment, this method has the potential to produce high yields of homogeneous plant material of excellent quality (Bouchard and Dion, 2009).

At present, basic research information about regulating the biosynthesis of secondary metabolites of medical cannabis is lacking because of legal restrictions in most countries (Aguilar et al., 2018). With respect to the internal and external factors influencing the secondary metabolite content and spectrum of cannabinoids, the main determining internal factor is the genetics of *Cannabis sativa* L. subsp. *sativa* and subsp. *indica* (Janatová et al., 2018; Mcpartland, 2018). This directly impacts the chemotype, habitus, cannabinoid, and terpene profile of the cultivated cannabis plant (Aizpurua-Olaizola et al., 2016). However, the genetics and the plant phenotypes are strongly influenced by external factors, with growing conditions playing a crucial role in productivity and quality. The main external parameters include light (Danziger and Bernstein, 2021), irrigation (Caplan et al., 2019), carbon dioxide concentration (Chandra et al., 2011), and nutrition (Malík et al., 2021). Nutrients play a central role in many aspects of plant metabolism. There is a wealth of experimental evidence to support the effects of nutrients, especially nitrogen (Saloner and Bernstein, 2021), phosphorus (Shiponi and Bernstein, 2021a), and potassium (Yep et al., 2021), on secondary metabolites of medical cannabis plants. The cannabinoid and terpene profile of medical cannabis can be influenced by the concentration and ratio of these major nutrients (Caplan et al., 2017a; Bernstein et al., 2019). Although emphasis is placed on the availability of sufficient amounts of these major plant nutrients, the potential effects of micronutrients and plant biostimulants must also be considered (Bernstein et al., 2019).

Several studies have used protein hydrolysates and amino acids (AAs) as plant biostimulants. The mechanism of their action on plants is thought to involve modulating nitrogen absorption and assimilation by regulating the enzymes and structural proteins involved in these processes. AA biostimulants also affect nitrogen uptake by the roots through modulation of specific signaling pathways. By controlling the enzymes of the Krebs (citric acid) cycle, they contribute to crosstalk between carbon and nitrogen metabolites (Colla et al., 2014; Du Jardin, 2015). The beneficial effect of chelation by some AAs has also been reported. In this way, certain AAs can protect plants from heavy metals, but they also contribute to the mobility and acquisition of micronutrients by the roots. AAs can also reduce environmental stress by scavenging free oxygen radicals, thereby contributing to antioxidant activity (Calvo et al., 2014). The stem and leaves of cannabis, like other plants, contain various concentrations of incorporated AAs (Audu et al., 2014). Plants can absorb and incorporate nitrogen in the form of intact AAs (Persson and Nasholm, 2001; Sauheitl et al., 2009), and thus, solutions of protein hydrolysates and AAs can increase plant growth (El-Ghamry et al., 2009; Talukder et al., 2018) and the nitrogen content of above-ground biomass (Matsumoto et al., 1999). Supplementing plants with environmentally friendly AA biostimulants can reduce the use of inorganic fertilizers (Ugolini et al., 2015).

Several commercial products derived from protein hydrolysates of plant and animal origin have already been marketed. Various results have been reported for agricultural and horticultural crops, but their application has led to significant improvements in yield and quality parameters (Calvo et al., 2014). So far, however, there have been no publications about their effects on plant secondary metabolism. Therefore, in this study, we focused on the physiological and chemical responses of medical cannabis plants to supplementation with a spectrum of AAs in a nutrient solution and subsequently compare the outcomes with two different hydroponic nutritional cycles. We proposed the following hypotheses: (1) the nutritional AA supplement causes a change in the amount of above-ground biomass and affects the inflorescence yield of medical cannabis plants; (2) the nutritional AA supplement causes a change in the medical cannabis plants cannabinoid and terpene profile; (3) the induced changes will be correlated with the contents of macro- and micro-elements in plant organs (leaves, stems, flowers); and (4) the induced changes will differ in each nutrition systems (recirculation vs. drain-to-waste). To test the hypotheses, we monitored the effects of AA supplementation in the nutrient solution of both systems on the amount of above-ground biomass and growth of leaves, stems, and flowers, the concentration of cannabinoids and terpenes, and the tissue ionome of the medical cannabis plant.

MATERIALS AND METHODS

Basic Parameters of the Growing Space

Cannabis plants were grown on tables in a room with controlled conditions. Each 2 m^2 (1×2 m) table supported a separate experiment with an independent 100 l tank for the nutrient solution. The container was made of inert plastic certified for food industry use. Each table held a maximum of 55 black conical square pots made of polypropylene (PP), each with a volume of 3.45 l with dimensions: TOP - 15 cm x 15 cm, BASE - 11.5 cm x 11.5 cm, HEIGHT –20 cm. Irrigation was provided by capillaries, which were placed in each pot to reach every plant separately using a needle applicator. The pump's timer was set for nine irrigation cycles, each lasting 60 s. During one cycle, 94 ml of nutrient solution was supplied to each plant (846 ml per plant per day). The growing tables allowed us to choose the irrigation method--either recirculation of the nutrient solution or drain-to-waste system, where the spent solution went to a separate waste tank and was no longer mixed with the original solution. Microclimatic parameters were provided by an air ventilation unit that maintained and recorded the set parameters (relative

humidity, temperature, CO_2 level). Enrichment of the atmosphere of the growing space with CO_2 was made possible by a generator that burned methane. Six double-ended high-pressure sodium lamps provided a suitable spectrum of light at a power of 1,000 W. Based on the photosynthetic photon flux density (PPFD), the lamps provided 1,029 μmol/m^2 s at a power of 6,000 W. The light mode was also recorded every minute using a data logger.

Plants and Growing Conditions

The plants used in the experiments came from the mother plants of the medical cannabis genotype with the working name "McLove." Plants are classified as chemotype I - high Δ^9-tetrahydrocannabinolic acid/cannabidiolic acid (THCA/CBDA) ratio (>>1.0). Appropriate mother plants were kept in a separate growing room with controlled conditions. A total of 220 cuttings were made (110 cuttings per cycle) and cultivated for 21 days in a rock-wool cube (4×4 cm). Rooted clones were moved to a growing room, where they were placed in 3.45-liter pots filled with three liters of Euro Pebbles (expanded clay) growing medium. The light mode was set to 18 h of light and 6 h of darkness, temperature in the light phase was 25°C, the relative humidity was 60%, and the CO_2 concentration was 550 ppm (1,065 mg/m^3). The dark phase temperature was reduced to 22°C with the same humidity. The vegetative phase lasted 7 days, after which the cultivation regime was adjusted to the generative phase. The light period was set at 12 h light and 12 h dark, the temperature and CO_2 concentration was left the same as the vegetative phase, and the relative humidity was reduced to 40%. From the 10th week, plants were irrigated with demineralized water (DMW). Plant density was 27.5 plants per m^2 (55 plants/table/treatment).

Treatments

Compared to the controls (CN), the experimental plants (ET) were exposed to one enhanced nutrition treatment with two separate nutritional cycles. The first cycle (1C) was performed with recirculated nutrient solution, and the second cycle (2C) used the drain-to-waste system. The enhanced treatments were set up for both nutritional cycles and received the AA biostimulant (**Table 1**) added from the 2nd week for the last 24 h at a volume 2 ml/l before changing the nutrient solution. The new nutrient solution was prepared from reverse osmosis water every 7 days from the first day of the experiment. The pH of the nutrient solution was adjusted to 5.9 (Velazquez et al., 2013). In the recirculation system the nutrient solution was adjusted to this value every day. The pH and electrical conductivity (EC) were recorded when mixing the new solution and on the last day before changing it. After preparing the fresh nutrient solution, a sample was taken from each treatment for analysis. The measured composition of the control treatment (CN) nutrient solution is shown in **Table 2**, and the composition of the enhanced treatment (ET) nutrient solution with the addition of AAs is shown in **Table 3**.

Sampling Plant Material

Three plants were harvested from each treatment, one plant randomly selected from each highlighted sector 1–3 (**Figure 1**), every 7 days during the entire vegetation cycle. Subsequently, a random plant from the edge (outside the sectors) was transferred to an empty space in each sector. Plants were uprooted, weighed whole fresh, and divided into leaves, stems, and flowers, which were weighed fresh separately for all plants. The materials were then dried at 25°C to constant moisture (8–10%) and re-weighed. To determine the dry matter, a reference amount of each part of the plant was dried at 105°C to constant weight. The plant parts were homogenized just before analysis. The flowers (including the leaves until the 4th week) were frozen in liquid nitrogen and then ground in a mortar and pestle. The dried leaves (from the 5th week) and stems were ground in a grinder.

TABLE 1 | Amino acid content in biostimulant.

AA	mg/L
Lys	0.071
His	0.00483
Arg	0.04615
Asp	0.0327
Thr	0.00954
Ser	0.0175
Glu	0.062
Pro	0.0828
Gly	0.1449
Ala	0.05569
Cys	0.036
Val	0.01401
Met	0.0039
Ile	0.00966
Leu	0.01836
Tyr	0.0016
Phe	0.01305

Dry Decomposition and Elemental Analysis

To determine the content of macroelements (except nitrogen), microelements, and trace elements in the plant, the leaves, stems, and flowers were analyzed separately. Weighed and homogenized plant biomass in a beaker was covered with a watch glass, placed on a hotplate 160°C, and the temperature was raised to 350°C over 4 h during which the samples gradually decomposed. The samples were next transferred to a muffle furnace, where they remained at 450–500°C for 12 h (Miholová et al., 1993). One ml of 65% HNO_3 was then added to the cooled beakers, which were placed on a 120°C hot plate for 60 min. The samples were then annealed for 90 min in an oven at 500°C and suspended in 1.5% HNO_3 with stirring in an ultrasonic bath. Elemental analysis of the samples was performed by flame atomic absorption spectrometry (FAAS) on a Varian 280FS with inductively coupled plasma optical emission spectrometry (ICP-OES) by Varian Vista-PRO instrument (Varian, Mulgrave, Australia; Hoenig, 2003).

Determination of Nitrogen in Plant Material by the Kjeldahl Method

For nitrogen determination, 0.5 g of plant material was weighed and put into a distillation tube. The samples were then mineralized by boiling with 95% H_2SO_4. After alkalization with sodium

TABLE 2 | Composition of control treatment (CN) nutrient solution (mg/L).

Elements	Weeks				
	1	2	3, 5	4, 6–9	10–13
N	100.85 ± 1.64	116.00 ± 1.85	130.00 ± 1.75	150.00 ± 1.92	DMW[a]
P	32.01 ± 0.75	39.40 ± 0.82	43.88 ± 0.59	51.73 ± 0.79	DMW[a]
K	124.93 ± 1.85	151.00 ± 1.38	173.11 ± 1.92	193.25 ± 1.58	DMW[a]
Ca	98.53 ± 1.32	119.00 ± 1.35	132.38 ± 1.42	146.00 ± 1.28	DMW[a]
Mg	25.17 ± 0.38	30.50 ± 0.42	34.94 ± 0.48	39.13 ± 0.45	DMW[a]
S	21.75 ± 0.25	26.72 ± 0.29	31.34 ± 0.34	34.53 ± 0.38	DMW[a]
Fe	0.91 ± 0.09	1.11 ± 0.09	1.21 ± 0.11	1.44 ± 0.08	DMW[a]
Mn	0.66 ± 0.07	0.74 ± 0.05	0.83 ± 0.08	0.99 ± 0.07	DMW[a]
Zn	0.21 ± 0.03	0.27 ± 0.03	0.28 ± 0.04	0.33 ± 0.03	DMW[a]
Cu	0.07 ± 0.01	0.09 ± 0.01	0.11 ± 0.01	0.13 ± 0.02	DMW[a]
B	0.14 ± 0.02	0.19 ± 0.01	0.22 ± 0.02	0.25 ± 0.02	DMW[a]
Mo	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	DMW[a]
EC	0.97 ± 0.01	1.19 ± 0.01	1.46 ± 0.01	1.74 ± 0.01	DMW[a]

[a]*demineralized water.*

TABLE 3 | Composition of enhanced treatment (ET) nutrient solution with the addition of AAs (mg/L).

Elements	Weeks				
	1	2	3, 5	4, 6–9	10–13
N	100.00 ± 1.59	300.00 ± 2.94	331.00 ± 3.01	353.00 ± 3.52	DMW[a]
P	32.20 ± 0.49	40.17 ± 0.52	44.18 ± 0.92	52.09 ± 0.57	DMW[a]
K	125.00 ± 1.56	151.51 ± 1.27	174.17 ± 1.38	194.26 ± 1.95	DMW[a]
Ca	98.50 ± 1.32	120.58 ± 1.24	133.15 ± 1.49	146.83 ± 1.56	DMW[a]
Mg	25.30 ± 0.34	31.00 ± 0.38	34.06 ± 0.43	40.03 ± 0.37	DMW[a]
S	21.49 ± 0.31	51.80 ± 0.52	56.27 ± 0.61	61.84 ± 0.85	DMW[a]
Fe	0.93 ± 0.08	1.14 ± 0.07	1.19 ± 0.09	1.47 ± 0.07	DMW[a]
Mn	0.64 ± 0.06	0.75 ± 0.03	0.81 ± 0.04	1.01 ± 0.07	DMW[a]
Zn	0.22 ± 0.04	0.27 ± 0.01	0.29 ± 0.02	0.36 ± 0.03	DMW[a]
Cu	0.07 ± 0.01	0.09 ± 0.02	0.11 ± 0.02	0.13 ± 0.01	DMW[a]
B	0.15 ± 0.01	0.20 ± 0.02	0.22 ± 0.02	0.26 ± 0.01	DMW[a]
Mo	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	DMW[a]
EC	0.97 ± 0.01	1.38 ± 0.01	1.71 ± 0.01	2.14 ± 0.01	DMW[a]

[a]*demineralized water.*

hydroxide, the free ammonia was distilled with water steam into H_3BO_3. Its content was determined by titration with HCl (0.5 mol/l) and then measured by Gerhardt Vapodest 30s (Königswinter, Germany; Baker and Thompson, 1992; Velechovský et al., 2021).

Phytocannabinoid Extraction, Identification, and Quantification

Phytocannabinoids from ground homogenized flowers (including the leaves until the 4th week) were extracted by the optimized method of dynamic maceration (Brighenti et al., 2017). Samples (0.30 g) from each experiment group were mixed with 10 ml of 96% ethanol and macerated for 60 min with constant stirring at 300 rpm. Mixtures were then filtered under vacuum using a Morton filter device (porosity S4/P16), and the filtrate was collected. The flowers were removed from the filter and mixed with another 10 ml of solvent. This step was repeated twice, and the filtrates were pooled. Aliquots of 0.5 ml of each sample were diluted to 10 ml with 96% ethanol and filtered once more through nylon syringe filters (0.22 μm) into vials. Samples of the extracts were injected into high-performance liquid chromatography system equipped with diode array detection (HPLC-DAD; Agilent 1,260, Agilent Technologies Inc., United States) and a Luna® C18 column (2) 250 × 3 mm, particle size 3 μm (Phenomenex, United States). The isocratic mobile phase consisted of acetonitrile/H_2O (31:9, v/v) with 0.1% HCOOH (v/v) and 0.1 mol/l NH_4COOH (without pH adjustment), flow rate was 0.55 ml/min, temperature 37°C, sample injection volume 8 μl, and UV detection at 275 nm (Križman, 2020). The instrument was externally calibrated using cannabinolic acid (CBNA) in the range of 0.3–10 mg/l and THCA, 0.3–100 mg/l, (Sigma-Aldrich, Czech Republic) as standards. Data were analyzed using OpenLAB CDS software, ChemStation Edition, Rev. C.01.5.

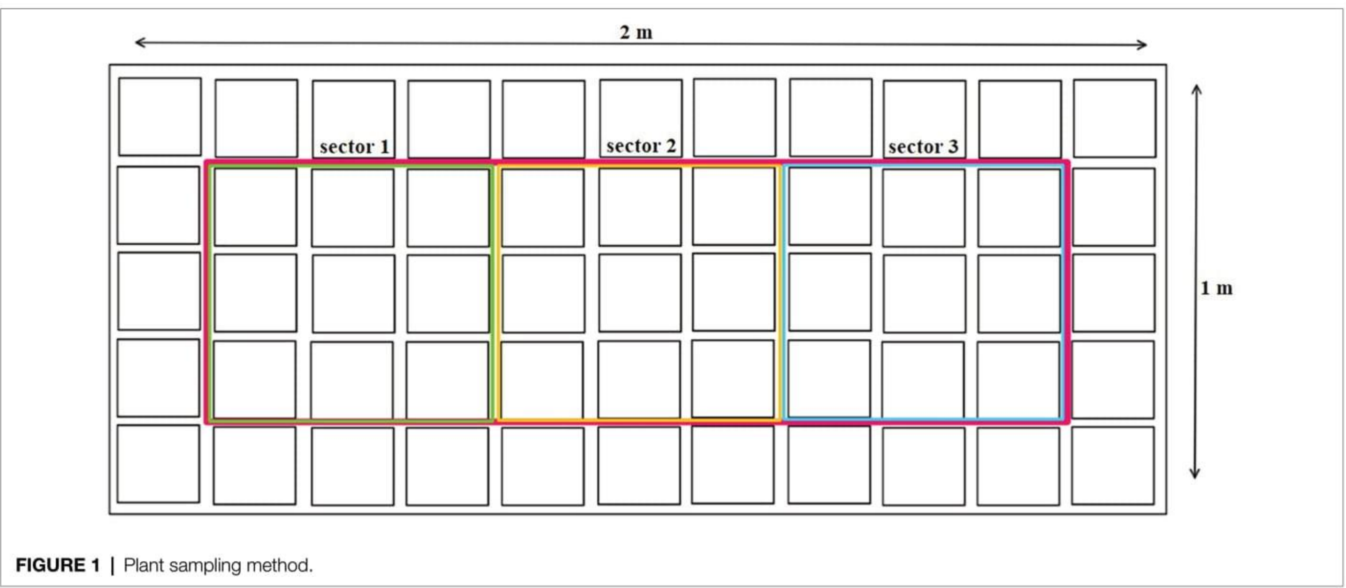

FIGURE 1 | Plant sampling method.

Terpene Extraction, Identification, and Quantification

Terpenes from ground and homogenized mature flowers (week 8–10, vegetation) were extracted with hexane. Plant samples (0.1 g) were mixed with 1 ml of hexane and pentadecane was added to a final concentration of 1 mg/ml as an internal standard. The samples were vortexed and placed in an ultrasonic bath for 30 min. Subsequently, the samples were centrifuged and filtered through polytetrafluoroethylene (PTFE) syringe filters (0.22 µm) into vials. Filtered samples (1.5 µl) were first injected into a gas chromatograph with a flame-ionization detector (GC-FID; Agilent Technologies 7890A, Palo Alto, CA). The GC-FID conditions were: column DB5 30 m × 0.25 mm × 0.25 µm film thickness, inlet temperature 230°C, detector temperature 300°C, and nitrogen flow rate of 1 ml/min. The initial temperature was 60°C, which was increased at the rate of 3.5°C/min until a temperature of 150°C was reached, and then at a rate of 30°C/min until a final temperature of 300°C was reached. Samples were also injected into a gas chromatograph connected to a mass spectrometer (GC–MS; Agilent Technologies 5975C, Palo Alto, CA). The GC–MS conditions were: column HP-5MS 30 m × 0.25 mm × 0.25 µm film thickness, inlet temperature 230°C, detector temperature 300°C, and helium flow rate of 1 ml/min. The initial temperature was 60°C, which was increased at the rate of 3.5°C/min until a temperature of 150°C was reached, and then at a rate of 30°C/min until a final temperature of 300°C was reached. Compounds detected by GC–MS were identified by comparing the mass spectrum and relative retention index with the published values of the National Institute of Standards and Technology (NIST) database, and the values for the standards, β-myrcene and limonene (Sigma-Aldrich, Czech Republic). The GC-FID data revealed the relative concentration of the identified substances, based on the peak area of the monitored substance relative to the total area of all detected substances.

Statistical Analyses

Data were subjected to ANOVA followed by Tukey's HSD test. The analysis was performed using IBM SPSS Statistics software (version 25, 2017, Chicago, Illinois, United States).

RESULTS

The AA nutritional supplement and the variable nutritional cycles (1C and 2C) induced changes in the tissue ionome of medical cannabis plants. The content of nitrogenous compounds was lowest in the stems and highest in the flowers (**Figure 2**). The concentrations of N in the leaves and flowers of control (CN) and enhanced treatment (ET) plants with AA supplement in the recirculation (1C) cycle began to differ significantly from the 5th week. The most significant differences in N concentrations between control and AA treatment were 34% for flowers at week 6 (CN, 44.26 mg/g; ET, 59.19 mg/g; **Figure 2A**). In contrast to 1C, the concentration of N in the stems and leaves of CN and ET began to differ significantly from week 2 to 4 in the drain-to-waste (2C) nutritional cycle; but, from week 5 to 13, fewer significant differences were observed with 2C than 1C. The most significant differences in N concentrations between nutritional treatments were 7% for flowers at week 7 (CN, 43.02 mg/g; ET, 45.85 mg/g; **Figure 2B**). The N concentration also differed between 1C and 2C of ETs with AA supplement, and the differences were evident beginning at week 2. The most significant differences in N concentrations in ETs between nutritional cycles were 31% (6% between CNs) for flowers at week 5 (1C, 61.63 mg/g; 2C, 47.18 mg/g; **Figure 2C**).

The calcium content was lowest in the stems and highest in the leaves, and showed a cumulative trend over time (**Figure 3**). The Ca concentration in the leaves of CN and ET in 1C began to differ significantly from the third week. The most significant differences in Ca concentration between nutritional treatments were 60% for leaves at week 11 (CN,

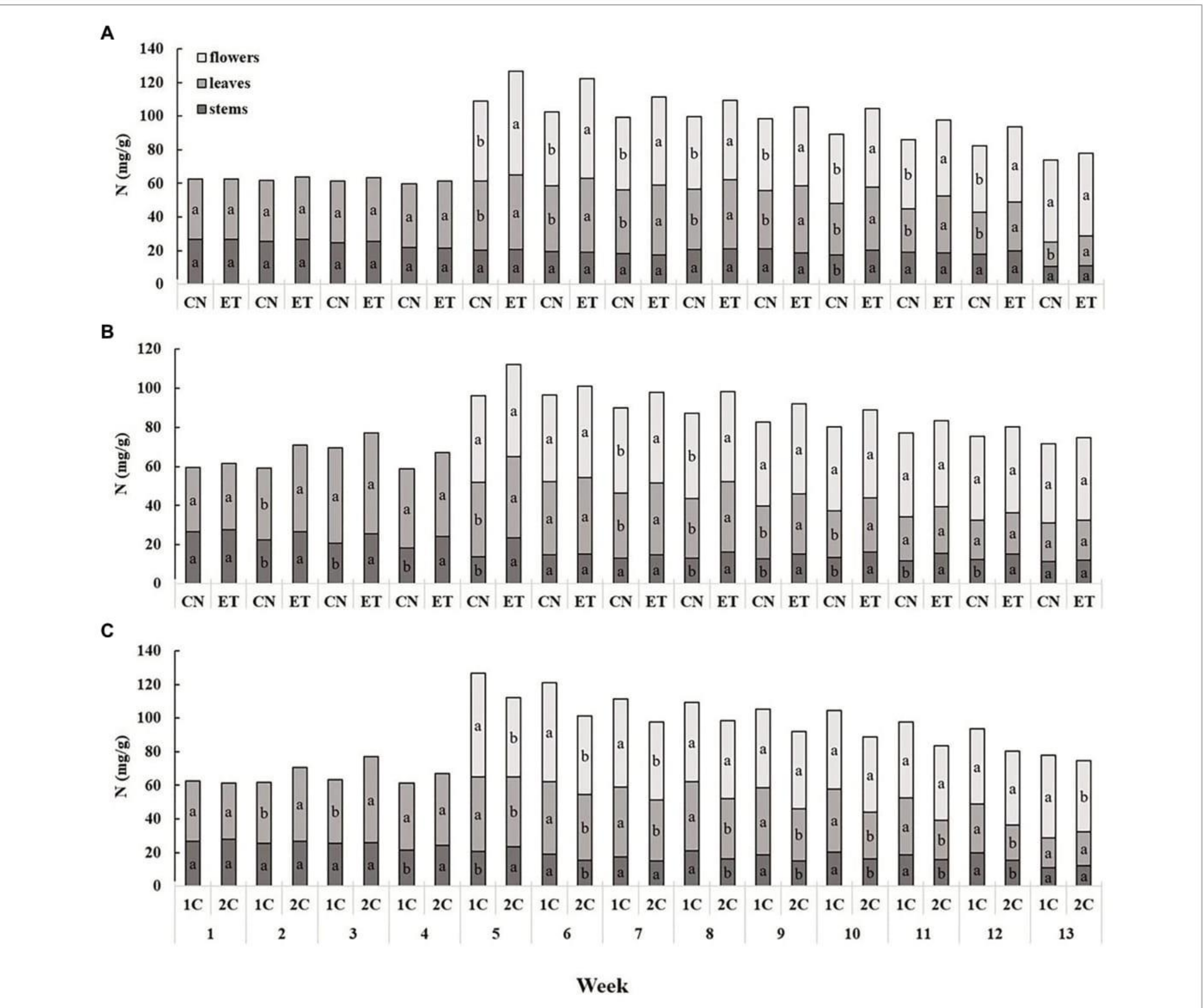

FIGURE 2 | Distribution of nitrogen among the organs of medical cannabis plants as affected by amino acid (AA) supplementation and nutrient cycle. N concentration of control (CN) and enhanced treatment (ET) with AAs nutritional supplement in recirculation (1C) growing cycle **(A)**, in drain-to-waste (2C) growing cycle **(B)**, ETs in 1C and 2C **(C)** in stems, leaves, and flowers. Data are means ± SE (n=3). The different small letters inside the bars represent significant differences within the plant organs (stems, leaves, and flowers) between the variants in a particular week according to Tukey's HSD test at α=0.05.

85.17 mg/g; ET, 53.13 mg/g; **Figure 3A**). In contrast to 1C, the Ca concentration in the CN and ET leaves differed significantly as early as week 2 in 2C. The most significant differences in Ca concentrations between CN and ET were 32% for leaves at week 7 (CN, 79.60 mg/g; ET, 60.13 mg/g; **Figure 3B**). The Ca concentration in ET also varied between 1C and 2C, beginning at week 1. The most significant differences in Ca concentrations in ETs between 1C and 2C in the last weeks of vegetation growth were 67% (11% between CNs) for leaves at week 11 (1C, 53.13 mg/g; 2C, 88.87 mg/g; **Figure 3C**).

The content of sulfur compounds was the lowest in the stems and the highest in the leaves (**Figure 4**). The concentration of S in the stems and leaves for CN and ET with 1C began to differ significantly from the third week. The most significant differences in S between CN and ET were 28% for leaves in week 8 (CN, 2375 mg/kg; ET, 3029 mg/kg; **Figure 4A**). In contrast to 1C, the concentration of S in the stems and leaves of CN and ET began to differ significantly from the second week for 2C; however, fewer significant differences than in 1C were observed. The most significant differences in S concentrations between nutritional treatments were 23% for leaves at week 5 (CN, 1834 mg/kg; ET - 2,260 mg/kg; **Figure 4B**). The S concentration also varied between 1C and 2C of ETs but was almost identical till the 3rd week. The most significant differences in S concentrations in ETs between 1C and 2C were 46% (27% between CNs) for leaves at week 8 (1C, 3,029 mg/kg; 2C, 2068 mg/kg; **Figure 4C**).

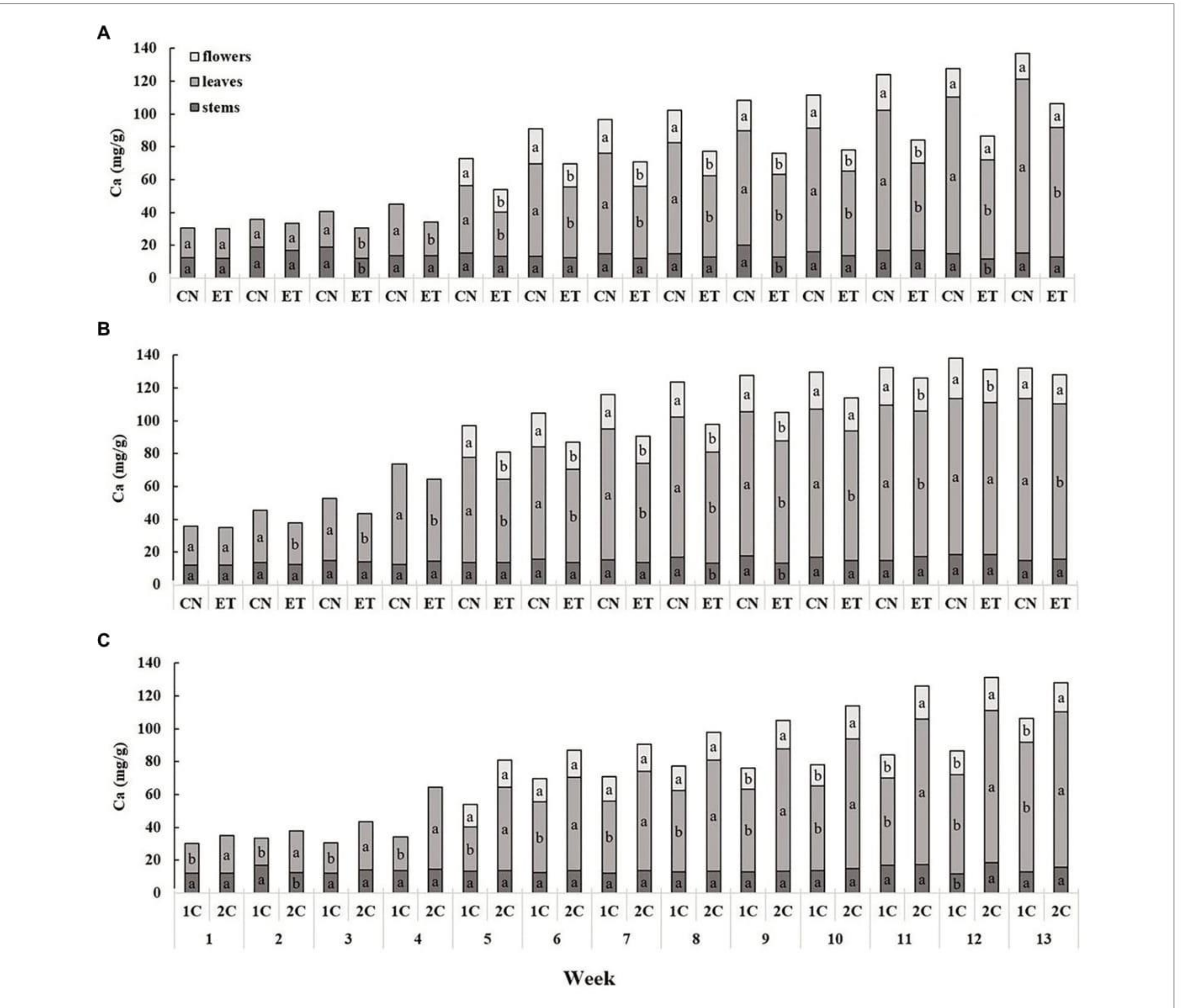

FIGURE 3 | Distribution of calcium among the organs of medical cannabis plants as affected by amino acid (AA) supplementation and nutrient cycle. Ca concentration in control (CN) and enhanced treatment (ET) plants with AAs nutritional supplement in recirculation (1C) growing cycle **(A)**, in drain-to-waste (2C) growing cycle **(B)**, ETs in 1C and 2C **(C)** in stems, leaves, and flowers. Data are means ± SE ($n = 3$). The different small letters inside the bars represent significant differences within the plant organs (stems, leaves, and flowers) between the variants in a particular week according to Tukey's HSD test at $\alpha = 0.05$.

The iron content was the lowest in leaves and highest in stems and showed a cumulative trend over time (**Figure 5**). The concentration of Fe in the stems for CN and ET in 1C began to differ significantly from week 6 to 13. The most significant differences in Fe between CN and ET were 79% for stems at week 8 (CN, 609.5 mg/kg; ET, 340.6 mg/kg; **Figure 5A**). In contrast to 1C, the concentration of Fe in the stems of the CN and ET began to differ significantly from week 3 to 13 in 2C. The most significant difference in Fe concentrations between CN and ET was 139% for stems at week 8 (CN, 666.4 mg/kg; ET, 279.3 mg/kg; **Figure 5B**). The Fe concentration also varied between 1C and 2C of ETs by the first week. The most significant difference in Fe concentrations in ETs between 1C and 2C in the last weeks of vegetation growth was 45% (40% between CNs) for stems at week 13 (1C, 844.2 mg/kg; 2C, 584.4 mg/kg; **Figure 5C**).

Nutritional supplementation with AAs in the two different nutritional cycles caused some change in growth of medical cannabis plants. Up to week 5, the increase in biomass was relatively slow, but was sharply increased from week 6. The largest weekly dry weight gain was recorded for flowers (**Figure 6**). The increase in biomass of stems, leaves, and flowers for CNs and ETs in 1C was almost identical until week 7. From week 8 to 12, leaf and flower biomass differed somewhat (**Figure 6A**). In contrast to 1C, stems, leaves, and flowers of CN and ET plants in 2C increased significantly from week 9. ET reached maximum dry plant biomass at week 11, and CN by week 12 (**Figure 6B**). Biomass also

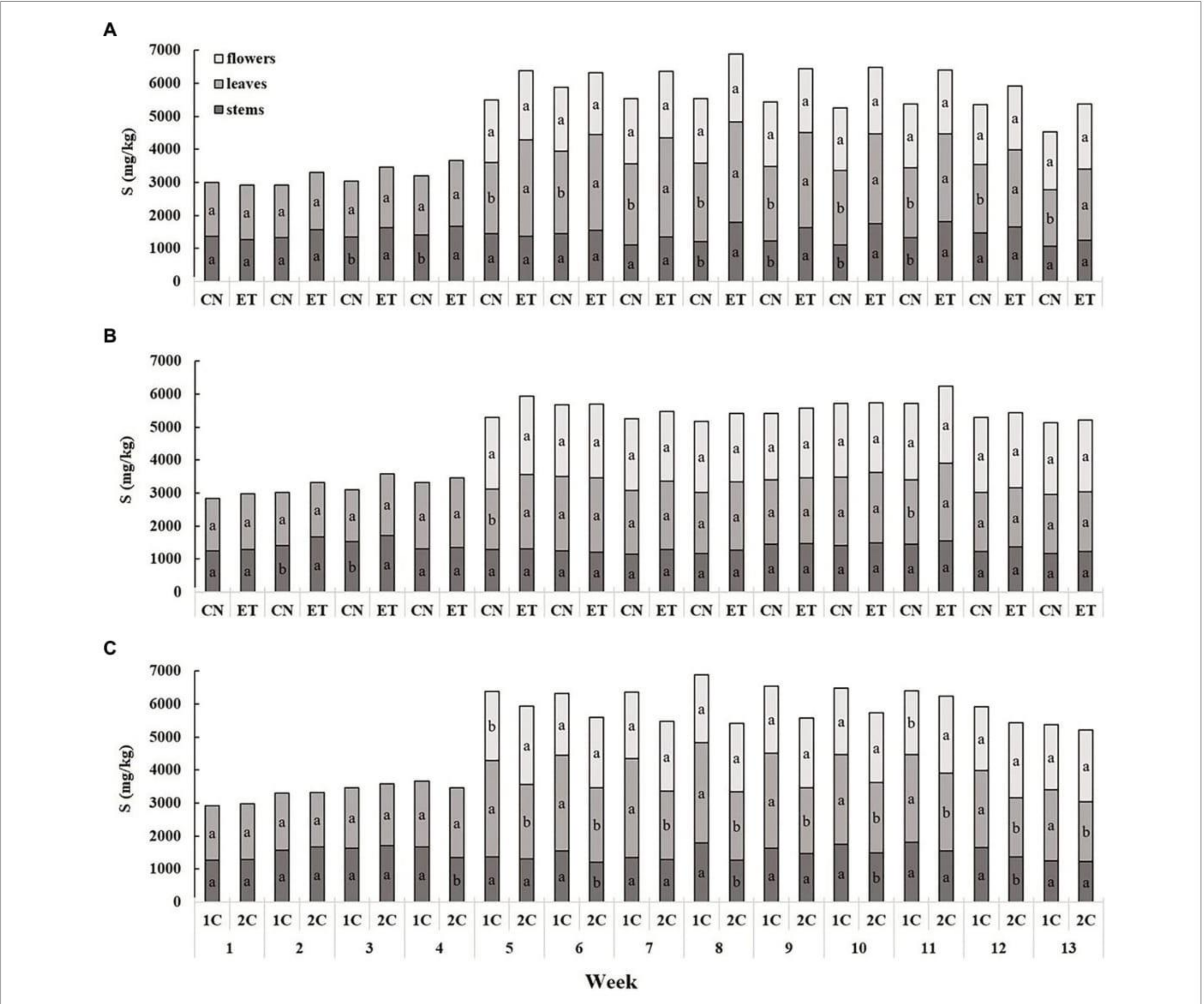

FIGURE 4 | Distribution of sulfur among the organs of medical cannabis plants as affected by amino acid (AA) supplementation and nutrient cycle. S concentration in control (CN) and enhanced treatment (ET) plants with AAs nutritional supplement in recirculation (1C) growing cycle **(A)**, in drain-to-waste (2C) growing cycle **(B)**, ETs in 1C and 2C **(C)** in stems, leaves, and flowers. Data are means ± SE ($n = 3$). The different small letters inside the bars represent significant differences within the medical cannabis plant organs (stems, leaves, and flowers) between the variants in a particular week according to Tukey's HSD test at $\alpha = 0.05$.

varied between 1C and 2C of ETs, but from week 7 (**Figure 6C**).

AA supplementation and nutritional cycle changed the concentration of THCA and CBNA in the flowers of cannabis plants, but concentration curves of both cannabinoid acids were similar for the same nutritional cycle and treatment (**Figure 7**). THCA in leaves and flowers slowly increased in both treatments until week 4, but from week 5, the concentration of THCA began to differ significantly because only flowers were analyzed (**Figures 7A–C**). The CN and ET concentrations of THCA began to differ significantly from week 5 to 13 in 1C, and CN (18.2%) and ET (16.0%) reached maximum at week 11 (**Figure 7A**). In contrast to 1C, the concentration of THCA in CN and ET (2C) differed significantly by the third week, but the differences were smaller. THCA peaked at week 9 for CN (15.4%) and week 7 for ET (15.4%; **Figure 7B**). The THCA levels in 1C and 2C of ETs differed significantly from week 5–13 (**Figure 7C**). The CBNA concentration in CN and ET in 1C began to vary significantly between week 5 and 13. CBNA peaked at week 11 in both treatments and differed significantly by 44% (**Figure 7D**). In contrast to 1C, the CBNA concentration in CN and ET did not differ significantly in 2C until weeks 5 and 10. CBNA in CN reached two maxima in 2C: at week 9, where it differed significantly by 41%, and at week 11 where it differed significantly by 44%. The CBNA for ET also reached two maxima in 2C: at week 7 where it differed significantly by 17%, and at week 11, the same as CN (**Figure 7E**). CBNA concentrations between 1C and 2C

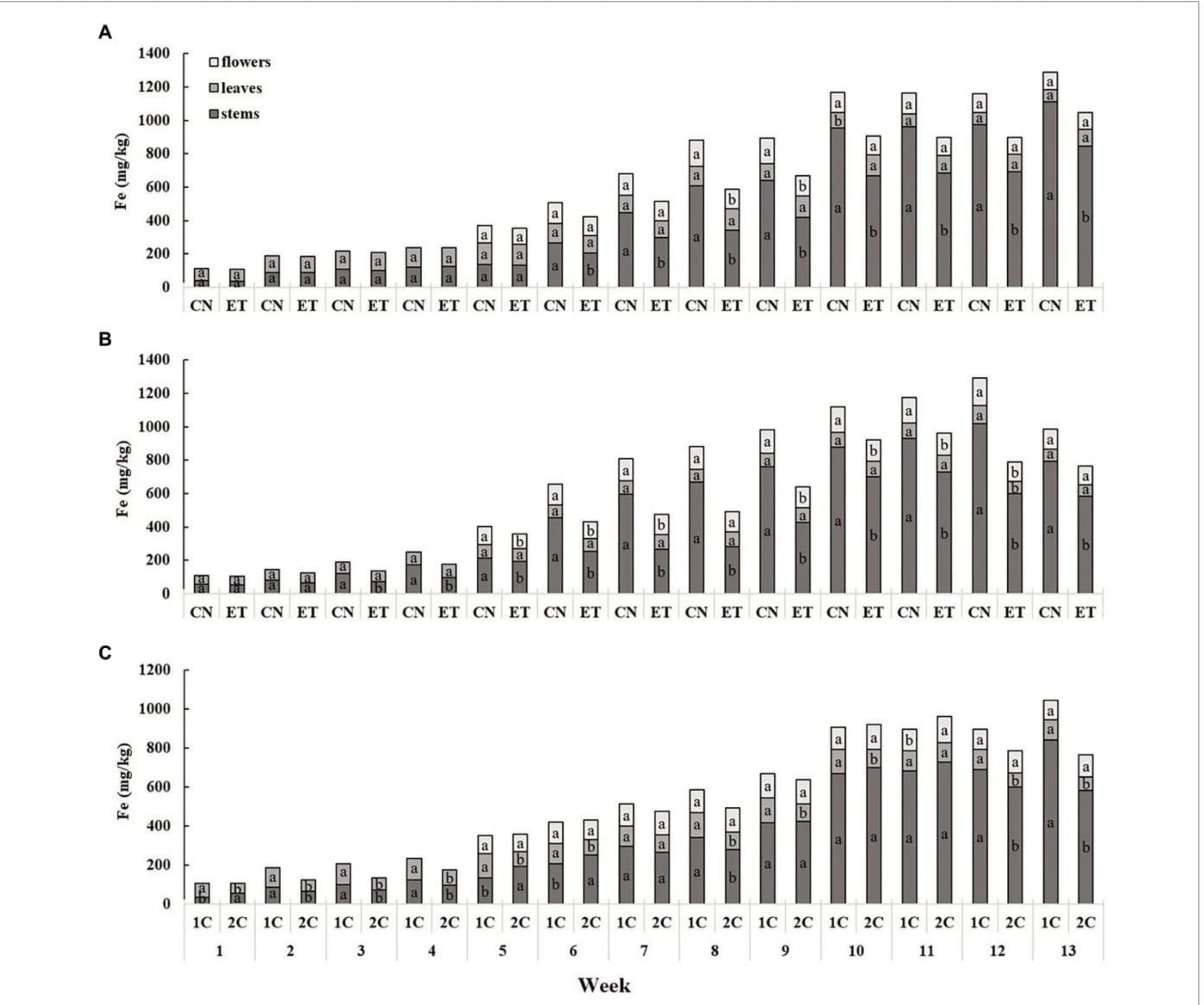

FIGURE 5 | Distribution of iron among the organs of medical cannabis plants as affected by amino acid (AA) supplementation and nutrient cycle. Fe concentration in control (CN) and enhanced treatment (ET) plants with AAs nutritional supplements in recirculation (1C) growing cycle **(A)**, in drain-to-waste (2C) growing cycle **(B)**, ETs in 1C and 2C **(C)** in stems, leaves, and flowers. Data are means ± SE ($n = 3$). The different small letters inside the bars represent significant differences within the medical cannabis plant organs (stems, leaves, and flowers) between the variants in a particular week according to Tukey's HSD test at $\alpha = 0.05$.

of ETs were almost identical until weeks 5 and 9. As stated above, the CBNA concentration of ET reached maximum at week 11 in 1C, when it differed significantly by 33% (also 33% for CNs) and at week 7 in 2C, when it differed significantly by 83% (7% for CNs; **Figure 7F**).

THCA is the most concentrated cannabinoid in our medical cannabis plant chemotype. The THCA yield per plant from dried flowers over time and the effect of the AA supplement and variable nutritional cycle was measured (**Figure 8**). THCA yields were almost identical for CN and ET with 1C until week 6 but differed significantly from week 7–13. The largest significant difference (46%) between the nutritional treatments was achieved at week 11, but the highest yield for both treatments was at week 13 (**Figure 8A**). The THCA yield for CN with 2C compared to 1C reached its maximum at week 12 (significant difference, 34%) and for ET at week 11 (significant difference, 10%; **Figure 8B**). As stated above, the THCA yield for ET with 1C reached a maximum at week 13 (significant difference between ETs, 279%) and at week 11 for ET with 2C (difference between ETs, 28%; **Figure 8C**).

The concentrations of limonene and β-myrcene in the flowers were also affected by AA supplementation and nutrient cycle (**Figure 9**). Limonene peaked at week 9 for CN (1.33 mg/g) and at week 10 for ET (2.12 mg/g). The most significant difference in limonene concentration between these two treatments was 81% reached at week 8 in 1C (**Figure 9A**). As in 1C, limonene concentration peaked at week 9 for CN (0.94 mg/g) but at week 8 for ET (1.58 mg/g) in 2C. The largest

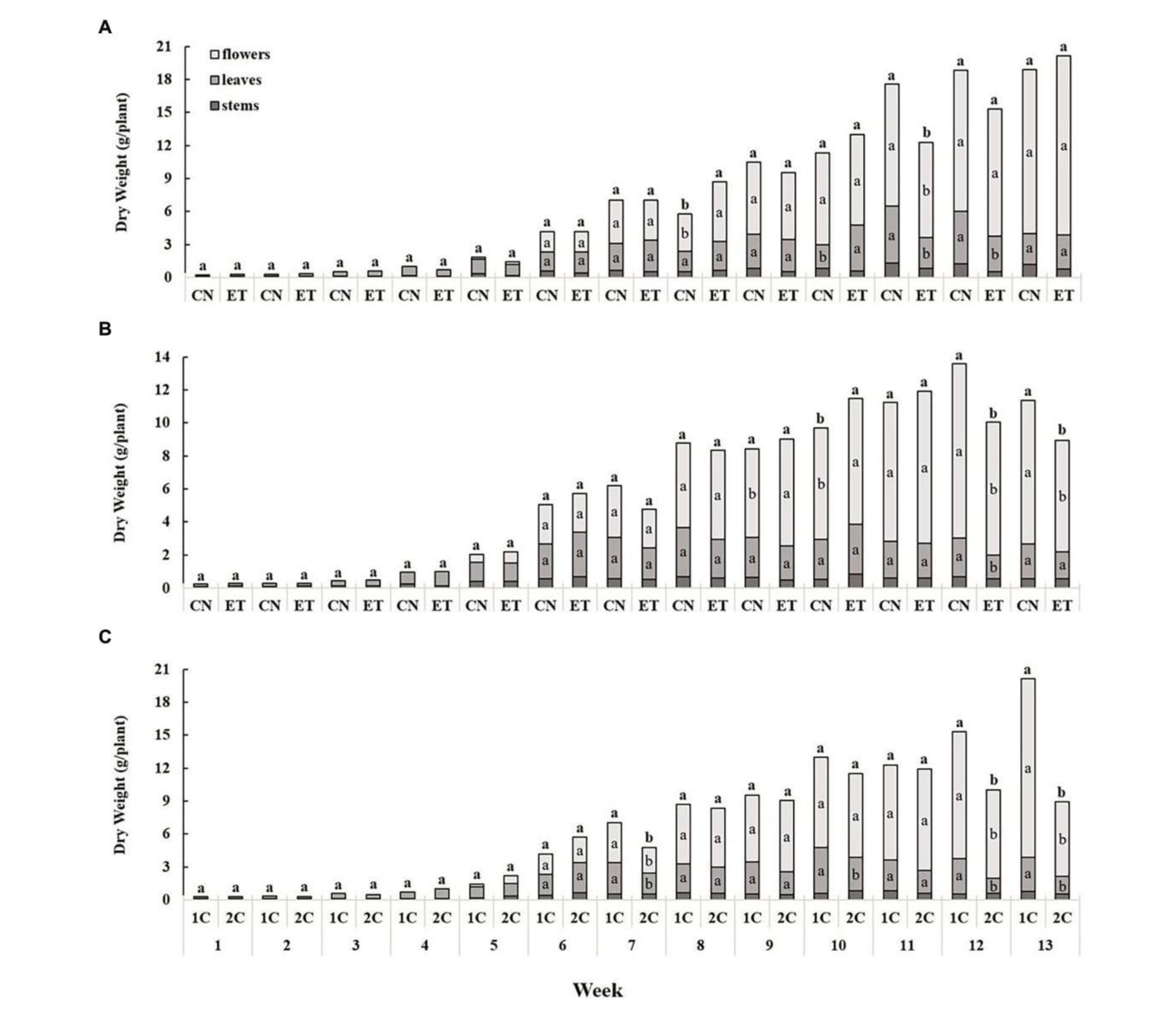

FIGURE 6 | The effect of amino acid supplementation (AAs) and growing nutritional cycle on medical cannabis plant biomass. Dry biomasses of stems, leaves, and flowers in control (CN) and enhanced treatment (ET) plants with AAs nutritional supplements in recirculation (1C) growing cycle **(A)**, in drain-to-waste (2C) growing cycle **(B)**, ETs in 1C and 2C **(C)**. Data are means ± SE ($n=3$). The different small letters inside the bars and small bold letters above the bars represent significant differences within the medical cannabis plant organs (leaves and flowers) and the whole plant biomass between the variants in a particular week according to Tukey's HSD test at $\alpha=0.05$.

significant difference in limonene concentration between these two treatments was 123% reached at week 10 (**Figure 9B**). Comparing limonene concentrations of ETs for 1C and 2C, the largest significant difference between these two cycles was 37% at week 10 (**Figure 9C**). β-myrcene levels peaked at week 9 for CN (0.89 mg/g) and at week 10 for ET (1.46 mg/g). The largest significant difference in β-myrcene concentration between these two treatments was 139% at week 8 in 1C (**Figure 9D**). As in 1C, β-myrcene peaked at week 9 for CN (0.61 mg/g), but at week 8 for ET (1.38 mg/g) in 2C. The largest significant difference in β-myrcene concentration between these two treatments was 167% at week 8 (**Figure 9E**). Comparing β-myrcene concentration in ETs for 1C and 2C, the most significant difference between these two cycles was 28% reached at week 10 (**Figure 9F**).

DISCUSSION

Nutrition is undoubtedly an important factor in the development, function, and metabolism of all plant organs and tissues. Data are already known regarding the optimal levels of individual macronutrients, such as N, P, and K, for normal function and development of the root system and above-ground biomass (Saloner et al., 2019; Saloner and Bernstein, 2020; Shiponi and Bernstein, 2021b) and formation of the desirable secondary

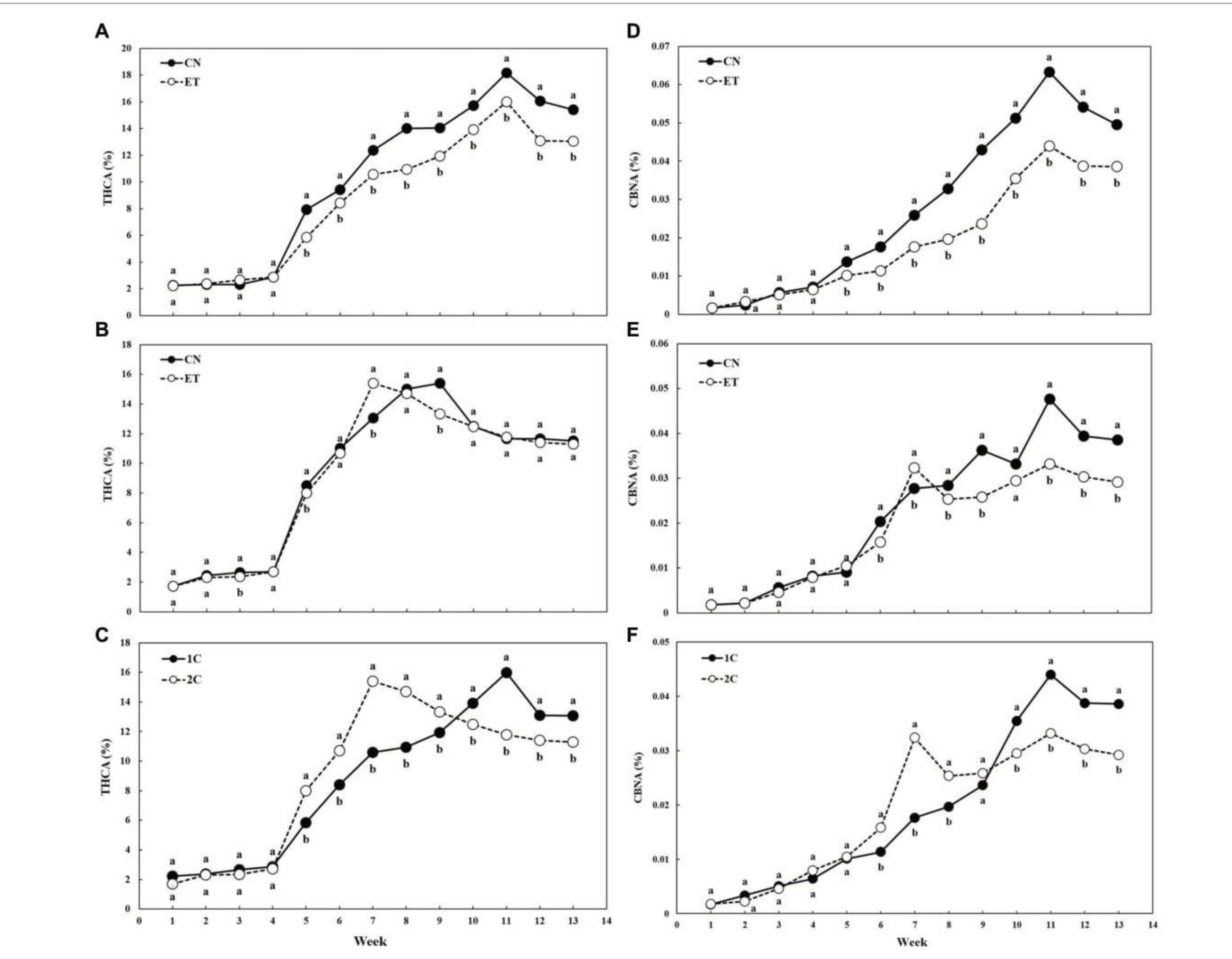

FIGURE 7 | Concentrations of tetrahydrocannabinolic acid (THCA) and cannabinolic acid (CBNA) in the flowers of medical cannabis plants as affected by amino acid (AA) supplementation and nutrient cycle. THCA concentration in control (CN) and enhanced treatment (ET) plants with AAs nutritional supplements in recirculation (1C) growing cycle **(A)**, in drain-to-waste (2C) growing cycle **(B)**, ETs in 1C and 2C **(C)**. CBNA concentration of control (CN) and enhanced treatment (ET) with AAs nutritional supplements in recirculation (1C) growing cycle **(D)**, in drain-to-waste (2C) growing cycle **(E)**, ETs in 1C and 2C **(F)**. The whole inflorescence of the plant was analyzed. Data are means ± SE ($n=3$). Different bold small letters represent significant differences in cannabinoid concentration between the variants in a particular week according to Tukey's HSD test at $\alpha=0.05$.

metabolites of medical cannabis plants (Caplan et al., 2017a; Bernstein et al., 2019; Saloner and Bernstein, 2021; Yep et al., 2021; Shiponi and Bernstein, 2021a). However, there is still emphasis on the availability of sufficient quantities of these major plant macronutrients in an optimal ratio. The effects of micronutrients (Yep et al., 2021) and plant biostimulants (Bernstein et al., 2019) must also be considered.

Nutritional treatment with AA supplements in different nutrient cycles clearly affected the concentrations of macro- and micro-elements in cannabis plants. Antagonistic and synergistic interactions between nutrient anions and cations during root cell membrane transport have been relatively well reported. However, the timing of replenishment of AAs and variations in pH, as in the case of the recirculation cycle, 1C, could affect their accessibility from the nutrient solution and thus the subsequent physiological and metabolic response of plants. The enhanced treatment (ET) with AA supplementation resulted in significantly greater nitrogen accumulation (**Figures 2A,B**) in all three plant organs, but mostly in flowers and leaves. This finding is consistent with claims that plants can absorb and incorporate intact amino acids directly (Matsumoto et al., 1999; Persson and Nasholm, 2001; Jämtgård et al., 2008). AAs can also modulate the assimilation and absorption of N in plants by regulating the enzymes and structural proteins involved in these processes. AAs also affect N uptake signaling pathways in roots and promote transfer between nitrogen and carbon metabolites by controlling enzymes of the tricarboxylic acid cycle (Colla et al., 2014; Du Jardin, 2015). When comparing nutritional cycles (**Figure 2C**), higher N concentrations were observed in the above-ground organs of plants, especially in leaves and flowers from ET plants in 1C. This was probably due

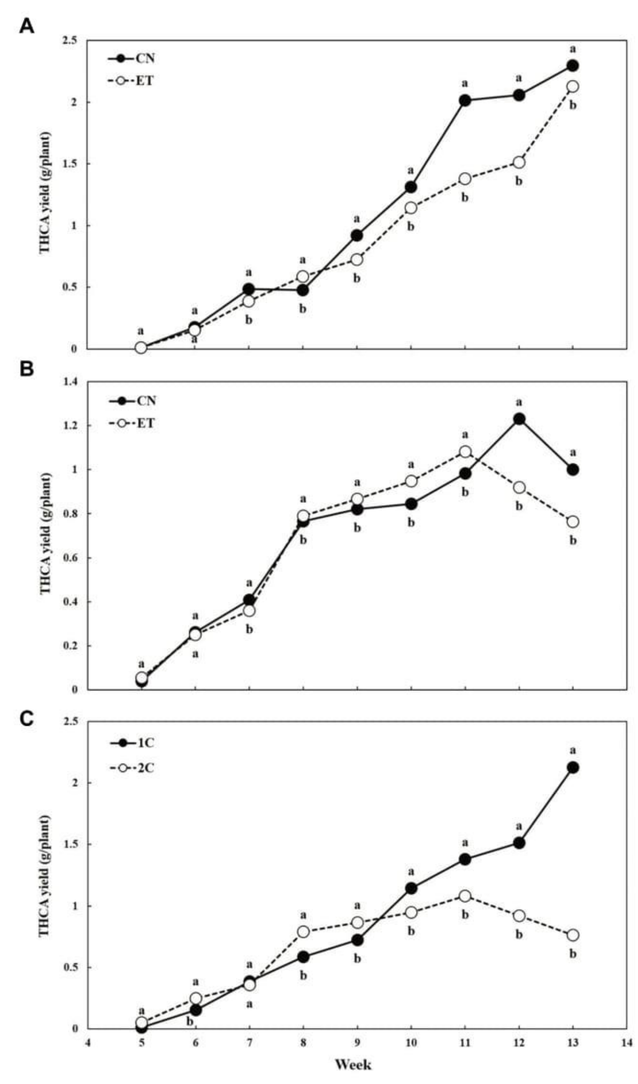

FIGURE 8 | Tetrahydrocannabinolic acid (THCA) yield per plant as affected by amino acid (AA) supplementation and nutrient cycle. THCA yield per plant in control (CN) and enhanced treatment (ET) with AAs nutritional supplements in recirculation (1C) nutrient cycle **(A)**, in drain-to-waste (2C) nutrient cycle **(B)**, ETs in 1C and 2C **(C)**. The whole inflorescence of the plant was analyzed. Data are means ± SE ($n=3$). Different bold small letters represent significant differences in cannabinoid concentration between the variants in a particular week according to Tukey's HSD test at $\alpha=0.05$.

to fluctuations in the pH of the 1C nutrient solution from addition of AAs, which increased the pH to 8.05 after 24 h. The initial pH of the nutrient solution, 5.9 (the constant pH of the 2C nutrient solution), was close to the isoelectric point of most AAs (Pogliani, 1992), but recirculation may have resulted in the formation of a partial charge on some AA molecules. At pH 5.9, most AAs were in the neutral zwitterionic form, making them less able to enter plant cells because of lipophilic interactions during membrane transport (Trapp, 2004). Sulfur showed an accumulation trend similar to N, but at a lower concentration (**Figure 4**), because of the sulfur-containing AAs, cysteine and methionine (**Table 1**). In 2C (**Figure 4B**), the S concentrations were almost identical in both treatments, probably because of lower solubility of the sulfur AAs at pH 5.9 and reduced absorption.

Calcium accumulation followed an opposite trend (**Figure 3**). In the ET group, the AA supplementation significantly lowered calcium accumulation (**Figures 3A,B**) in all three plant organs, but mostly in leaves and flowers. The same trend was observed for magnesium accumulation (*data not shown*), but with minor differences because of lower concentration. This was probably due to the coordination of calcium with the carboxyl, hydroxyl, thiol, and amino groups of the AAs to form complexes with limited accessibility (Maeda et al., 1990). When comparing nutritional cycles (**Figure 3C**), higher calcium concentrations were observed in above-ground parts, especially leaves and flowers, from ET plants in 2C. This was probably due to the stable 5.9 pH of the 2C nutrient solution, in which the AAs were in the form of zwitterions that did not complex with *Ca*. The increased formation of root exudates containing negatively charged or free electron pair groups capable of coordinating and binding Ca from the nutrient solution might also have contributed to this process. It is probable that more exudates were excreted in 1C because of the pH change in the cytosol and also from the increase in TCA cycle function after uptake of negatively charged AAs (Ryan et al., 2001). In the case of 2C, replenishment with fresh nutrient solution also contributed to increased calcium ions. Iron showed an accumulation trend similar to calcium, only at lower concentrations, where it occurred mainly in the stem due to low mobility (**Figure 5**). However, when comparing nutritional cycles (**Figure 5C**), a higher Fe concentration was observed at some weeks in above-ground organs, especially leaves and stems, of ET plants in 1C. This may have resulted from the Fe levels of ET plants in 2C reaching a maximum at week 11 compared to week 13 in 1C, and also, from the chelating effects of some AAs, which could contribute to mobility and micronutrient acquisition by roots (Calvo et al., 2014). The levels of phosphorus and potassium (*data not shown*) did not differ in nutrient solutions, nor did they show many significant differences in accumulation in the above-ground organs of both treatments, so they were not discussed.

The changed accessibility and supply of individual nutrients within CN and ET plants during different nutritional cycles also affected the yield of dry biomass of stems, leaves, and flowers (**Figure 6**). In CN with 1C, only a slight increase in the weight of above-ground biomass was observed from week 11 to 13, whereas in ET we saw a sharp increase in total dry matter, especially in flowers, in the last weeks (**Figure 6A**). This was probably caused by an increased supply of nitrogenous and possibly other compounds in the root cells of ET plants and their subsequent transport to flowers during the so-called rinsing period (watering only with DMW; **Table 3**) from week 10–13 (Pratelli and Pilot, 2014; Yao et al., 2020). In CN plants with 2C, the maximum increase in biomass was reached at week 12, and in ET a week earlier (**Figure 6B**). This probably resulted from earlier maturation of the plants with 2C compared to 1C. The differences in dry biomass in the CN and ET groups in

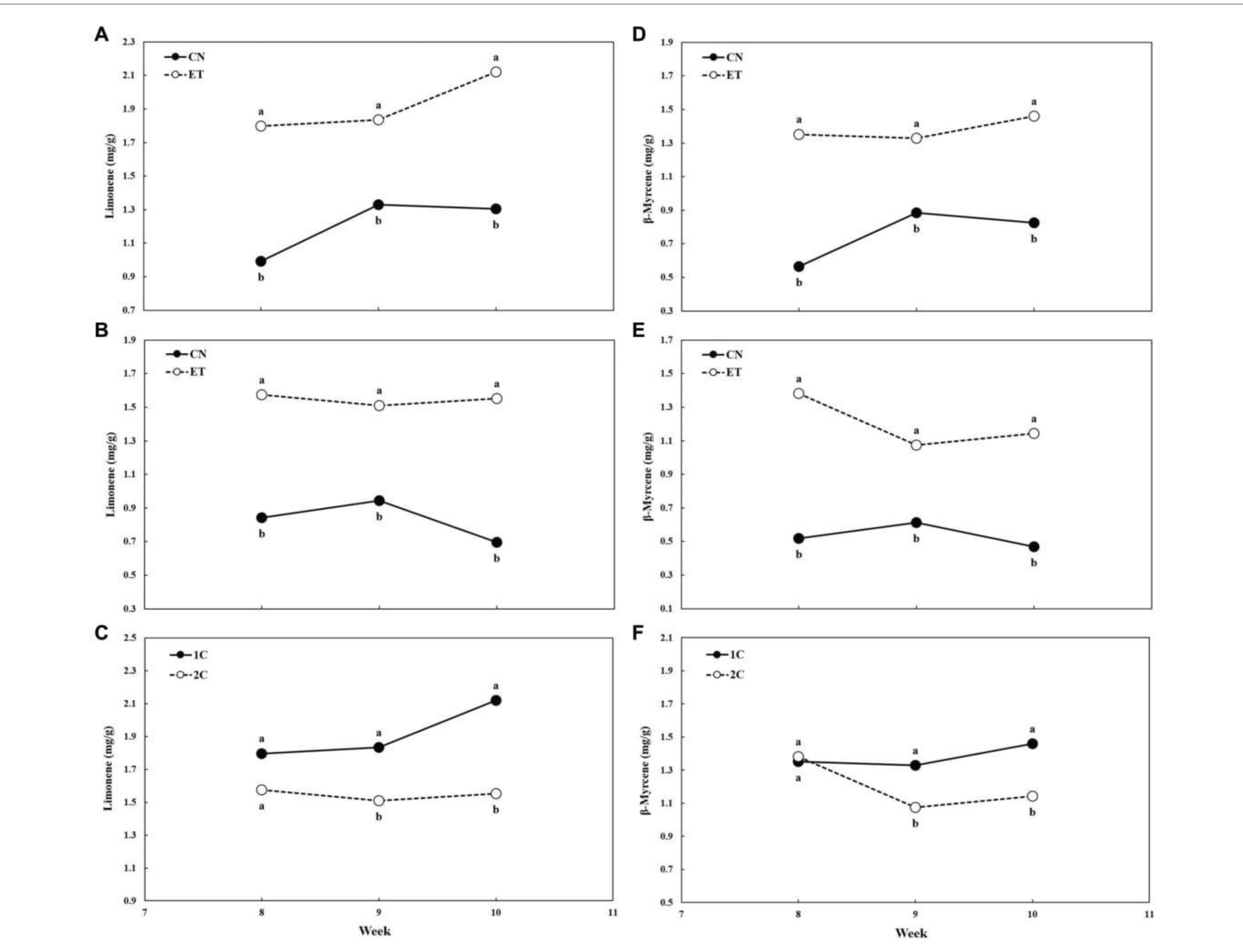

FIGURE 9 | Concentration of limonene and β-myrcene in flowers of medical cannabis plants as affected by amino acid (AA) supplementation and nutrient cycle. Limonene concentration in control (CN) and enhanced treatment (ET) plants with AAs nutritional supplements in recirculation (1C) nutrient cycle **(A)**, in drain-to-waste (2C) nutrient cycle **(B)**, ETs in 1C and 2C **(C)**, the β-myrcene concentration of control (CN) and enhanced treatment (ET) with AAs nutritional supplements in recirculation (1C) growing cycle **(D)**, in drain-to-waste (2C) growing cycle **(E)**, ETs in 1C and 2C **(F)**. The whole inflorescence of the plant was analyzed. Data are means ± SE ($n = 3$). Different bold small letters represent significant differences in cannabinoid concentration between the variants in a particular week according to Tukey's HSD test at $\alpha = 0.05$.

both cycles were mainly due to the different N doses from AAs delivered to ET plants from the second (first blooming) week (**Tables 2** and **3**). According to Saloner and Bernstein (2021), the optimal dose of mineral N for medical cannabis in bloom is 160 mg/l. In our experiments, the amount of mineral N in the nutrient solution was gradually increased from 116 mg/l (week 2) to 150 mg/l (weeks 4 and 6–9) in both CN and ET. Caplan et al. (2017a) stated that the optimal dose of N in organic fertilizers for maximum biomass of medical cannabis plants in bloom was 283 mg/l. In our experiments, the amount of organic N in the nutrient solution for ET plants was gradually increased from 184 mg/l (week 2) to 203 mg/l (weeks 4 and 6–9). However, the amount of total N supplied in the nutrient solution for ET ranged from 300 mg/l (week 2) to 353 mg/l (weeks 4 and 6–9; **Table 3**). Therefore, this amount of total nitrogen in the nutrient solution may already have exceeded the optimal dosage for medical cannabis plants, especially with 2C (Albornoz, 2016).

This hypothesis was partially supported by the premature ripening of plants based on the concentration of THCA in ET in 2C (**Figure 7B**), but this could also be caused by increased abiotic stress from high N doses (Gepstein and Glick, 2013). Conversely, the higher dose of nutrients in 2C compared to 1C ensured optimal fertigation, which can shorten the ripening time of cannabis (Caplan et al., 2017b). This hypothesis was supported by the nearly identical trend of increasing THCA concentration with 2C in both treatments, although ET peaked at week 7 compared to CN at week 9 (**Figure 7B**). When comparing ET results at 1C and 2C, the difference was 4 weeks because the ET plants with 1C did not reach their maximum THCA concentrations until the 11th week (**Figure 7C**). Differences in THCA concentrations in both treatments and cycles, but especially in 1C, could

be explained by the previously discovered positive correlation of calcium with Δ^9-tetrahydrocannabinol (Δ^9-THC), which is a decarboxylation product of THCA (**Figures 3**, **7**; Pate, 1994). Its oxidation product, CBNA, had a similar course and maxima as THCA, but reversed (**Figures 7D–F**), probably because of the antioxidant activity of AAs, which reduced environmental stress by scavenging free oxygen radicals (Calvo et al., 2014).

The combination of the dry weight of flowers and the concentration of THCA was reflected in the yield of THCA. In 1C, an almost linear dependence of THCA yield on time could be seen for both treatments (**Figure 8A**) because of the lower amount of total nutrients supplied in 1C compared to 2C, and thus the delay in ripening time. However, when comparing ETs from both cycles at the weeks of their maximum THCA yield (week 13 for 1C and week 11for 2C), the THCA yield with 1C was more than twice as high (**Figure 8C**). This may have been a result of the increased production of abscisic acid (ABA) in response to stress, which slows plant growth and increases THCA production (Mansouri et al., 2009). It was also likely to cause oxidative stress (Jiang and Zhang, 2001), thus indirectly increasing CBNA production (**Figure 7F**).

The final concentration of monoterpenes showed the same trend in the respective weeks in both cycles and treatments as the concentration of THCA (**Figure 9**). This was consistent with Aizpurua-Olaizola et al. (2016) who claimed that this could be explained by the fact that monoterpenes were synthesized in the same glandular trichomes as cannabinoids (Meier and Mediavilla, 1998). Similar to Saloner and Bernstein (2021), our results showed that the increased N in the nutrient solution decreased THCA concentration proportionally. But conversely when exceeding a specific limit of nitrogen fertilization, as 160 mg N/L in the case of Saloner and Bernstein (2021), a reversible increase in limonene and myrcene concentration was observed. This was in agreement with studies showing a positive dependence of isoprene unit formation on N fertilization (Mccullough and Kulman, 1991; Close et al., 2004). High N concentrations in leaves promoted photosynthetic activity, which increased the availability of assimilated carbon used to generate metabolites *via* the methylerythritol pyrophosphate (MEP) pathway (Ormeno and Fernandez, 2012). Two biosynthetic pathways contributed to the early steps in the production of plant terpenes. The first is the cytosolic mevalonic acid (MVA) pathway, which is involved in the biosynthesis of sesquiterpenes and triterpenes. The second, plastid-localized methylerythritol phosphate (MEP) pathway, is involved in the biosynthesis of monoterpenes, diterpenes, and tetraterpenes (Bouvier et al., 2005). Phytocannabinoids are synthesized from isoprenoid precursors combined with fatty acids (Dewick, 2002). However, the geranyl pyrophosphate necessary for the production of the terpenoid part of cannabinoids is predominantly (>98%) synthesized by the MEP pathway in plastids (Fellermeier et al., 2001). Because limonene, β-myrcene, and the terpenoid part of THCA are synthesized *via* the same MEP pathway and exhibit a concentration response in medical cannabis flowers opposite to that from addition of AAs to the nutrient solution, which increases N levels, it can be concluded that the biosynthesis of the ketide (fatty acid) part of the THCA molecule may be affected (Tedesco and Duerr, 1989). However, further research will be needed to draw relevant conclusions.

CONCLUSION

This study investigated the effects of amino acid supplementation and two different nutritional cycles (systems) on medical cannabis growth. The exact relationship between the content of secondary metabolites and the nutritional supplements remains unclear. This connection is complex and involves several parameters, including nutrient availability, biosynthetic conditions, and physiological signals. The amino acid-based nutritional supplement significantly increased the nitrogen and sulfur content and reduced the accumulation of calcium and iron in both cycles throughout the plant. It caused earlier maturation in plants as reflected in the THCA concentration in the drain-to-waste cycle and reduced the CBNA content in flowers. Furthermore, in both nutritional cycles, it significantly increased the content of monoterpenes, limonene and β-myrcene. When comparing the nutritional cycles of treatments with the amino acid supplement, it can be seen that a significantly higher content of nitrogen and sulfur was achieved in the recirculation cycle, but a lower content of calcium in the whole plant. In the drain-to-waste cycle, medical cannabis plants matured about a month earlier, based on THCA concentration, but at the expense of half-maximal THCA yield in flowers and significantly lower concentrations of limonene and β-myrcene than with the recirculation cycle. This study clearly shows the advantages and disadvantages of the amino acid-based biostimulant and of the different nutritional cycles. In the recirculation cycle, higher yields of secondary metabolites were achieved with much lower total nutrient consumption, but over a more extended time. On the contrary, the drain-to-waste cycle allowed better control of the nutrient solution, stable supply of accurate nutrient concentration, and accelerated plant ripening, but with higher fertilizer consumption and lower overall yield of secondary metabolites. This study examined a high-yield THCA variety classified as chemotype I grown hydroponically in Euro Pebbles (expanded clay) medium. Therefore, it would be interesting to carry out these studies on cannabis varieties of different chemotypes.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

MM designed the study, wrote the manuscript, controlled the cultivation scheme, and performed physiological, chemical, and data analyses. JV designed the study, controlled the cultivation scheme, and performed physiological and chemical analyses. LP and AJ performed chemical analyses. ZK controlled the cultivation scheme. PK supervised the study. PT designed and supervised the study. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the European Regional Development Fund (project NUTRISK no. CZ.02.1.01/0.0/0.0/16_019/0000845).

ACKNOWLEDGMENTS

We thank Dr. Gary Bentley for proofreading and improving the English in the manuscript.

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4.4. Comparison of Recirculation and Drain-to-Waste Hydroponic Systems in Relation to Medical Cannabis (*Cannabis sativa* L.) Plants

Autoři: Malík M, Praus L, Tlustoš, P

Rok publikace: "Under Review"

Časopis: Industrial Crops & Products

1 **Comparison of Recirculation and Drain-to-Waste Hydroponic Systems in Relation to**
2 **Medical Cannabis (*Cannabis sativa* L.) Plants**
3
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11 **Abstract**

12 Soon, rising temperatures, higher water consumption for irrigation, and the discharge of
13 agricultural wastewater may adversely impact water resources and, thus, the entire
14 environment. Agriculture is one of the sectors that consume an enormous amount of water.
15 Therefore, there is growing evidence to explore the possible recirculation and reuse of
16 nutrient solutions to reduce the associated environmental and economic costs in hydroponics,
17 which is currently one of the fastest-growing techniques in horticulture. This study focused on
18 the comparison of two different nutrient hydroponic cultivation systems, a recirculation (RS)
19 and a drain-to-waste system (DS), and the effect on the ionome, growth, and concentration of
20 secondary metabolites in medical cannabis (*Cannabis sativa* L.) plants. Elemental analysis of
21 plant organs was performed using the dry decomposition method with flame atomic
22 absorption spectroscopy (FAAS) and inductively coupled plasma optical emission
23 spectroscopy (ICP-OES). Nitrogen content was measured using the Kjeldahl method. The
24 phytocannabinoid profile was analyzed by high-performance liquid chromatography equipped
25 with a diode array detector (HPLC-DAD) in ethanolic flower extracts. The content of terpenic
26 compounds in hexane extracts was analyzed using gas chromatography with flame ionization
27 detection (GC-FID) and gas chromatography with mass spectrometry (GC-MS). During the
28 cultivation of plants in the RS, a significantly higher accumulation of nitrogen and
29 phosphorus was observed in the stems. Conversely, calcium and magnesium contents were
30 lower in whole above-ground biomass. Among the micronutrients, more manganese, copper,
31 and molybdenum were also accumulated there, but less zinc and boron. Medical cannabis
32 plants grown in the DS matured two weeks earlier but at the expense of an 87 % lower
33 maximum yield of tetrahydrocannabinolic acid (THCA) in the flowers and a significantly
34 lower concentration of monoterpene compounds than in the RS. Overall, much less nutrients
35 were consumed, but increased plant stress and extended growing time were observed in the
36 RS.

37

38 **Keywords:** Fertigation; Hydroponics; *Cannabis sativa* L.; Plant nutrition;
39 Phytocannabinoids; Expanded clay

1. Introduction

Agriculture, including horticulture, is one of the industries that consume the largest amounts of water. In the near future, rising temperatures, a higher need of water for irrigation, and the discharge of agricultural wastewater may have an adverse impact on water resources and the whole environment. Therefore, there is a growing need to explore the possible recirculation and reuse of nutrient solutions to reduce ecological and economic costs (Atzori et al., 2019; Hogeboom, 2020). Hydroponics is an alternative to conventional plant management and is currently one of the fastest-growing methods in the horticultural industry. Nutrients are supplied directly to a plant's root system as an aqueous solution. Due to the possibility of year-round plant growth in a controlled growth chamber, this method has the potential to produce high-quality, homogeneous plant material. Growers have already begun to widely use hydroponics not only for growing tomatoes and cucumbers but also for cannabis (*Cannabis sativa* L.). In many aspects, techniques required for cannabis growth are similar to those used for other plant species (Bouchard and Dion, 2009; Vanhove et al., 2011).

However, one of the weakest points in hydroponics is the lack of information about nutrient solution management. Many growers and researchers still drain nutrient solutions and refill them several times weekly for more precise control of nutrient delivery. However, the rapid depletion and addition of some nutrients to nutrient solutions can cause the amounts of supplied and subsequently received nutrients to be toxic to plants (Bugbee, 2004). Nutrients play a central role in many aspects of plant metabolism. There is already experimental evidence on the beneficial effects of an optimal level of supplied nutrients, especially for the group of plant macronutrients such as nitrogen (Saloner and Bernstein, 2020, 2021, 2022b), phosphorus (Shiponi and Bernstein, 2021a; Shiponi and Bernstein, 2021b), and potassium (Saloner and Bernstein, 2022a; Saloner et al., 2019; Yep and Zheng, 2021) on the secondary metabolites of medical cannabis plants. The cannabinoid and terpene profiles of medical cannabis can be influenced by the concentration and relative ratio of nutrients in the nutrient solutions (Bernstein et al., 2019; Bevan et al., 2021; Caplan et al., 2017a, b; Malík et al., 2021).

However, there is still a lack of information in the scientific literature concerning the management of nutrient solutions in the soilless cultivation of medical cannabis. Therefore, this study focused on supplying nutrients using two different hydroponic systems, recirculation and drain-to-waste. The study aimed to verify the amount and potential effects of supplemented nutrients on the growth and subsequent physiological and chemical reactions of medical cannabis plants in both nutrient hydroponic systems. The experiments were based on

74 the following hypotheses: (1) the amount of supplied nutrients will cause a change in the
75 growth of the above-ground biomass and will affect the flower yield; (2) the amount of
76 supplied nutrients will cause a change in the cannabinoid and terpenic profile of medical
77 cannabis plants; (3) changes induced will be in accordance with the macro and micronutrient
78 contents in above-ground plant organs (stems, leaves, and flowers); and (4) induced changes
79 will be different in individual hydroponic nutrient systems. To verify the hypotheses were
80 monitored the effects of nutrient supplementation in the nutrient solutions of each of the two
81 hydroponic systems on (1) the amount of total above-ground biomass and individual plant
82 organs (stems, leaves, and flowers); (2) type and concentration of cannabinoid and terpenic
83 compounds; and (3) the amount of nutrients utilized and the medical cannabis plant tissue
84 ionome.
85

86 **2. Material and methods**

87 2.1 Parameters of the cultivation area

88 Soilless cultivation of cannabis plants in pots filled with expanded clay (Euro Pebbles)
89 growing medium placed on tables took place in a controlled condition room. Each cultivation
90 table measured 2 m^2 (1 × 2 m) and introduced a different hydroponic system with an
91 independent nutrient solution tank with a volume of 100 L. Each table could hold up to 55
92 polypropylene (PP) conical, square 3.45-liter pots with dimensions: base 11.5 cm × 11.5 cm,
93 top 15 cm × 15 cm, and height 20 cm. Capillaries provided drip irrigation. The timer of the
94 water pump was set for 9 irrigation cycles, each lasting 60 s and all occurring during the
95 daylight phase. During one irrigation cycle, a volume of 94 mL of the nutrient solution was
96 supplied to each plant for a total of 846 mL per plant per day. Microclimatic parameters were
97 maintained using an air conditioning unit that maintained and recorded the set parameters
98 every minute using a data logger. A constant carbon dioxide (CO_2) concentration was
99 maintained using a methane-burning generator. Six high-pressure sodium lamps, each rated at
100 1000 W provided a suitable light spectrum. According to photosynthetic photon flux density,
101 lamps provided 1029 μmol/m^2/s at 6000 W.

102 2.2 Chemical phenotype and cultivation conditions of plants

103 In the experiment, clones (cuttings) were used, obtained from mother plants by vegetative
104 propagation. The medical cannabis genotype had a chemical phenotype classified as
105 chemotype I, meaning a high tetrahydrocannabinolic acid/cannabidiolic acid (THCA/CBDA)
106 ratio (> 1.0). The cuttings were cultivated for about three weeks in Rockwool cubes (4 × 4 cm)

107 under light-emitting diodes. Rooted clones were then transferred to PP pots with a volume of
108 3.45 L filled with 3 liters of expanded clay. The density of plants was 27.5 plants per m^2 (55
109 plants/table/hydroponic system). The light regimen was set to 18 h of light and 6 h of darkness
110 for the first week during the vegetative (growth) phase. The light phase temperature was kept
111 at 25°C and was reduced to 22°C during the dark phase. Relative air humidity was maintained
112 at 60%, and CO_2 concentration at 1065 mg/m^3 (1.065 mg/L). From the second week, the
113 cultivation regimen was adjusted to the generative (flowering) phase. The photoperiod was set
114 to 12 h of light and 12 h of darkness. The CO_2 concentration and temperature were kept the
115 same as in the vegetative phase but with a reduced 40% relative humidity due to mold risk.

116 2.3 Nutrient hydroponic systems and treatment

117 Plants were exposed to one treatment with two different nutrient hydroponic systems. The
118 first was a system with a recirculated (RS) nutrient solution, and the second was a drain-to-
119 waste system (DS) where the applied solution was used only once, then went into a separate
120 waste tank, and was no longer mixed with the fresh solution. A new nutrient solution was
121 prepared from demineralized water (DMW) with reverse osmosis every seven days starting
122 from the 1st day of the experiment. The nutrient content was increased according to the
123 growing age of the plants. From the 10th week, the plants were irrigated only with DMW.
124 The pH of a nutrient solution was adjusted to a value of 5.9 (Velazquez et al., 2013), and the
125 recirculation system solution was checked and adjusted daily. The electrical conductivity
126 (EC) was recorded while mixing the new solution. After preparing the new nutrient solution, a
127 sample was collected for analysis every week. The measured nutrient composition of the fresh
128 solution is shown in **Table 1** (Malík et al., 2022).
129

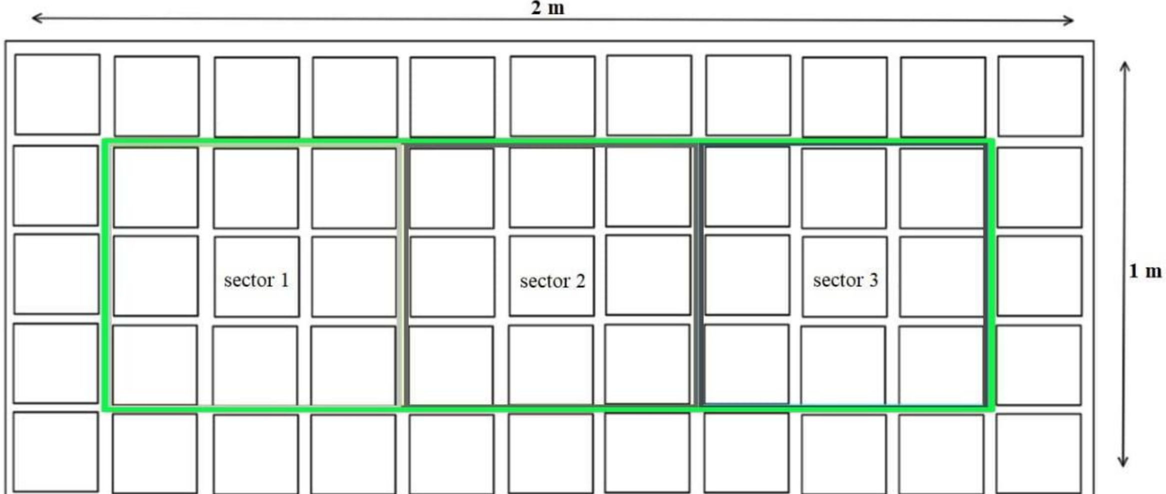
Table 1. Nutrient composition of fresh solution (mg/L).

Nutrients	Weeks				
	1	2	3, 5	4, 6-9	10-13
N	100.85 ± 1.64	116.00 ± 1.85	130.00 ± 1.75	150.00 ± 1.92	DMW[a]
P	32.01 ± 0.75	39.40 ± 0.82	43.88 ± 0.59	51.73 ± 0.79	DMW[a]
K	124.93 ± 1.85	151.00 ± 1.38	173.11 ± 1.92	193.25 ± 1.58	DMW[a]
Ca	98.53 ± 1.32	119.00 ± 1.35	132.38 ± 1.42	146.00 ± 1.28	DMW[a]
Mg	25.17 ± 0.38	30.50 ± 0.42	34.94 ± 0.48	39.13 ± 0.45	DMW[a]
S	21.75 ± 0.25	26.72 ± 0.29	31.34 ± 0.34	34.53 ± 0.38	DMW[a]
Fe	0.91 ± 0.09	1.11 ± 0.09	1.21 ± 0.11	1.44 ± 0.08	DMW[a]
Mn	0.66 ± 0.07	0.74 ± 0.05	0.83 ± 0.08	0.99 ± 0.07	DMW[a]
Zn	0.21 ± 0.03	0.27 ± 0.03	0.28 ± 0.04	0.33 ± 0.03	DMW[a]
B	0.14 ± 0.02	0.19 ± 0.01	0.22 ± 0.02	0.25 ± 0.02	DMW[a]
Cu	0.07 ± 0.01	0.09 ± 0.01	0.11 ± 0.01	0.13 ± 0.02	DMW[a]
Mo	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	DMW[a]
EC[b]	0.97 ± 0.01	1.19 ± 0.01	1.46 ± 0.01	1.74 ± 0.01	DMW[a]

144 [a] demineralized water
145 [b] electrical conductivity (mS/cm)
146

147 2.4 Plant sampling and drying

148 The plants were harvested weekly in 3 replicants from each nutrient hydroponic system
149 throughout the vegetative cycle. Plants were selected randomly from each highlighted sector
150 1-3 (**Figure 1**). Subsequently, a random plant outside the sectors was moved to the resulting
151 space to minimize the possible edge effect (Antolinos et al., 2020; Malík et al., 2022). The
152 above-ground biomass of plants was clipped, weighed whole fresh, and then divided into
153 stems, leaves, and flowers which were again weighed fresh separately for each plant. Above-
154 ground plant organs were dried to constant moisture (8-10%) at 25°C and reweighed dry. The
155 reference amount of each plant part was then dried at 105°C to a constant weight to determine
156 the dry matter. Specific plant parts were always homogenized just before the analysis. Dried
157 flowers (including leaves up to the 4th week) were frozen in liquid nitrogen and ground in the
158 mortar and pestle. Dried stems and leaves (from the 5th week) were ground separately in an
159 automatic grinder.



160

161 **Fig. 1.** Plant harvesting sectors.

162

163 2.5 Elemental analysis using the dry decomposition (ashing) method

164 Homogenized weighed plant organs (0.5 g) in beakers were covered with a watch glass and
165 placed on a hot plate at 160°C where the temperature was gradually increased every hour,
166 over 4 h, to 350°C. The samples were then transferred to a muffle furnace, where remained at
167 a temperature of 450-500°C for 12 h. Subsequently, 1 mL of 65% nitric acid (HNO_3) was
168 added to the cooled beakers were placed on a hob at 120°C for 60 min. After evaporation, the
169 samples were annealed in an oven at 500°C for 90 min and finally suspended in a 1.5% HNO_3
170 solution with stirring in an ultrasonic bath. The measurement of the concentration of the
171 monitored elements was carried out using flame atomic absorption spectroscopy (FAAS) on a
172 Varian 280FS and inductively coupled plasma optical emission spectroscopy (ICP-OES) on a
173 Varian Vista-PRO device (Varian, Mulgrave, Australia). This method was used to determine
174 the content of macroelements (except nitrogen), microelements, and trace elements (Hoenig,
175 2003; Miholová et al., 1993).

176 2.6 Determination of nitrogen content using the Kjeldahl method

177 Weighed plant material (0.5 g) was inserted into the distillation tube. Subsequently, 2 g of
178 catalyst (a mixture of 100 g K_2SO_4, 1 g $CuSO_4$, and 0.1 g Selenium powder) were added, and
179 the samples were mineralized by boiling for 90 min at 420°C with 95% sulfuric acid (H_2SO_4).
180 After alkalization with sodium hydroxide (NaOH), free ammonia was steam distilled to
181 orthoboric acid (H_3BO_3). Its content was determined by hydrochloric acid (HCl) titration
182 (0.5 M) and then measured on a Gerhardt Vapodest 30s device (Königswinter, Germany)
183 (Baker and Thompson, 1992).

184 2.7 Extraction and measurement of phytocannabinoids

185 Cannabinoids from the ground homogenized flowers (including leaves up to the 4th week)
186 were extracted by an optimized dynamic maceration method (Brighenti et al., 2017). Weighed
187 samples (0.3 g) were mixed with 10 mL of 96% ethanol and macerated for 1 h at room
188 temperature under constant magnetic stirring at 300 rpm. Subsequently, the mixtures were
189 filtered under a vacuum, and the filtrates were collected. The flowers were removed from the
190 filter and then mixed with another 10 mL of solvent. This step was two times repeated, and
191 the filtrates were combined. Aliquots of 0.5 mL of each sample were diluted with 96%
192 ethanol to the volume of 10 mL and once more filtered through the nylon syringe filters
193 (0.22 μm) into the vials. Extracted samples were injected into a high-performance liquid
194 chromatography system equipped with a diode array detector (HPLC-DAD; Agilent 1260,
195 Agilent Technologies Inc., USA) and a Luna® C18 column (2) 250 × 3 mm, the particle size
196 of 3 μm (Phenomenex, USA). The isocratic mobile phase consisted of acetonitrile/water
197 (31:9, v/v) with 0.1% formic acid (v/v) and 0.1 M ammonium formate (without pH
198 adjustment). The flow rate was 0.55 mL/min, and the temperature was 37°C. The sample
199 injection volume was 8 μL, and UV detection was performed at 275 nm (Križman, 2019). The
200 instrument was externally calibrated using THCA, 0.3-100 mg/L, and other
201 phytocannabinoids, 0.3-10 mg/L (Sigma-Aldrich, Czech Republic) as the standards. Data
202 were analyzed with OpenLAB CDS, ChemStation Edition, Rev. C.01.5.

203 2.8 Extraction and measurement of terpenic compounds

204 Terpenic compounds from homogenized ripe flowers (7th-11th week of vegetation growth)
205 were extracted with hexane, and 1 mL was added to each sample (0.1 g). Pentadecane was
206 used as an internal standard to the final concentration of 1 mg/mL. The samples were vortexed
207 and then placed for 30 min into an ultrasonic bath. After that, samples were centrifuged and
208 filtered via polytetrafluoroethylene syringe filters (0.22 μm) into vials. Filtered samples
209 (1.5 μL) were injected into a gas chromatography system equipped with a flame ionization
210 detection (GC-FID; Agilent Technologies 7890A, Palo Alto, USA) and a DB5 column,
211 30 m × 0.25 mm × 0.25 μm film thickness. The inlet temperature was 230°C, the detector
212 temperature was 300°C, and the nitrogen flow rate was 1 mL/min. The initial temperature of
213 60°C was increased at a rate of 3.5°C/min till the temperature of 150°C was reached and
214 subsequently at a rate of 30°C/min till the final temperature of 300°C was reached. The
215 samples were further injected into the gas chromatography system equipped with mass
216 spectrometry (GC-MS; Agilent Technologies 5975C, Palo Alto, USA) and an HP-5MS
217 column, 30 m × 0.25 mm × 0.25 μm film thickness. The inlet temperature was 230°C, the

218 detector temperature was 300°C, and the helium flow rate was 1 mL/min. The initial
219 temperature of 60°C was increased at a rate of 3.5°C/min till the temperature of 150°C was
220 reached and subsequently at a rate of 30°C/min till the final temperature of 300°C was
221 reached. Compounds detected by GC-MS were identified by mass spectrum and relative
222 retention index comparing with published values of the National Institute of Standards and
223 Technology database and values for standards, limonene, β-myrcene, and β-caryophyllene
224 (Sigma-Aldrich, Czech Republic). Data from GC-FID revealed a relative concentration of
225 identified compounds according to the peak area of the monitored compound relative to the
226 total area of all detected compounds.

227 2.9 Statistical analysis

228 Data were subjected to one-way ANOVA with post-hoc Tukey's HSD test. Analysis was
229 performed using SPSS Statistics (version 25, 2017, IBM, Armonk, NY, USA).
230

231 **3. Results and discussion**

232 3.1 Nutrient consumption from nutrient solutions

233 Due to the reduction of ecological and economic costs concerning water management, the
234 need for recirculation and reuse of nutrient solutions is increasing. However, many cannabis
235 growers using hydroponics still drain once-used nutrient solutions into the waste and refill
236 them at almost daily intervals. Based on the lack of practical information in the scientific
237 literature regarding the management of nutrient solutions in the soilless cultivation of medical
238 cannabis, this experiment with different hydroponic systems was proposed. It aims to verify
239 whether it is possible to recirculate nutrient solutions without potential loss of yield of the
240 main secondary metabolites.

241 Variable nutrient hydroponic systems (RS and DS) resulted in different nutrient utilization
242 from nutrient solutions and induced changes in the medical cannabis plant tissue ionome.
243 **Table 2** shows the measured composition of the remaining nutrient solution after recirculation
244 at the end of each week. Approximately 80 liters of the fresh nutrient solution was always
245 mixed at the beginning of each week in the RS. **Table 3** describes the composition of the
246 waste solution from the hydroponic drain-to-waste system. Approximately 100 liters of the
247 fresh nutrient solution was always mixed at the beginning of each week in the DS. This fresh
248 nutrient solution was mixed within this volume 2-3 times a week according to the number of
249 cultivated plants. When comparing **Tables 2** and **3**, it can be seen that this is not necessary.
250 According to the composition of the nutrient solution remaining in the tank after recirculation

251 at the end of each week in **Table 2**, there were still enough nutrients left in the RS.
252 Maintaining high nutrient concentrations in a solution can cause excessive intake leading to
253 nutrient imbalance. The plants thus quickly take the daily dose of some nutrients while other
254 nutrients accumulate in the solution. Rapid depletion of some nutrients often causes toxic
255 amounts of these nutrients to be subsequently added to the solution (Bugbee, 2004; Ho and
256 Adams, 1995; Sambo et al., 2019).
257
258 **Table 2.** Nutrient composition of recirculation system remaining solution (mg/L).

Nutrients	Weeks									
	1	2	3	4	5	6	7	8	9	10-13
N	125.25 ± 1.72	138.50 ± 2.06	130.50 ± 1.21	123.75 ± 1.29	186.52 ± 1.56	195.25 ± 1.83	223.34 ± 2.24	245.51 ± 2.62	248.25 ± 1.93	WW[a]
P	26.70 ± 0.56	26.10 ± 0.71	47.90 ± 1.01	52.80 ± 0.57	66.40 ± 1.05	62.37 ± 1.26	71.32 ± 1.13	68.76 ± 0.85	65.34 ± 0.43	WW[a]
K	167.29 ± 1.42	202.08 ± 1.02	209.61 ± 3.56	310.24 ± 3.02	326.95 ± 3.28	368.36 ± 2.15	371.81 ± 2.46	427.07 ± 3.15	435.12 ± 3.03	WW[a]
Ca	133.02 ± 2.23	156.12 ± 1.53	136.32 ± 2.46	189.15 ± 1.85	164.69 ± 2.51	167.27 ± 2.44	196.14 ± 2.19	199.24 ± 1.94	201.45 ± 2.23	WW[a]
Mg	34.30 ± 0.68	39.60 ± 0.68	40.60 ± 0.69	52.25 ± 0.59	57.25 ± 0.87	54.91 ± 0.59	67.92 ± 0.75	70.72 ± 0.72	73.51 ± 0.67	WW[a]
S	29.82 ± 0.35	36.21 ± 0.51	39.63 ± 0.28	55.15 ± 0.72	57.31 ± 0.53	52.54 ± 0.48	69.76 ± 0.57	67.46 ± 0.47	65.55 ± 0.52	WW[a]
Fe	0.82 ± 0.02	1.12 ± 0.02	1.32 ± 0.03	1.58 ± 0.03	1.80 ± 0.04	1.79 ± 0.03	2.06 ± 0.04	2.09 ± 0.05	2.12 ± 0.04	WW[a]
Mn	0.43 ± 0.01	0.59 ± 0.01	0.72 ± 0.02	0.57 ± 0.01	0.58 ± 0.02	0.77 ± 0.02	0.67 ± 0.02	0.65 ± 0.03	0.63 ± 0.03	WW[a]
Zn	0.25 ± 0.01	0.30 ± 0.00	0.34 ± 0.00	0.43 ± 0.00	0.43 ± 0.01	0.41 ± 0.00	0.43 ± 0.02	0.41 ± 0.02	0.42 ± 0.01	WW[a]
B	0.01 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.00	WW[a]
Cu	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	WW[a]
Mo	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	WW[a]
volume[b]	52.82	53.13	47.22	40.49	35.55	34.76	30.81	28.98	23.80	WW[a]
pH[c]	6.30	6.63	6.08	6.08	6.05	5.95	5.96	5.96	6.36	WW[a]

259 [a] waste water (demineralized water with leached nutrients from expanded clay)
260 [b] volume of solution (L)
261 [c] pH value weekly averaged
262

263 **Table 3.** Nutrient composition of drain-to-waste system waste solution (mg/L).

Nutrients	Weeks								
	1	2	3	4	5	6	7	8	9	10-13
N	104.25 ± 1.85	120.07 ± 2.22	132.58 ± 0.51	144.54 ± 0.53	138.27 ± 2.04	152.16 ± 3.95	166.09 ± 2.12	166.00 ± 2.16	159.30 ± 2.86	WW[a]
P	29.88 ± 0.98	37.10 ± 0.80	41.85 ± 0.05	47.50 ± 0.80	40.70 ± 2.20	49.00 ± 1.40	49.10 ± 0.40	49.15 ± 0.15	50.00 ± 0.20	WW[a]
K	131.28 ± 2.28	160.26 ± 1.11	182.94 ± 4.14	212.84 ± 4.13	192.61 ± 3.08	215.31 ± 0.01	225.06 ± 3.18	226.83 ± 4.50	229.88 ± 2.63	WW[a]
Ca	102.96 ± 2.56	127.19 ± 0.61	138.52 ± 2.43	161.60 ± 1.22	142.16 ± 4.86	149.64 ± 3.04	154.92 ± 0.61	157.35 ± 1.82	159.78 ± 0.61	WW[a]
Mg	25.59 ± 0.52	31.05 ± 0.74	34.16 ± 0.16	38.89 ± 0.47	33.53 ± 0.79	37.11 ± 0.53	39.68 ± 0.11	39.74 ± 0.37	40.32 ± 0.32	WW[a]
S	22.55 ± 0.39	27.90 ± 0.60	33.15 ± 0.05	35.70 ± 0.70	33.25 ± 0.25	36.75 ± 0.05	38.90 ± 0.10	38.30 ± 0.40	38.65 ± 0.25	WW[a]
Fe	0.78 ± 0.03	1.00 ± 0.03	1.17 ± 0.02	1.36 ± 0.02	1.23 ± 0.04	1.40 ± 0.04	1.37 ± 0.01	1.37 ± 0.02	1.41 ± 0.02	WW[a]
Mn	0.58 ± 0.02	0.63 ± 0.02	0.74 ± 0.01	0.84 ± 0.01	0.72 ± 0.03	0.82 ± 0.02	0.77 ± 0.00	0.81 ± 0.01	0.83 ± 0.01	WW[a]
Zn	0.21 ± 0.01	0.28 ± 0.00	0.30 ± 0.00	0.34 ± 0.00	0.30 ± 0.01	0.33 ± 0.00	0.33 ± 0.00	0.33 ± 0.00	0.34 ± 0.00	WW[a]
B	0.11 ± 0.00	0.15 ± 0.00	0.20 ± 0.00	0.21 ± 0.00	0.21 ± 0.01	0.23 ± 0.00	0.23 ± 0.01	0.21 ± 0.01	0.21 ± 0.00	WW[a]
Cu	0.06 ± 0.00	0.07 ± 0.00	0.08 ± 0.00	0.12 ± 0.00	0.09 ± 0.00	0.12 ± 0.00	0.13 ± 0.00	0.13 ± 0.00	0.13 ± 0.00	WW[a]
Mo	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	WW[a]
volume[b]	90.12 ± 2.66	89.38 ± 3.13	86.88 ± 3.13	85.63 ± 2.13	84.38 ± 2.88	85.38 ± 3.38	81.13 ± 2.13	79.63 ± 3.13	77.25 ± 3.25	WW[a]

264 [a] waste water (demineralized water with leached nutrients from expanded clay)
265 [b] volume of solution (L)

266

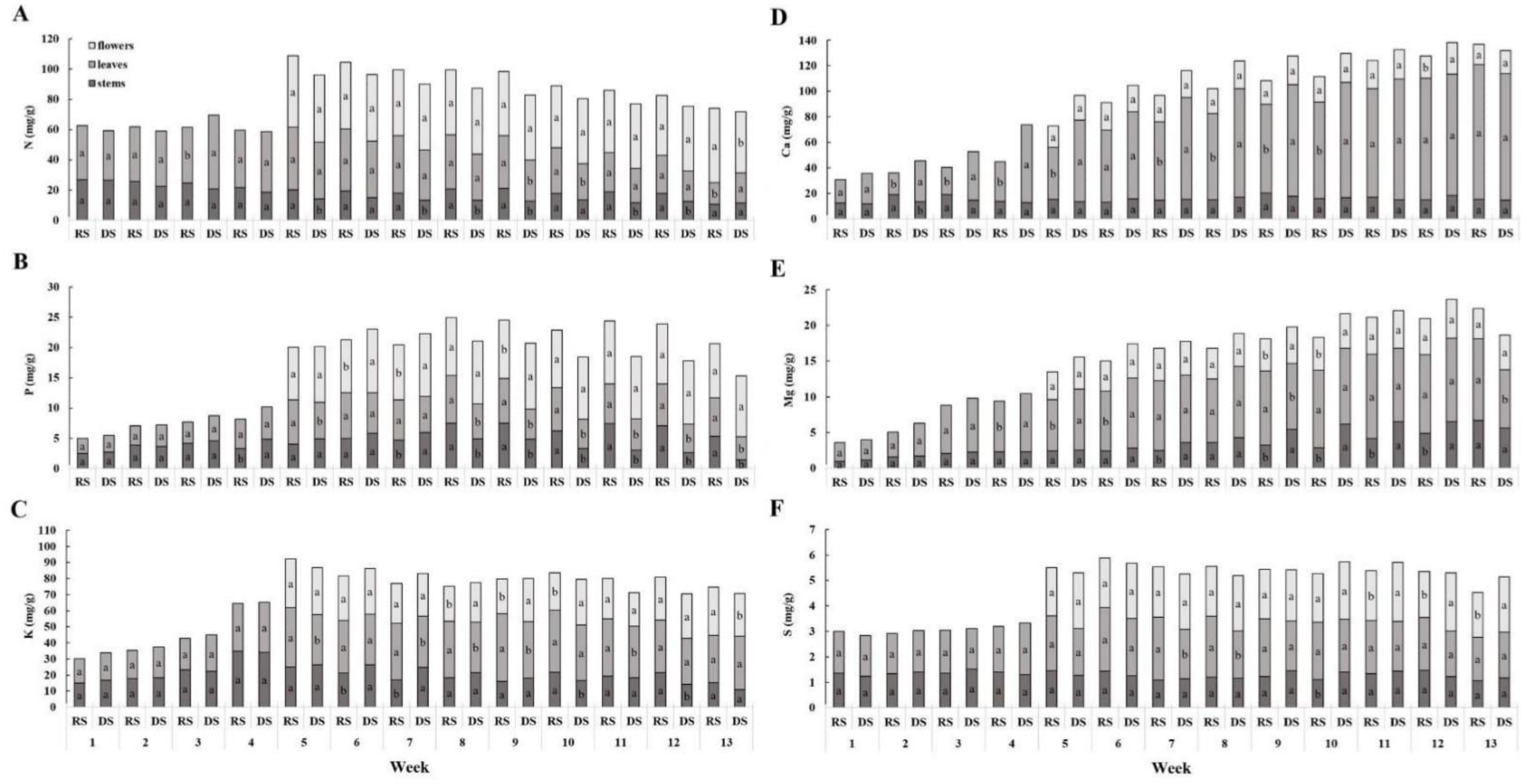
267 3.2 Plant tissue ionomes

268 Different hydroponic cultivation systems affected the concentrations of macro- and
269 microelements in the tissues of medical cannabis plants. Interactions between cations and
270 anions of nutrients during root cell membrane transport have been relatively well described.
271 However, fluctuations in the pH of the nutrient solution, as in the case of the RS, and the
272 larger amount of nutrients offered in the DS, could affect the accessibility of some nutrients
273 and the subsequent physiological and metabolic responses of the plants.

274 3.2.1 Macroelement content in above-ground plant organs

275 Macroelement content was generally low in stems and high in leaves and flowers (**Figure 2**).
276 The concentration of nitrogenous compounds (N) was lowest in stems and highest in flowers.
277 When comparing the macroelement ionome of medical cannabis grown in two different
278 hydroponic systems, RS and DS, significantly higher concentrations of N were observed in
279 several cases in above-ground organs. However, RS and DS most often differed significantly
280 in stem N concentrations at six weeks, specifically in the 5th, 7th, 8th, 9th, 11th, and 12th
281 weeks. In flowers, the variations in N concentration differed only in the last (13th) week. In
282 both cases, the RS variant reached higher N concentrations (**Figure 2A**). This was probably
283 due to pH fluctuation in the nutrient solution due to recirculation which, on average, increased
284 to pH 6.36 after 24 hours without adjustment. The increase in nutrient solution pH to a value

285 of 6.5 leads to higher absorption of the ammonium form of nitrogen (Dyhr-Jensen and Brix,
286 1996), which is also taken up faster by plants in hydroponics than the nitrate form, but is less
287 mobile. Therefore, nitrogen was only increased in the stems and probably also in the roots,
288 but these were not analyzed (Boschiero et al., 2018). The phosphorus compound content (P)
289 was highest in flowers and lower in leaves and stems. Most often, RS and DS differed
290 significantly in stem P concentrations from the 7th to the 13th week. The P content began to
291 be significantly higher in RS compared with DS from the 8th week, especially in stems and
292 leaves. In contrast, the flowers had lower P concentrations in RS compared with DS at three
293 weeks, in the 6th, 7th, and 9th weeks (**Figure 2B**). The higher concentration of P in RS was
294 also probably caused by the increased pH, as in the experiment of Kerwin et al. (2017).
295 Conversely, a higher pH of the nutrient solution can also cause the precipitation of phosphates
296 with calcium (Ca^{2+}) and magnesium (Mg^{2+}) into insoluble and unavailable salts in
297 hydroponics (Lee et al., 2017), which is shown by the overall lower contents in RS compared
298 with DS. Calcium (Ca) content was the lowest in the stems and the highest in the leaves, and
299 showed a cumulative trend depending on time. Most often, RS and DS differed significantly
300 in leaf Ca concentrations at eight weeks, specifically from the 2nd to the 5th and from the 7th
301 to the 10th weeks. In all these cases, the DS variant achieved higher Ca concentrations
302 (**Figure 2D**). A similar cumulative trend was also shown for the magnesium (Mg) content,
303 which was also highest in the leaves. Hydroponic systems most often differed significantly in
304 stem and leaf Mg concentrations in both cases at five different weeks (**Figure 2E**). The
305 potassium (K) content was highest in leaves and lower in flowers and stems. Hydroponic
306 systems most often differed significantly in K concentrations of the leaves, at five weeks,
307 specifically in the 5th, 7th, 8th, 9th, and 11th weeks (**Figure 2C**). The concentration of sulfur
308 compounds (S) was high in leaves and flowers. Most often, RS and DS differed significantly
309 in flower S content in the last three weeks. From the 11th to the 13th week, the DS achieved
310 higher S concentrations (**Figure 2F**). The concentrations of K and S were similar in both
311 hydroponic systems. This can be due to the ion-pairing nature of the counterions in the
312 solution. An anion that is taken up relatively slowly can reduce the rate of uptake of its
313 counterion, as observed for the effect of SO_4^{2-} on K^+ absorption (Marschner, 2012).



314

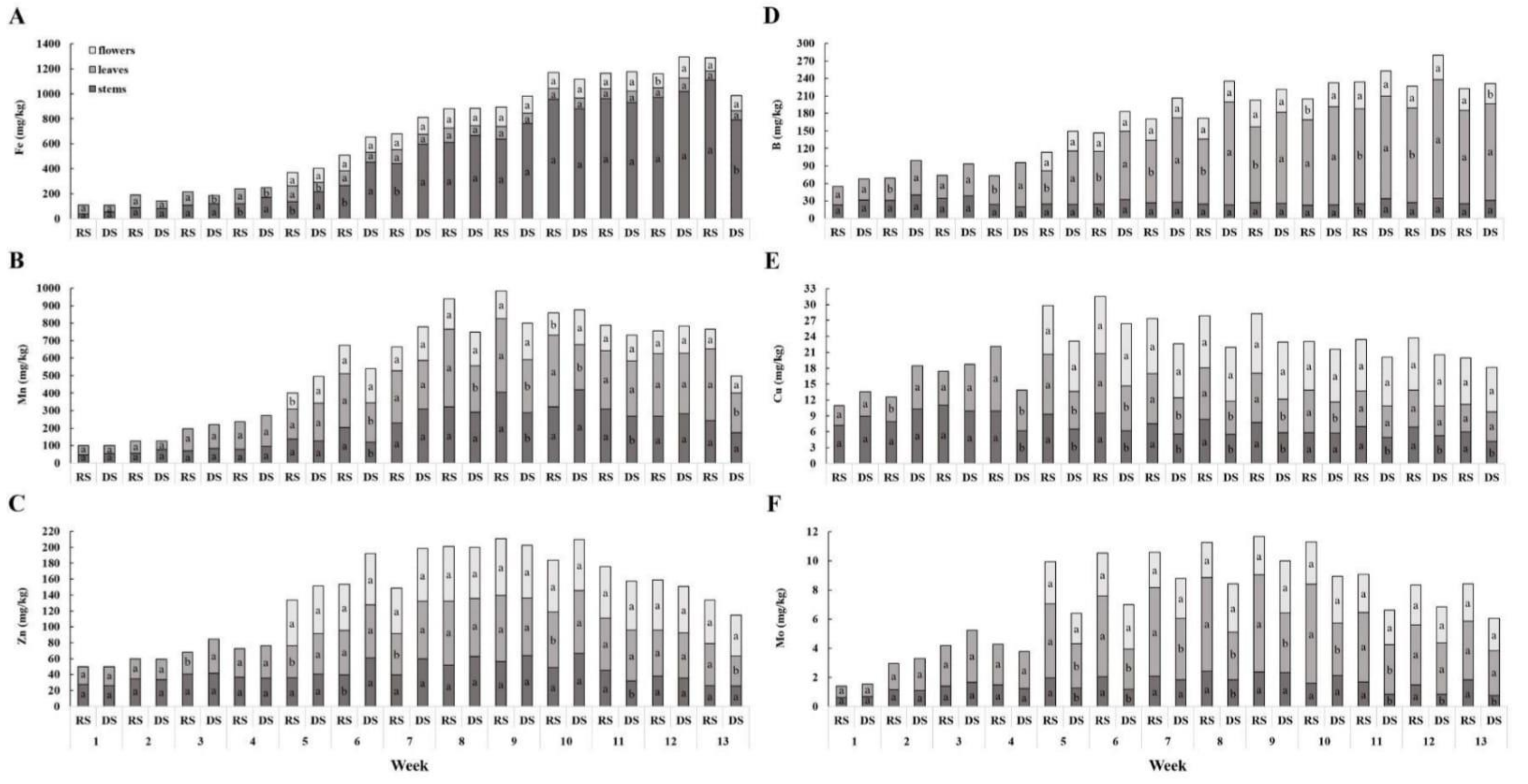
315 **Fig. 2.** Macroelement distribution among above-ground organs of medical cannabis plants
316 affected by a nutrient hydroponic system. Concentrations of N (A), P (B), K (C), Ca (D), Mg
317 (E), and S (F) for recirculation (RS) and drain-to-waste (DS) hydroponic systems throughout
318 the plant vegetation cycle in stems, leaves, and flowers. Data are means ± SE ($n = 3$).
319 Different lowercase letters inside bars represent significant differences within plant organs
320 between variants in a particular week by Tukey's HSD test at $\alpha = 0.05$.

321

322 3.2.2 Microelement content in above-ground plant organs

323 The microelement content was plant tissue-specific (**Figure 3**). The iron (Fe) content was the
324 highest in the stems, where it showed a cumulative trend depending on time. Fe showed an
325 accumulation trend similar to Ca and Mg only at lower concentrations, mainly in stems, due
326 to low plant mobility. The Fe contents were identical in both hydroponic systems, except for
327 the last (13th) week, when a significant decrease in the concentration in DS was recorded,
328 probably because the concentration reached a maximum from the earlier plant maturation
329 (**Figure 3A**). Manganese (Mn) concentration was lowest in flowers and high in leaves and
330 stems. Most often, RS and DS significantly differed in leaf Mn concentrations at five weeks,
331 specifically in the 6th, 8th, 9th, 10th, and 13th weeks. In all cases, the RS variant achieved
332 higher Mn concentrations (**Figure 3B**). The zinc (Zn) content was lowest in stems, and higher
333 in flowers and leaves. Most often, RS and DS differed significantly in leaf Zn concentrations

334 at five weeks, specifically in the 3rd, 5th, 7th, 10th, and 13th weeks. Except for the last week,
335 a higher concentration was achieved in DS (**Figure 3C**). According to Gillespie et al. (2020),
336 Mn and Zn content in hydroponically grown plants increased proportionally with increasing
337 pH. In dicotyledonous plants, the plasma membrane protein iron-regulated transporter 1
338 (IRT1) can, in addition to Fe (II), absorb and transport other bivalent cations, such as Mn^{2+}.
339 However, the transport of Mn^{2+} can be inhibited by Zn^{2+} since these two elements can
340 compete in this case (Korshunova et al., 1999). The boron (B) content was the highest in the
341 leaves, where it also showed a cumulative trend depending on time. Hydroponic systems
342 differed significantly in B concentrations in leaves at nine weeks, namely in the 2nd week,
343 from the 4th to the 9th week, and in the 11th and 12th weeks. In all these cases, the DS
344 variant achieved higher concentrations of B (**Figure 3D**). The absorption of boron is
345 relatively passive in plants and depends on the amount of dissolved boron ions (Dannel et al.,
346 2002). In DS, where the nutrient solution was replenished several times a week, the B supply
347 was much higher than in RS. The concentration of copper (Cu) was highest in flowers, but in
348 all plant organs from the 6th week, it showed a dilution effect depending on time. Most often,
349 RS and DS significantly differed in stem Cu concentrations at nine weeks, specifically from
350 the 4th to the 9th week and from the 11th to the 13th week. In all cases, the RS variant
351 achieved higher Cu concentrations (**Figure 3E**). This was due to the mutual competition of
352 Cu/Zn and similar uptake mechanisms (Stuckey et al., 2021). Molybdenum (Mo) content was
353 lowest in stems and highest in leaves. Hydroponic systems most often differed significantly in
354 leaf Mo concentrations at seven weeks, specifically from the 5th to the 11th week. In all these
355 cases, the RS variant achieved higher Mo concentrations (**Figure 3F**). This was due to the
356 fluctuation and increase of pH in RS compared with the stable pH in DS. At higher pH,
357 molybdenum becomes more soluble and is accessible to plants mainly in its anionic form as
358 MoO_4^{2-}. At pH< 5 it occurs mainly as $HMoO_4^-$, which is less accessible (Kaiser et al., 2005;
359 Lawson-Wood et al., 2021; Smedley and Kinniburgh, 2017).



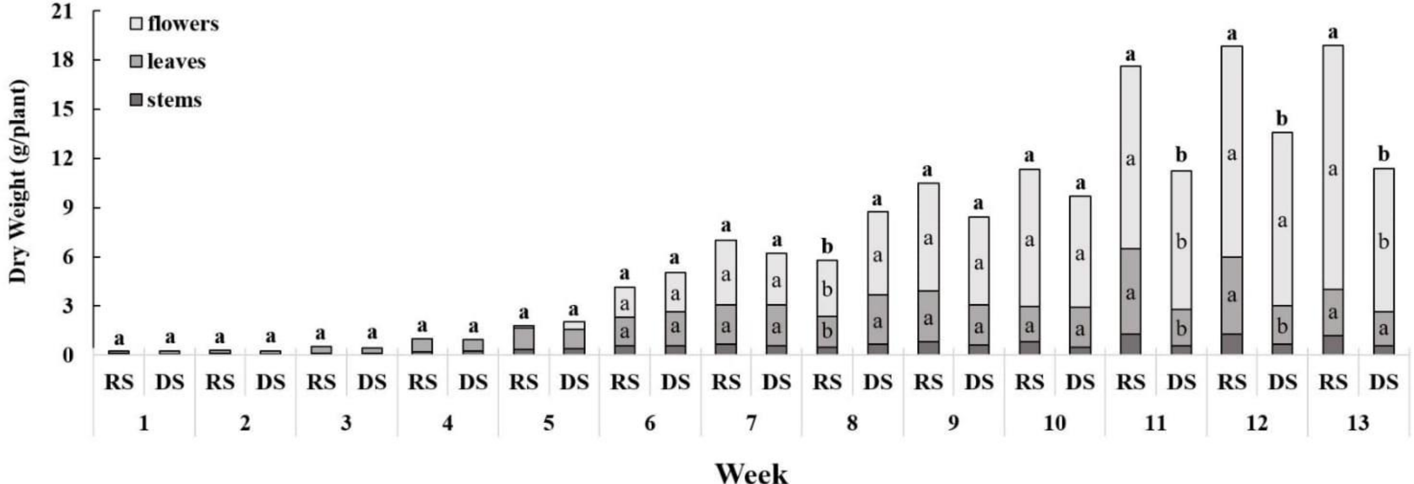
360

361 **Fig. 3.** Microelement distribution in above-ground organs of medical cannabis plants affected
362 by a nutrient hydroponic system. Concentrations of Fe (A), Mn (B), Zn (C), B (D), Cu (E),
363 and Mo (F) for recirculation (RS) and drain-to-waste (DS) hydroponic systems throughout the
364 plant vegetation cycle in stems, leaves, and flowers. Data are means ± SE ($n = 3$). Different
365 lowercase letters inside bars represent significant differences within plant organs between
366 variants in a particular week by Tukey's HSD test at $\alpha = 0.05$.

367

368 3.3 Dry above-ground biomass yields

369 The different nutrient hydroponic systems (RS and DS) caused a change in the growth of
370 medical cannabis plants. Until the 5th week, the increase in biomass was relatively slow; but
371 from the 6th week, it increased sharply. The most significant weekly increase was recorded in
372 dry weight of flowers. The increase in biomass of stems, leaves, and flowers was almost
373 identical in both hydroponic systems until the 10th week. In the last three weeks, specifically
374 from the 11th to the 13th week, the biomass, especially of the flowers, began to differ
375 significantly. This was probably caused by increased mobilization and translocation of
376 substances to leaves and flowers from roots (Ludewig and Frommer, 2002), where were
377 previously accumulated due to nutrient supply (**Figure 2**, **3**). The maximum yield of dry
378 flowers was achieved in DS in the 12th week but in RS in the last (13th) week (**Figure 4**).



379

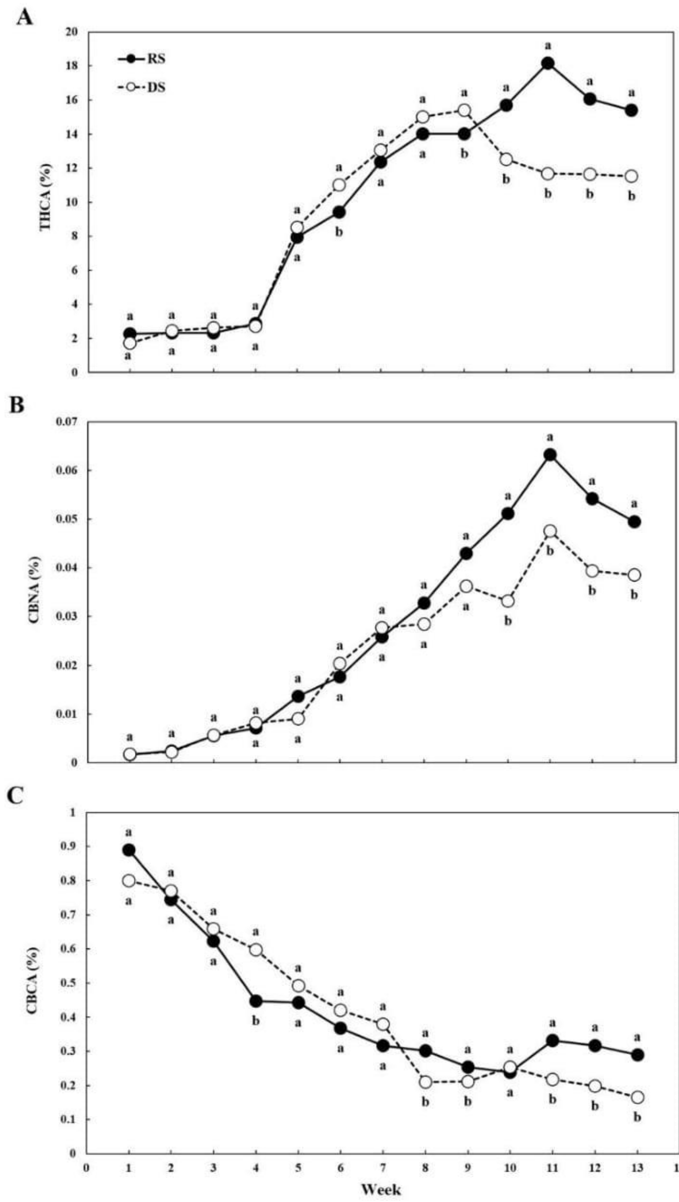
380 **Fig. 4.** The effect of nutritional hydroponic systems on medical cannabis plant above-ground
381 biomass. Dry biomass of stems, leaves, and flowers in recirculation (RS) and drain-to-waste
382 (DS) hydroponic systems. Data are means ± SE ($n = 3$). The different small letters inside the
383 bars and small bold letters above the bars represent significant differences within the medical
384 cannabis plant organs (leaves and flowers) and the whole plant biomass between the variants
385 in a particular week by Tukey's HSD test at $\alpha = 0.05$.

386

387 3.4 Cannabinoid concentration in flowers

388 Different nutrient hydroponic growing systems affected the development and subsequent
389 concentration of THCA, cannabinolic acid (CBNA), and cannabichromenic acid (CBCA) in
390 cannabis plant flowers (**Figure 5**). The concentration of THCA in leaves and flowers
391 increased slowly in both variants (RS and DS) up to the 4th week. From the 5th week, it
392 started to increase sharply because only the flowers were analyzed. In RS and DS, THCA
393 concentrations began to differ significantly from the 9th to the 13th week. In DS, it reached its
394 maximum (15.4 %) in the 9th week, but in RS, it reached its maximum (18.2 %) in the 11th
395 week (**Figure 5A**). After taking into account the volume of the nutrient solution and the fact
396 that the fresh nutrient solution was mixed 2-3 times a week in the DS. This could be due
397 either to increased abiotic stress from high doses of nutrients in the DS (Gepstein and Glick,
398 2013; Gong et al., 2020) or, on the contrary, a higher supply of nutrients in the DS that
399 ensured optimal fertilization, which can shorten the maturation time of cannabis (Caplan et
400 al., 2017b). This second hypothesis was partially supported by the lower concentration of
401 CBNA, which is the oxidation product of THCA, in DS compared with RS. The mutual ratio
402 of Cu to Zn probably also influenced the increased oxidative stress in RS. A high copper
403 concentration causes abiotic stress in plants, but a high zinc concentration can reverse this

404 effect. A higher Cu content was recorded in plants grown in RS and, conversely, a higher Zn
405 content in plants in DS (Thounaojam et al., 2014; Upadhyay and Panda, 2010). In RS and DS,
406 CBNA concentrations began to differ significantly from the 10th to the 13th week. In the 11th
407 week, CBNA peaked for both variants, but the concentrations significantly differed with RS
408 by 33% compared with DS (**Figure 5B**). The CBCA concentration showed the opposite time-
409 dependent trend to the THCA and CBNA concentrations. The CBCA concentrations were
410 similar in RS and DS treatments and began to differ significantly in the 8th, 9th, and from the
411 11th to 13th weeks (**Figure 5C**). The CBCA concentration decreased with time in both
412 hydroponic systems, which is a common phenomenon since the content of this cannabinoid
413 decreases with plant age (Morimoto et al., 1997).



414

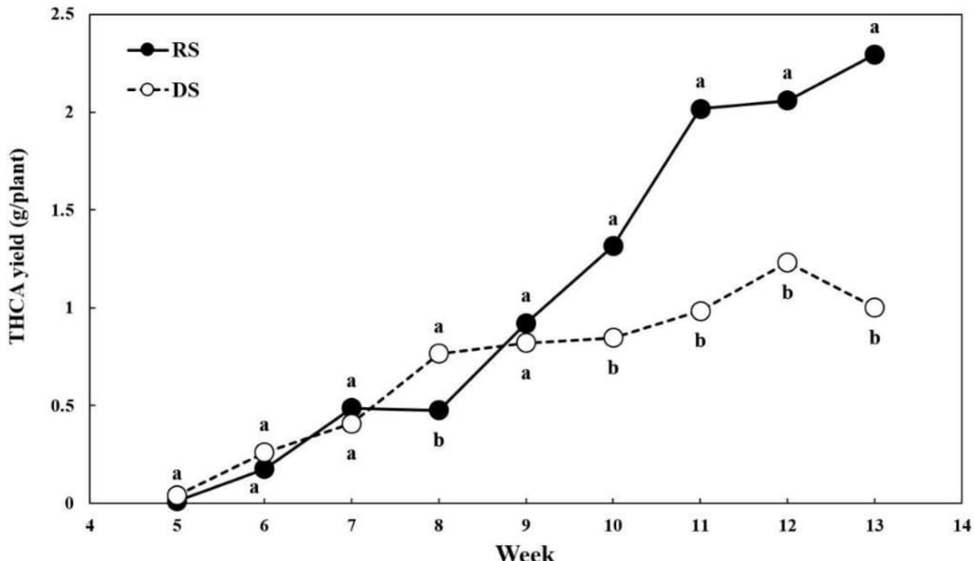
415 **Fig. 5.** Concentrations of tetrahydrocannabinolic acid (THCA), cannabinolic acid (CBNA),
416 and cannabichromenic acid (CBCA) in the flowers of medical cannabis plants grown with

417 different nutritional hydroponic systems. THCA concentration (A), CBNA concentration (B),
418 and CBCA concentration (C) of cannabis flowers cultivated in recirculation (RS) and drain-
419 to-waste (DS) hydroponic systems. The whole flowers of the plant were analyzed. Data are
420 means ± SE ($n = 3$). Different bold small letters represent significant differences in
421 cannabinoid concentration between the variants in a particular week by Tukey's HSD test at
422 $\alpha = 0.05$.

423

424 ## 3.5 Tetrahydrocannabinolic acid (THCA) yields

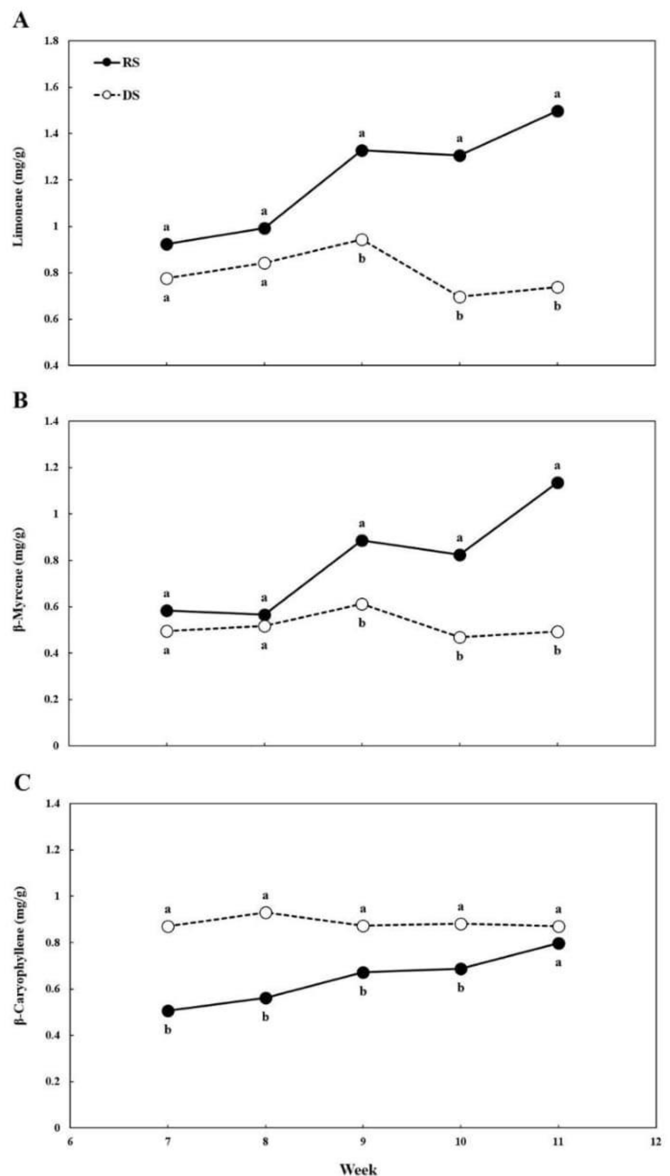
425 The most concentrated cannabinoid in this medical cannabis chemical phenotype I was
426 THCA. The combination of THCA concentration and flower dry weight was reflected in
427 THCA yield. In dried flowers, THCA yield per plant was measured weekly in both nutrient
428 hydroponic systems. An almost linear dependence of THCA yield on time was seen in RS.
429 For both RS and DS, THCA yields were almost identical up to the 9th week. But began to
430 differ significantly in the last vegetation weeks, specifically from the 10th to the 13th week.
431 The yield of THCA in DS reached its maximum in the 12th week, but in RS in the 13th week.
432 When comparing both hydroponic cultivation systems in the weeks of maximum THCA yield
433 (13th week for RS and 12th week for DS), the THCA yield was almost twice as high in RS.
434 This could be due to increased abscisic acid (ABA) production in response to stress, which
435 slows plant growth and increases THCA production, concentration, and yield (Caplan et al.,
436 2019; Mansouri et al., 2009). The enormous significant difference (129 %) between both
437 hydroponic systems was achieved in the last (13th) week with the RS (**Figure 6**).



438

439 **Fig. 6.** Tetrahydrocannabinolic acid (THCA) yield per plant affected by nutritional
440 hydroponic systems. The whole flowers of the plant were analyzed. Data are means ± SE

441 ($n = 3$). Different bold small letters represent significant differences in THCA yield per plant
442 between the variants in a particular week by Tukey's HSD test at $\alpha = 0.05$.
443
444 3.6 Terpenic compound concentration in flowers
445 Hydroponic cultivation systems also affected the concentration of limonene, β-myrcene, and
446 β-caryophyllene in flowers (**Figure 7**). Limonene concentration in RS gradually increased
447 with time and peaked in the 11th week (1.50 mg/g). In DS, it peaked in the 9th week (0.94
448 mg/g). The most significant difference in limonene concentration between the two variants
449 was 103 % achieved in the 11th week (**Figure 7A**). β-myrcene levels had a similar time-
450 dependent trend as limonene concentrations in both variants. In DS, it peaked in the 9th week
451 (0.61 mg/g), and in RS, it peaked in the 11th week (1.14 mg/g). The most significant
452 difference in β-myrcene concentration between the two variants was 130 %, achieved in the
453 11th week (**Figure 7B**). The content of the monoterpenes, limonene, and β-myrcene, showed
454 the same trend in the measured weeks, specifically from the 7th week to the 11th week in both
455 hydroponic cultivation systems as THCA concentration (**Figure 7A**, **B**, and **5A**). This was
456 consistent with the finding of Aizpurua-Olaizola et al. (2016) for cannabis chemical
457 phenotype I. The reason may be that monoterpenes are biosynthesized in the same glandular
458 trichomes and partly by a similar biochemical pathway as cannabinoids, consisting of
459 alkylresorcinol and monoterpene groups (Booth et al., 2017; Livingston et al., 2020;
460 Sirikantaramas et al., 2007). On the contrary, the sesquiterpene, β-caryophyllene, showed a
461 higher and constant content in DS in almost all measured weeks (**Figure 7C**). β-
462 caryophyllene concentration in DS peaked in the 8th week (0.93 mg/g), and in RS, it peaked
463 in the 11th week (0.80 mg/g). The most significant difference in β-caryophyllene
464 concentration between the two variants was 72 % achieved in the 7th week (**Figure 7C**). This
465 difference between the content of monoterpenes and sesquiterpenes may be because the two
466 biosynthetic pathways contribute to the synthesis of plant terpenes in early steps. First, the
467 cytosolic mevalonic acid (MVA) pathway is involved in the biosynthesis of sesquiterpenes
468 and triterpenes. The second, plastid-localized methylerythritol phosphate (MEP) pathway is
469 involved in synthesizing monoterpenes, diterpenes, and tetraterpenes. These pathways are
470 regulated by different substrates (Bouvier et al., 2005; Eisenreich et al., 1998). This was
471 probably also influenced by ABA, the concentration of which, as already indicated, could be
472 increased in RS and which reduces the content of sesquiterpenes in cannabis plants (Mansouri
473 and Asrar, 2012).



474

475 **Fig. 7.** Concentration of terpenic compounds in flowers of medical cannabis plants affected
476 by nutritional hydroponic systems. Limonene concentration (A), β-myrcene concentration
477 (B), and β-caryophyllene concentration (C) of cannabis flowers cultivated in recirculation
478 (RS) and drain-to-waste (DS) hydroponic systems. The whole flowers of the plant were
479 analyzed. Data are means ± SE ($n = 3$). Different bold small letters represent significant
480 differences in terpenic compound concentration between the variants in a particular week by
481 Tukey's HSD test at $\alpha = 0.05$.

482

483 **4. Conclusions**

484 In this study, were compared two different nutrient hydroponic systems, a recirculation (RS)
485 and a drain-to-waste system (DS), and individual effects on the ionome, growth, and content
486 of natural compounds from chemotype I medical cannabis plants. Plants grown in DS

487 accumulated more calcium and magnesium in leaves and flowers and also more zinc and
488 boron in leaves. With regard to THCA concentration, these plants matured two weeks earlier
489 than the plants grown in RS, which was reflected in above-ground biomass, especially in
490 flowers, and subsequently also in the yield of THCA from the plants. The content of
491 monoterpenes, specifically limonene and β-myrcene, was also lower, but a significantly
492 higher concentration of the sesquiterpene, β-caryophyllene, was achieved with the DS system.
493 Plants cultivated with the RS system accumulated more nitrogen and phosphorus in the stems
494 and more manganese, copper, and molybdenum in the leaves. Due to later ripening, a higher
495 concentration of THCA and above-ground biomass was achieved, which significantly affected
496 the subsequent yield of THCA. The content of monoterpenes was also higher in the flowers of
497 these plants. This study clearly shows the advantages and disadvantages of different
498 nutritional hydroponic cultivation systems in growing medical cannabis. In RS, higher yields
499 of the main cannabinoid of medical cannabis chemotype I, THCA, were achieved with much
500 lower total water and nutrient consumption, and with the longer cultivation period, a higher
501 concentration of CBNA, and a lower concentration of the sesquiterpene, β-caryophyllene. On
502 the contrary, DS enabled better control over the nutrient solution with a stable delivery of the
503 exact concentration of nutrients and probably accelerated plant maturation, but at the cost of
504 higher water and fertilizer consumption and a significantly lower total yield of monoterpenes
505 and THCA. From an economic point of view, it would be interesting for the horticultural
506 industry to carry out similar studies with the continuous replenishment of limiting nutrients
507 and balancing the pH of the nutrient solution until it is eventually consumed.
508

509 **Declaration of Competing Interest**

510 The authors declare that the research was conducted without any commercial or financial
511 relationships that could be construed as a potential conflict of interest.
512

513 **Acknowledgments**

514 We thank Dr. Lucie Malá for creating the graphical abstract with BioRender.com and Dr.
515 Gary Bentley of On the Mark Scientific Editing for improving the manuscript's English.
516

517 **Funding**

518 This research was supported by the European Regional Development Fund (project
519 NUTRISK no. CZ.02.1.01/0.0/0.0/16_019/0000845).
520

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4.5. Selective Cytotoxicity of Medical Cannabis (*Cannabis sativa* L.) Extracts Across the Whole Vegetation Cycle Under Various Hydroponic and Nutritional Treatments

Autoři: Malík M, Doskočil I, Pavlík J, Ulman M, Praus L, Kouřimský P, Lampová B, Kuklina A, Tlustoš P

Rok publikace: 2022

Časopis: Cannabis and Cannabinoid Research **0**:1-12

Cannabis and Cannabinoid Research
Volume X, Number X, 2022
© Mary Ann Liebert, Inc.
DOI: 10.1089/can.2022.0243

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Selective Cytotoxicity of Medical Cannabis (*Cannabis sativa* L.) Extracts Across the Whole Vegetation Cycle Under Various Hydroponic and Nutritional Treatments

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Abstract

Introduction: The use of *Cannabis sativa* L. in health care requires stringent care for the optimal production of the bioactive compounds. However, plant phenotypes and the content of secondary metabolites, such as phytocannabinoids, are strongly influenced by external factors, such as nutrient availability. It has been shown that phytocannabinoids can exhibit selective cytotoxicity against various cancer cell lines while protecting healthy tissue from apoptosis.

Research Aim: This study aimed to clarify the cytotoxic effect of cannabis extracts on colorectal cell lines by identifying the main active compounds and determining their abundance and activity across all developmental stages of medical cannabis plants cultivated under hydroponic conditions.

Materials and Methods: Dimethyl sulfoxide extracts of medical cannabis plants bearing the genotype classified as chemotype I were analyzed by high-performance liquid chromatography, and their cytotoxic activity was determined by measuring cell viability by methylthiazolyldiphenyl-tetrazolium bromide assay on the human colon cancer cell lines, Caco-2 and HT-29, and the normal human epithelial cell line, CCD 841 CoN.

Results: The most abundant phytocannabinoid in cannabis extracts was tetrahydrocannabinolic acid (THCA). Its maximum concentrations were reached from the 7th to the 13th plant vegetation week, depending on the nutritional cycle and treatment. Almost all extracts were cytotoxic to the human colorectal cancer (CRC) cell line HT-29 at lower concentrations than the other cell lines. The phytocannabinoids that most affected the cytotoxicity of individual extracts on HT-29 were cannabigerol, Δ^9-tetrahydrocannabinol, cannabidiol, cannabigerolic acid, and THCA. The tested model showed almost 70% influence of these cannabinoids. However, THCA alone influenced the cytotoxicity of individual extracts by nearly 65%.

Conclusions: Phytocannabinoid extracts from plants of the THCA-dominant chemotype interacted synergistically and showed selective cytotoxicity against the CRC cell line, HT-29. This positive extract response indicates possible therapeutic value.

Keywords: *Cannabis sativa* L.; cytotoxic activity; hydroponics; phytocannabinoids; plant nutrition; medical cannabis

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Introduction

Colorectal cancer (CRC) is one of the most common cancers worldwide. Despite the fact that health systems have been improved and screening programs have been implemented recently, every year, one to two million new cases are diagnosed, making CRC the third most common cancer diagnosis and the fourth most common cause of cancer-related deaths.[1] Thus, many scientific teams are trying to find new active compounds with cytostatic and cytotoxic effects.

Studies have already shown that phytocannabinoids and prenylated polyketides of mixed biosynthetic origin[2] can prevent proliferation, angiogenesis, and metastasis, and induce apoptosis in various types of human cancers, including breast,[3] pancreatic,[4] prostate,[5] and intestinal.[6,7] Acting through cannabinoid receptor and nonreceptor signaling pathways, phytocannabinoids exhibit specific cytotoxicity against tumor cells and simultaneously protect healthy tissue from apoptosis.[8] Until recently, phytocannabinoids had been predominantly used to treat nausea and vomiting in cancer patients undergoing chemotherapy. There is growing experimental evidence *in vitro* and in animal models supporting the anticancer activity of individual cannabinoids through modulation of key cell signaling pathways involved in the control of cancer cell proliferation and survival, inhibition of angiogenesis, and reduction of metastasis in various types of tumors.[9,10]

Nevertheless, their utilization in oncology is likely to be limited, for now, because clinical evidence is still lacking. Research is hampered by variability and insufficient standardization in trial design, drug formulation, and pharmacodynamics.[11,12] The success of such treatments will depend on the dose, the individual, the tumor stage, and many other circumstances.[13,14]

The use of cannabis in health care and pharmacy puts strict demands on growers raising plants with optimal production of the required compounds.[15] Cultivation can be done either by growing in soil or hydroponically.[16] In hydroponic cultivation, nutrients are supplied in an aqueous solution directly to the plant's roots fixed in an inert growing medium.[17] At present, basic research information on regulation of the biosynthesis of secondary metabolites of *Cannabis sativa* L. is lacking due to legal restrictions in most countries.[18] Concerning internal and external factors influencing the phytocannabinoid spectrum, the main determining internal factors are the plant's genotype and vegetation or harvest phase.[19] However, plant phenotypes are also strongly influenced by external factors such as light,[20] carbon dioxide level,[21] irrigation,[22] and nutrition.[23]

In this study, we focused on the cytotoxicity of extracts of hydroponically grown medical cannabis of chemotype I, which had a Δ^9-tetrahydrocannabinolic acid/cannabidiolic acid (THCA/CBDA) ratio ($>>1.0$), at different stages of plant maturity collected throughout the vegetative cycle.

The following hypotheses were proposed: (1) the spectrum and concentration of individual phytocannabinoids in extracts will change during the vegetation cycle of plants cultivated under hydroponic conditions; (2) cannabis extracts will exhibit selective cytotoxicity on specific human intestinal cell lines; and (3) the cytotoxicity of individual extracts will vary during the plant's vegetation cycle cultivated under hydroponic conditions.

To test the hypotheses on a sufficiently large dataset and precisely control the expected responses, we monitored the cytotoxicity of cannabis extracts on three cell lines within 13 vegetation weeks of hydroponically grown cannabis in four nutrient treatments and two nutrient systems. The first hydroponic system was a recirculating nutrient solution, and the second was a "drain-to-waste" system.

Materials and Methods

Plants and treatments

The plants used in the experiments came from the vegetative propagation of mother plants of the medical cannabis genotype with the working name, "McLove," classified as chemotype I, which has a high THCA/CBDA ratio ($\gg$1.0). The fresh cuttings were cultivated for 21 days in rockwool cubes. Rooted clones were moved to a growing room, where test plants were grown hydroponically in Euro Pebbles (expanded clay) on tables in a room with controlled light, temperature, and relative humidity. Plant density was 27.5 plants per m^2 (55 plants/table/treatment). The light mode was set to 18 h of light and 6 h of darkness. The vegetative phase lasted 7 days, after which the cultivation regimen was adjusted to the generative phase. The light period was reset to 12 h light and 12 h dark.

Plants were subjected to three enhanced nutritional treatments with two different nutrient cycles, recirculation, and drain to waste, compared with control treatment A (AT) (Table 1). In the recirculation cycle (1C), fresh nutrient solution was added every week and recirculated for 1 week with no addition of other nutrients, and the pH was adjusted to 5.9 daily.[24]

Table 1. Nutrient Composition of Control (AT) and Enhanced Treatments (BT, CT, DT): Solutions (mg/L)

		Weeks						
Treatments	**Elements**	**1**	**2**	**3**	**4**	**5**	**6–9**	**10–13**
A[a], C[c] B[b], D[d]	N	100 ± 1	115 ± 1 302 ± 2	129 ± 2 333 ± 3	150 ± 2 352 ± 3	129 ± 2 332 ± 3	150 ± 2 353 ± 3	DMW[f] DMW[f]
A[a], B[b] C[c], D[d]	P	32.2 ± 0.5	39.9 ± 0.6	44.0 ± 0.8	51.9 ± 0.6	44.0 ± 0.8 92.0 ± 1.8	51.9 ± 0.7 92.9 ± 1.8	DMW[f] DMW[f]
A[a], B[b] C[c], D[d]	K	125 ± 2	151 ± 1	174 ± 2	194 ± 2	174 ± 2 258 ± 2	194 ± 2 266 ± 3	DMW[f] DMW[f]
all[e]	Ca	98.3 ± 1.2	120 ± 2	133 ± 2	147 ± 2	133 ± 1	144 ± 2	DMW[f]
all[e]	Mg	25.3 ± 0.3	30.9 ± 0.3	34.0 ± 0.5	40.0 ± 0.4	33.0 ± 0.4	38.6 ± 0.6	DMW[f]
A[a], C[c] B[b], D[d]	S	21.5 ± 0.2	26.0 ± 0.9 51.1 ± 0.6	30.9 ± 0.4 56.0 ± 0.5	33.7 ± 0.3 62.0 ± 0.9	30.8 ± 0.4 55.9 ± 0.6	34.9 ± 0.3 62.0 ± 0.8	DMW[f] DMW[f]
A[a], B[b] C[c], D[d]	Fe	0.92 ± 0.10	1.13 ± 0.08	1.20 ± 0.09	1.47 ± 0.08	1.20 ± 0.10 12.2 ± 0.3	1.46 ± 0.08 13.7 ± 0.9	DMW[f] DMW[f]
all[e]	Mn	0.65 ± 0.05	0.74 ± 0.04	0.80 ± 0.06	1.00 ± 0.08	0.76 ± 0.07	0.94 ± 0.09	DMW[f]
all[e]	Zn	0.21 ± 0.05	0.28 ± 0.01	0.28 ± 0.03	0.35 ± 0.04	0.29 ± 0.05	0.33 ± 0.05	DMW[f]
all[e]	Cu	0.07 ± 0.01	0.08 ± 0.01	0.10 ± 0.01	0.13 ± 0.01	0.11 ± 0.02	0.11 ± 0.01	DMW[f]
all[e]	B	0.16 ± 0.01	0.19 ± 0.02	0.21 ± 0.01	0.25 ± 0.02	0.22 ± 0.02	0.24 ± 0.02	DMW[f]
all[e]	Mo	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.01	0.02 ± 0.01	DMW[f]
A[a] C[c] B[b] D[d]	EC	0.97 ± 0.01	1.20 ± 0.01 1.38 ± 0.01	1.46 ± 0.01 1.71 ± 0.01	1.74 ± 0.01 2.14 ± 0.01	1.46 ± 0.01 2.05 ± 0.02 1.71 ± 0.01 2.30 ± 0.02	1.74 ± 0.01 2.34 ± 0.06 2.14 ± 0.01 2.74 ± 0.02	DMW[f] DMW[f] DMW[f] DMW[f]

[a]Control treatment (AT).
[b]Enhanced treatment B (BT) with the addition of amino acids.
[c]Enhanced treatment C (CT) with the addition of P, K, and Fe.
[d]Enhanced treatment D (DT) with the addition of amino acids, P, K, and Fe.
[e]Control (AT) and enhanced treatments (BT, CT, DT).
[f]Demineralized water.

In the drain-to-waste nutrient cycle (2C), the spent solution went to a separate waste tank and was no longer mixed with a fresh nutrient solution. The enhanced treatments were set up for both nutritional cycles. The enhanced treatment B (BT) received an amino acid biostimulant (composition previously described)[25] added from the 2nd week for the last 24 h at a volume of 2 mL/L before changing the nutrient solution. The enhanced treatment C (CT) received increased amounts of P (P_2O_5), K (K_2O), and Fe (chelated) added from the 5th week. The enhanced treatment D (DT) received a mixture of the two enhanced treatments (BT and CT) (Table 1). The new nutrient solution was prepared with reverse osmosis water every 7 days from the first day of the experiment. From the 10th week, plants were irrigated with demineralized water.

Sampling of plant material

Three plants were harvested continuously from each treatment group every 7 days during the entire vegetative cycle. Plant samples were divided into leaves, stems, and flowers. The flowers were then separately dried at 25°C to constant moisture (8–10%). A reference amount was dried at 105°C to constant weight to determine the dry matter. Subsequently, the flowers (including the leaves until the 4th week) were frozen in liquid nitrogen and ground in a mortar and pestle.

Extraction and identification of phytocannabinoids

Phytocannabinoids from homogenized flowers (including the leaves until the 4th week) were extracted by the

optimized method of dynamic maceration.[26] Samples (0.30 g) from each experimental group were mixed with 10 mL of 96% ethanol and macerated for 1 h at room temperature with constant stirring at 300 rpm. Mixtures were then filtered, and the filtrates were collected. The flowers were removed from the filter and mixed with another 10 mL of solvent. This step was repeated twice, and the filtrates were pooled. Aliquots of 20 mL of each sample were dried in a vacuum evaporator (Heidolph 4000, Germany) to constant weight.

Subsequently, the dried matter was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 8.129 mg/mL. Aliquots of 0.5 mL of each sample were diluted to 10 mL with DMSO and filtered through a nylon syringe filter (0.22 μm) into vials. Samples of the extracts were injected into a high-performance liquid chromatography system equipped with diode array detection (Agilent 1260; Agilent Technologies, Inc., USA) and a Luna® C18 column (2) 250×3 mm^2, particle size 3 μm (Phenomenex, USA).

The isocratic mobile phase consisted of acetonitrile/H_2O (31:9, *v*/*v*) with 0.1% HCOOH (*v*/*v*) and 0.1 M NH_4HCO_2 (without pH adjustment). The flow rate was 0.55 mL/min and temperature was 37°C. The sample injection volume was 8 μL, and UV detection was at 275 nm.[27] The instrument was externally calibrated using THCA from 0.3 to 100 mg/L and other phytocannabinoids, 0.3–10 mg/L (Sigma-Aldrich, Czech Republic) as standards. Data were analyzed using OpenLAB CDS software, ChemStation Edition, Rev. C.01.5, and are presented as mean ± standard deviation.

Cell cultures

Human CRC cell lines Caco-2 (ATCC HTB 37), HT-29 (ATCC HTB 38), and normal human colon epithelial cell line CCD 841 CoN (ATCC CRL 1790) (ATCC, Rockville, MD, USA) were cultured in Eagle's Minimal Essential Medium (EMEM) supplemented with 10% fetal bovine serum, 1% sodium bicarbonate, 1% sodium pyruvate, 5 mM glutamine, 1% MEM nonessential amino acids (without CCD 841 CoN), and 1% penicillin–streptomycin solution (10,000 units/mL of penicillin and 10 mg/mL of streptomycin) (Biowest, Nuaille, France). Cells were incubated at 37°C with 5% CO_2 and media were replenished every 2–3 days, with passaging every 7 days.

Cytotoxicity assay

Cell viability was measured using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) cytotoxicity assay originally developed by Mosmann (1983)[28] with modification according to Doskočil et al.[29] Briefly, Caco-2 and HT-29 cells were seeded in 96-well plates at a density of 2.5×10^3 per well and CCD 841 CoN cells were seeded at 2.5×10^5 cells/well in a 96-well plate for 24 h. Samples were added in double serial dilution (4–128 μg/mL, originally in DMSO) for 72 h.

Thereafter, the MTT reagent (1 mg/mL) in EMEM was added to the wells and incubated for 2 h at 37°C with 5% CO_2. The medium with MTT was removed, and cells were dissolved in 100 μL of DMSO. The absorbance was measured at 555 nm using a Tecan Infinite M200 spectrometer (Tecan Group, Männedorf, Switzerland), and the percent viability (IC_{50} value) was calculated and compared with negative control, cells in complete culture medium with DMSO vehicle only. Three independent experiments (two replicates each) were performed for every test. Data are presented as mean ± standard deviation. The solvents did not affect the viability of normal or CRC cell lines at the tested concentration ($\leq 1\%$).

Statistical analyses

Measured data were statistically analyzed with STATISTICA software suite (version 14, 2020; TIBCO Software, Inc., Palo Alto, California, USA) using multifactor linear regression. The explanatory variables were the values for measured cannabinoid concentrations, while the dependent variables were percent viability of Caco-2, HT-29, and CCD 841 CoN cells.

The base models included all the explanatory variables and were further enhanced by removing statistically nonrelevant variables using stepwise selection. A combination of both forward-stepwise and backward-stepwise adjustments was performed to establish a set of most relevant explanatory variables. The significance of each variable was measured by a standard *t*-test with a p-value < 0.05 as a threshold. The overall model's statistical significance was verified using the F-test, and each model's goodness of fit was ascertained with the adjusted R-squared statistic. We also evaluated the residuals (differences between observed and predicted values) for selected models to determine if they followed a normal distribution using the Shapiro–Wilk test aided by observations of the respective Q–Q plots.

Results

Cannabinoid content in cannabis extracts

The content of cannabinoids in cannabis extracts was measured after individual treatments (AT, BT, CT, DT) in both nutritional cycles (1C, 2C) every week for the

Table 2. Cannabinoid Content (μg/mL) in Cannabis Extracts from Different Treatments (AT, BT, CT, DT) of both Nutritional Cycles (1C, 2C) by Vegetation Weeks (1–13)

Nutritional cycles	Treatments	Weeks	Cannabinoids									
			CBDVA	CBDA	CBGA	CBG	CBD	CBNA	Δ^9-THC	Δ^8-THC	THCA	CBCA
1[a]	all[c]	1	9.3 ± 3.8	12.4 ± 4.5	20.4 ± 2.3	30.7 ± 5.9	20.7 ± 1.9	2.3 ± 0.3	28.8 ± 6.3	85.4 ± 4.3	129 ± 12	108 ± 6
2[b]			8.9 ± 0.2	3.2 ± 0.6	9.7 ± 1.3	15.5 ± 2.7	13.7 ± 0.6	2.3 ± 0.4	19.4 ± 2.0	79.4 ± 4.7	17.0 ± 1.8	24.1 ± 2.6
1[a]	A[d], C[e]	2	12.1 ± 7.1	11.2 ± 2.0	26.4 ± 5.9	17.5 ± 6.4	19.6 ± 1.2	8.1 ± 3.9	28.1 ± 5.4	122 ± 5	151 ± 29	59.6 ± 2.9
2[b]			9.6 ± 0.4	3.2 ± 0.6	5.4 ± 0.3	16.7 ± 4.9	13.5 ± 0.6	0.3 ± 0.1	13.4 ± 1.5	62.7 ± 2.8	1.8 ± 0.1	25.1 ± 0.2
1[a]	B[f], D[g]		2.3 ± 0.4	7.1 ± 0.7	31.2 ± 5.5	22.8 ± 12.1	12.6 ± 0.6	2.9 ± 0.1	29.5 ± 9.6	25.6 ± 1.1	107 ± 9	41.7 ± 1.2
2[b]			14.6 ± 0.5	3.8 ± 0.1	15.4 ± 0.7	10.4 ± 0.4	4.4 ± 0.2	2.8 ± 0.2	22.6 ± 0.7	64.9 ± 2.6	47.7 ± 3.1	66.8 ± 6.3
1[a]	A[d], C[e]	3	13.3 ± 1.7	7.8 ± 0.8	17.2 ± 1.0	19.8 ± 7.2	10.8 ± 0.5	11.1 ± 1.7	20.9 ± 5.2	60.9 ± 3.7	155 ± 9	44.4 ± 2.9
2[b]			12.7 ± 0.5	4.1 ± 0.4	13.5 ± 0.6	16.3 ± 1.2	11.3 ± 0.7	1.7 ± 0.2	30.7 ± 1.2	64.3 ± 6.8	38.5 ± 1.6	52.7 ± 0.2
1[a]	B[f], D[g]		10.2 ± 5.2	7.3 ± 1.2	17.8 ± 0.5	17.7 ± 7.1	11.0 ± 0.4	3.4 ± 1.3	26.6 ± 5.9	36.5 ± 1.6	132 ± 10	50.4 ± 2.3
2[b]			11.1 ± 0.3	4.0 ± 0.4	15.6 ± 1	10.8 ± 2.2	5.1 ± 0.2	1.8 ± 0.1	27.3 ± 0.1	63.1 ± 4.7	48.1 ± 1.4	47.4 ± 1.5
1[a]	A[d], C[e]	4	7.1 ± 2.2	6.1 ± 0.2	23.1 ± 4.0	14.3 ± 7.2	9.5 ± 0.6	2.8 ± 0.4	49.3 ± 4.2	39.6 ± 2.8	157 ± 18	72.4 ± 8.5
2[b]			13.0 ± 0.7	5.7 ± 0.1	53.9 ± 0.7	17.0 ± 0.3	29.4 ± 1.3	12.6 ± 0.4	77.2 ± 8.0	130 ± 4	369 ± 8	41.3 ± 0.7
1[a]	B[f], D[g]		4.9 ± 0.3	5.8 ± 0.2	13.5 ± 1.2	12.2 ± 6.2	9.4 ± 0.5	3.5 ± 0.7	29.2 ± 5.2	47.4 ± 2.3	134 ± 11	41.8 ± 4.2
2[b]			15.6 ± 0.6	8.1 ± 0.1	81.4 ± 2.6	12.8 ± 1.8	16.9 ± 2.9	18.7 ± 0.1	71.1 ± 1.6	153 ± 1	709 ± 4	62.1 ± 6.0
1[a]	A[d]	5	17.3 ± 3.6	6.8 ± 0.2	116 ± 8	16.7 ± 3.7	24.6 ± 1.3	21.7 ± 1.1	64.1 ± 7.5	215 ± 8	915 ± 30	86.9 ± 7.2
2[b]			9.0 ± 0.7	14.4 ± 0.7	91.3 ± 5.4	11.5 ± 4.1	47.4 ± 14.1	34.8 ± 1.2	69.1 ± 7.3	161 ± 6	808 ± 27	37.9 ± 1.1
1[a]	B[f]		13.3 ± 4.7	8.4 ± 0.5	64.2 ± 0.7	51.6 ± 5.8	17.6 ± 0.6	12.9 ± 1.5	95.2 ± 11.1	126 ± 5	634 ± 27	54.3 ± 2.7
2[b]			7.7 ± 0.4	12.2 ± 1.0	66.0 ± 4.9	10.0 ± 0.4	35.0 ± 7.6	24.2 ± 3.0	105 ± 9	170 ± 5	799 ± 100	35.1 ± 3.0
1[a]	C[e]		12.5 ± 1.7	6.2 ± 0.2	71.2 ± 5.4	12.6 ± 0.5	28.0 ± 2.2	17.6 ± 1.1	76.4 ± 7.1	130 ± 7	752 ± 42	66.0 ± 5.6
2[b]			7.0 ± 0.3	11.9 ± 3.3	78.7 ± 2.1	9.9 ± 0.4	28.2 ± 2.4	23.8 ± 2.6	97.4 ± 10.5	151 ± 4	755 ± 74	35.6 ± 1.0
1[a]	D[g]		9.8 ± 8.0	7.3 ± 0.6	108 ± 8	16.9 ± 5.6	28.1 ± 5.0	16.3 ± 1.1	88.2 ± 6.7	161 ± 7	766 ± 63	86.4 ± 2.1
2[b]			7.4 ± 0.6	12.4 ± 0.4	78.3 ± 3.7	11.9 ± 0.3	46.9 ± 13.8	21.0 ± 1.0	48.6 ± 3.4	124 ± 9	854 ± 42	41.9 ± 2.8
1[a]	A[d]	6	31.4 ± 1.6	9.9 ± 0.3	64.3 ± 8.1	117 ± 11	151 ± 7	26.1 ± 0.6	24.8 ± 1.7	132 ± 5	1079 ± 41	33.6 ± 1.2
2[b]			6.8 ± 0.3	14.1 ± 0.7	67.4 ± 3.9	20.0 ± 2.6	57.2 ± 9.4	38.1 ± 2.0	98.5 ± 8.5	207 ± 17	886 ± 66	35.6 ± 2.3
1[a]	B[f]		10.6 ± 2.0	11.2 ± 1.3	71.0 ± 0.9	94.7 ± 6.1	25.2 ± 1.4	16.4 ± 1.3	96.0 ± 4.4	145 ± 6	999 ± 57	37.6 ± 0.9
2[b]			7.7 ± 0.4	14.8 ± 1.5	66.3 ± 6.7	19.0 ± 5.5	54.5 ± 20.5	32.4 ± 2.9	101 ± 13	200 ± 5	700 ± 74	32.2 ± 2.0
1[a]	C[e]		9.4 ± 4.1	13.4 ± 0.4	115 ± 5	13.8 ± 5.9	44.9 ± 3.6	30.7 ± 3.0	132 ± 11	172 ± 11	1448 ± 77	47.0 ± 6.6
2[b]			6.2 ± 0.4	11.7 ± 0.3	52.7 ± 1.1	20.9 ± 1.3	50.2 ± 8.0	25.6 ± 1.6	85.7 ± 5.6	158 ± 6	644 ± 37	25.5 ± 1.9
1[a]	D[g]		10.7 ± 3.8	13.5 ± 2.0	121 ± 13	15.8 ± 4.7	46.2 ± 3.4	33.3 ± 4.0	121 ± 5	264 ± 7	1469 ± 155	57.9 ± 3.1
2[b]			7.1 ± 0.4	16.0 ± 1.4	58.5 ± 6.2	27.2 ± 2.0	46.8 ± 14.5	26.8 ± 4.4	115 ± 12	190 ± 8	737 ± 107	25.8 ± 3.3
1[a]	A[d]	7	9.9 ± 2.3	12.9 ± 1.1	81.4 ± 3.7	33.6 ± 5.3	10.0 ± 0.8	29.9 ± 2.0	46.5 ± 4.6	527 ± 30	1399 ± 45	39.7 ± 2.8
2[b]			8.0 ± 0.3	20.8 ± 2.5	81.2 ± 2.4	12.7 ± 4.8	37.7 ± 2.7	35.7 ± 1.4	79.9 ± 1.3	142 ± 43	1344 ± 74	38.2 ± 8.6
1[a]	B[f]		20.4 ± 4.8	14.2 ± 1.4	58.7 ± 4.3	129 ± 7	42.6 ± 2.8	22.2 ± 4.7	49.2 ± 3.6	137 ± 6	1001 ± 63	28.8 ± 1.2
2[b]			6.5 ± 0.3	20.3 ± 0.1	78.1 ± 1.7	11.6 ± 2.7	36.3 ± 0.3	31.6 ± 0.5	113 ± 6	181 ± 21	1170 ± 24	33.3 ± 0.3
1[a]	C[e]		8.4 ± 0.6	22.5 ± 1.6	93.7 ± 9.1	13.2 ± 1.5	49.5 ± 3.2	30.8 ± 5.3	130 ± 6	128 ± 6	1534 ± 83	50.1 ± 2.0
2[b]			6.4 ± 0.4	26.0 ± 1.3	61.5 ± 3.6	13.4 ± 1.3	76.5 ± 21.6	31.2 ± 3.1	134 ± 21	147 ± 6	1003 ± 95	33.1 ± 5.4
1[a]	D[g]		7.5 ± 4.2	12.2 ± 0.8	67.2 ± 1.3	10.9 ± 4.9	43.7 ± 3.5	31.2 ± 3.2	94.1 ± 7.8	243 ± 7	1165 ± 44	31.4 ± 0.6
2[b]			7.4 ± 0.4	19.5 ± 0.8	69.6 ± 2.6	12.4 ± 0.6	58.7 ± 9.3	32.0 ± 1.5	143 ± 6	180 ± 5	1000 ± 42	25.3 ± 7.4

(continued)

Table 2. (Continued)

Nutritional cycles	Treatments	Weeks	Cannabinoids									
			CBDVA	CBDA	CBGA	CBG	CBD	CBNA	Δ^9-THC	Δ^8-THC	THCA	CBCA
1[a]	A[d]	8	7.8 ± 0.4	21.7 ± 1.9	75.8 ± 5.8	14.0 ± 2.5	27.6 ± 1.5	27.4 ± 3	92.3 ± 20.3	143 ± 9	1562 ± 123	48.1 ± 2.2
2[b]			7.1 ± 0.3	24.6 ± 2.3	36.6 ± 0.6	8.4 ± 1.8	42.3 ± 9.7	32.5 ± 6.6	126 ± 36	129 ± 32	1172 ± 115	34.8 ± 3.0
1[a]	B[f]		4.0 ± 1.7	31.1 ± 1.0	64.9 ± 2.1	23.8 ± 6.4	19.7 ± 1.2	24.6 ± 4.0	23.2 ± 0.9	99.4 ± 4.1	1199 ± 47	37.6 ± 2.3
2[b]			7.0 ± 0.6	25.4 ± 3.4	47.4 ± 6.3	7.8 ± 1.4	30.0 ± 8.1	32.4 ± 1.8	123 ± 18	122 ± 24	1248 ± 58	29.5 ± 2.8
1[a]	C[e]		8.2 ± 7.0	16.8 ± 4.0	52.7 ± 2.8	13.0 ± 2.4	50.5 ± 5.6	33.9 ± 3.5	139 ± 15	133 ± 8	1636 ± 52	38.8 ± 1.9
2[b]			5.2 ± 0.6	23.9 ± 3.5	45.9 ± 4.2	14.7 ± 3.8	68.6 ± 3.0	25.1 ± 3.5	136 ± 33	123 ± 62	1041 ± 120	31.1 ± 2.8
1[a]	D[g]		9.0 ± 0.7	19.9 ± 2.3	72.4 ± 1.6	18.7 ± 5.5	61.5 ± 5.6	39.3 ± 1.5	123 ± 5	155 ± 7	1653 ± 56	45.7 ± 3.2
2[b]			6.8 ± 0.3	32.0 ± 4.6	51.0 ± 2.2	7.7 ± 2.1	79.3 ± 36.9	31.7 ± 2.4	196 ± 46	121 ± 62	1348 ± 107	31.3 ± 1.6
1[a]	A[d]	9	13.1 ± 3.8	19.9 ± 1.7	53.8 ± 4.9	13.6 ± 3.9	7.5 ± 0.7	31.3 ± 3.6	69.4 ± 2.4	79.1 ± 8.1	1514 ± 43	45.7 ± 1.4
2[b]			6.5 ± 0.2	23.1 ± 2.0	53.8 ± 2.2	12.1 ± 3.7	24.9 ± 10.1	23.7 ± 4.0	139 ± 21	150 ± 64	1272 ± 107	37.9 ± 3.1
1[a]	B[f]		26.0 ± 5.1	22.9 ± 1.7	30.6 ± 0.6	4.4 ± 0.4	22.4 ± 1.1	24.7 ± 3.3	58.5 ± 6.2	178 ± 6	1088 ± 54	25.3 ± 1.2
2[b]			6.4 ± 0.3	23.0 ± 2.6	56.7 ± 6.3	16.1 ± 6.9	16.1 ± 3.3	19.2 ± 2.3	87.0 ± 8.1	120 ± 23	1206 ± 144	31.0 ± 0.4
1[a]	C[e]		7.7 ± 2.4	18.1 ± 5.0	42.3 ± 0.9	11.3 ± 1.7	50.0 ± 4.2	38.0 ± 4.4	111 ± 5	86.7 ± 3.9	1607 ± 61	33.0 ± 1.0
2[b]			5.2 ± 0.3	16.3 ± 0.8	41.5 ± 6.1	9.0 ± 4.4	37.8 ± 3.0	24.3 ± 2.5	119 ± 17	151 ± 35	737 ± 112	22.5 ± 2.1
1[a]	D[g]		7.9 ± 4.1	20.4 ± 3.3	49.4 ± 7.9	11.3 ± 2.2	41.9 ± 3.8	39.1 ± 5.9	119 ± 11	148 ± 6	1481 ± 146	36.6 ± 0.6
2[b]			6.9 ± 0.3	26.9 ± 3.3	58.0 ± 0.9	23.1 ± 6.6	77.5 ± 17.5	31.1 ± 2.2	192 ± 31	133 ± 24	1106 ± 69	27.6 ± 1.1
1[a]	A[d]	10	8.2 ± 1.8	24.5 ± 1.4	45.7 ± 3.9	12.4 ± 3.0	30.4 ± 3.6	39.4 ± 5.1	85.3 ± 17.7	85.4 ± 15.7	1640 ± 28	48.0 ± 2.5
2[b]			10.1 ± 6.0	47.0 ± 3.0	39.3 ± 3.7	24.0 ± 4.9	56.4 ± 11.0	26.7 ± 4.5	179 ± 39	148 ± 64	741 ± 10	18.6 ± 1.8
1[a]	B[f]		12.2 ± 3.1	10.1 ± 0.8	38.9 ± 1.6	5.3 ± 0.4	29.3 ± 2.0	20.7 ± 2.0	112 ± 32	36.6 ± 2.0	1213 ± 69	36.4 ± 6.1
2[b]			4.8 ± 0.3	20.5 ± 0.6	51.0 ± 0.9	8.1 ± 1.3	28.2 ± 4.3	29.6 ± 2.1	134 ± 10	173 ± 20	1002 ± 16	31.4 ± 0.7
1[a]	C[e]		7.7 ± 2.0	17.8 ± 3.0	46.2 ± 0.3	14.0 ± 1.4	43.9 ± 2.7	37.2 ± 2.3	128 ± 11	95.6 ± 5.2	1656 ± 34	42.4 ± 1.7
2[b]			5.2 ± 0.4	20.7 ± 1.5	52.8 ± 2.8	12.8 ± 3.3	49.6 ± 2.8	34.3 ± 2.6	140 ± 9	164 ± 26	1153 ± 79	29.7 ± 6.0
1[a]	D[g]		6.9 ± 2.8	20.7 ± 2.4	48.1 ± 2.8	10.8 ± 0.9	53.6 ± 4.2	39.2 ± 2.9	135 ± 11	131 ± 7	1570 ± 32	42.3 ± 3.0
2[b]			13.0 ± 2.9	47.0 ± 0.9	54.7 ± 0.9	23.9 ± 15.3	68.0 ± 6.8	28.9 ± 2.4	167 ± 30	134 ± 1.1	1271 ± 15	28.3 ± 1.3
1[a]	A[d]	11	4.2 ± 1.5	8.6 ± 0.7	74.1 ± 4.7	1.1 ± 0.1	10.9 ± 0.7	33.2 ± 3.1	190 ± 14	26.7 ± 2.0	1989 ± 173	57.9 ± 2.8
2[b]			4.0 ± 1.4	20.8 ± 1.9	44.0 ± 5.3	19.2 ± 7.7	44.9 ± 10.4	27.5 ± 2.6	159 ± 13	121 ± 32	1115 ± 126	30.4 ± 2.6
1[a]	B[f]		4.6 ± 3.0	28.1 ± 4.8	57.6 ± 9.1	37.1 ± 11.5	59.6 ± 15.3	40.3 ± 4.0	99.7 ± 14.2	176 ± 47	1391 ± 241	38.5 ± 5.4
2[b]			4.4 ± 1.1	21.0 ± 0.6	44.4 ± 4.0	14.1 ± 1.2	24.3 ± 4.4	28.8 ± 2.3	172 ± 8	143 ± 9	1010 ± 81	29.2 ± 0.9
1[a]	C[e]		7.4 ± 2.0	20.2 ± 7.7	46.5 ± 1.2	16.0 ± 1.6	5.7 ± 1.0	35.0 ± 2.1	118 ± 5	94.6 ± 6.6	1738 ± 69	46.2 ± 8.4
2[b]			4.7 ± 0.5	20.2 ± 3.1	42.9 ± 3.6	14.6 ± 3.6	27.9 ± 2.7	28.7 ± 1.6	149 ± 19	142 ± 13	778 ± 60	25.0 ± 4.7
1[a]	D[g]		6.9 ± 2.5	22.9 ± 2.0	58.1 ± 2.0	10.6 ± 3.7	59.3 ± 12.3	39.1 ± 2.9	135 ± 6	140 ± 28	1433 ± 118	32.3 ± 2.3
2[b]			5.5 ± 0.6	25.8 ± 4.5	39.1 ± 4.5	12.3 ± 2.0	49.0 ± 9.1	28.6 ± 2.2	279 ± 68	141 ± 40	1210 ± 123	29.9 ± 1.7
1[a]	A[d]	12	3.8 ± 1.1	37.2 ± 8.2	65.7 ± 9.4	39.1 ± 14.8	39.4 ± 5.1	36.4 ± 6.1	152 ± 46	105 ± 41	1840 ± 273	47.1 ± 5.5
2[b]			4.6 ± 0.9	24.8 ± 6.2	44.6 ± 3.9	20.2 ± 9.7	33.5 ± 4.0	24.2 ± 1.3	217 ± 61	104 ± 27	1082 ± 107	25.7 ± 0.8
1[a]	B[f]		3.0 ± 0.3	17.8 ± 0.4	40.8 ± 2.3	21.4 ± 5.5	23.0 ± 2.3	28.0 ± 2.0	111 ± 12	124 ± 34	1023 ± 30	26.5 ± 1.2
2[b]			3.4 ± 0.4	21.0 ± 2.2	36.7 ± 1.9	22.1 ± 2.9	29.1 ± 3.5	26.6 ± 1.2	223 ± 30	124 ± 15	798 ± 26	23.4 ± 0.8
1[a]	C[e]		6.5 ± 0.9	23.0 ± 0.8	54.4 ± 2.4	14.7 ± 2.4	34.8 ± 6.5	40.9 ± 5.2	148 ± 42	92.2 ± 9.5	1583 ± 59	40.1 ± 1.9
2[b]			5.6 ± 1.4	32.8 ± 3.4	32.2 ± 2.9	23.8 ± 4.3	34.2 ± 13.6	24.0 ± 2.4	193 ± 77	108 ± 50	859 ± 33	26.9 ± 1.3
1[a]	D[g]		5.8 ± 1.1	16.0 ± 0.9	48.1 ± 2.9	11.1 ± 3.6	41.1 ± 9.7	34.8 ± 1.5	131 ± 8	128 ± 34	1219 ± 61	27.0 ± 0.9
2[b]			5.1 ± 1.0	19.7 ± 1.1	42.4 ± 0.3	20.2 ± 4.6	40.9 ± 8.8	26.7 ± 2.8	232 ± 13	122 ± 6	1167 ± 88	29.7 ± 2.5

(continued)

Table 2. (Continued)

Nutritional cycles	Treatments	Weeks	Cannabinoids									
			CBDVA	CBDA	CBGA	CBG	CBD	CBNA	Δ^9-THC	Δ^8-THC	THCA	CBCA
1[a]	A[d]	13	7.8 ± 0.4	27.9 ± 1.7	73.2 ± 3.9	17.7 ± 5.3	19.9 ± 0.4	36.2 ± 1.5	146 ± 15	111 ± 17.4	2029 ± 157	48.5 ± 2.7
2[b]			5.4 ± 1.4	33.9 ± 1.3	35.2 ± 7.9	20.5 ± 0.9	45.0 ± 13.7	24.3 ± 2.5	183 ± 29	74.5 ± 9.3	802 ± 116	22.7 ± 3.2
1[a]	B[f]		8.6 ± 2.4	27.9 ± 0.5	45.8 ± 5.0	30.5 ± 12.2	23.4 ± 2.5	24.2 ± 2.4	92.0 ± 4.1	72.4 ± 9.2	1014 ± 106	35.5 ± 8.9
2[b]			6.2 ± 0.6	31.9 ± 2.7	43.8 ± 4.5	24.7 ± 4.5	23.4 ± 1.2	29.8 ± 3.6	153 ± 25	114 ± 7	891 ± 20	26.0 ± 1.7
1[a]	C[e]		6.8 ± 1.7	19.7 ± 1.9	59.9 ± 6.6	15.6 ± 3.2	26.4 ± 4.9	35.9 ± 3.7	144 ± 25	80.0 ± 5.0	1625 ± 156	45.5 ± 10.4
2[b]			6.4 ± 2.1	34.2 ± 5.7	42.0 ± 9.5	23.7 ± 12.1	26.1 ± 7.3	30.7 ± 6.4	185 ± 36	109 ± 36	1110 ± 230	28.2 ± 7.4
1[a]	D[g]		7.2 ± 1.3	21.5 ± 0.4	47.6 ± 6.4	30.7 ± 7.9	33.0 ± 6.4	24.4 ± 4.1	84.0 ± 8.0	22.9 ± 1.8	1064 ± 82	28.1 ± 1.4
2[b]			7.7 ± 0.8	29.1 ± 1.0	41.3 ± 1.5	37.4 ± 2.4	45.3 ± 9.1	25.0 ± 1.0	188 ± 7	93.6 ± 4.7	1051 ± 29	24.3 ± 0.4

[a]Recirculation cycle.
[b]Drain-to-waste cycle.
[c]Control (AT) and enhanced treatments (BT, CT, DT).
[d]Control treatment (AT).
[e]Enhanced treatment C (CT) with the addition of P, K, and Fe.
[f]Enhanced treatment B (BT) with the addition of amino acids.
[g]Enhanced treatment D (DT) with the addition of amino acids, P, K, and Fe.
Δ^8-THC, Δ^8-tetrahydrocannabinol; Δ^9-THC, Δ^9-tetrahydrocannabinol; CBCA, cannabichromenic acid; CBD, cannabidiol; CBDA, cannabidiolic acid; CBDVA, cannabidivarinic acid; CBG, cannabigerol; CBGA, cannabigerolic acid; CBNA, cannabinolic acid; THCA, tetrahydrocannabinolic acid.

entire vegetation cycle (13 weeks). Seventeen phytocannabinoids were identified and measured. The ten with the highest concentration are described in this study.

As this is chemotype I, the most abundant cannabinoid in plants and subsequently also in cannabis extracts was THCA. THCA content in plants increased with time, and its maximum concentration was reached at full maturity of the plants, from the 7th to the 13th week, depending on the nutritional cycle and treatment (Table 2). The highest average concentration of THCA in the cannabis extract was achieved in 1C of AT at week 13 (2029 ± 157 μg/mL). The concentration of THCA decarboxylation products such as Δ^9-tetrahydrocannabinol (Δ^9-THC) and Δ^8-tetrahydrocannabinol (Δ^8-THC) and THCA oxidative degradation products, such as cannabinolic acid, increased proportionally with the THCA content. Furthermore, the concentration of cannabichromenic acid gradually decreased in proportion to the age of the plant. Other phytocannabinoids did not show a consistent trend over time.

Cytotoxicity of cannabis extracts

Cannabis cytotoxicity was tested on three cell lines. The tested extracts showed no cytotoxic effect on the Caco-2 cell line. For this reason, it was not considered further. Almost all extracts were cytotoxic to cell line HT-29 at lower concentrations than other cell lines (Table 3). The selective cytotoxicity of the extracts was compared with the normal cell line CCD 841 CoN, where in most cases, a much higher extract concentration was required for IC_{50}. The most effective extracts on HT-29 were from the final weeks of growth, where the lowest average concentration of cannabis extract was achieved in 1C of AT at week 13 (31.6 ± 5.0 μg/mL).

Statistical evaluation of the dependence of cytotoxicity on individual phytocannabinoids

The statistical analysis was based on a linear regression model of the dependence of the IC_{50} value of CRC cell line HT-29 on concentrations of individual cannabinoids in cannabis extracts. The phytocannabinoids that most influenced the cytotoxicity of individual extracts on HT-29 according to the p-value were cannabigerol (CBG) and Δ^9-THC (Table 4).

Based on forward and backward stepwise selections, a custom model was created that included all crucial variables from the group of cannabinoids in individual cannabis extracts (Table 5). In addition to the already mentioned CBG and Δ^9-THC, cannabidiol (CBD), cannabigerolic acid (CBGA), and THCA were also included.

Table 3. Inhibitory Concentrations (IC_{50}, µg/mL) of Cannabinoids from Cannabis Extracts on Selected Cell Line Types

Nutritional cycles	Treatments	Weeks	Cell line types: HT-29[a]	CCD 841 CoN[b]	SI[c]
1[d]	all[f]	1	73.6 ± 2.6	> 128	0.57
2[e]			91.3 ± 1.0	> 128	0.71
1[d]	A[g], C[h]	2	85.2 ± 5.7	> 128	0.67
2[e]			87.0 ± 0.9	> 128	0.68
1[d]	B[i], D[j]		93.8 ± 30.4	> 128	0.73
2[e]			113 ± 3	> 128	0.88
1[d]	A[g], C[h]	3	117 ± 2	> 128	0.92
2[e]			106 ± 10	> 128	0.83
1[d]	B[i], D[j]		87.6 ± 10.5	> 128	0.68
2[e]			102 ± 3	> 128	0.79
1[d]	A[g], C[h]	4	86.6 ± 8.4	> 128	0.68
2[e]			74.1 ± 2.3	> 128	0.58
1[d]	B[i], D[j]		86.7 ± 0.5	> 128	0.68
2[e]			66.2 ± 3.3	> 128	0.52
1[d]	A[g]	5	40.4 ± 7.9	> 128	**0.32**
2[e]			58.7 ± 1.1	78.8 ± 8.8	0.75
1[d]	B[i]		42.2 ± 8.8	> 128	**0.33**
2[e]			66.6 ± 11.0	67.3 ± 34.7	0.99
1[d]	C[h]		37.3 ± 3.6	> 128	**0.29**
2[e]			88.0 ± 7.0	> 128	0.69
1[d]	D[j]		63.0 ± 0.7	46.9 ± 12.8	1.36
2[e]			51.7 ± 3.3	75.7 ± 12.0	0.68
1[d]	A[g]	6	37.6 ± 5.2	> 128	**0.29**
2[e]			35.3 ± 0.8	82.1 ± 14.3	**0.43**
1[d]	B[i]		58.1 ± 1.5	> 128	**0.45**
2[e]			58.0 ± 6.5	67.2 ± 0.2	0.86
1[d]	C[h]		35.2 ± 1.4	42.9 ± 8.4	0.82
2[e]			68.7 ± 4.3	> 128	0.54
1[d]	D[j]		37.5 ± 4.8	43.6 ± 9.9	0.86
2[e]			53.4 ± 10.0	83.4 ± 23.1	0.64
1[d]	A[g]	7	52.5 ± 2.0	> 128	**0.41**
2[e]			49.3 ± 4.1	> 128	**0.39**
1[d]	B[i]		36.0 ± 4.4	> 128	**0.28**
2[e]			52.0 ± 8.1	> 128	**0.41**
1[d]	C[h]		35.4 ± 3.4	35.4 ± 1.9	1.00
2[e]			58.0 ± 8.0	> 128	**0.45**
1[d]	D[j]		46.6 ± 2.0	> 128	**0.36**
2[e]			41.8 ± 3.1	> 128	**0.33**
1[d]	A[g]	8	48.6 ± 0.1	> 128	**0.38**
2[e]			44.1 ± 1.2	> 128	**0.34**
1[d]	B[i]		47.6 ± 7.7	> 128	**0.37**
2[e]			50.3 ± 8.6	> 128	**0.39**
1[d]	C[h]		31.8 ± 1.5	> 128	**0.25**
2[e]			56.5 ± 9.3	> 128	**0.44**
1[d]	D[j]		30.8 ± 2.2	> 128	**0.24**
2[e]			50.4 ± 1.0	> 128	**0.39**
1[d]	A[g]	9	49.8 ± 4.6	> 128	**0.39**
2[e]			52.0 ± 6.6	63.3 ± 33.2	0.82
1[d]	B[i]		47.7 ± 2.1	> 128	**0.37**
2[e]			50.9 ± 6.0	> 128	**0.40**
1[d]	C[h]		63.3 ± 28.2	> 128	**0.49**
2[e]			58.8 ± 7.4	> 128	**0.46**
1[d]	D[j]		39.1 ± 3.5	> 128	**0.31**
2[e]			41.0 ± 5.9	> 128	**0.32**
1[d]	A[g]	10	35.3 ± 0.2	> 128	**0.28**
2[e]			57.2 ± 0.6	76.8 ± 33.7	0.74
1[d]	B[i]		51.1 ± 12.2	> 128	**0.40**
2[e]			52.6 ± 6.5	> 128	**0.41**
1[d]	C[h]		38.2 ± 2.7	45.6 ± 13.9	0.84
2[e]			51.2 ± 0.3	> 128	**0.40**
1[d]	D[j]		33.7 ± 0.1	> 128	**0.26**
2[e]			39.7 ± 2.2	> 128	**0.31**
1[d]	A[g]	11	34.1 ± 2.4	> 128	**0.27**
2[e]			57.1 ± 8.2	51.7 ± 20.2	1.10
1[d]	B[i]		35.6 ± 7.6	> 128	**0.28**
2[e]			67.1 ± 10.7	> 128	0.52
1[d]	C[h]		32.3 ± 2.1	78.1 ± 19.8	**0.43**
2[e]			36.2 ± 0.7	> 128	**0.28**
1[d]	D[j]		40.8 ± 9.9	> 128	**0.32**
2[e]			32.0 ± 2.1	> 128	**0.25**
1[d]	A[g]	12	55.0 ± 1.5	> 128	**0.43**
2[e]			54.7 ± 6.5	50.3 ± 18.8	1.09
1[d]	B[i]		61.6 ± 8.9	> 128	**0.48**
2[e]			45.6 ± 3.7	> 128	**0.36**
1[d]	C[h]		35.3 ± 1.6	> 128	**0.20**
2[e]			34.9 ± 1.4	> 128	**0.27**
1[d]	D[j]		45.8 ± 9.0	> 128	**0.36**
2[e]			34.0 ± 1.7	> 128	**0.27**
1[d]	A[g]	13	31.6 ± 5.0	> 128	**0.25**
2[e]			54.7 ± 4.6	64.6 ± 34.3	0.85
1[d]	B[i]		39.7 ± 0.9	> 128	**0.31**
2[e]			49.7 ± 3.7	> 128	**0.39**
1[d]	C[h]		37.3 ± 4.5	> 128	**0.29**
2[e]			47.7 ± 3.3	> 128	**0.37**
1[d]	D[j]		60.2 ± 9.7	> 128	**0.47**
2[e]			45.6 ± 4.3	> 128	**0.36**

The bold ratio values below 0.5 mean a strong selectivity index.
[a]Human colorectal cell line.
[b]Normal human colon epithelial cell line.
[c]Selectivity Index as the ratio of IC_{50} of cancer cell line HT-29 to that of normal cell line CCD 841 CoN.
[d]Recirculation cycle.
[e]Drain-to-waste cycle.
[f]Control (AT) and enhanced treatments (BT, CT, DT).
[g]Control treatment (AT).
[h]Enhanced treatment C (CT) with the addition of P, K, and Fe.
[i]Enhanced treatment B (BT) with the addition of amino acids.
[j]Enhanced treatment D (DT) with the addition of amino acids, P, K, and Fe.

This model showed almost 70% influence by these phytocannabinoids. Graphic processing of the custom model was performed (Fig. 1). Based on the *t* test, we found that THCA had the strongest cytotoxic effects on HT-29. Therefore, a model was created with THCA as the only variable, and it was proven to affect the cytotoxicity of individual extracts by almost 65% (Table 6).

Table 4. Regression Summary for Dependent Variable, HT-29

	$R = 0.84510450$, $R^2 = 0.71420162$, adjusted $R^2 = 0.69971860$, $F(15.296) = 49.313$, $p < 0.0000$, standard error of estimate: 13.274					
***N*=312**	***b****	**Standard error of *b****	***b***	**Standard error of *b***	***t* (296)**	***p*-Value**
Intercept			**28.89609**	**3.724151**	**7.75911**	**0.000000**
CBDVA	−0.013060	0.037223	−0.06282	0.179052	−0.35085	0.725949
CBDA	−0.005716	0.054948	−0.01389	0.133544	−0.10402	0.917221
CBGA	0.103478	0.059680	0.09740	0.056177	1.73387	0.083982
CBG	**0.104163**	**0.037768**	**0.13319**	**0.048294**	**2.75797**	**0.006178**
CBD	0.064347	0.045732	0.06829	0.048531	1.40705	0.160463
CBNA	0.114210	0.098886	0.21232	0.183830	1.15496	0.249039
Δ^9-THC	**0.207536**	**0.062231**	**0.08023**	**0.024059**	**3.33496**	**0.000962**
Δ^8-THC	0.013216	0.042499	0.00486	0.015612	0.31098	0.756037
THCA	0.241006	0.172996	0.01032	0.007410	1.39312	0.164627
CBCA	0.039728	0.040485	0.04954	0.050480	0.98131	0.327241

*b** indicates standardized beta coefficient that indicates the relative change of the variable.
Bold value indicates the variables that are statistically significant based on the *p*-values.

Since the maximum IC_{50} value at which cytotoxicity was tested was 128 μg/mL, inverted values were used in our model. Thus, an IC_{50} value > 128 μg/mL was designated as 0 and the higher the inverted values obtained for individual extracts, the more cytotoxic they were. This model was not applied to the CRC cell line Caco-2 or to the normal human colon epithelial cell line, CCD 841 CoN, because many of the extracts required concentrations of > 128 μg/mL to reach IC_{50}.

Discussion

Cannabis research has developed in recent years,[30,31] but mostly toward CBD strains, so-called hemp, with the psychoactive component in the form of Δ^9-THC at a concentration less than 0.3%.[32] Currently, there is still a lack of basic research information on the regulation of the biosynthesis of THCA and the secondary metabolites in the THCA-predominant chemotype of medical cannabis, and its pharmaceutical potential because of legal restrictions in most countries.[18]

This study provides evidence of selective cytotoxic activity of *C. sativa* extracts against colon cancer cells across all plant vegetation stages (1st–13th week), different treatments (AT, BT, CT, DT), and nutritional cycles (1C, 2C). This variety of medical cannabis is classified as chemical phenotype I, in which THCA is the main active component.[33] Depending on the time when the plants were harvested, these cannabis extracts also contain a large amount of THCA decarboxylation products in the form of Δ^9-THC and its thermodynamically more stable isomer Δ^8-THC.[34]

Furthermore, there is a large concentration of CBGA, the primary precursor of phytocannabinoid biosynthesis, and its decarboxylation product CBG, and also, a significant amount of CBD (Table 2). Cannabinoids are mainly found in the form of acids in plants.[23] The larger amount of decarboxylated phytocannabinoids contained in these extracts was probably due to the evaporation of ethanol in a vacuum evaporator and the subsequent dissolving of the dry matter in DMSO.[35–37] However, from a statistical point of view, this contributed to the more significant heterogeneity of the cannabis extracts and the subsequent proof of their possible effect.

Phytocannabinoids act by modulating signaling pathways critical to controlling cell proliferation and survival. Many *in vitro*, *ex vivo*, and *in vivo* experiments

Table 5. Custom Model of Regression Summary for Dependent Variable, HT-29

	$R = 0.83947197$, $R^2 = 0.70471319$, adjusted $R^2 = 0.69988824$, $F(5.306) = 146.06$, $p < 0.0000$, standard error of estimate: 13.270					
***N*=312**	***b****	**Standard error of *b****	***b***	**Standard error of *b***	***t* (306)**	***p*-Value**
Intercept			**28.35014**	**1.936394**	**14.64068**	**0.000000**
CBGA	**0.139695**	**0.041953**	**0.13149**	**0.039490**	**3.32981**	**0.000975**
CBG	**0.096768**	**0.033627**	**0.12374**	**0.043000**	**2.87767**	**0.004288**
CBD	**0.082724**	**0.038581**	**0.08779**	**0.040943**	**2.14415**	**0.032808**
Δ^9-THC	**0.205607**	**0.042835**	**0.07949**	**0.016560**	**4.79995**	**0.000002**
THCA	**0.545248**	**0.050703**	**0.02336**	**0.002172**	**10.75383**	**0.000000**

*b** indicates standardized beta coefficient that indicates the relative change of the variable.
Bold value indicates the variables that are statistically significant based on the *p*-values.

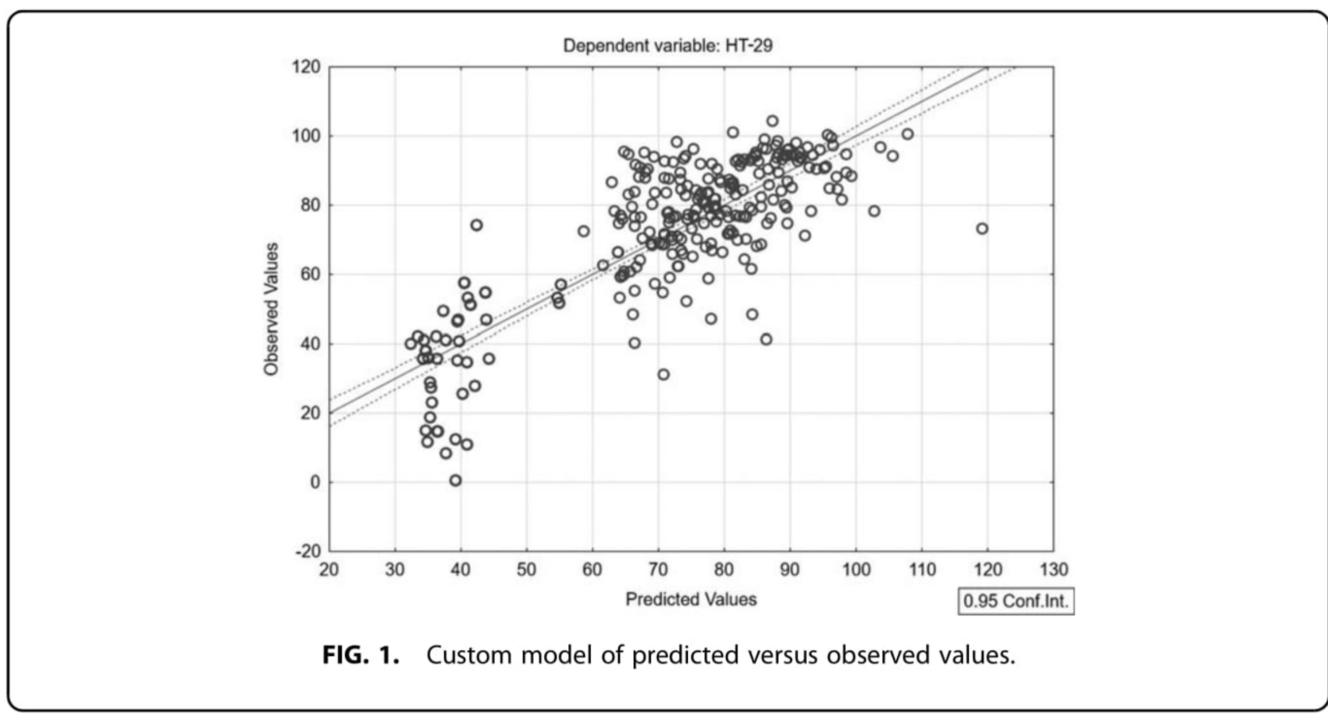

FIG. 1. Custom model of predicted versus observed values.

have shown that phytocannabinoids inhibit cancer cell proliferation and induce their apoptosis.[38–42] Cannabis extracts showed the highest selective cytotoxicity according to IC_{50} against HT-29 CRC cell line in the last weeks of vegetation (Table 3), when the THCA concentration also reached its maximum depending on the nutritional cycle and treatment. THCA alone contributed to the selective cytotoxicity of cannabis extracts against HT-29 by almost 65% (Table 6).

Several cell line-based experiments have shown that THCA interacts with various molecular targets and exhibits potential neuroprotective, anti-inflammatory, immunomodulatory, cytotoxic, and antineoplastic properties.[43–45] In addition, several *in vivo* studies showed that this compound exhibits pharmacological effects in rodents, possibly by the involvement of cannabinoid type 1 (CB_1) receptors.[44] There was also an effort to include all important variables from the cannabinoid group in individual cannabis extracts.

Based on forward and backward stepwise selections, we obtained the other phytocannabinoids, CBGA, CBG, CBD, and Δ^9-THC. These four phytocannabinoids contributed an additional 5% to selective cytotoxicity. Altogether, these five phytocannabinoids influenced the cytotoxicity effect of the extracts by almost 70% (Table 5). This confirms the previously proposed synergism between the active compounds in the cannabis plant. Therefore, the unrefined content of flower extracts with their cocktail of various extracted compounds may have an advantage over the activity of isolated compounds.[46] Mixtures of different phytocannabinoids of *C. sativa* show a specific interaction for cellular cytotoxic activity and are more biologically active than single phytocannabinoids.[39–41]

Conclusions

This study provides evidence of selective cytotoxic activity of *C. sativa* extracts from a THCA-predominant

Table 6. Tetrahydrocannabinolic Acid Model of Regression Summary for Dependent Variable, HT-29

	R =0.80550029, R^2 =0.64883071, adjusted R^2 =0.64769791, $F(1.310)$=572.77, $p<0.0000$, standard error of estimate: 14.378					
***N*=312**	***b****	**Standard error of *b****	**B**	**Standard error of *b***	***t* (310)**	***p*-Value**
Intercept			**38.08467**	**1.483696**	**25.66878**	**0.00**
THCA	**0.805500**	**0.033657**	**0.03450**	**0.001442**	**23.93251**	**0.00**

*b** indicates standardized beta coefficient that indicates the relative change of the variable.
Bold value indicates the variables that are statistically significant based on the *p*-values.

chemotype against CRC cell lines at all vegetative stages (1st–13th week), different nutritional cycles (1C, 2C), and treatments (AT, BT, CT, DT). Cannabis extracts showed the highest selective cytotoxicity effect, according to IC_{50}, against HT-29 CRC cells in the last weeks of growth, when THCA concentration also reached its maximum depending on the nutritional cycle and treatment. THCA alone contributed almost 65% to the selective cytotoxicity effect of cannabis extracts against HT-29. CBGA, CBG, CBD, and Δ^9-THC contributed an additional 5% to the selective cytotoxicity. Altogether, these five mentioned phytocannabinoids affected the cytotoxicity of the extracts by almost 70%. This confirms the previously proposed synergistic or additive effect between cannabinoid compounds produced by medical cannabis plants.

Acknowledgments

The authors thank Dr. Gary Bentley of On the Mark Scientific Editing for editing and improving the article's English.

Authors' Contributions

M.M. designed the study, wrote the article, controlled the cultivation scheme, and performed physiological, chemical, and data analyses. I.D. coordinated and executed the cytotoxicity assay and data analysis. J.P. and M.U. performed the statistical data processing. L.P. performed chemical analyses. P.K. and B.L. performed the cytotoxicity assay. A.K. provided sample dilution and filtration. P.T. supervised the study.

Author Disclosure Statement

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding Information

This work was partly supported by the Internal Grant Agency of the Faculty of Economics and Management CZU Prague, grant no. 20171023. The METROFOOD-CZ research infrastructure project (MEYS Grant No.: LM2018100) included access to its facilities.

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Cite this article as: Malík M, Doskočil I, Pavlík J, Ulman M, Praus L, Kouřimský P, Lampová B, Kuklina A, Tlustoš P (2022) Selective cytotoxicity of medical cannabis (*Cannabis sativa* L.) extracts across the whole vegetation cycle under various hydroponic and nutritional treatments, *Cannabis and Cannabinoid Research* X:X, 1–12, DOI: 10.1089/can.2022.0243.

Abbreviations Used

Δ^8-THC = Δ^8-tetrahydrocannabinol
Δ^9-THC = Δ^9-tetrahydrocannabinol
CBD = cannabidiol
CBDA = cannabidiolic acid
CBG = cannabigerol
CBGA = cannabigerolic acid
CRC = colorectal cancer
DMSO = dimethyl sulfoxide
EMEM = Eagle's Minimal Essential Medium
MTT = methylthiazolyldiphenyl-tetrazolium bromide
THCA = tetrahydrocannabinolic acid

5. SOUHRNNÁ DISKUSE

Tato kapitola je rozdělena do tří hlavních podkapitol dle jednotlivých cílů práce. V podkapitole 5.1. jsou shrnuty zásadní informace z práce 4.1. a dosažené výsledky experimentů z prací 4.2., 4.3. a 4.4. týkajících se vlivu různé koncentrace a poměru živin a biostimulantů na obsah sekundárních metabolitů, především fytokanabinoidů a terpenických sloučenin, tkáňový ionom, tvorbu květenství a biomasy rostlin konopí. Podkapitola 5.2. pojednává o biosyntéze kanabinoidních látek obsažených především v květech v průběhu vegetačního pěstebního cyklu rostlin léčebného konopí a vychází z poznatků prací 4.1., 4.3. a 4.4. Poslední podkapitola 5.3. diskutuje výsledky, kterých bylo dosaženo během *in vitro* pokusu selektivní cytotoxicity různých konopných extraktů za účelem ověření potencionálního antikarcinogenního účinku konopí. Diskutovaná data jsou v plném rozsahu obsažena v publikovaném článku 4.5.

5.1. Vliv výživy na ionom, biomasu a obsah sekundárních metabolitů rostlin konopí

Výživa je bezpochyby důležitým faktorem ve vývoji, funkci a metabolismu všech rostlinných orgánů a tkání. Pro rostliny konopí (*Cannabis sativa* L.) jsou již známy experimentální údaje o optimálních hladinách jednotlivých makroživin, jako je N, P a K, pro normální funkci a vývoj kořenového systému, nadzemní biomasy (Caplan et al. 2017b; Saloner et al. 2019; Saloner & Bernstein 2020; Shiponi & Bernstein 2021b) a tvorbu žádoucích sekundárních metabolitů (Caplan et al. 2017a; Bernstein et al. 2019a; Saloner & Bernstein 2021; Shiponi & Bernstein 2021a; Saloner & Bernstein 2022b, a). Stále je však za potřebí prozkoumat vliv jednotlivých forem makroživin na dostupnost jejich dostatečného množství v optimálním vzájemném poměru, ať už pro rostliny konopí pěstované v půdě či v jiných pěstebních médiích. Taktéž je třeba vzít v úvahu vliv mikroživin (Yep & Zheng 2021) a rostlinných biostimulantů (Bernstein et al. 2019b; Malík et al. 2022), které bývají často opomíjeny.

Zemědělství, včetně zahradnictví, je jedním z odvětví, které spotřebovává největší množství vody. Roste proto rovněž potřeba prozkoumat možnou recirkulaci a opětovné použití živných roztoků ke snížení ekologických a ekonomických dopadů (Atzori et al. 2019; Hogeboom 2020). Vzhledem k rostoucímu významu půdní ekologie a udržitelnému využívání půdy by rostliny konopí mohly dále působit jako vhodná plodina k výrobě

bioplynu v uzavřeném okruhu. Vedlejšího produktu z bioplynové stanice by se využilo jako hnojiva pro pěstované konopí a získaná biomasa by byla využita pro další produkci bioplynu. S ohledem na výtěžnost produkovaného bioplynu, problematické využití a likvidaci zbytků rostlinného materiálu léčebného konopí se jejich anaerobní digesce může jevit jako bezpečné a efektivní zpracování.

5.1.1. Vliv výživy na tkáňový ionom

Zásadní vliv při hydroponickém pěstování konopí má hodnota pH živného roztoku. V hydroponické kultuře je doporučené rozmezí pH mezi 5,5-6,0 (Velazquez et al. 2013). Při porovnání dvou různých hydroponických systémů, recirkulačního a průtokového, ovlivnilo kolísání pH živného roztoku v případě recirkulačního systému (RS) a dále též větší nabídka živin v průtokovém systému (PS), dostupnost některých živin a následné fyziologické a metabolické reakce rostlin.

Z hlediska makroprvkového ionomu rostlin konopí pěstovaných ve dvou různých hydroponických systémech RS a PS a při dvou různých výživách byly v několika případech pozorovány odlišné koncentrace v nadzemních orgánech. U dusíku byla pozorována významně vyšší kumulace v rostlinách pěstovaných v RS. Toto bylo ještě umocněno ve výživové variantě se suplementací aminokyselin (AMK), kde se pH živného roztoku zvýšilo za 24 hod až na hodnotu 8,05. Počáteční pH živného roztoku, 5,9, bylo blízké izoelektrickému bodu většiny AMK (Pogliani 1992), kdy se vyskytovaly ve formě obojetných iontů, díky čemuž byl jejich vstup do rostlinných buněk ztížen kvůli lipofilním interakcím během membránového transportu (Trapp 2004). Recirkulace ale mohla vést k vytvoření částečného náboje na některých molekulách AMK. U fosforu byla taktéž pozorována celkově vyšší koncentrace v nadzemních orgánech u rostlin pěstovaných v RS, což bylo rovněž pravděpodobně způsobeno zvýšeným pH jako v experimentu Kerwin et al. (2017). Naopak vyšší pH živného roztoku mohlo způsobit i vysrážení fosforečnanů s vápníkem (Ca^{2+}) a hořčíkem (Mg^{2+}) na nerozpustné, a pro rostliny tím pádem nedostupné soli (Lee et al. 2017), což se projevilo celkově nižšími obsahy těchto prvků u rostlin pěstovaných v RS ve srovnání s PS. Tento rozdíl mezi hydroponickými systémy ještě eskaloval ve výživové variantě s AMK, což bylo pravděpodobně způsobeno koordinací vápníku s karboxylovými, hydroxylovými, thiolovými a aminoskupinami AMK za vzniku komplexů s omezenou dostupností pro rostliny (Maeda et al. 1990). K tomuto procesu mohla také přispět zvýšená tvorba kořenových exsudátů obsahujících záporně nabité skupiny

schopné koordinovat a vázat vápník a hořčík ze živného roztoku. Je pravděpodobné, že v RS bylo vylučováno více exsudátů kvůli změně pH v cytosolu a také díky zvýšené funkci citrátového cyklu po vychytávání negativně nabitých AMK (Ryan et al. 2001). Koncentrace draslíku a síry byly v obou hydroponických systémech podobné. To mohlo být způsobeno párováním opačně nabitých iontů v roztoku. Anion, který je vychytáván relativně pomalu, může částečně snížit rychlost vychytávání svého protiiontu, jak bylo pozorováno u účinku SO_4^{2-} na absorpci K^+ (Marschner 2012). Při suplementování AMK síra vykazovala trend akumulace podobný dusíku, ale v nižší koncentraci. Toto bylo způsobeno AMK obsahujícími síru, cysteinem a methioninem.

Koncentrace jednotlivých mikroprvků je pro rostliny orgánově specifická. Obsah železa vykazoval kumulativní trend v závislosti na čase, podobný Ca a Mg, v obou hydroponických systémech i výživových variantách. Kumulace byla hlavně ve stoncích, kvůli nízké mobilitě Fe v rostlinách. U manganu byla pozorována významně vyšší kumulace v rostlinách pěstovaných v RS, ale naopak u zinku bylo dosaženo vyšší koncentrace v konopí pěstovaném v PS. Dle Gillespie et al. (2020) obsah Mn a Zn v hydroponicky pěstovaných rostlinách roste úměrně se zvyšujícím se pH. U dvouděložných rostlin může membránový protein IRT1 kromě Fe (II) přenášet taktéž další dvojmocné kationty, jako je například Mn^{2+}. Tento transport Mn^{2+} však může být dále inhibován Zn^{2+}, protože tyto dva prvky si mohou v tomto případě konkurovat (Korshunova et al. 1999). Obsah bóru byl nejvyšší v listech, kde také vykazoval kumulativní trend v závislosti na čase. Bylo dosaženo jeho celkově vyšších koncentrací v rostlinách pěstovaných v PS. Absorpce boru je totiž u rostlin relativně pasivní a závisí tedy na množství rozpuštěných iontů boru (Dannel et al. 2002). V PS, kde byl živný roztok doplňován několikrát týdně, byla nabídka bóru několikanásobně vyšší než v RS. U mědi byla pozorována významně vyšší kumulace v rostlinách pěstovaných v RS. To bylo pravděpodobně způsobeno vzájemnou kompeticí Cu/Zn a jejich podobnými mechanismy příjmu (Stuckey et al. 2021). U molybdenu bylo dosaženo vyšších koncentrací u konopí pěstovaného v RS. To bylo způsobeno kolísáním a občasným zvýšením pH v RS ve srovnání se stabilním pH v PS. Při vyšším pH se molybden stává rozpustnější a je pro rostliny přístupný především ve své aniontové formě jako MoO_4^{2-}. Při pH <5 se molybden vyskytuje hlavně jako $HMoO_4^-$, který je pro rostliny hůře dostupný (Kaiser et al. 2005; Smedley & Kinniburgh 2017; Lawson-Wood et al. 2021). Dle získaných výsledků je patrné, že tkáňový ionom rostlin konopí pěstovaných v hydroponii

za kontrolovaných podmínek lze významně ovlivnit výživou. Toto má pak sekundární efekt na tvorbu biomasy a obsah sekundárních metabolitů rostlin konopí.

5.1.2. Vliv výživy na tvorbu biomasy

Změněná dostupnost a zásoba jednotlivých živin také ovlivnila výnos suché biomasy. V polním pokusu aplikace jednotlivých složek digestátu jako zdroje dusíku v porovnání s minerálním NPK hnojivem nebyly zjištěny žádné signifikantní rozdíly při srovnání průměrného výnosu sušiny rostlin. K tomuto pravděpodobně částečně přispěly také poměrně vysoké rozdíly v hmotnosti sklizených rostlin. Podobné výsledky uvedli Tsachidou et al. (2019), kteří experimentálně prokázali, že aplikace zbytků anaerobní digesce jako zdroje dusíku prokázaly schopnost udržet výnosy píce na podobné úrovni jako při použití minerálního NPK hnojiva. Současně se v této praxi snižuje environmentální riziko spojené s vyplavováním nitrátového dusíku.

Různé pěstební hydroponické systémy (RS a PS) způsobily taktéž změnu v dynamice růstu rostlin. Nejvýraznější nárůst byl od 11. vegetačního týdne postupně zaznamenán u suché hmotnosti květů. To bylo pravděpodobně způsobeno zvýšenou mobilizací a translokací látek z kořenů do listů a květů (Ludewig & Frommer 2002), kde se dříve hromadily v důsledku přísunu živin. Maximálního výnosu suchých květů bylo v PS dosaženo ve 12. týdnu, ale v RS až ve 13. týdnu. Obdobných výsledků bylo dosaženo u rostlin pěstovaných v PS s AMK aditivem. Toto dřívější dozrávání rostlin v PS oproti RS bylo pravděpodobně způsobeno vyšší dávkou dodávaných živin v PS, což zajistilo optimální hnojení, které může zkrátit dobu dozrání konopí (Caplan et al. 2017b). Tuto hypotézu částečně podpořilo předčasné dozrávání rostlin i s ohledem na maximální koncentrace THCA.

5.1.3. Vliv výživy na obsah sekundárních metabolitů

Různé hydroponické pěstební systémy dále ovlivnily vývoj a následnou koncentraci THCA, kanabinolové kyseliny (CBNA) a CBCA v květech rostlin konopí. Dle maximální dosažené koncentrace THCA rostliny pěstované v PS dozrály dříve než ty pěstované v RS. To bylo ještě umocněno u výživové varianty s AMK. Při zohlednění dodaného množství živin v živném roztoku u rostlin pěstovaných v PS a skutečnosti, že čerstvý živný roztok zde byl míchán 2-3x týdně. Toto mohlo být způsobeno buď zvýšeným abiotickým stresem z

vysokých dávek živin (Gepstein & Glick 2013; Gong et al. 2020) nebo tím, že vyšší přísun živin v PS zajistil optimální hnojení, které mohlo zkrátit dobu zrání konopí (Caplan et al. 2017b). Tato druhá hypotéza byla částečně podpořena i nižší koncentrací CBNA, což je oxidační produkt THCA, u rostlin pěstovaných v PS ve srovnání s těmi pěstovanými v RS. U výživové varianty s AMK toto bylo ještě pravděpodobně posíleno antioxidační aktivitou AMK, které snižovaly stres z prostředí vychytáváním volných kyslíkových radikálů (Calvo et al. 2014). Vzájemný poměr Cu a Zn pravděpodobně také přispěl ke zvýšenému oxidačnímu stresu u rostlin pěstovaných v RS. Vysoká koncentrace Cu totiž může v rostlinách způsobit abiotický stres, ale vysoká koncentrace zinku může tento efekt zvrátit. Vyšší obsah Cu byl zaznamenán u rostlin pěstovaných v RS, ale vyšší obsah Zn naopak u rostlin v PS (Upadhyay & Panda 2010; Thounaojam et al. 2014). Koncentrace CBCA nebyly výživou takto radikálně ovlivněny, a byly v obou hydroponických systémech podobné.

Rozdílné hydroponické kultivační systémy ovlivnily také koncentraci terpenických sloučenin, konkrétně limonenu, β-myrcenu a β-karyofylenu ve zralých květech rostlin konopí. Obsah monoterpenů, limonenu a β-myrcenu, vykazoval v měřených týdnech stejný trend v obou hydroponických systémech i výživových variantách jako koncentrace THCA. Toto je v souladu se zjištěním Aizpurua-Olaizola et al. (2016) pro konopný chemický fenotyp I. Důvodem může být to, že monoterpeny jsou biosyntetizovány ve stejných glandulárních trichomech a částečně podobnou biochemickou cestou jako kanabinoidy, sestávající se z alkylresorcinolových a monoterpenových skupin (Sirikantaramas et al. 2007; Booth et al. 2017; Livingston et al. 2020). Ve výživové variantě s AMK podobně jako u Saloner a Bernstein (2021) naše výsledky ukázaly, že nadměrně zvýšená hladina dusíku v živném roztoku úměrně snižuje koncentraci THCA. Ale naopak při překročení specifické hraniční koncentrace, což bylo v případě Saloner a Bernstein (2021) 160 mg N/l, lze pozorovat reverzibilní zvýšení koncentrace limonenu a myrcenu. Toto je v souladu se studiemi ukazujícími pozitivní závislost tvorby monoterpenů na hnojení dusíkem (McCullough & Kulman 1991; Close et al. 2004). Vysoké koncentrace N v listech podporovaly fotosyntetickou aktivitu, což zvýšilo dostupnost asimilovaného uhlíku využívaného k tvorbě metabolitů MEP dráhou (Ormeno & Fernandez 2012). K raným krokům v produkci terpenických sloučenin v rostlinách přispívají dvě biosyntetické cesty. První je MVA cesta, která se podílí na biosyntéze seskviterpenů a triterpenů. Druhá, v plastidech lokalizovaná MEP cesta, se účastní biosyntézy monoterpenů, diterpenů a tetraterpenů (Bouvier et al. 2005). Fytokanabinoidy jsou syntetizovány z izoprenoidních

prekurzorů v kombinaci s polyketidy (Dewick 2002). Geranylpyrofosfát nezbytný pro produkci terpenoidní části kanabinoidů je však převážně (>98 %) syntetizován MEP cestou v plastidech (Fellermeier et al. 2001). Vzhledem k tomu, že limonen, β-myrcen a terpenoidní část THCA jsou syntetizovány stejnou biochemickou cestou a vykazují opačnou koncentrační odezvu v květech konopí po přidání AMK do živného roztoku, lze dojít k závěru že je tímto ovlivněna biosyntéza ketidové části molekuly THCA (Tedesco & Duerr 1989). Naopak seskviterpen, β-karyofylen, vykazoval vyšší a konstantní obsah u rostlin pěstovaných v PS téměř ve všech měřených týdnech. Tento rozdíl mezi obsahem monoterpenů a seskviterpenů mohl být způsoben tím, že dvě odlišné výše zmíněné biosyntetické dráhy přispívají k syntéze rostlinných terpenů v raných krocích. Tyto dráhy jsou regulovány různými substráty (Eisenreich et al. 1998; Bouvier et al. 2005). Toto bylo pravděpodobně ovlivněno i abscisovou kyselinou (ABA), jejíž koncentrace mohla být u rostlin pěstovaných v RS zvýšena, a která snižuje obsah seskviterpenů v rostlinách konopí (Mansouri & Asrar 2012).

5.2. Biosyntéza kanabinoidních látek během vývoje konopného květenství

Nejkoncentrovanějším kanabinoidem v tomto chemickém fenotypu I léčebného konopí byla THCA. Optimální zralost rostlin byla tedy určována dle maximální dosažené koncentrace tohoto kanabinoidu v určitém vegetačním týdnu. Koncentrace THCA v listech a květech se u rostlin pěstovaných v obou hydroponických systémech i za všech výživových variant pomalu zvyšovala až do 4. týdne. Od 5. týdne se začala prudce zvyšovat, protože květy byly již dostatečně narostlé a mohly být analyzovány samostatně. Maxima tohoto kanabinoidu bylo dosaženo při optimální zralosti rostlin. Oxidační produkt THCA, CBNA, měl podobný průběh tvorby a maxima jako THCA. Koncentrace třetího měřeného kanabinoidu, CBCA, ale vykazovala opačný časově závislý trend než koncentrace THCA a CBNA. V obou hydroponických systémech koncentrace s časem klesala, což je pro tento kanabinoid běžný jev, protože jeho obsah standardně klesá s věkem rostlin (Morimoto et al. 1997).

Kombinace koncentrace THCA a suché hmotnosti květů se odrazila ve výnosu THCA z rostliny. U sušených květů byl hodnocen výtěžek THCA na rostlinu týdně v obou pěstebních hydroponických systémech i výživových variantách. U rostlin pěstovaných v RS byla pozorována téměř lineární závislost výnosu THCA na čase pro obě výživové varianty.

Naopak výtěžnost THCA u rostlin pěstovaných v PS dosáhla maxima dříve, což bylo ještě umocněno u výživové varianty s AMK. Každopádně při porovnání obou hydroponických kultivačních systémů i výživových variant v týdnech maximálního výnosu THCA byl výnos THCA u rostlin pěstovaných v RS cca dvojnásobný oproti výnosu THCA z rostlin pěstovaných v PS. To by mohlo být způsobeno zvýšenou produkcí ABA v reakci na stres, která zpomaluje růst rostlin a zvyšuje produkci, koncentraci a výnos THCA (Mansouri et al. 2009; Caplan et al. 2019).

5.3. Potencionální antikarcinogenní účinky konopí

Byla testována cytotoxicita extraktů hydroponicky pěstovaného léčebného konopí chemotypu I s poměrem obsahových kanabinoidů THCA/CBDA >>1,0, v různých fázích zralosti rostlin odebraných během vegetačního cyklu. Studie poskytla důkazy o selektivní cytotoxické aktivitě extraktů konopí proti buňkám kolorektálního karcinomu ve všech vegetačních stádiích rostlin (1. až 13. týden), při různých variantách výživy a při pěstování v obou hydroponických systémech (RS, PS). Extrakty z konopí vykazovaly nejvyšší selektivní cytotoxicitu dle poloviny maximální inhibiční koncentrace (IC_{50}) proti buněčné linii HT-29 odvozené od adenokarcinomu tlustého střeva v posledních týdnech vegetace, kdy také koncentrace THCA dosáhla svého maxima v závislosti na hydroponickém pěstebním systému a výživě. Samotné THCA přispělo k selektivní cytotoxicitě konopných extraktů vůči HT-29 téměř 65 %. Několik experimentů založených na buněčných liniích ukázalo, že THCA interaguje s různými molekulárními cíli a vykazuje potenciální neuroprotektivní, protizánětlivé, imunomodulační, cytotoxické a antineoplastické vlastnosti (Moreno-Sanz 2016; Nallathambi et al. 2017; Nallathambi et al. 2018). Kromě toho několik studií *in vivo* ukázalo, že tato sloučenina vykazuje farmakologické účinky u hlodavců, pravděpodobně zapojením kanabinoidních receptorů typu 1 (Moreno-Sanz 2016).

Na základě postupných selekcí byly zahrnuty další fytokanabinoidy, konkrétně CBGA, CBG, CBD a Δ^9-THC. Tyto čtyři fytokanabinoidy přispěly dalšími 5 % k selektivní cytotoxicitě. Dohromady těchto pět výše zmíněných fytokanabinoidů ovlivnilo cytotoxický účinek extraktů téměř ze 70 %. Toto potvrzuje dříve navrhovaný synergismus mezi účinnými látkami v rostlinách konopí (Russo 2011). Směsi různých fytokanabinoidů vykazují specifickou interakci podporující buněčnou cytotoxickou aktivitu a jsou biologicky

aktivnější než jednotlivě izolované fytokanabinoidy (Namdar et al. 2019; Mazuz et al. 2020; Peeri & Koltai 2022).

6. ZÁVĚR

Tématem této disertační práce je studium vlivu výživy na tvorbu biomasy a obsah terapeuticky využitelných sekundárních metabolitů rostlin konopí (*Cannabis sativa* L). Použití konopí ve zdravotnictví vyžaduje přísnou kontrolu optimální produkce bioaktivních látek. Rostlinné fenotypy a obsah sekundárních metabolitů, jako jsou fytokanabinoidy, jsou silně ovlivněny vnějšími faktory, jako je dostupnost a příjem živin. Tato práce je souborem pěti vědeckých článků, které jsou rozděleny do tří hlavních částí. V první části se práce zabývá vlivem koncentrace a poměru živin a biostimulantů na tkáňový ionom, tvorbu biomasy, především květů, a na obsah fytokanabinoidů a terpenických sloučenin rostlin konopí. Druhá část práce se věnuje biosyntéze kanabinoidních látek obsažených především v květech v průběhu pěstebního cyklu rostlin léčebného konopí. Poslední okruh je zaměřen na ověření potencionálních antikarcinogenních účinků rostlin konopí pomocí testování selektivní cytotoxicity různých konopných extraktů *in vitro*.

Ze srovnání dosažených výsledků a vlastností vedlejších produktů z anaerobní digesce a minerálního NPK hnojiva lze říci, že vedlejší produkty z anaerobní digesce mohou být použity jako vhodná alternativa k minerálním NPK hnojivům v polní produkci konopí. Rostliny dosahovaly srovnatelných výnosů a prokázaly vyšší míru akumulace živin v jednotlivých rostlinných pletivech. Tyto odpadní materiály jsou však variabilní jak ve složení konkrétních živin, tak v jejich dostupnosti pro rostliny. Tato variabilita je dána odlišností vstupních surovin do procesu anaerobní digesce. Dále byly demonstrovány výhody a nevýhody dvou různých pěstebních hydroponických systémů při dvou různých systémech výživy léčebného konopí. Při pěstování rostlin v recirkulačním hydroponickém systému bylo dosaženo vyšších výtěžků hlavního kanabinoidu léčebného konopí chemotypu I, tetrahydrokanabinolové kyseliny (THCA), s mnohem nižší celkovou spotřebou vody a živin, ale za delší dobou kultivace, vyšších koncentrací kanabinolové kyseliny (CBNA) a nižších koncentrací seskviterpenu, β-karyofylenu, v květech. Naopak průtokový hydroponický systém umožnil lepší kontrolu nad živným roztokem se stabilním dodáváním přesné koncentrace živin, čímž pravděpodobně urychlil dozrávání rostlin, ovšem za cenu vyšší spotřeby vody a hnojiv a výrazně nižšího celkového výnosu monoterpenů a THCA. Výživový doplněk na bázi aminokyselin výrazně zvýšil obsah dusíku a síry, ale snížil akumulaci vápníku a železa v celé rostlině konopí při pěstování v obou zmíněných hydroponických systémech. Dále způsobil dřívější dozrávání rostlin, což se následně

projevilo v koncentraci a výtěžku THCA, ale snížil obsah CBNA v květech. V obou pěstebních hydroponických systémech také významně zvýšil obsah monoterpenů, limonenu a β-myrcenu. Tato práce převážně zkoumala jen na THCA bohatý konopný chemotyp I pěstovaný hydroponicky v médiu „Euro Pebbles“ (keramzit). Proto by bylo do budoucna určitě zajímavé provést tyto experimenty s rostlinami konopí dalších chemotypů kultivovaných v odlišných pěstebních substrátech. Z ekonomického hlediska by též mohlo být pro zahradnický průmysl potencionálně využitelné provést obdobné studie s průběžným doplňováním omezujících živin a vyrovnáváním pH živného roztoku až do jeho konečné spotřeby.

Tato práce v neposlední řadě poskytla důkaz o selektivní cytotoxické aktivitě extraktů z rostlin konopí (chemotyp I) proti buněčným liniím kolorektálního karcinomu ve všech vegetačních stádiích rostlin za pěstování v různých hydroponických systémech při odlišné výživě. Extrakty z konopí vykazovaly nejvyšší selektivní cytotoxický účinek v posledních týdnech růstu, kdy také koncentrace THCA zpravidla dosáhla svého maxima. Samotné THCA přispělo téměř 65 % k selektivnímu cytotoxickému účinku konopných extraktů proti buněčné linii HT-29 odvozené od adenokarcinomu tlustého střeva. Kanabigerolová kyselina (CBGA), kanabigerol (CBG), kanabidiol (CBD) a Δ^9-tetrahydrokanabinol (Δ^9-THC) přispěly k selektivní cytotoxicitě dalšími 5 %. Dohromady těchto pět zmíněných fytokanabinoidů ovlivnilo cytotoxicitu extraktů téměř ze 70 %. To potvrzuje již dříve navrhovaný synergický či aditivní účinek mezi kanabinoidními sloučeninami produkovanými rostlinami konopí.

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