Fizjologiczne podstawy syntezy wybranych metabolitów wtórnych w elicytowanych i transformowanych kulturach tkankowych roślin *Dionaea muscipula* J. Ellis i ich właściwości antybakteryjne.

Praca doktorska

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Spis treści:

1. Spis skrótów

CAT	(ang. *catalase*), katalaza
DPPH	2,2-difenylo-1-pikrylohydrazyl
HPLC	(ang. *high-performance liquid chromatography*), wysokosprawna chromatografia cieczowa
MBC	(ang. *minimum bactericidal concentration*), minimalne stężenie antybakteryjne
MDA	(ang. *malondialdehyde*), aldehyd dimalonowy
MIC	(ang. *minimum inhibitory concentration*), minimalne stężenie hamujące
MS	pożywka do hodowli roślin wg. Murashige i Skooga
PCR	(ang. *polimerase chain reaction*), reakcja łańcuchowa polimerazy
ROS	(ang. *reactive oxygen species*), reaktywne formy tlenu
SOD	(ang. *superoxide dismutase*), dysmutaza ponadtlenkowa
T-DNA	(ang. *transfer* DNA), DNA transferowe

2. Wykaz publikacji wchodzących w skład pracy doktorskiej

Makowski, W.✉, Tokarz, K.M., Tokarz, B., Banasiuk, R., Witek, K., Królicka, A.✉ (2020). Elicitation-based method for increasing the production of antioxidant and bactericidal phenolic compounds in *Dionaea muscipula* J. Ellis tissue. *Molecules*, *25*(8), 1794.

IF$_{2019}$: 3,267
IF$_{5\text{-letni}}$: 4,588
MNiSW 2019: 100 pkt.
liczba cytowań wg WoS: 10

Mój wkład w powstanie tej publikacji polegał na: zaprojektowaniu doświadczeń i sformułowaniu hipotez badawczych, założeniu i przeprowadzeniu doświadczeń, przeprowadzeniu analiz biometrycznych, spektrofotometrycznych i mikrobiologicznych, analizie statystycznej uzyskanych wyników, interpretacji uzyskanych wyników, napisaniu pierwotnej wersji manuskryptu oraz odpowiedzi na uwagi recenzentów i poprawie manuskryptu.

Makowski, W.✉, Królicka, A.✉, Nowicka, A., Zwyrtková, J., Tokarz, B., Pecinka, A., Banasiuk R., Tokarz, K.M. (2021). Transformed tissue of *Dionaea muscipula* J. Ellis as a source of biologically active phenolic compounds with bactericidal properties. *Applied Microbiology and Biotechnology*, *105*(3), 1215-1226.

IF$_{2020}$: 4,813
IF$_{5\text{-letni}}$: 4,697
MNiSW 2020: 100 pkt.
liczba cytowań wg WoS: 2

Mój wkład w powstanie tej publikacji polegał na: opracowaniu koncepcji badań i sformułowaniu hipotez badawczych, zaprojektowaniu doświadczeń, transformacji roślin, wykonaniu analiz PCR oraz Southern Blotting, przeprowadzeniu analiz biometrycznych, spektrofotometrycznych i mikrobiologicznych, analizie statystycznej uzyskanych wyników, interpretacji uzyskanych wyników, napisaniu pierwotnej wersji manuskryptu oraz odpowiedzi na uwagi recenzentów i poprawie manuskryptu.

Makowski, W.✉, Królicka, A.✉, Tokarz, B., Miernicka, K., Kołton, A., Pięta, Ł., Malek, K., Ekiert, H., Szopa, A., Tokarz, K. M.✉ (2021). Response of physiological parameters in *Dionaea muscipula* J. Ellis teratomas transformed with *rol*B oncogene. *BMC Plant Biology*, *21*(564), 1-13.

IF$_{2021}$: 4,215
IF$_{5\text{-letni}}$: 4,960
MEiN2021: 140 pkt.
liczba cytowań wg WoS: 0

Mój wkład w powstanie tej publikacji polegał na: opracowaniu koncepcji badań i sformułowaniu hipotez badawczych, zaprojektowaniu doświadczeń, przeprowadzeniu analiz spektrofotometrycznych, analizie statystycznej uzyskanych wyników, interpretacji uzyskanych wyników, napisaniu pierwotnej wersji manuskryptu, odpowiedzi na uwagi recenzentów i poprawie manuskryptu.

Sumaryczny IF: 12,295; Sumaryczna punktacja MEiN: 340 pkt.

wg WoS – według Web of Science

✉ autor korespondencyjny

3. Wstęp teoretyczny

3.1. Muchołówka amerykańska (*Dionaea muscipula* J. Ellis) – niemodelowy gatunek w badaniach nad syntezą, akumulacją i właściwościami biologicznie czynnymi związków fenolowych

Rośliny mięsożerne (zwane potocznie owadożernymi) od setek lat stanowiły obiekt badań biologów, którzy starali się zrozumieć ich osobliwe przystosowanie do środowiska, jakim jest mięsożerność. Za prekursora badań nad mięsożernością u roślin uznaje się Charlesa Darwina, który w 1875 roku wydał książkę *Insectivorous Plants*, będącą pierwszym na świecie opracowaniem naukowym poświęconym tej grupie roślin [Darwin i Darwin 1915]. Rośliny mięsożerne mają zdolność do produkcji barwników fotosyntetycznie czynnych (chlorofili i karotenoidów), co powoduje, że są one zdolne do przeprowadzania efektywnego procesu fotosyntezy. Ponadto w toku ewolucji rośliny te wykształciły zdolność do suplementowania pierwiastków biogennych, takich jak azot, fosfor czy potas dzięki trawieniu owadów, mięczaków, a czasem nawet małych kręgowców [Juniper 1989]. Jest to ściśle związane z faktem, iż w naturze rośliny te występują zwykle na wilgotnych, nasłonecznionych siedliskach ubogich w składniki mineralne [Givnish i in. 1984]. Możliwość czerpania składników pokarmowych z innego źródła niż roztwór glebowy warunkuje bardziej efektywny wzrost, kwitnienie i wydanie większej ilości nasion. Niemniej jednak zdobycie i strawienie ofiary przez rośliny mięsożerne nie jest warunkiem koniecznym do ich przetrwania [Pavlovic i Saganova 2015].

Jedną z najbardziej popularnych roślin mięsożernych jest muchołówka amerykańska (*Dionaea muscipula* J. Ellis), która należy do rodziny rosiczkowatych (Droseraceae). Ze względu na budowę swoich pułapek liściowych i zdolność do wykonywania jednego z najszybszych ruchów indukowanych bodźcem mechanicznym w świecie roślin, to właśnie muchołówka stanowi główny obiekt badań botaników pracujących nad syndromem mięsożerności u roślin [Pavlovic i in. 2017]. Roślina ta jest byliną, która naturalnie występuje jedynie w Karolinie Północnej i Południowej Stanów Zjednoczonych Ameryki, a rodzaj *Dionaea* jest taksonem monotypowym, obejmującym wyłącznie jeden gatunek [Hook 2001]. Ze względu na jej biologię, muchołówkę amerykańską (Fot. 1) można więc uznać za gatunek modelowy do badań nad mięsożernością wśród roślin [Pavlovic i in. 2017] i wraz z czułkiem

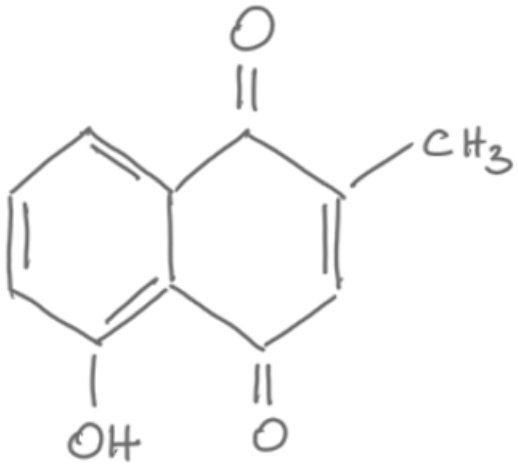
wstydliwym (*Mimosa pudica* L.) za model w badaniach nad elektrofizjologią roślin [Guo i in. 2015; Suda i in. 2020]. Jednakże dotychczas gatunek ten nie był rozpatrywany jako model w badaniach poświęconych fizjologii i biotechnologii roślin.

Fotografia 1. Rośliny muchołówki amerykańskiej (*Dionaea muscipula* J. Ellis).

Rośliny należące do rodziny Droseraceae od wieków wykorzystywane były w medycynie ludowej [Królicka i in. 2008]. Ze swoich właściwości leczniczych znane były przede wszystkim różne rosiczki. Rodzaj *Drosera* sp. (rosiczka) reprezentowany jest przez ponad 180 gatunków roślin na całym świecie i w wielu krajach ziele rosiczek wykorzystywane było do leczenia kaszlu, próchnicy zębów, stanów zapalnych i infekcji grzybiczych oraz bakteryjnych [Didry i in. 1998; Putalun i in. 2010; Kawiak i in. 2011]. Dziś wiadomo, że szerokie spektrum właściwości biologicznie czynnych, jakie wykazują roślin mięsożerne z rodziny Droseraceae, wynika z ich zdolności do syntezy różnych metabolitów wtórnych z grupy związków fenolowych [Gaascht i in. 2013].

Roślinne metabolity wtórne są produktami szlaków niezwiązanych z procesami metabolizmu pierwotnego (oddychanie komórkowe i fotosynteza) i syntetyzowane są w roślinach w odpowiedzi na działanie różnych czynników środowiskowych [Caretto i in. 2015]. Metabolity należące do grupy związków fenolowych wykorzystywane są przez rośliny w reakcjach odpornościowych, będących następstwem stresu środowiskowego [Cheynier i in.

2013]. Uznaje się, że naturalna zdolność roślin mięsożernych z rodziny Droseraceae do syntezy rozmaitych pochodnych związków fenolowych wynika z faktu, iż związki te zabezpieczają pułapki liściowe roślin mięsożernych przed procesami gnilnymi w trakcie trawienia złapanej przez nie ofiary [Widhalm i Rhodes 2016]. Ponadto wykazano, że metabolity te mogą zabezpieczać organy roślin mięsożernych przed nadmiernym natężeniem promieniowania świetlnego lub zbyt wysoką energią fali, niesioną przez promieniowanie krótkofalowe [Tokarz i in. 2018]. Dzięki temu rośliny te mogą z powodzeniem zasiedlać stanowiska o bezpośredniej ekspozycji słonecznej [Tkalec i in. 2015]. Oprócz kwasów fenolowych, fenylopropanoidów, flawonoli czy antocyjanów rosiczkowate akumulują w swoich tkankach liczne pochodne 1,4-naftochinonów, z których najpopularniejszą pochodną jest plumbagina (5-hydroksy-2-metylo-1,4-naftochinon), której wzór strukturalny pokazano na Rys. 1 [Babula i in. 2009].



Rysunek 1. Wzór strukturalny plumbaginy.

Dotychczas najbogatszym poznanym źródłem plumbaginy są właśnie rośliny muchołówki amerykańskiej [Makowski i in. 2020]. Poza tym wcześniejsze badania roślin *D. muscipula* wykazały, że charakteryzuje się ona bardzo szerokim spektrum akumulowanych pochodnych fenolowych, a przez to unikatowym składem biochemicznym [Gaascht i in. 2013]. Z tego też względu muchołówka amerykańska została wybrana jako model do badań nad podstawami syntezy związków fenolowych u roślin poddanych działaniu elicytora i transformacji

genetycznej w prezentowanej rozprawie doktorskiej. Założono bowiem, że ten niemodelowy gatunek może dostarczyć nowych informacji na temat fizjologii roślin leczniczych oraz właściwości biologicznie czynnych związków fenolowych pochodzenia roślinnego, w tym rzadko spotykanych metabolitów z grupy 1,4-naftochinonów.

3.2. Związki fenolowe – synteza, właściwości, znaczenie i potencjalne zastosowanie

Metabolity wtórne należące do grupy związków fenolowych to jedna z najliczniejszych grup związków chemicznych pochodzenia roślinnego [Kovacik i in. 2012]. Obecnie znanych jest kilka tysięcy związków fenolowych, które syntetyzowane są przez rośliny wyższe. Przyjmuje się, że około 2% węgla w metabolizmie roślinnym pożytkowane jest na syntezę związków wtórnych z grupy polifenoli [Cheynier i in. 2013]. Z tego też względu stanowią one jedną z głównych składowych odpowiedzi roślin na biotyczne i abiotyczne czynniki stresowe, takie jak: stres radiacyjny, atak patogenów, uszkodzenia tkanki przez roślinożerców, stres niskich i wysokich temperatur, suszy, zasolenia i innych [Caretto i in. 2015]. Zgodnie z przyjętą nomenklaturą związki fenolowe to naturalne metabolity wtórne powstające w cyklu kwasu szikimowego i/lub w poliketydowym szlaku octanowo-malonianowym. Produktem pierwszego z nich są fenylopropanoidy, a dokładnie kwas cynamonowy, który stanowi substrat w dalszych przemianach biochemicznych, prowadzących do syntezy innych grup związków fenolowych (flawonoidów czy antocyjanów) [Cheynier i in. 2013]. Poliketydowy szlak octanowo-malonianowy odpowiada za dostarczanie fenoli prostych, jak również licznych polifenoli. Szlak ten jest postulowany jako miejsce produkcji 1,4-naftochinonów, jednak kompletna droga ich syntezy w roślinach wyższych nie została jeszcze w pełni opisana [Widhalm i Rhodes 2016].

Ze względu na swoją budowę chemiczną i wysoki potencjał oksydo-redukcyjny związki fenolowe biorą udział w neutralizacji reaktywnych form tlenu (ROS), które powstają w konsekwencji działania stresu środowiskowego [Makowski i in. 2019]. Stanowią więc one część nieenzymatycznego systemu antyoksydacyjnego, zabezpieczając komórki roślinne przed peroksydacją błon biologicznych. Ta właściwość związków fenolowych powoduje, że większość z nich uznawana jest za substancje prozdrowotne, o szerokim zastosowaniu

w dietetyce, kosmetologii czy farmakologii [Metsamuuronen i Siren 2019]. Poza tym związki te pełnią szereg innych funkcji w ekofizjologii roślin: mogą być chelatorami metali ciężkich [Tokarz i in. 2020], działać przeciwko patogenom bakteryjnym i grzybiczym, zapobiegając rozwojowi infekcji u roślin [Bhattacharya i in. 2010], czy działać jako metabolity ekranujące, neutralizując nadmiar energii świetlnej docierającej do organów roślinnych [Tokarz i in. 2018]. Duża część związków fenolowych to także barwniki roślinne, które barwią płatki kwiatów lub powodują przebarwienia w komórkach epidermy liści roślin ozdobnych [Widhalm i Rhodes 2016]. Z tego też względu badania prowadzące do zrozumienia podstaw syntezy związków fenolowych w kontekście działania stresów środowiskowych wydają się znaczące dla rozwoju szeroko pojętej biologii roślin.

Spośród związków fenolowych zidentyfikowanych w zielu muchołówki amerykańskiej najwyższą aktywność biologicznie czynną przypisuje się 1,4-naftochinonom, a w szczególności plumbaginie, będącej metabolitem wiodącym w obrębie tej grupy [Makowski i in. 2020]. Wykazano bowiem, że 1,4-naftochinony wykazują wysoką aktywność cytotoksyczną. Związki te mają zdolność do interkalowania pomiędzy nici DNA, mogą zaburzać gospodarkę ROS i prowadzić do zwiększenia produkcji wolnych rodników, a co najważniejsze – zaburzają działanie licznych białek regulujących szlaki sygnalizacji komórkowej, co prowadzić może do indukowania procesów śmierci komórkowej [Kumagai i in. 2012]. Pomimo dużego potencjału biologicznie czynnego związków z grupy 1,4-naftochinonów, niewiele wiadomo na temat ich syntezy w roślinach mięsożernych oraz możliwości zwiększania ich puli w materiale roślinnym [Widhalm i Rhodes 2016]. Ponadto wysoka aktywność biologicznie czynna tej grupy związków fenolowych predysponuje je do badań nad możliwością ich wykorzystania w przemyśle farmaceutycznym [Krychowiak i in. 2014].

Jednym z problemów współczesnej medycyny jest rosnąca oporność bakterii chorobotwórczych w stosunku do większości komercyjnie dostępnych antybiotyków. Zjawisko to dotyczy zarówno izolatów klinicznych, jak również środowiskowych [Krychowiak i in. 2018]. Z tego względu współczesna biotechnologia poszukuje związków chemicznych pochodzenia naturalnego, o silnym potencjale bakteriobójczym, które nie będą wywoływać oporności wśród bakterii [Michalak i in. 2021]. Biorąc pod uwagę wskazane powyżej właściwości związków fenolowych, w pracy doktorskiej założono, że ekstrakty

pozyskane z tkanek muchołówki amerykańskiej, zasobne w związki fenolowe, będą cechować się dużym potencjałem bakteriobójczym.

3.3. Kultury *in vitro*, elicytacja i transformacja genetyczna – techniki biotechnologiczne wykorzystywane do badań nad muchołówką amerykańską

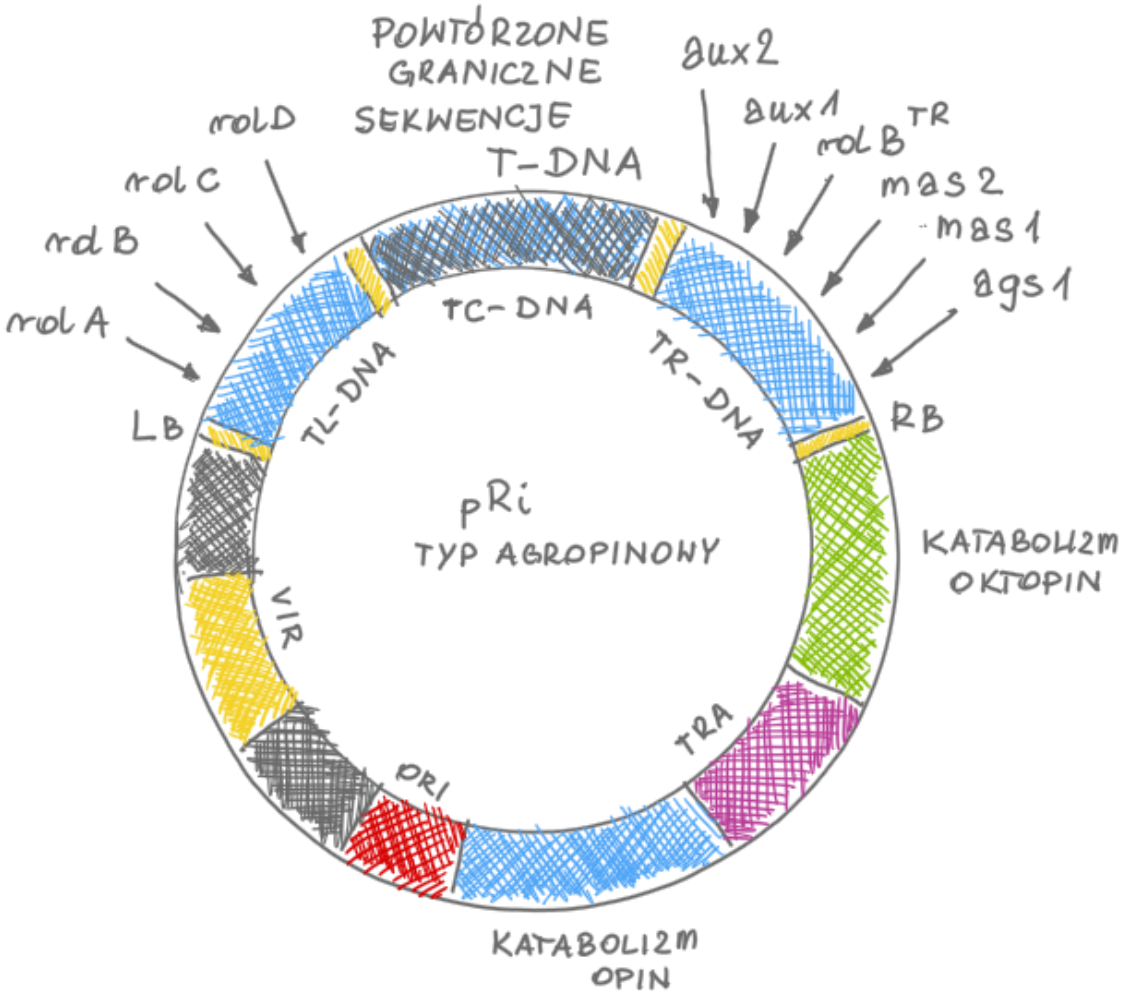
Większość roślin z rodziny Droseraceae znajduje się pod ścisłą ochroną gatunkową. Pozyskiwanie roślin muchołówki amerykańskiej ze stanowisk naturalnych jest niemożliwe, dlatego w celu wydajnej propagacji wyrównanego materiału roślinnego zostały opracowane protokoły do namnażania klonalnego roślin mięsożernych w warunkach *in vitro* [Banasiuk i in. 2012]. Roślinne kultury *in vitro* to technika służąca do mnożenia materiału roślinnego w warunkach sterylnych, na sztucznie skomponowanych pożywkach, w plastikowych lub szklanych pojemnikach. Kultury tkankowe od wielu lat służą do propagowania materiału roślinnego w różnych celach: komercyjnych i badawczych [Dias i in. 2016]. Zaletą tej techniki rozmnażania roślin jest możliwość stosowania ściśle określonych warunków uprawy, włączając w to skład pożywki, warunki oświetlenia, temperaturę, fotoperiod itp. [Tokarz i in. 2018]. Ponadto kultury *in vitro* roślin dają możliwość przeprowadzania różnych zabiegów biotechnologicznych na materiale roślinnym, których nie umożliwiają tradycyjne metody uprawy roślin. Kultury tkankowe powszechnie wykorzystywane są w biotechnologii roślin leczniczych, z uwagi na fakt, że wiele gatunków roślin o właściwościach prozdrowotnych nie może być pozyskiwanych ze stanu naturalnego, cechuje się trudnościami w rozmnażaniu generatywnym, bądź w uprawie tradycyjnej daje surowiec roślinny o zmiennych cechach, co z punktu widzenia przemysłu zielarskiego jest cechą niepożądaną [Niazian 2019]. W prezentowanej pracy doktorskiej wszystkie eksperymenty zostały przeprowadzone na materiale roślinnym rozmnażanym w warunkach *in vitro*.

Roślinne kultury tkankowe dają możliwość prowadzenia elicytacji metabolitów wtórnych o szerokich właściwościach leczniczych. Elicytacja to celowe indukowanie odpowiedzi obronnej roślin w celu zwiększenia zawartości wtórnych metabolitów roślinnych. W zależności od typu elicytora (czynnika stresotwórczego), wyróżniamy elicytację abiotyczną lub biotyczną [Narayani i Srivastava 2017]. Dostępna literatura naukowa wskazuje, że elicytacja jest jedną z najpowszechniejszych technik służącą do zwiększania

poziomu cennych metabolitów roślinnych w kulturach tkankowych *in vitro*. Jednakże aby skutecznie indukować (elicytować) związki pochodzenia roślinnego, zarówno elicytor, jak również jego dawka i czas stosowania, muszą być dobrane eksperymentalnie do każdego gatunku roślin [Lim i in. 2013; Silja i Satheeshkumar 2015; Jesionek i in. 2018]. Elicytacja może służyć zarówno do otrzymywania materiału roślinnego bogatego w biologicznie czynne metabolity wtórne, jak również stanowić narzędzie do indukowania reakcji odpornościowej roślin w badaniach fizjologicznych i biochemicznych [Makowski i in. 2019]. Według współczesnej fizjologii roślin stres jest zjawiskiem następującym w wyniku działania czynnika, który zmienia procesy metaboliczne rośliny, wywołując reakcję aklimatyzacyjną [Cheynier i in. 2013]. W związku z tym, stresorem (a w biotechnologii roślin elicytorem) może być dowolny czynnik pochodzenia biologicznego, chemicznego czy fizycznego, który indukować będzie u roślin reakcje biochemiczne inne niż występujące w warunkach optymalnych [Tokarz i in. 2021].

Jako narzędzie służące do zwiększania puli cennych metabolitów wtórnych biotechnologia roślin wykorzystuje również transformację genetyczną za pośrednictwem dzikich szczepów bakterii *Rhizobium rhizogenes* (wcześniej *Agrobacterium rhizogenes*) [Królicka i in. 2010]. Bakterie te posiadają naturalną zdolność wbudowywania swojego T-DNA (DNA transferowe) zawartego w DNA plazmidowym do genomu rośliny – gospodarza [Makowski i in. 2021]. W naturze zakażenie roślin bakteriami z gatunku *R. rhizogenes* prowadzi do wystąpienia syndromu korzeni włośnikowych (ang. *hairy root syndrome*) lub transformowanych pędów (teratomów). W przypadku infekcji *Rhizobium tumefaciens* tworzą się na korzeniach guzowate narośla [Veremeichik i in. 2016]. Jest to skutek uboczny zaburzeń w gospodarce hormonalnej roślin, która w wyniku ekspresji genów bakteryjnych przekierowana zostaje na syntezę opin – związków, które stanowią pokarm dla bakterii z rodzaju *Rhizobium* [Bulgakov i in. 2018]. Biotechnologia roślin wykorzystuje zdolność przekazania genów bakteryjnych do tkanek roślinnych w celu wprowadzenia zmian w genomie roślinnym. DNA przekazywane przez bakterie zawiera między innymi geny z rodziny *rol* (Rys. 2), których obecność w genomie roślinnym może indukować zmiany fenotypowe i metaboliczne [Bulgakov i in. 2012]. Zwykle w kulturach tkankowych rośliny transformowane genami *rol* ujawniają fenotyp korzeni włośnikowych, ale znane są przypadki, że transformacja prowadzi do powstania teratomów [Królicka i in. 2010]. Ten typ transformacji prowadzi do powstania stabilnych genetycznie i szybko rosnących kultur

pędowych. Co więcej, geny z rodziny *rol* indukują u roślin zmiany metaboliczne, które mogą manifestować się zwiększoną syntezą wtórnych metabolitów roślinnych [Tusevski i in. 2019]. Z tego też względu niektórzy autorzy uznają geny *rol* za endogenne, działające w sposób ciągły elicytory cennych metabolitów roślinnych [Tusevski i in. 2017].



Rysunek 2. Schemat plazmidu bakterii *R. rhizogenes*.

4. Główne cele i zadania badawcze pracy doktorskiej

Synteza i akumulacja związków fenolowych w tkankach roślin wyższych są kluczowe z punktu widzenia mechanizmów aklimatyzacyjnych roślin do warunków stresowych. Związki fenolowe, ze względu na swoje właściwości biologicznie czynne, stanowią grupę substancji o potencjalnym zastosowaniu w przemyśle farmaceutycznym.

W związku z powyższym głównym celem pracy doktorskiej było **opracowanie efektywnych narzędzi biotechnologicznych: elicytacji oraz transformacji genetycznej, które będą prowadziły do zwiększenia syntezy związków fenolowych w kulturach tkankowych roślin muchołówki amerykańskiej**. **Elicytowane i transformowane rośliny muchołówki amerykańskiej zostały wybrane jako model w badaniach nad syntezą związków fenolowych w warunkach stresu oraz materiał roślinny zawierający związki biologicznie czynne wykazujące aktywność przeciwbakteryjną.**

Aby zrealizować powyższe cele wyznaczono następujące zadania badawcze:

1. Zbadanie, w jaki sposób uprawa roślin muchołówki amerykańskiej w płynnej pożywce z wytrząsaniem orbitalnym zmieni poziom akumulacji biomasy i związków fenolowych w tkance roślinnej.
2. Określenie, czy elicytacja z wykorzystaniem lizatów bakteryjnych z *Cronobacter sakazakii* doprowadzi do indukcji reakcji odpornościowej u roślin muchołówki amerykańskiej manifestującej się zwiększoną syntezą związków fenolowych.
3. Sprawdzenie, czy wybrana strategia elicytacyjna doprowadzi do zmian w aktywności biologicznie czynnych metabolitów wtórnych zawartych w ekstraktach uzyskanych z roślin poddanych elicytacji.
4. Przeprowadzenie transformacji genetycznej muchołówki amerykańskiej z wykorzystaniem dzikich szczepów bakterii *Rhizobium rhizogenes*.
5. Zbadanie zmian w akumulacji biomasy, syntezie związków fenolowych oraz właściwościach antybakteryjnych transformowanych roślin muchołówki amerykańskiej.
6. Zbadanie odpowiedzi roślin muchołówki amerykańskiej na obecność bakteryjnego genu *rol* wbudowanego w genomowe DNA roślin, z uwzględnieniem wybranych

parametrów fizjologicznych: produkcji aldehydu dimalonowego, proliny, aktywności enzymów antyoksydacyjnych, akumulacji glutationu, karotenoidów, kwasów fenolowych oraz zmian w gospodarce tłuszczowej i cukrowej.

5. Omówienie szczegółowych celów i wyników poszczególnych prac wchodzących w skład rozprawy doktorskiej

Optymalizacja metod uprawy roślin leczniczych warunkuje wydajność produkcji biomasy oraz syntezy związków chemicznych pochodzenia naturalnego [Niazian 2019]. Uprawa roślin w warunkach *in vitro* umożliwia precyzyjną modyfikację warunków kultury, która prowadzić może do uzyskania roślin o pożądanych cechach [Tokarz i in. 2018]. Poza tym jednym z narzędzi biotechnologicznych, które poprzez indukowanie reakcji stresowej może prowadzić do zwiększenia syntezy roślinnych metabolitów wtórnych, jest elicytacja. Jednakże aby w precyzyjny sposób stymulować (elicytować) syntezę poszczególnych grup roślinnych metabolitów wtórnych czy ich określone pochodne, należy dla danego gatunku rośliny opracować rodzaj i sposób elicytacji [Narayani i Srivastava 2017].

Pierwsza praca wchodząca w skład rozprawy doktorskiej, która stanowi element cyklu połączonych ze sobą tematycznie publikacji: **„Elicitation-based method for increasing the production of antioxidant and bactericidal phenolic compounds in *Dionaea muscipula* J. Ellis tissue”**, opisuje dwa powiązane ze sobą eksperymenty, których nadrzędnym celem było zwiększenie syntezy związków fenolowych w kulturach tkankowych roślin muchołówki amerykańskiej, a w konsekwencji zwiększenie ich właściwości antyoksydacyjnych i antybakteryjnych. W pierwszym etapie badań sprawdzono, jak uprawa roślin muchołówki amerykańskiej w kulturach płynnych z wytrząsaniem orbitalnym przełoży się na przyrost biomasy i syntezę związków fenolowych. Pierwsza hipoteza badawcza zakładała, że pożywka płynna ½ MS [Murashige i Skoog 1962] będzie stanowić efektywny system rozmnażania roślin muchołówki amerykańskiej, ze względu na fakt, że w naturze rośliny te zasiedlają tereny podmokłe. Dodatkowo zastosowanie pożywki płynnej z wytrząsaniem miało na celu zapewnienie roślinom lepszego dostępu do składników pokarmowych i intensywniejszego natlenienia systemu korzeniowego w porównaniu do pożywki zestalonej agarem (kontrola). Druga hipoteza badawcza zakładała, że wytrząsanie roślin w płynnej pożywce będzie stanowić bodziec mechaniczny dla wrażliwych na dotyk liści pułapkowych muchołówki, co

zaindukuje reakcję obronną, manifestującą się zwiększoną syntezą związków fenolowych. Badana modyfikacja warunków uprawy *D. muscipula* skutkowała około 15% zwiększeniem przyrostu biomasy roślin w porównaniu do kontroli, czyli hodowli na pożywkach ½ MS zestalonych 0,75% agarem. Kultury w pożywkach płynnych z wytrząsaniem cechowały się również zwiększoną całkowitą zawartością związków fenolowych (o 38%), fenylopropanoidów (o 35%), flawonoli (o 37%) i antocyjanów (o 49%). Z tego też względu kolejne eksperymenty prowadzano z wykorzystaniem orbitalnym kultur płynnych całych roślin.

Drugi etap badań prezentowanych w pierwszej publikacji dotyczył elicytacji biotycznej związków fenolowych w kulturach roślin muchołówki amerykańskiej z wykorzystaniem lizatu z bakterii *Cronobacter sakazakii* (wcześniej *Enterobacter sakazakii*). Pomimo faktu, że ta Gram-ujemna, względnie beztlenowa pałeczka jest patogenem wywołującym zapalenie opon mózgowych i posocznicę u noworodków [Korpysa-Dzirba i in. 2007], literatura naukowa wskazywała, że lizat z tej bakterii może wywoływać reakcje obronne również u roślin [Staniszewska i in. 2003]. Ponadto w toku badań wstępnych wykazano, że lizaty z tej bakterii dają lepsze efekty elicytacyjne w porównaniu do lizatów uzyskanych z patogenów roślinnych, takich jak *Pseudomonas syringae*. W pierwszej hipotezie założono, że lipopolisacharydy będące endotoksynami zawartymi w błonie komórkowej bakterii *C. sakazakii* będą dla roślin muchołówki bodźcem stresowym, a odpowiedź rośliny będzie manifestować się zwiększoną syntezą niektórych związków fenolowych w jej tkankach. Druga hipoteza zakładała, że efektywność elicytacji związków fenolowych w kulturach roślin *D. muscipula* będzie zależna od stężenia elicytora i czasu trwania elicytacji. W trzeciej hipotezie założono, że elicytacja związków fenolowych doprowadzi do zwiększenia właściwości antyoksydacyjnych i przeciwbakteryjnych ekstraktów pozyskanych z kultur muchołówki amerykańskiej.

Uzyskane w toku badań rezultaty wykazały, że elicytacja lizatem *C. sakazakii*, niezależnie od czasu trwania i stężenia elicytora, nie zmieniała dynamiki przyrostu biomasy badanych roślin. Jest to pierwszy ważny parametr przy wyborze efektywnie działających elicytorów stosowanych w kulturach *in vitro*. Wynika to z faktu, że często elicytory wywołujące reakcje odpornościowe manifestujące się zwiększoną syntezą cennych związków chemicznych powodują równocześnie redukcję przyrostu biomasy roślinnej [Jesionek i in. 2018]. Zgodnie z koncepcją Lattanzio i in. [2018] w warunkach stresu zasoby węgla będącego budulcem

biomasy roślinnej zużyte mogą być w reakcjach metabolizmu wtórnego. Prowadzić to może do redukcji szybkości wzrostu roślin i zwiększonej syntezy metabolitów wtórnych [Caretto i in. 2015].

Badania wykazały, że zastosowanie lizatu z bakterii *C. sakazakii* w hodowli płynnej *D. muscipula* przełożyło się na syntezę związków fenolowych zawartych w tkankach w zależności od czasu działania elicytora i jego stężenia. Związane jest to prawdopodobnie z tym, że poszczególne pochodne fenolowe syntetyzowane są na różnych etapach odpowiedzi roślin na czynnik stresowy [Makowski i in. 2020]. Ponadto ekstrakty uzyskane z roślin poddanych elicytacji wykazywały również zwiększoną aktywność biologiczną, w zależności od czasu działania elicytora. Wraz z wydłużającym się czasem ekspozycji roślin na działanie lizatów bakteryjnych (5, 6 lub 7 dni) wzrastał ich potencjał przeciwutleniający i aktywność przeciwbakteryjna w stosunku do bakterii Gram-dodatniej *Staphylococcus aureus*. Zdolność ekstraktów roślinnych do redukcji ROS badana metodą z wykorzystaniem rodnika DPPH [Sharma i Bhat 2009], z roślin, które elicytowane były przez 7 dni elicytorem o stężeniu 1,5, 2,5 oraz 5%, wzrosła kolejno o: 88, 84 i 89% w porównaniu z kontrolą. Aktywność przeciwbakteryjną ekstraktów roślinnych badano, wyznaczając ich wartość minimalnych stężeń bakteriobójczych (MBC), korzystając z metody mikrorozcieńczeń pożywki (ang. *broth microdilutions method*) [Królicka i in. 2008]. Ekstrakty uzyskane z roślin traktowanych elicytorem przez 6 dni wykazywały zwiększoną aktywność bakteriobójczą o 17%, a elicytowane przez 7 dni o 34% (niezależnie od jego stężenia) w stosunku do roślin nieelicytowanych (kontrolnych). W przypadku zastosowania ekstraktów z *D. muscipula* na bakterie Gram-ujemne *Escherichia coli*, również zaobserwowano wzrost aktywności przeciwbakteryjnej w porównaniu do kontroli (o 20%), ale był on niezależny od stężenia i czasu działania elicytora.

Kolejnym narzędziem biotechnologicznym, które może być pomocne do zwiększenia syntezy metabolitów wtórnych w materiale roślinnym, jest transformacja genetyczna z wykorzystaniem dzikich szczepów bakterii *R. rhizogenes* [Tusevski i in. 2015]. Dostępna literatura naukowa wskazuje, że spośród genów, które bakterie *R. rhizogenes* wbudowują do genomu roślinnego w wyniku transformacji, gen *rol*B znajdujący się w lewej części bakteryjnego T-DNA (Rys. 2) jest najsilniejszym induktorem roślinnego metabolizmu wtórnego [Bulgakov 2008]. Ten typ transformacji genetycznej został dotychczas wykorzystany u różnych gatunków roślin leczniczych, jednak doniesień związanych

z transformacją genetyczną w obrębie rodziny Droseraceae jest zaledwie kilka [Hirsikorpi i in. 2002; Królicka i in. 2010; Blehova i in. 2015; Suda i in. 2020]. Ponadto w literaturze naukowej brak było informacji na temat transformacji roślin *D. muscipula* z użyciem dzikich szczepów bakterii *R. rhizogenes*. Z tego względu nadrzędnym celem w badaniach prowadzonych w ramach projektu Preludium 16, zatytułowanego: „Synteza związków fenolowych w transformowanych kulturach roślin *Dionaea musipula* J. Ellis i ich właściwości przeciwbakteryjne", realizowanego przeze mnie w latach 2019-2021, była transformacja genetyczna roślin muchołówki amerykańskiej. Klony roślin uzyskanych w wyniku transformacji miały posłużyć do badań nad profilem zmian w obrębie związków fenolowych oraz aktywnością biologiczną ekstraktów uzyskanych z transformowanego materiału roślinnego. Ponadto w kolejnym etapie badań transformowane klony muchołówki amerykańskiej miały zostać zbadane pod kątem zmian wybranych parametrów fizjologicznych zachodzących pod wpływem wbudowania genu *rol*B z *R. rhizogenes* do komórek *D. muscipula*.

Artykuł drugi, który wchodzi w skład powiązanego ze sobą tematycznie cyklu, zatytułowany: **„Transformed tissue of *Dionaea muscipula* J. Ellis as a source of biologically active phenolic compounds with bactericidal properties"** jest pierwszym doniesieniem naukowym, w którym opisano uzyskanie transformowanych klonów roślin muchołówki amerykańskiej z użyciem bakterii *R. rhizogenes*. W pierwszej hipotezie badawczej postawionej w badaniach nad transformacją założono, że wykorzystanie agropinowych szczepów bakterii *R. rhizogenes*: LBA 9402 (NCPPB 1855), ATCC 15834 i A4 (ATCC 31798) oraz transformacja kłącza *D. muscipula*, które zawiera stosunkowo niską zawartość metabolitów wtórnych wykazujących aktywność bakteriobójczą, pozwoli na skuteczną transformację i uzyskanie kultur korzeni włośnikowych i/lub teratomów (transformowanych pędów). Druga hipoteza badawcza zakładała, że wbudowane do genomu roślinnego bakteryjne T-DNA zadziała jak endogenny elicytor niektórych metabolitów wtórnych, co w konsekwencji może doprowadzić do zwiększenia syntezy wybranych związków fenolowych i zwiększenia potencjału antybakteryjnego transformowanych klonów muchołówki amerykańskiej w stosunku do roślin nietransformowanych (kontrola).

Przeprowadzone badania wykazały, że transformacja genetyczna muchołówki amerykańskiej z użyciem dzikich szczepów bakterii *R. rhizogenes* LBA 9402 i ATCC 15834 pozwoliła na uzyskanie kultur teratomów (transformowanych pędów), bez wystąpienia

syndromu korzeni włośnikowych. Efektywność transformacji dla szczepu LBA 9402 wynosiła 14% i 16% dla szczepu ATCC 15834. Do dalszych badań wybrano dwa klony powstałe w wyniku transformacji szczepem LBA 9402 (klony P i K) oraz dwa uzyskane z wykorzystaniem szczepu ATCC 15834 (klony L i E), które zostały poddane charakterystyce molekularnej. Przy użyciu reakcji łańcuchowej polimerazy (PCR) [Królicka i in. 2010] zbadano, czy w wyniku transformacji doszło do wbudowania do DNA roślinnego genów *rol*B oraz *rol*C, pochodzących z lewej sekwencji bakteryjnego T-DNA. Ponadto, aby wykluczyć obecność bakterii *R. rhizogenes* w kulturach roślin muchołówki amerykańskiej, sprawdzano obecność genu *vir*G, występującego w plazmidzie bakteryjnym, ale nieulegającego wbudowaniu do genomu roślinnego na drodze transformacji. Wykazano, że u wszystkich badanych klonów roślin *D. muscipula* doszło do inkorporacji genu *rol*B. Wykorzystując hybrydyzację typu Southern [Nowicka i in. 2020], pokazano, że gen *rol*B został wbudowany do DNA genomowego klonu P, K, L i E w jednej kopii.

Obecność poszczególnych genów z rodziny *rol* w genomie roślinnym i liczba kopii, w jakich zostaną one wbudowane, jest jednym z głównych czynników determinujących fenotyp rośliny transformowanej [Królicka i in. 2010]. Jedną z cech, które często charakteryzują rośliny poddane transformacji genetycznej z użyciem bakterii *R. rhizogenes*, jest bardzo szybki przyrost biomasy [Tusevski i in. 2019]. Spośród czterech badanych klonów muchołówki jedynie klon L cechował się szybszym przyrostem biomasy o 20% w stosunku do roślin nietransformowanych (kontrola). Klon K natomiast charakteryzował się zmniejszoną akumulacją biomasy w stosunku do roślin kontrolnych o 39%. Wykorzystując analizę zawartości związków fenolowych metodami spektrofotometrycznymi według Swaina i Hillisa [1959] oraz Fukumoto i Mazza [2000], wykazano, że trzy z badanych klonów (P, L i E) charakteryzowały się zwiększoną całkowitą zawartością związków fenolowych, jak również fenylopropanoidów, flawonoidów i antocyjanów. Natomiast klon K akumulował mniej fenylopropanoidów w porównaniu do roślin nietransformowanych. Analiza zawartości wybranych pochodnych fenolowych z użyciem wysokosprawnej chromatografii cieczowej (HPLC) [Makowski i in. 2019] wykazała, że wśród klonów P, L i E transformacja genetyczna doprowadziła do zwiększonej syntezy plumbaginy (u klonu P o 33%, klonu L o 46% i klonu E o 53%) i badanych kwasów fenolowych. Transformacja nie spowodowała zwiększonej syntezy mirycetyny i kwercetyny, a poziom hiperozydu obniżył się w porównaniu do roślin nietransformowanych. Zmiany w syntezie metabolitów wtórnych w transformowanych klonach P, L i E manifestowało się zwiększeniem potencjału bakteriobójczego ekstraktów

uzyskanych z poszczególnych teratomów *D. muscipula*. W przypadku bakterii *Enterococcus faecalis, S. aureus* i *E. coli* minimalna ilość transformowanej tkanki niezbędna do ich całkowitej eliminacji (MBC) zmalała o 33% w porównaniu do kontroli. Z kolei klony L i E wykazały również zwiększenie potencjału bakteriobójczego w stosunku do bakterii G (-) - *Pseudomonas aeruginosa* o 7%.

Aby w skuteczny i efektywny sposób wykorzystywać narzędzia biotechnologiczne, które prowadzą do zwiększenia syntezy cennych związków biologicznie czynnych pochodzenia roślinnego, kluczowe jest rozumienie procesów fizjologicznych, jakie za nimi stoją [Bulgakov i in. 2018]. Z tego też względu w toku badań realizowanych w ramach grantu Preludium 16 oprócz analizy, jak transformacja z wykorzystaniem dzikich szczepów bakterii *R. rhizogenes* przełoży się na akumulację związków fenolowych i właściwości biologicznie czynne transformowanych roślin, zbadano również wybrane parametry fizjologiczne odpowiedzi roślin na stres związany z transformacją genetyczną. Dotychczas większość badań nad transformacją roślin leczniczych przy użyciu *R. rhizogenes* ograniczała się do określenia, czy inkorporacja genów bakteryjnych do genomu roślinnego ma wpływ na wzrost zawartości metabolitów wtórnych w transformowanych tkankach oraz czy zmienia się aktywność biologiczna pozyskanych z nich ekstraktów. W związku z powyższym, nadrzędnym celem trzeciej pracy wchodzącej w skład rozprawy doktorskiej, zatytułowanej: **„Response of physiological parameters in *Dionaea muscipula* J. Ellis teratomas transformed with *rol*B oncogene”** było opisanie potencjalnych strategii aklimatyzacyjnych roślin muchołówki amerykańskiej do obecności w jej genomie obcych genów bakteryjnych. Na podstawie wyników uzyskanych w drugiej publikacji do badań fizjologicznych wybrano dwa transformowane klony muchołówki amerykańskiej (klon K i L), które różniły się tempem wzrostu, co przekładało się na różnice w świeżej i suchej masie oraz zawartością związków fenolowych, pomimo obecności jednej kopii genu *rol*B w ich genomie.

W pierwszej hipotezie badawczej założono, że obecność genu *rol*B w genomowym DNA muchołówki amerykańskiej będzie prowadzić do zmian w metabolizmie reaktywnych form tlenu, co doprowadzi do zmian w enzymatycznym i nieenzymatycznym systemie antyoksydacyjnym. Wyniki uzyskane w drugiej publikacji, które wykazały, że klon K cechuje się wolniejszym przyrostem biomasy i akumuluje więcej suchej masy niż rośliny kontrolne, a klon L przyrasta szybciej, akumulując więcej związków fenolowych, były podstawą do

postawienia hipotezy drugiej. Założono w niej, że transformacja genetyczna może prowadzić również do zmian w metabolizmie pierwotnym. W trzeciej hipotezie badawczej założono, że odpowiedź wybranych klonów muchołówki amerykańskiej na transformację będzie różna pomimo obecności genu *rol*B inkorporowanego w jednej kopii do genomu obu z nich.

Prowadzone badania wykazały, że wyselekcjonowane klony transformowane muchołówki amerykańskiej cechują się dwoma różnymi strategiami odpowiedzi na obecność genu *rol*B. Analizy spektrofotometryczne metodą wg Hwanga i in. [1999] wykazały, że w stosunku do roślin nietransformowanych, klon K cechował się zwiększoną o 392% aktywnością enzymu dysmutazy ponadtlenkowej (SOD), należącej do enzymatycznego systemu antyoksydacyjnego. Ponadto wykorzystując metodę wg Quevala i Noctora [2007], wykazano zwiększoną o 94% zawartość całkowitej puli glutationu i jego formy utlenionej o 30%, działającego jako nieenzymatyczny antyutleniacz drobnocząsteczkowy. W odpowiedź klonu K na transformację zaangażowane były również karotenoidy, których akumulacja zwiększyła się o 27% w porównaniu do roślin nietransformowanych. Zawartość karotenoidów badano przy użyciu metody Tokarz i in. [2020], wykorzystując do obliczeń wzory Wellburna [1994]. Dzięki zastosowaniu spektroskopii absorpcyjnej w podczerwieni [Trivedi i in. 2015] wykazano zwiększenie o 79% stosunku wolnych kwasów tłuszczowych do lipidów całkowitych i obniżenie o 24% zawartości tyrozyny. Uzyskane wyniki mogą świadczyć o tym, że obecność genu *rol*B w DNA genomowym klonu K doprowadziła do zmian w gospodarce tłuszczowej i aminokwasowej, będących składową szlaków metabolizmu pierwotnego. Z kolei klon L cechował się zwiększoną o 46% (w stosunku do kontroli) akumulacją aldehydu dimalonowego (MDA), będącego produktem peroksydacji błon lipidowo-białkowych w stresie oksydacyjnym [Dhindsa i in. 1981]. Ponadto w wyniku transformacji *D. muscipula* w klonie L, dzięki zastosowaniu techniki wg. Bates i in. [1973] wykazano zwiększenie syntezy proliny o 47%. Dodatkowo klon L cechował się wyższą o 399% aktywnością SOD oraz drugiego z enzymów antyoksydacyjnych – katalazy (CAT) [Aebi 1984] o 54% w porównaniu do roślin nietransformowanych. Badania fizjologiczne wykazały również, że u klonu L doszło do zwiększenia o 107% akumulacji całkowitej puli glutationu oraz stosunku jego formy zredukowanej do utlenionej o 78%. Analizy biochemiczne z wykorzystaniem HPLC [Sułkowska-Ziaja i in. 2017, Szopa i in. 2020] pozwoliły stwierdzić, że klon L akumulował więcej kwasu galusowego (o 73%), kwasu chlorogenowego (o 36%), kwasu p-kumarowego (o 101%) i kwasu ferulowego (o 283%),

przy jednoczesnym spadku zawartości kwasu protokatechowego (o 17%) oraz kemferolu (o 69%) w porównaniu do kontroli. Co więcej, w badaniach z użyciem spektroskopii absorpcyjnej w podczerwieni wykazano, że wbudowanie genu *rol*B do genomu *D. muscipula* (klon L) skutkowało zwiększeniem o 454% stosunku trójglicerydów do lipidów, sacharozy o 16%, związków fenolowych o 49%, tyrozyny o 20% oraz fruktozy i glukozy o 19%. Jednocześnie akumulacja skrobi w klonie L obniżyła się o 28% w stosunku do roślin nietransformowanych.

6. Wnioski – weryfikacja postawionych hipotez badawczych

1) System kultur tkankowych w pożywce płynnej z wytrząsaniem orbitalnym jest wydajniejszym systemem do uprawy roślin muchołówki amerykańskiej w porównaniu do tradycyjnych kultur na pożywkach zestalonych agarem.
2) Lizat z bakterii *Cronobacter sakazakii* może stanowić użyteczny elicytor związków fenolowych w kulturach tkankowych roślin muchołówki amerykańskiej, natomiast efektywność jego działania w stosunku do poszczególnych pochodnych związków fenolowych zależy od zastosowanego stężenia i czasu ekspozycji roślin na jego działanie.
3) Rezultaty elicytacji zależne od czasu i stężenia elicytora związane są prawdopodobnie z tym, że poszczególne pochodne fenolowe syntetyzowane są na różnych etapach odpowiedzi roślin na czynnik stresowy. Adaptacja muchołówki amerykańskiej do warunków stresowych jest procesem ciągłym, dlatego opracowanie specyficznych warunków elicytacji dla poszczególnych metabolitów wtórnych jest bardzo trudne.
4) Elicytacja związków fenolowych w roślinach muchołówki amerykańskiej z użyciem lizatów z bakterii *C. sakazakii* prowadzi do zmian w aktywności biologicznej ekstraktów uzyskanych z roślin poddanych elicytacji.
5) Bakterie Gram-ujemne są mniej wrażliwe na działanie ekstraktów z muchołówki amerykańskiej, bogatych w związki fenolowe w porównaniu do bakterii Gram-dodatnich. Fakt ten może mieć ściśle związek z tym, że ściana komórkowa bakterii G-ujemnych pokryta jest lipopolisacharydową otoczką, która zmniejsza przepuszczalność biologicznie aktywnych związków do wnętrza komórki bakteryjnej.

6) Transformacja genetyczna z wykorzystaniem dzikich szczepów bakterii *Rhizobium rhizogenes* jest użytecznym narzędziem biotechnologicznym do otrzymywania tkanek roślinnych *D. muscipula* bogatych w związki fenolowe.
7) Obecność plazmidowego genu *rol*B *R. rhizogenes* wbudowanego do genomu roślinnego muchołówki amerykańskiej w jednej kopii nie zapewnia uzyskania tego samego fenotypu różnych jej klonów.
8) Gen *rol*B wbudowany do genomowego DNA rośliny w jednej kopii może działać jak endogenny elicytor związków fenolowych (w tym 1,4-naftochinonów) i prowadzić do zwiększenia potencjału antybakteryjnego transformowanych roślin w stosunku do Gram-dodatnich i Gram-ujemnych patogenów bakteryjnych.
9) Wbudowanie genu *rol*B do genomu roślinnego *Dionaea muscipula* prowadzi do zmian na poziomie roślinnego metabolizmu pierwotnego oraz wtórnego – efekt plejotropowy.
10) Obecność genu *rol*B inkorporowanego do genomu roślinnego w jednej kopii nie indukuje takiej samej strategii aklimatyzacyjnej u wszystkich uzyskanych klonów (teratomów) muchołówki amerykańskiej.

7. Podsumowanie

Wyniki badań uzyskane w ramach przedstawionej rozprawy doktorskiej przyczyniły się do poszerzenia wiedzy w zakresie syntezy wtórnych metabolitów roślinnych w warunkach stresu. Ponadto opracowane metody elicytacji i transformacji genetycznej wzbogaciły wiedzę z zakresu szeroko pojętej biotechnologii roślin leczniczych. Prezentowana praca doktorska pokazała, że niemodelowy gatunek roślin, jakim jest *D. muscipula*, może stanowić użyteczny obiekt w badaniach nad syntezą i wykorzystaniem związków fenolowych.

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9. Streszczenie rozprawy doktorskiej w języku polskim

Związki fenolowe stanowią jedną z najliczniejszych grup roślinnych metabolitów wtórnych, które syntetyzowane są przez rośliny w warunkach stresu środowiskowego. Ze względu na swoją budowę chemiczną niektóre związki fenolowe cechuje szerokie spektrum właściwości biologicznie czynnych, wśród których jedną z najważniejszych w kontekście problemów współczesnej medycyny jest aktywność przeciwdrobnoustrojowa. Ze względu na rosnącą lekooporność bakterii poszukuje się nowych źródeł związków chemicznych, w tym również metabolitów wtórnych zawartych w tkankach roślinnych, które posiadają potencjał przeciwbakteryjny i nie indukują oporności wśród zwalczanych mikroorganizmów.

Muchołówka amerykańska (*Dionaea musipula* J. Ellis) jest rośliną mięsożerną, która jest monotypowym rodzajem w obrębie rodziny rosiczkowatych (Droseraceae). Z uwagi na swoje specyficzne przystosowanie do środowiska muchołówka amerykańska ma zdolność do syntezy i akumulacji bardzo dużych ilości różnych pochodnych fenolowych: fenylopropanoidów, flawonoidów, antocyjanów czy 1,4-naftochinonów. Z tego względu stanowi ona użyteczny model do badań nad syntezą związków fenolowych w warunkach stresu oraz ich właściwościami biologicznie czynnymi.

Prezentowana praca doktorska składa się z cyklu trzech powiązanych tematycznie, oryginalnych publikacji naukowych. Tematem przewodnim opublikowanych prac była synteza związków fenolowych w kulturach tkankowych roślin muchołówki amerykańskiej, które poddano elicytacji i transformacji genetycznej. Równocześnie przedmiotem badań było opisanie odpowiedzi fizjologicznej roślin *D. muscipula* na transformację genetyczną oraz określenie właściwości biologicznie czynnych metabolitów wtórnych zawartych w ekstraktach uzyskanych z roślin w procesie elicytacji i transformacji.

Głównymi celami pracy doktorskiej było: (1) opracowanie efektywnej metody elicytacji związków fenolowych w kulturach tkankowych muchołówki amerykańskiej, w celu podwyższenia aktywności przeciwutleniających i antybakteryjnych ekstraktów uzyskanych z badanych roślin, (2) uzyskanie transformowanych roślin *D. muscipula* dzięki wykorzystaniu transformacji wektorowej – *Rhizobium rhizogenes,* (3) ocena transformowanych klonów muchołówki amerykańskiej pod kątem akumulacji związków fenolowych i ich właściwości

antybakteryjnych, (4) zbadanie wybranych parametrów fizjologicznych odpowiedzi roślin muchołówki amerykańskiej na transformację genetyczną.

Aby zrealizować postawione cele badawcze, zastosowano elicytor biotyczny w postaci lizatu z bakterii *Cronobacter sakzakii*, o stężeniu: 1,5, 2,5 i 5% w hodowli płynnej *D. muscipula*. Transformację genetyczną wykonano przy użyciu trzech szczepów bakterii *R. rhizogenes* LBA 9402, ATCC 15834 i A4, które posiadają naturalną zdolność inkorporacji fragmentu DNA plazmidowego (T-DNA) do genomu roślinnego. Rośliny muchołówki amerykańskiej poddane elicytacji i transformacji badano w kontekście akumulacji biomasy, związków fenolowych (metodami spektrofotometrycznymi i wysokosprawnej chromatografii cieczowej) oraz ich właściwości antyoksydacyjnych i antybakteryjnych w stosunku do antybiotykoopornych patogenów bakteryjnych: *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli* i *Pseudomonas aeruginosa.* Transformację genetyczną *D. muscipula* potwierdzono na poziomie molekularnym dzięki zastosowaniu reakcji PCR. Wykazano obecność genu *rol*B w DNA uzyskanych teratomów (transformowanych pędów) muchołówki amerykańskiej. Dodatkowo z użyciem hybrydyzacji typu Southern wykazano, że bakteryjny gen *rol*B został wbudowany do DNA klonów *D. muscipula* w jednej kopii. Transformowane klony muchołówki amerykańskiej zbadano pod kątem ich zmian w akumulacji aldehydu dimalonowego (markera stresu oksydacyjnego), proliny, karotenoidów, kwasów fenolowych, glutationu i aktywności enzymów antyoksydacyjnych w porównaniu do roślin nietransformowanych. Ponadto w teratomach oznaczono zawartość lipidów i cukrów, będących składową metabolizmu pierwotnego.

Uzyskane wyniki wykazały, że: (1) efektywność elicytacji związków fenolowych w kulturach tkankowych muchołówki amerykańskiej z użyciem lizatu z bakterii *C. sakazakii* jest zależna od stężenia i czasu działania elicytora, (2) zastosowana strategia elicytacyjna skutecznie podnosi właściwości przeciwutleniające i przeciwbakteryjne roślin muchołówki amerykańskiej, (3) bakteryjny gen *rol*B w DNA genomowym roślin muchołówki może działać jak endogenny elicytor związków fenolowych i tym samym prowadzić do zwiększenia właściwości przeciwbakteryjnych ekstraktów uzyskanych z roślin transformowanych, (4) transformacja roślin *D. muscipula* wywołuje efekt plejotropowy, prowadząc do zmian w gospodarce reaktywnych form tlenu, metabolizmie pierwotnym i wtórnym roślin, (5) stabilne genetycznie organizmy transformowane mogą stanowić dobry model do badań nad syntezą i akumulacją związków fenowych w roślinach leczniczych.

10. Streszczenie rozprawy doktorskiej w języku angielskim

Phenolic compounds are one of the most numerous groups of plant secondary metabolites that are synthesized by plants under environmental stress. Due to their chemical structure phenolic compounds are characterized by a wide spectrum of biologically active properties. One of the most important in the context of the problems of modern medicine is antibacterial activity. Due to the increasing drug resistance of bacteria, new sources of chemical compounds are sought, including secondary metabolites contained in plant tissues, which have antibacterial potential and do not induce resistance among the target microorganisms.

Venus flytrap (*Dionaea musipula* J. Ellis) is a carnivorous plant which is the monotypic genus within the Droseraceae family. Due to its specific adaptation to the environment, the Venus flytrap has the ability to synthesize and accumulate large amounts of various phenolic derivatives: phenylpropanoids, flavonoids, anthocyanins or 1,4-naphthoquinones. For this reason, it is a useful model for research into the synthesis of phenolic compounds under stress and their biologically active properties.

The presented doctoral dissertation consists of a series of three thematically related original scientific publications. The main topic of the published works was the synthesis of phenolic compounds in tissue cultures of Venus flytrap plants, which were subjected to elicitation and genetic transformation. At the same time, the subject of the research was to study the physiological response of *D. muscipula* plants to genetic transformation and to determine the properties of biologically active secondary metabolites contained in extracts obtained from plants in the process of elicitation and transformation.

The main objectives of the dissertation were: (1) to develop an effective method of eliciting phenolic compounds in the tissue cultures of the Venus flytrap in order to increase the antioxidant and antibacterial activity of extracts obtained from the studied plants, (2) to obtain transformed *D. muscipula* plants through the use of vector transformation – *Rhizobium rhizogenes*, (3) evaluation of transformed Venus flytrap clones in terms of the accumulation of phenolic compounds and their antibacterial properties, (4) examination of selected physiological parameters of Venus flytrap plant responses to genetic transformation.

To achieve the research objectives, a biotic elicitor was used in the form of a lysate from *Cronobacter sakazakii* bacteria, at a concentration of: 1.5, 2.5 and 5% in *D. muscipula* liquid culture. The genetic transformation was performed using three strains of *R. rhizogenes* LBA

9402, ATCC 15834 and A4, which have the natural ability to incorporate a plasmid DNA fragment (T-DNA) into the plant genome. Plants of the Venus flytrap, subjected to elicitation and transformation, were studied in the context of the accumulation of biomass, phenolic compounds (using spectrophotometric methods and high-performance liquid chromatography) and their antioxidant and antibacterial properties in relation to antibiotic-resistant bacteria: *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa* . The genetic transformation of *D. muscipula* was confirmed on the molecular level by means of the PCR reaction. The presence of the *rol*B gene was demonstrated in the genome of the obtained teratomas (transformed shoots) of the Venus flytrap. In addition, it was shown using Southern blot hybridization that the bacterial *rol*B gene was integrated into the DNA of the Venus flytrap clones in a single copy. The transformed clones were examined for their changes in the accumulation of malonedialdehyde (an oxidative stress marker), proline, carotenoids, phenolic acids, glutathione, and antioxidant enzyme activity compared to untransformed plants. Moreover, the content of lipids and sugars, which are a component of primary metabolism, was determined in teratomas.

The obtained results showed that: (1) the efficiency of elicitation of phenolic compounds in Venus flutrap tissue cultures with the use of *C. sakazakii* lysate depends on the concentration and duration of the elicitor, (2) the applied elicitation strategy effectively increases the antioxidant and antibacterial properties of *D. muscipula* plants, (3) the bacterial *rol*B gene in the genomic DNA of Venus flytrap plants may act as an endogenous elicitor of phenolic compounds and thus lead to an increase in the antibacterial properties of extracts obtained from transformed organisms, (4) the transformation of *D. muscipula* plants causes a pleiotropic effect, leading to changes in the reactive oxygen, primary and secondary metabolism of plants, (5) genetically stable transformed organisms can be a good model for research on the synthesis and accumulation of phenolic compounds in medicinal plants.

11. Wykaz publikacji i doniesień konferencyjnych niewchodzących w zakres pracy doktorskiej

11.1. Wykaz opublikowanych rozdziałów w monografiach naukowych

M1. Tokarz K., Piwowarczyk B., **Makowski W.** (2020). Mechanisms involved in photosynthetic apparatus protection against lead toxicity. [W:] *Lead in Plants and the Environment*. [Red.] Gupta D.K., Chatterjee S., Walther C., Springer, Cham, Szwajcaria, pp. 117-128.

$MNiSW_{2020}$: 20 pkt.

11.2. Wykaz opublikowanych artykułów w czasopismach naukowych

A1. Piwowarczyk B., Tokarz K., **Makowski W.,** Łukasiewicz A. (2017). Different acclimatization mechanisms of two grass pea cultivars to osmotic stress in *in vitro* culture. *Acta Physiologiae Plantarum, 39*(4): 96.

IF_{2017}: 1,438; $MNiSW_{2017}$: 25 pkt.

A2. Tokarz K., **Makowski W.,** Banasiuk R., Krolicka A., Piwowarczyk B. (2018). Response of *Dionaea muscipula* J. Ellis to light stress in *in vitro*: physiological study. *Plant Cell, Tissue and Organ Culture, 134*(1), 65–77.

IF_{2018}: 2,200; $MNiSW_{2017}$: 35 pkt.

A3. Piwowarczyk, B., Tokarz, K., Muszyńska, E., **Makowski, W.,** Jędrzejczyk, R., Gajewski, Z., Hanus-Fajerska, E. (2018). The acclimatization strategies of kidney vetch (*Anthyllis vulneraria* L.) to Pb toxicity. *Environmental Science and Pollution Research, 25*(20), 19739–19752.

IF_{2018}: 2,914; $MNiSW_{2018}$: 30 pkt.

A4. Tokarz, K., Piwowarczyk, B., Wysocka, A., Wójtowicz, T., **Makowski, W.,** Golemiec, E. (2019). Response of grass pea (*Lathyrus sativus* L.) photosynthetic apparatus to short-term intensive UV-A: Red radiation. *Acta Physiologiae Plantarum, 41*(10), 168.

IF_{2019}: 1,760; $MNiSW_{2019}$: 70 pkt.

A5. **Makowski, W.,** Tokarz B., Banasiuk, R., Królicka, A., Wojciechowska, R., Dziurka, M., Tokarz, K.M. (2019). Is a blue–red light a good elicitor of phenolic compounds in the family Droseraceae? A comparative study. *Journal of Photochemistry & Photobiology, B: Biology, 201,* 111679.

IF_{2019}: 4,383; $MNiSW_{2019}$: 100 pkt.

A6. Tokarz, K.M., **Makowski, W.,** Tokarz, B., Hanula, M., Sitek, E., Muszyńska, E., Jędrzejczyk R. Banasiuk R., Chajec Ł. & Mazur, S. (2020). Can Ceylon leadwort (*Plumbago zeylanica* L.) acclimate to lead toxicity? – studies of photosynthetic apparatus efficiency. *International Journal of Molecular Sciences*, *21*(5), 1866.

IF_{2020}: 5,923; $MNiSW_{2020}$: 140 pkt.

A7. Tokarz, B., Wójtowicz, T., **Makowski, W.,** Jędrzejczyk, R. J., Tokarz, K.M. (2020). What is the Difference between the Response of Grass Pea (*Lathyrus sativus* L.) to Salinity and Drought Stress? – A Physiological Study. *Agronomy*, *10*(6), 833

IF_{2019}: 3,417; $MNiSW_{2020}$: 100 pkt.

A8. Tokarz, K.M., Wesołowski, W. Tokarz, B., **Makowski, W.,** Wysocka, A., Jędrzejczyk, R.J., Chrabaszcz, K., Malek, K. & Kostecka-Gugała, A. (2021). Stem photosynthesis–a key element of grass pea (*Lathyrus sativus* L.) acclimatisation to salinity. *International Journal of Molecular Sciences*, *22*, 685.

IF_{2020}: 5,923; $MNiSW_{2020}$: 140 pkt.

11.3. Wykaz doniesień naukowych na konferencjach polskich i międzynarodowych

D1. **Makowski W.,** Tokarz K., Piwowarczyk B. 2015. Reaction of *Drosera spatulata* Labill. on the light stress in *in vitro* culture conditions. Acta Physiologiae Plantarum 38:17, pp. 100.

D2. **Makowski W.,** Tokarz K., Płachno B., Piwowarczyk B. 2016. Changes in the structure of *Drosera spatulata* Labill. cells as response on various spectral composition of radiation in *in vitro* cultures. Acta Biologica Cracoviensia. Series Botanica. Supplement 58(1), 85.

D3. Piwowarczyk B., Tokarz K., **Makowski W.** 2016. Charakterystyka systemu antyoksydacyjnego siewek lędźwianu siewnego (*Lathyrus sativus* L.) rosnących

w warunkach *in vitro*. Streszczenia Referatów i Plakatów 57 Zjazdu PTB 'Botanika - tradycja i nowoczesność', 27 czerwca-3 lipca 2016, Lublin, Polska:78.

D4. **Makowski W.,** Tokarz K., Piwowarczyk B., Wojciechowska R., Banasiuk R., Królicka A. 2016. Synthesis of pharmacologically active compounds of plants from the Droseraceae family in response to light stress in *in vitro* conditions. Acta Biologica Cracoviensia. Series Botanica. Supplement 58(2), 64.

D5. **Makowski W.,** Piwowarczyk B., Banasiuk R., Królicka A., Hanula M., Tokarz K. 2017. Acclimatization strategy of *Dionaea muscipula* J. Ellis to light stress in *in vitro* – physiological study. Abstract Book: EUROBIOTECH „6th Central European Congress of Life Science" September 11-14th, 2017: 87.

D6. Tokarz K., Piwowarczyk B., **Makowski W.,** Hanus-Fajerska E. 2017. Photosynthetic apparatus acclimatization of *Anthyllis vulneraria* L. to Pb toxicity in *in vitro* conditions. Book of Abstracts: 8th Conference of the Polish Society of Experimental Plant Biology, 12–16 September 2017, Białystok, 163.

D7. **Makowski W.,** Hanula M., Piwowarczyk B., Jędrzejczyk R., Tokarz K.M. 2018. *Plumbago zeylanica* L. - is it really Pb accumulator? The new insight in the context of the latest data. Acta Physiologiae Plantarum 40: 193, pp. 81.

D8. Tokarz K.M., **Makowski W.,** Hanula M., Piwowarczyk B., Jędrzejczyk R.J. 2018. Response of *Plumbago zeylanica* L. to Pb in *in vitro*: physiological study. Acta Physiologiae Plantarum 40: 193, pp. 55.

D9. Piwowarczyk B., Tokarz K., **Makowski W.,** Wyłupek I. 2018. Comparison of grass pea seedlings response to osmotic and chemical stress in *in vitro* conditions. BioTechnologia 99(3), 259.

D10. **Makowski W.,** Tokarz K., Piwowarczyk B., Banasiuk R., Królicka A., Dziurka M., Wojciechowska R. 2018. Different acclimatization strategies of two genus from the family Droseraceae to high light intensity cultivated *in vitro*. BioTechnologia 99(3), 312.

D11. Hanula M., Tokarz K., **Makowski W.,** Piwowarczyk B. 2018. Does Pb toxicity increase the accumulation of plumbagin in *Plumbago zeylanica*? Book of Abstracts: 13th International Seminar: "Biomolecules Identification and Functions", 19-20.10.2018, Krakow: 20.

D12. **Makowski W.,** Tokarz K., Piwowarczyk B., Witek K. 2018. Elicytation of phenolic compounds in tissue cultures of plants from Droseraceae family. Book of Abstracts: 13th International Seminar: "Biomolecules Identification and Functions", 19-20.10.2018, Krakow: 25.

D13. Piwowarczyk B., Tokarz K., **Makowski W.,** Wysocka A., Hanula M. 2018. The potential role of ODAP (β-N-oxalyl-α, β-diaminopropionic acid) in response of grass pea (*Lathyrus sativus* L.) to abiotic stress in *in vitro* conditions. Book of Abstracts: 13th International Seminar: "Biomolecules Identification and Functions", 19-20.10.2018, Krakow: 30.

D14. Wysocka A., Tokarz K., Piwowarczyk B., **Makowski W.** 2018. Response of grass pea (*Lathyrus sativus* L.) photosynthetic apparatus to salt stress. Book of Abstracts: 13th International Seminar: "Biomolecules Identification and Functions", 19-20.10.2018, Krakow: 37.

D15. **Makowski W.,** Królicka A., Tokarz K.M., Piwowarczyk B., Banasiuk R. 2019. Elicitation of phenolic compounds using bacterial lysates in *Drosera indica* L. and its antibacterial potential against human-pathogenic microorganisms. Book of Abstracts: Plant Biotechnology: Green for Good V, 10-13.06.2019, Ołomuniec, Czechy: 136.

D16. Piwowarczyk B., Tokarz K.M., **Makowski W.,** Wysocka A., Jędrzejczyk R. 2019. Potential strategies of grass pea (*Lathyrus sativus* L.) acclimatization to salinity. Book of Abstracts: Plant Biotechnology: Green for Good V, 10-13.06.2019, Ołomuniec, Czechy: 141.

D17. Tokarz K., Tokarz B., **Makowski W.,** Hanula M. 2019. May stem photosynthetic apparatus provide an effective alternative to leaves in acclimatization strategy to salinity stress (NaCl) in non-halophytes plants? - the case of grass pea. Book of Abstracts: 9th Conference of the Polish Society of Experimental Plant Biology 'New trends in plant reproduction and growth regulation', 9-12.09.2019, Toruń: 136.

D18. **Makowski W.,** Królicka A., Tokarz B., Banasiuk R., Tokarz K. 2019. *In vitro* and *ex vitro* cultured carnivorous plants as a source of biologically active compounds. Book of Abstracts 9th Conference of the Polish Society of Experimental Plant Biology 'New trends in plant reproduction and growth regulation', 9-12.09.2019, Toruń: 198.

D19. Wojciechowska W., Michalak A., **Makowski W.,** Rzeszotarski M., Krychowiak-Maśnicka M., Królicka A. *In vitro* culture of carnivorous plants with antioxidant activity. Acta Biologica Cracoviensia. Series Botanica. Supplement 61(1):71.

D20. Miernicka K, Tokarz B., **Makowski W.,** Banasiuk R., Królicka A., Tokarz K.M. 2021. Short-term reaction of *Dionaea muscipula* J. Ellis photosynthetic apparatus after UV-A radiation treatment. 1st International Electronic Conference on Agronomy, 3-17.05.2021, Online, Proceedings IECAG 2021, 68.

D21. Miernicka K., Banasiuk R., **Makowski W.,** Tokarz B., Królicka A., Tokarz K. 2021. Phenolic compounds profile of Dionaea muscipula J. Ellis leaves and traps after UV-A treatment. Polyphenols communications: XXX International Conference on Polyphenols – 13-15.07.2021, Turku, Finlandia.

D22. **Makowski W.,** Królicka A., Tokarz B., Ekiert H., Szopa A., Banasiuk R., Tokarz K.M. 2021. *Rol*B oncogene increased synthesis of phenolic compounds and bioactivity of *Dionaea muscipula* J. Ellis. Polyphenols communications: XXX International Conference on Polyphenols – 13-15.07.2021, Turku, Finlandia.

D23. Tokarz B, Mrzygłód K., **Makowski W.,** Miernicka K., Tokarz K.M. 2021. The response of grass pea (*Lathyrus sativus* L.) seedlings to low temperature stress – preliminary study Book of Abstracts: 110th biennial PSEPB Conference "Experimental plant

biology at various scales: from molecules to environment" 20-23.09.2021, Katowice:143.

D24. Tokarz K.M., Augustynowicz J., Tokarz B., Płachno B., **Makowski W.** 2021. Response of the long-styled water-starwort (*Callitriche cophocarpa* Sendtn.) photosynthetic apparatus to chromium stress. Book of Abstracts: 110th biennial PSEPB Conference "Experimental plant biology at various scales: from molecules to environment" 20-23.09. 2021, Katowice:144.

11.4. Informacje o projektach naukowych

Kierownik tematu nr 2516 w ramach projektu finansowanego z dotacji celowych na prowadzenie badań naukowych dla młodych pracowników oraz uczestników studiów doktoranckich w roku akademickim 2018/2019. Tytuł tematu: „Mechanizmy aklimatyzacji do warunków stresu świetlnego u wybranych przedstawicieli rodziny Droseraceae w kontekście syntezy biologicznie czynnych metabolitów wtórnych". Temat realizowany w Katedrze Botaniki, Fizjologii i Ochrony Roślin Wydziału Biotechnologii i Ogrodnictwa Uniwersytetu Rolniczego im. Hugona Kołłątaja w Krakowie.

Kierownik projektu Preludium 16 przyznanego przez Narodowe Centrum Nauki. Tytuł projektu: „Synteza związków fenolowych w transformowanych kulturach roślin *Dionaea musipula* J. Ellis i ich właściwości przeciwbakteryjne". Projekt realizowany na Wydziale Biotechnologii i Ogrodnictwa Uniwersytetu Rolniczego im. Hugona Kołłątaja w Krakowie, w terminie 27.07.2019 – 27.07.2021. Numer projektu: 2018/31/N/NZ9/00. Opiekun merytoryczny projektu: dr hab. inż. Aleksandra Królicka, prof. UG.

11.5. Informacje o odbytych stażach naukowych

01.02.2017 - 31.03.2017 Staż naukowy w Ośrodku Bioremediacji Małopolskiego Centrum Biotechnologii Uniwersytetu Jagiellońskiego w Krakowie (obecnie Grupa Badawcza Interakcji Mikroorganizmów z Roślinami MCB UJ w Krakowie).

12.12.2016 - 16.12.2020 5 wyjazdów naukowych po 1 do 2 tygodni do Pracowni Badania Związków Biologicznie Czynnych w Katedrze Biotechnologii Międzyuczelnianego Wydziału Biotechnologii Uniwersytetu Gdańskiego i Gdańskiego Uniwersytetu Medycznego (obecnie Zakład Badania Związków Biologicznie Czynnych Międzyuczelnianego Wydziału Biotechnologii UG i GUMed).

01.10.2019 - 15.12.2019 Staż naukowy w ramach programu Erasmus+ w Czeskiej Akademii Wiedzy w Instytucie Botaniki Eksperymentalnej, Ołomuniec, Republika Czeska.

11.6. Informacje o recenzowanych pracach naukowych w czasopismach międzynarodowych

Pięć recenzji wydawniczych manuskryptów przygotowanych dla czasopism o zasięgu międzynarodowym: *Acta Scientiarum Polonorum Series Hortorum Cultus* (1), *Scientific Reports* (1), *Biologia* (1), *BMC Plant Methods* (2).

INFORMACJE NAUKOMETRYCZNE

Sumaryczny IF **(zgodnie z rokiem publikacji)**	**Sumaryczna liczba cytowań** **wg WoS**	**Liczba cytowań bez autocytowań** **wg WoS**	**Indeks Hirscha** **wg WoS**	**Sumaryczna Punktacja MEiN** **(zgodnie z rokiem wydania)**
41,397	89	54	6	1000

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12. Cykl publikacji stanowiący pracę doktorską

Publikacja 1

molecules

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Elicitation-Based Method for Increasing the Production of Antioxidant and Bactericidal Phenolic Compounds in *Dionaea muscipula* J. Ellis Tissue

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Abstract: The carnivorous plant *Dionaea muscipula* J. Ellis (Venus flytrap) is a widely known medical herb, capable of producing various phenolic compounds known for their strong antioxidant and antibacterial properties. In the pharmaceutical industry, Venus flytrap is grown in tissue cultures, as the natural population of *D. muscipula* is very limited. Here, we describe an improved method to increase the quantity and quality of phenolic compounds produced in *D. muscipula.* This is achieved by combining biotic elicitation (using *Cronobacter sakazakii* bacteria lysate) of *D. muscipula* cultured with rotary shaking (hydromechanical stress), which we describe here for the first time. The antibacterial activity and the antioxidant properties of the obtained compounds were studied on two antibiotic-resistant human pathogenic bacteria. The proposed plant culture conditions resulted in an increase in fresh weight, as well as a higher total phenolic content, in comparison to traditional tissue cultures on agar-solidified medium. With the use of high-performance liquid chromatography, we demonstrated that the described elicitation strategy leads to an increased synthesis of myricetin, caffeic acid, ellagic acid and plumbagin in *D. muscipula* tissue. We also found that a higher level of antioxidant activity, exhibited by the plant extract, corresponded with its higher phenylpropanoid content. The bactericidal activity of the extract against *Staphylococcus aureus* was dependent on the duration of plant culture under described elicitation conditions, whereas neither elicitation condition (duration or elicitor concentration) seemed relevant for the bactericidal activity of the extract towards *Escherichia coli*. This suggest that Gram-negative bacteria are less sensitive to compounds derived from Venus flytrap tissue.

Keywords: antibiotic-resistant bacteria; biotic elicitation; *Cronobacter sakazakii*; DPPH; *Escherichia coli*; plant secondary metabolites; *Staphylococcus aureus*; Venus Flytrap

1. Introduction

Carnivorous plants from the family, Droseraceae, have been used in natural medicine around the world for centuries. The first known report on the application of *Drosera* sp. herb in natural therapy is dated in 12th century [1]. The medical properties of these plants arise from the ability to synthesize

various secondary metabolites from a group of phenolic compounds, especially 1,4-naphtoquinones derivatives, among which plumbagin (PLU) is the most common derivative [2,3]. Due to the chemical structure of PLU (5-hydroxy-2-methyl-1,4-naphthoquinone), this compound can undergo redox cycling and generate reactive oxygen species (ROS) in cells, resulting in its biological activity [2].

According to Gaascht et al. [2], secondary metabolites are highly diversified and complex group of plant derived chemicals, accumulated in very small amount [4], with various biological activities. As shown, the extracts from carnivorous plants from the family, Droseraceae, have strong antioxidant [5], antibacterial [1,6] and antifungal [7] properties. Recently, Kawiak et al. [8] showed that they also have anticancer properties. Due to the growing resistance for antibiotics of some human-pathogenic microbes, and the increasing demand for new drugs in cancer therapy, plant material with strong biological activity are in great demand.

The main source of PLU in the medical plants industry are in field growing *Plumbago* sp. plants, although characterized by the low concentration of secondary metabolites [9]. On the other hand, *Plumbago* sp. is known to be a heavy metals accumulator [10], so the root material from the natural environment can be contaminated and toxic. Furthermore, *D. muscipula* plants grown in vitro can accumulate between 15–20 times more PLU per gram of biomass than the *Plumbago* roots culture [11–14]. Despite this, *D. muscipula* is not using as an industrial source of PLU, because of limited availability of plant material.

In the family, Droseraceae, the most abundant in phenolic compounds and rich in PLU is *Dionaea muscipula* J. Ellis (Venus flytrap) [2,3]. This plant grows in a marshy, wet, nutrient-poor and sun-exposed areas, in North and South Carolina of the United States, being an endemit and the only species in genus *Dionaea* [13]. To survive in a natural environment, *D. muscipula* synthesizes phenolic compounds to protect against predators, UV radiation and putrefaction processes during digestion of prey [15]. Phenolic compounds are produced via shikimate/phenylpropanoid or polyketide acetate-malonate pathways and play a crucial role in plant-environment interactions [16]. The natural population of *D. muscipula* is very small, and obtaining plants from natural habitats is impossible. To create an environment-independent source of this valuable plant material, in vitro propagation protocols have been established [17]. Tissue culture technique makes it possible to obtain large amounts of plant biomass in a short time. The most common technique for in vitro plant propagation is the cultivation of whole plants or plant organs on solid medium with agar. However, modifications of this basic technique, such as culture in liquid media (LM) [18] or temporary immersion bioreactors [19,20], allow for greater plant biomass or plant-derived compounds production. In this study, for the first time, we show how cultivation of the Venus flytrap in LM with rotary shaking affects the plant's secondary metabolites accumulation and biomass production, in comparison to traditional solid medium (SM)—experiment 1. We hypothesised that, because of the physical features of LM and shaking, the plant will have better accessibility to medium resources and better conditions for efficient growth. On the other hand, rotary shaking can be a mechanical stress factor for *D. muscipula* and leads to increased synthesis of some phenolic compounds. Based on the results and observations from experiment 1, we have designed conditions for an experiment with elicitation—experiment 2.

Elicitation is a plant biotechnology technique based on exogenous addition of elicitors (biotic or abiotic) to the growth medium. This is one of the most effective ways to increase plant secondary metabolites production [21] and biological activity of plant-derived extract [1], although some medical plants are not always sensitive to elicitation [14,20]. Elicitors are perceived by specific receptors in the plasma membrane, trigger gene expression and induce a stress response in plants, which may result in production of higher amounts or new forms of valuable secondary metabolites [9]. However, stress modifies plant metabolism, growth or reproduction in a negative and/or positive way [16]. Stress response can be manifested on various levels of plant organization and usually has unspecific character, so elicitation strategies do not always work effectively and should be investigated.

To the extent of our knowledge, there are a few studies focused on elicitation strategies of *Drosera* sp. plants using various elicitors [18,22–24], but little is known about the possible elicitation

strategies for *D. muscipula* [1]. Due to the unique biochemical compositions and strong biological activity of Venus flytrap extract, studies on this species are valuable. Our previous study on this plant showed that abiotic elicitation with a blue-red LED light did not increase synthesis of PLU and did not affect total phenolic content (TPC) [14]. In this experiment, we examined the response of Venus flytrap in vitro plants grown in LM with rotary shaking to elicitation with lysate of *Cronobacter sakazakii* (former *Enterobacter sakazakii*) bacteria. This is the first report showing such an elicitation strategy, where for the first time, an elicitor has been used in a concentration and time gradient. Moreover, we present how such elicitation affects the antioxidant properties and antibacterial activity against two antibiotic-resistant human-pathogenic bacteria: *Staphyloccocus aureus* and *Escherichia coli.*

Bacterial-derived elicitors are proven to stimulate plant secondary metabolism in plant tissue cultures, and the preparation of these is easy and fast compared to other biotic elicitors, e.g., fungal derived elicitors [21]. *C. sakazakii* lysate has been chosen for our experiment based on literature data for other plant species [20,25]. *C. sakazakii* are the human-pathogenic, facultative, Gram-negative, non-spore forming and motile microorganisms (possess flagella) that belong to the *Gammaproteobacteria* class and the Enterobacteriaceae family [26]. Flagellin was found to be the main, if not the only, factor in the recognition of Gram-negative bacteria (e.g., various pathovars *Pseudomonas syringae*, *E. coli*, *Pseudomonas aeruginosa*) by plant cells [27]. Moreover, specific bacterial O-polysaccharides [28] can be a signal for the plant cell, which will trigger a stress response and will affect higher secondary metabolites production. Furthermore, elicitation in LM with rotary shaking may turn out to be a good system for elicitation studies, due to easier and faster elicitor action.

The aims of the study were; (1) evaluation of biometric and biochemical parameters of *D. muscipula* plants growing in LM with rotary shaking, (2) optimisation of elicitation protocol using lysate from *C. sakazakii* bacteria cells in the context of synthesis of medical active phenolic compounds belonging to 1,4-naphtoquinones, phenolic acids, phenylpropanoids, flavonoids and anthocyanins, (3) check antioxidant and antibacterial properties of extracts isolated from elicited plant tissue.

Present research indicate that LM system is more suitable for *D. muscipula* plant cultivation, than agar-solidified media. Elicitation with *C. sakazakii* lysate causes changes in biochemical composition of examined plants, and increase its biological activity against reactive oxygen species and antibiotic-resistance bacteria.

2. Results

2.1. Biometric and Biochemical Parameters of Plants Growing in LM (Experiment 1)

To evaluate effect of LM on *D. muscipula* growth and development the growth index (GI) were calculated. Plants cultivated in LM had significantly higher GI (69.55%) than plants from SM (54.98%) (Figure 1A,B). In turn, dry weigh (DW) accumulation did not vary between tested techniques (Figure 1C). Moreover, LM with rotary shaking affected accumulation of phenolic compounds in plant tissue. In comparison to plants from SM, plants cultivated in LM accumulated significantly more: TPC, phenylpropanoids (PHE), flavonoids (FLA) and anthocyanins (ANT) (Figure 2A–D).

2.2. Results of D. muscipula Elicitation with C. sakazakii Lysate (Experiment 2)

In this experiment we compared biometric parameters and accumulation of various phenolic derivatives in plants affected by biotic elicitation in comparison to untreated plants. In the Figure 3 A–D morphology of plants under various doses of elicitor (1.5; 2.5 or 5.0% of *C. sakazakii* lysate) and time of exposure (5, 6 or 7 days) is presented in comparison to untreated plants. The applied elicitor did not affect GI and DW content in *D. muscipula* (Figure 4A,B).

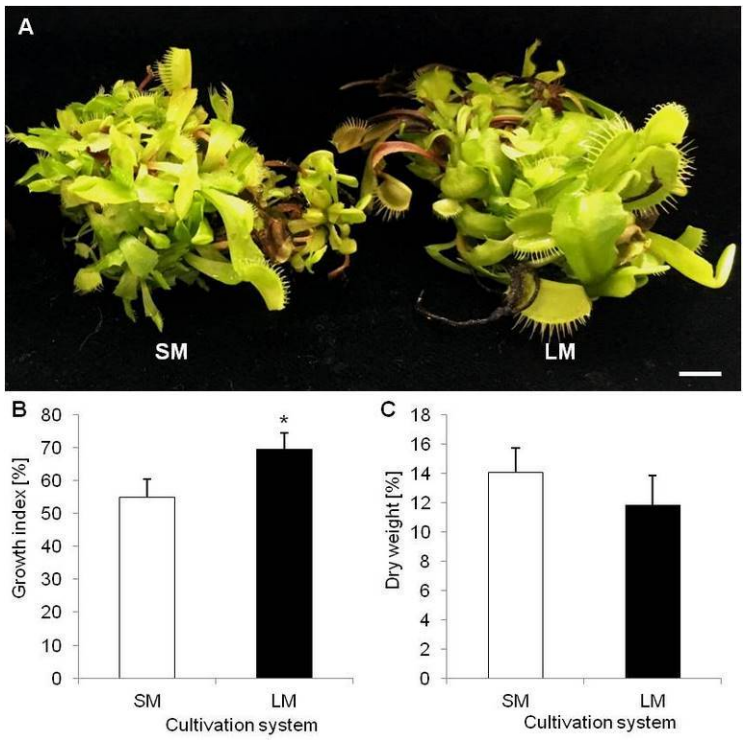

Figure 1. *Dionaea muscipula* plants in different cultivation systems: (**A**) plants cultivated on solid medium (SM) and in liquid medium with rotary shaking (LM); (**B**) growth index [%] of plants depending on cultivation system; (**C**) dry weight content [%] of plants depending on cultivation system; * significant difference between means at $p < 0.05$; bar—1 cm.

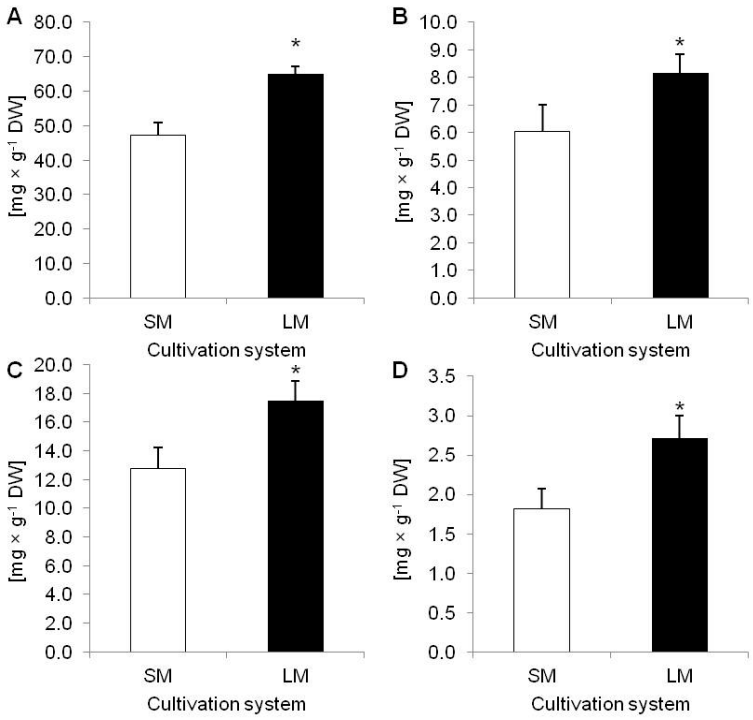

Figure 2. Accumulation of phenolic compounds in *Dionaea muscipula* plants cultivated on solid medium (SM) and in liquid medium with rotary shaking (LM); (**A**) total phenolic content; (**B**) phenylpropanoids; (**C**) flavonoids; and (**D**) anthocyanins depending on cultivation system; * significant difference between means at $p < 0.05$; bar—standard deviation.

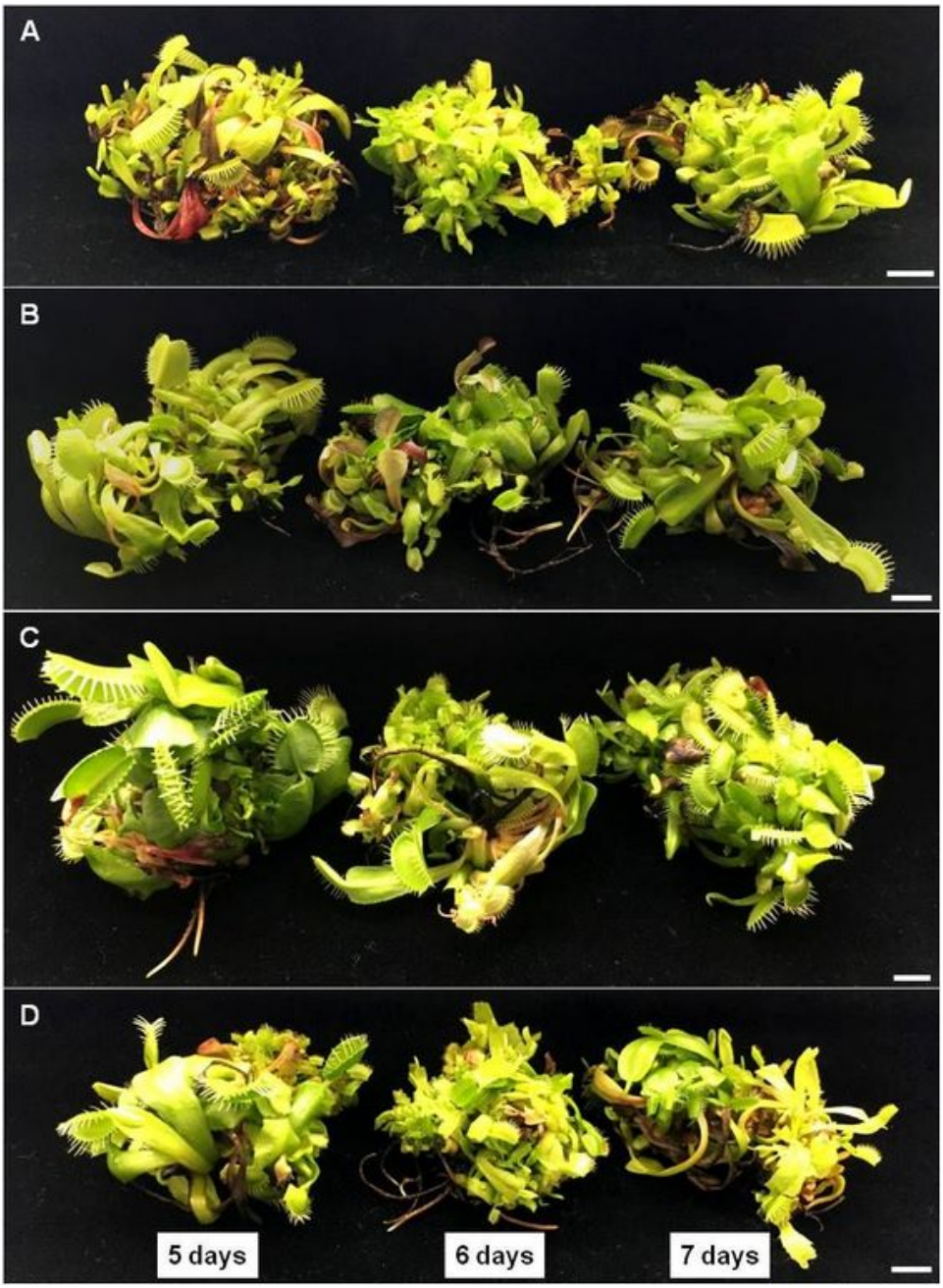

Figure 3. Morphology of *Dionaea muscipula* plants after 5, 6 and 7 days of elicitation with *Cronobacter sakazakii* lysate in different concentrations: (**A**) control (0%); (**B**) 1.5%; (**C**) 2.5%; (**D**) 5%; bar—1 cm.

In turn, TPC increased significantly in plants treated with 2.5% of elicitor after 5 and 6 days of exposure, as well as in those treated with 5% after 5, 6 and 7 days (Figure 5A). The highest level of TPC was noted in plants treated with 5% of bacterial lysate for 7 days, where concentration of phenolic compounds was 1.74-fold higher than in the control plants. Also, accumulation of PHE was affected by 5% of *C. sakazakii* lysate. After 5 days of treating, *D. muscipula* plants synthesized 2.43-fold more PHE than in control conditions (Figure 5B). On the other hand, FLA content grew significantly in plant tissues after 7 days of treatment with 1.5% and 2.5% of elicitor, regardless of the length of treatment (Figure 5C). Only lower doses of elicitor (1.5 and 2.5%) stimulated plants to higher ANT accumulation but only after 6 days of elicitation (Figure 5D).

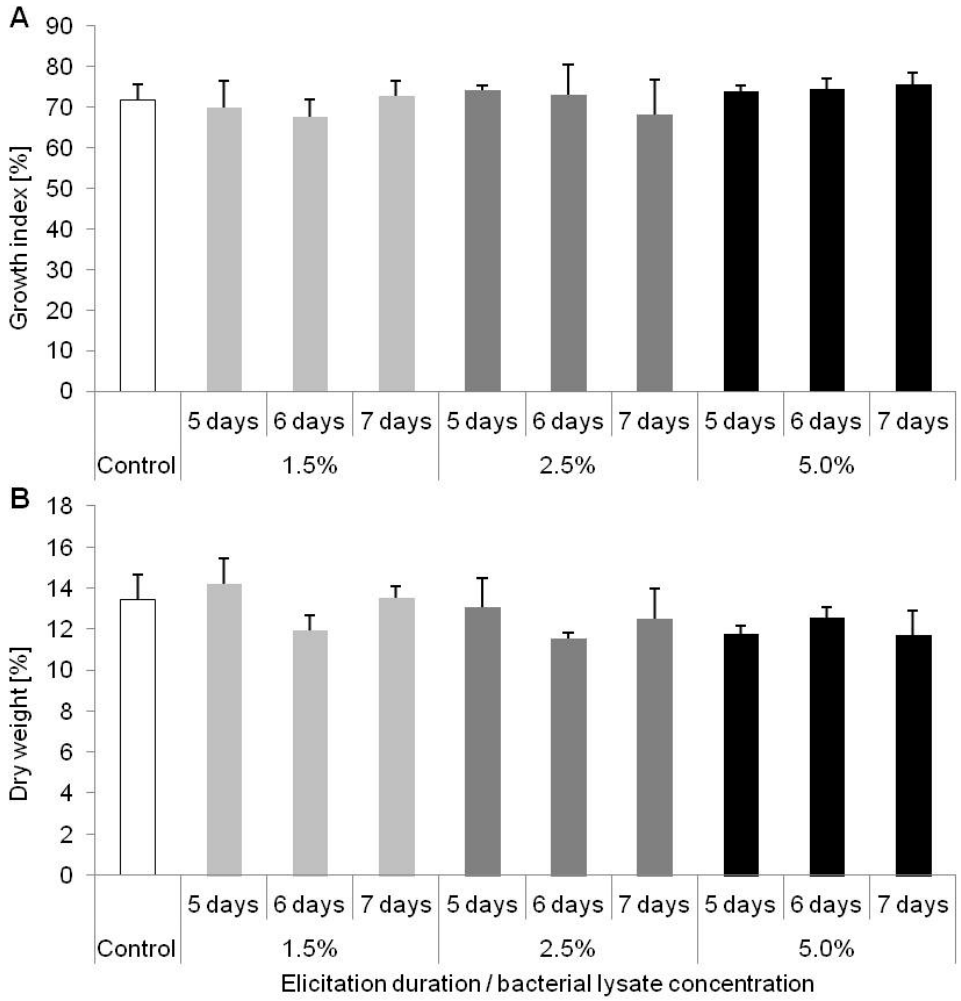

Figure 4. (**A**) Growth index (%) and (**B**) dry weight content (%) of *Dionaea muscipula* plants after 5, 6 and 7 days of elicitation with *Cronobacter sakazakii* lysate in different concentrations; no letters—no significant difference between means at $p < 0.05$; bar—standard deviation.

Applied elicitation also changed the accumulation of some phenolic derivatives in examined plants. Increased PLU content was noted after 5 days of treatment with 2.5% and 5% elicitor and after 6 days with 1.5% and 2.5% of the elicitor (Figure 6). The most effective results were noticed in plants treated with 2.5% elicitor for 6 days (69.82 mg × g^{-1} DW) (Figure 6).

Synthesis of caffeic acid (CA) significantly increased after 7 days of elicitation with 1.5 and 2.5% of the elicitor (2.31 and 2.43-fold higher synthesis, respectively) (Figure 7A). After 7 days of cultivation, accumulation of myricetin (MYR) also increased, not only in plants treated with 1.5 and 2.5% elicitor but also in those treated with 5% (Figure 7B). In turn, a higher level of ellagic acid (EA) was only recorded in plants treated with 2.5% elicitor for 6 days (Figure 7C). Interestingly, treatment with *C. sakazakii* lysate also caused a reduction of some phenolics accumulation (Figure 7B–F). MYR, salicylic acid (SA) and quercetin (QUE) content decreased in plants after 5 days of elicitation with 2.5% bacterial lysate (Figure 7 B,E,F). EA and hyperoside (HYP) accumulation were reduced after treatment with 1.5% of elicitor for 6 and 7 days, respectively (Figure 7C,D).

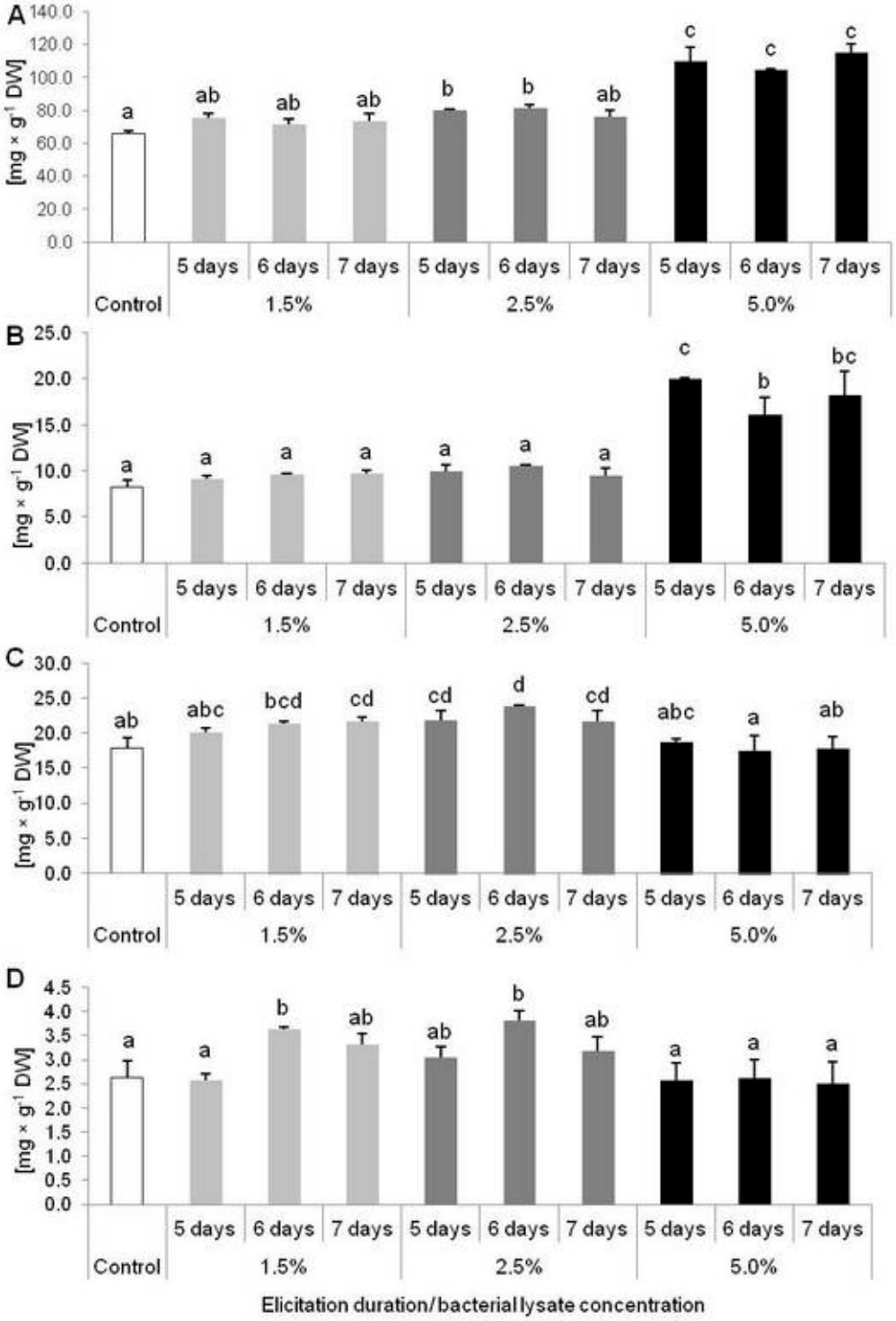

Figure 5. Accumulation of phenolic compounds in *Dionaea muscipula* plants after 5, 6 and 7 days of elicitation with *Cronobacter sakazakii* lysate in different concentrations: (**A**) total phenolic content; (**B**) phenylpropanoids; (**C**) flavonoids; (**D**) anthocyanins; different letters—significant difference between means at $p < 0.05$, bar—standard deviation.

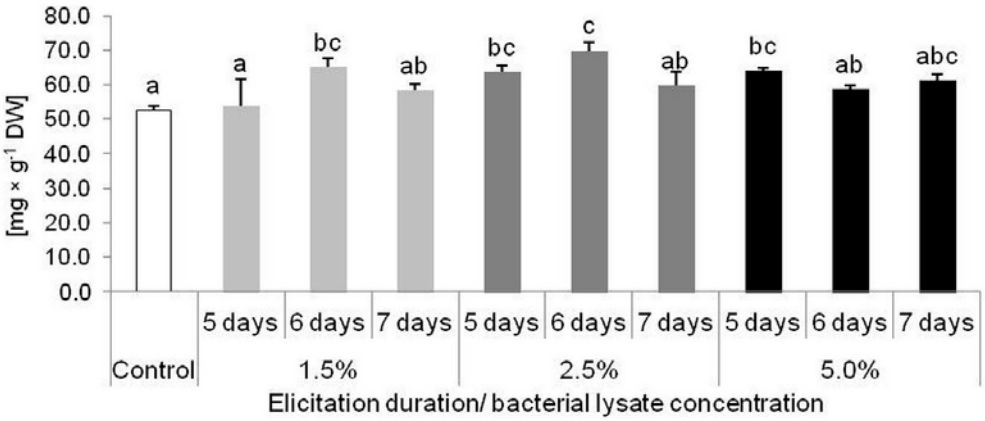

Figure 6. Accumulation of plumbagin in *Dionaea muscipula* plants after 5, 6 and 7 days of elicitation with *Cronobacter sakazakii* lysate in different concentrations; different letters—significant difference between means at $p < 0.05$; bar—standard deviation.

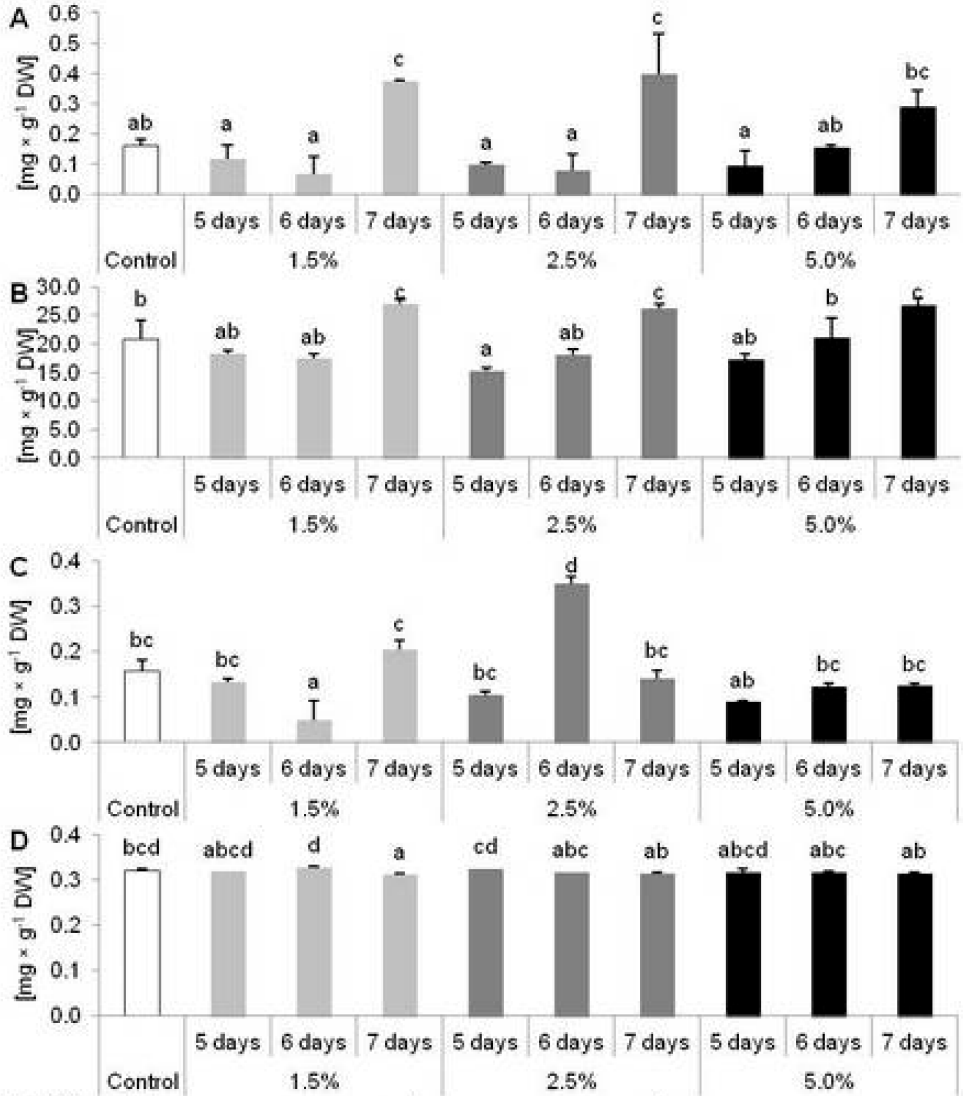

Figure 7. *Cont.*

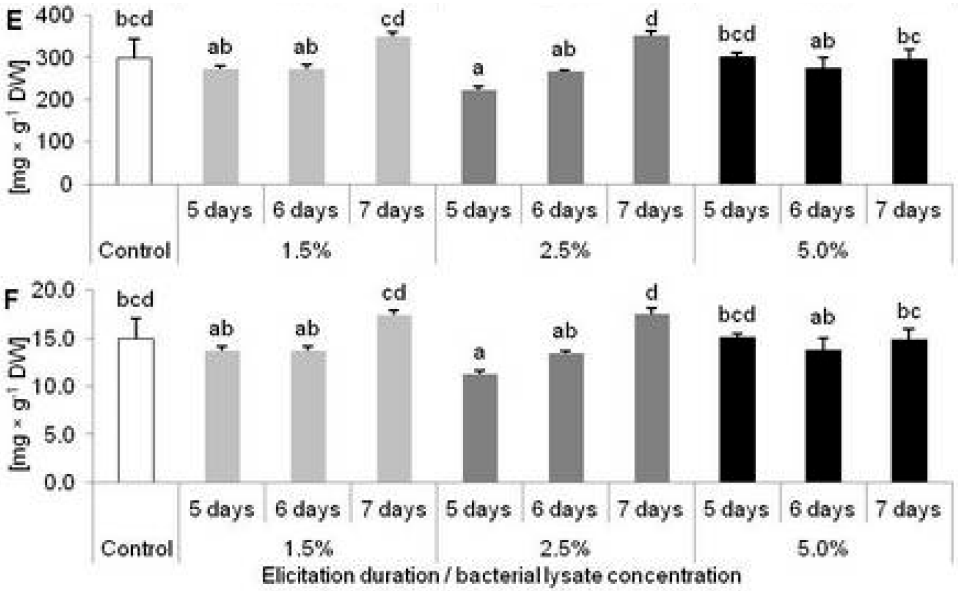

Figure 7. Accumulation of phenolic derivatives in *Dionaea muscipula* plants after five, six and seven days of elicitation with *Cronobacter sakazakii* lysate in different concentrations: (**A**) caffeic acid; (**B**) myricetin; (**C**) ellagic acid; (**D**) hyperoside; (**E**) salicylic acid; and (**F**) quercetin; different letters—significant difference between means at $p < 0.05$, bar—standard deviation.

2.3. Biological Activity of Extracts from D. muscipula Plants Elicited with C. sakazakii Lysate

2.3.1. Reactive Oxygen Species Scavenging Activity

The scavenging potential of extracts derived from *D. muscipula* tissue have been affected by some elicitor doses and exposure time. Significantly higher antioxidant activity have been noticed in plants treated 7 days by each concentration of elicitor, and also in plants treated 6 days with 5% of *C. sakazakii* lysate (Figure 8).

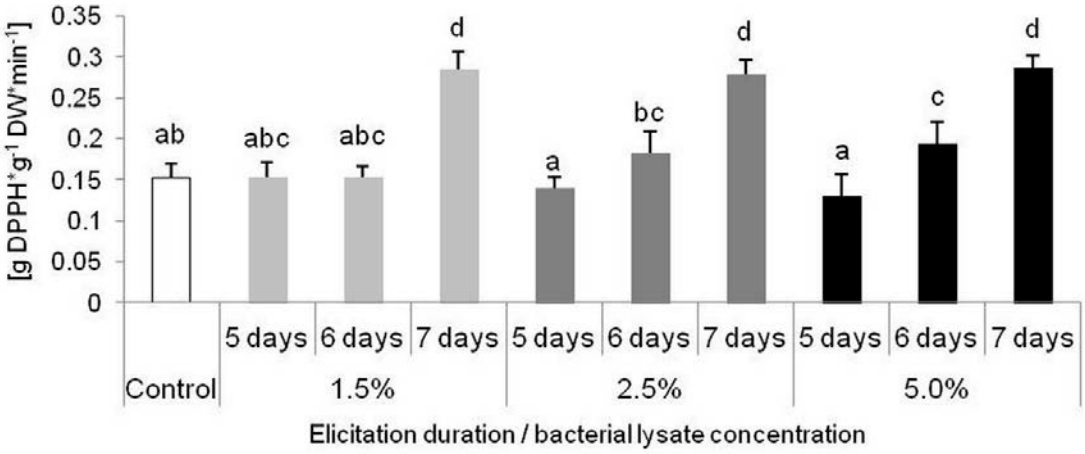

Figure 8. ROS scavenging activity (expressed as g DPPH reduced per g DW per minute) of *Dionaea muscipula* plants after 5, 6 and 7 days of elicitation with *Cronobacter sakazaki* lysate in different concentrations; different letters—significant difference between means at $p < 0.05$, bar—standard deviation.

2.3.2. Antibacterial Activity

In the presented study, the MBC of plant tissue cultivated under bacterial elicitation have been investigated. Regardless of the elicitor concentration and exposure time, elicited plants extracts had 20% stronger activity against Gram-negative bacteria *E. coli* comparing to control plants (Table 1).

Table 1. Minimal bactericidal concentration (MBC) of *S. aureus* and *E. coli* after treatment with extracts of *Dionaea muscipula* plants after five, six and seven days of elicitation with *Cronobacter sakazaki* lysate in different concentrations.

Concentration of *C. sakazaki* Lysate (%)	Days of Elicitation	*Staphylococcus aureus* ATCC 25923 MBC (μg DW × mL^{-1})	*Escherichia coli* ATCC 25922 MBC (μg DW × mL^{-1})
0.0 (Control)		501	2087.5
1.5	5	501	1670
	6	417.5	1670
	7	334	1670
2.5	5	417.5	1670
	6	417.5	1670
	7	334	1670
5.0	5	417.5	1670
	6	417.5	1670
	7	334	1670

Interestingly, in case of Gram-positive bacteria, *S. aureus,* the bactericidal properties of examined plants have been affected, depending on the elicitor concentration and time of elicitation. Tissue cultures treated with 1.5% of *C. sakazakii* lysate had 17% higher activity after 6 days of elicitation and about 34% increased bactericidal properties after 7 days of treatment. For treatment with 2.5 and 5% of elicitor the same effect has been observed (Table 1). After 5 and 6 days plant tissue had 17% higher activity against *S. aureus*, while after 7 days of treatment antibacterial strength increases 34% in compare to control plants.

3. Discussion

3.1. The Effect of Shaking on Plant Growth and Secondary Metabolite Levels

In the presented study, we focused on the possibility to produce a large amount of *D. muscipula* plant biomass with a high concentration of medical-active phenolic compounds. According to some authors, shaking technology has a lot of benefits for various types of cultures [29]. LM, with a rotary shaking system, is usually used for bacteria, cell suspensions or roots culture cultivation [29]. Due to fast oxygen and nutrient transfer from medium to living cells [29], lack of impurities from agar and the dilution of some exudates released from roots, like phenolic compounds [30], it is possible to obtain a faster growth and multiplication rate. This is the reason why, in the first experiment, we tested whether LM with rotary shaking will be a suitable system for Venus flytrap whole plants culture propagation. The results showed that such a cultivation system increased GI of the examined plants (by approx. 25%) in comparison to plants cultured on SM. Interestingly, our results are in agreement with observations by Liu et al. [31] that *Artemisia judaica* plants propagated in an LM flask culture with rotary shaking accumulated more FW and had higher proliferation rate than plants on SM. Also, Weathers et al. [32] revealed that *Artemisia annua* plants cultivated in LM with rotary shaking accumulated 25–50% more biomass than plants in bioreactors. Moreover, we conclude that LM conditions could be a good way for carnivorous plants to propagate because of their biology. In natural conditions, these plants occur in wet and flooded areas, which may result in a good acclimation mechanism to the physical properties of such a medium.

Once the culture conditions are optimized for higher biomass production of medical plants, the desired goal is to increase the amount of secondary metabolites in tissue. Unfortunately, these two objectives do not always occur in the tandem [32]. According to the results of Lattanzio et al. [33], under stress conditions, higher synthesis of phenolic compounds is strongly negatively correlated with the growth rate of plant tissue. This phenomenon results from the fact that a stress response is very costly for a plant and that acclimation to stress conditions requires a plant to use the basic metabolism

products for a defense response consisting of secondary metabolites production. Carbon skeletons produced in primary metabolites pathways are distributed to secondary metabolites production pathways. Lattanzio et al. [34] showed that increased amounts of phenolic compounds in suspension culture of *Cynara cardunculus* growing under nutritional stress, was connected with a decrease of suspension culture biomass. However, the cultivation of *D. muscipula* plants in LM with rotary shaking, the increase of GI was simultaneous with significantly higher accumulation of TPC, PHE, FLA and ANT. It may result from the fact that the composition of the medium, used in our experiment, does not cause nutrient deficit and plants do not have to manage the limited resources of nutrients [17]. On the other hand, increased production of phenolic compounds in Venus flytrap plants, in LM with rotary shaking, may result from the fact that such a system of cultivation induces hydromechanical stress [29]. The carnivorous plant leaf-traps, being very sensitive to mechanical stimulation, can sense mechanical stimulus as the potential for catching the prey, while it is known that secondary metabolites from the phenolic compounds group play as protectants in the process of prey digestion [15]. Moreover, hydromechanical stress in an LM system is connected with the intensity of culture' shaking. Perez-Hernandez et al. [35] examined suspension cell culture of medical plant *Sphaeralcea angustifolia* and revealed that cells grown with 200 rpm had the highest cell biomass and increased concentration of sphaeralcic acid. Growing cells with 100 rpm induces oxygen deficit stress, while 400 rpm negatively affects viability of cells, which was the consequence of hydrodynamic stress. Nevertheless, liquid cultures are one of the most important branches of tissue cultures in biotechnology of medical plants, and the selection of specific conditions is the crucial for both studying of plant response to stress factors and obtaining large quantities of plant material for medical purposes [36]. In the presented research, we proved that LM with rotary shaking is an effective system for *D. muscipula* propagation and synthesis of valuable, biologically active, phenolic compounds. This is the first report where shaking technology was used for propagation of a carnivorous plant from family Droseraceae. Based on the results from experiment 1, the culture conditions for experiment 2 were developed.

3.2. Impact of Biotic Elicitation on Plant Growth and Secondary Metabolite Levels

Many plant-derived chemicals, with importance in the pharmacological industry, can be overproduced in response to an external stimulus called an elicitor. As the elicitors do not act equally in every plant species, elicitation studies in various plants, with biological activity, need to be conducted [32]. On the other hand, this technique has some limitations. It has been shown that, despite the elicitor's contribution to increasing synthesis of secondary metabolites in plant tissue, the vitality of the in vitro culture can decline, resulting in decreased growth rate or conduct to plant death [32]. Gadzovska et al. [37] revealed that elicitation of phenolic compounds, using jasmonic acid in *Hypericum perforatum* suspension culture caused increase of TPC and FLA production with a simultaneous decrease of cells' viability. Jesionek et al. [20] showed that the elicitation of essential oil in a *Rhododendron tomentosum* bioreactor-grown microshoots culture, with aphid ethanol extract and bacteria lysates from *Candida albicans*, *C. sakazakii*, *Pectobacterium carotovorum* and *Dickeya dadantii* decreased the GI of plants, compared to untreated control shoots. In turn, *Ruta graveolens* shoots elicited with lysate of *Bacillus* sp. cells have been characterized by increased growth and accumulation of coumarin [38]. In the presented study, a tissue culture of *D. muscipula* was elicited in LM with rotary shaking using lysate from *C. sakazakii* cells, with various concentrations and exposure time to elicitors. Neither DW content nor GI was affected by elicitor treatments in comparison to control plants. It may be connected with a short time of elicitation (5, 6 or 7 days) and/or effective acclimation mechanisms of Venus flytrap plants for stress connected with bacterial elicitation. These results are in agreement with Krolicka et al. [1] findings, where FW of *D. muscipula* did not change under biotic elicitation with *Agrobacterium rhizogenes* lysate. On the other hand, in our previous studies, GI did not change in plants growing in higher light intensity or under white LED light in comparison to the fluorescence radiation [13], while blue-red LED light increased GI of *D. muscipula* and *Drosera peltata* cultivated in vitro [14].

Only few articles indicate the activity of lysate from *C. sakazakii* on the plant's secondary metabolism. Previously, Staniszewska et al. [39] and Krolicka et al. [25] reported that the elicitation of *Ammi majus* with *C. sakazakii* led to changes in metabolism of cumarins and can decrease the growth rate of tissue culture. Jesionek et al. [20] did not find any changes of essential oil content in *Rhododendron* culture elicited with lysate from *C. sakazakii*. In our study, for the first time, we presented application of this elicitor for increased production of phenolic compounds in the *D. muscipula* plant. Krolicka et al. [1] showed that, lysate from *A. rhizogenes*, increased content of PLU in a Venus flytrap tissue culture, with simultaneous higher antibacterial activity of extract derived from elicited plants. Moreover, Krolicka et al. [1] reported that such an elicitation strategy did not affect the synthesis of FLA: MYR and QUE. We noticed that accumulation of total FLA, PLU and MYR was affected significantly by some of elicitor's concentrations and exposure time, while the highest yield of PLU was obtained by a treatment with 2.5% of bacteria lysate for six days. Moreover, the highest TPC and PHE accumulation was obtained with 5% of *C. sakazakii* lysate, independently of the exposure time. In contrast, CA and MYR synthesis was affected by exposure time to the elicitor. The highest amount of these metabolites was obtained after seven days of elicitation, regardless the dose of bacterial lysate. Furthermore, elicitation with *C. sakazakii* led to decrease in content of some phenolic derivatives. After five days with 2.5% of bacteria lysate synthesis of SA, QUE and MYR decreased, after 6 days of treatment with 1.5% of elicitor level of EA decreased, while content of HYP was significantly lower after five days of treatment with 2.5% of elicitor. Such negative changes in secondary metabolites content can be also the consequence of the stress-related response of plants [20].

Based on these results, we can conclude that the chosen elicitor (containing the endotoxin O-antigen involved in bacterial pathogenesis) stimulates some of the phenolic derivatives in Venus flytraps; the response of this plant is also not specific and we cannot outline clear relationships between elicitation effect and content of all phenolic derivatives. It is worth noting that the O-antigen polysaccharide of the bacterial cell surface are mostly involved in a host specific immunological response [28] and clearly affects the content of secondary metabolites in Venus flytrap tissue. Moreover, flagellum protein synthesized by *C. sakazakii* is an important virulence factor for bacteria pathogenic to animals and plants [40], so it is possible that this protein acted as an elicitor in our study. Furthermore, we conclude that bacterial elicitation is a more suitable strategy to improve secondary metabolites production in *D. muscipula*, than light elicitation [13,14].

Other authors also showed some biotic elicitation strategies of phenolic compounds in different medical plant species. One of the most common agents used in elicitation of phenolic derivatives in plants is chitosan. Chitosan was reported to increase phytoalexin production in *R. graveolens* [41], stimulate lignans accumulation in *Schisandra chinensis* [19] and affect higher accumulation of TPC in *Orthosiphon stamineus* [42]. Moreover, the application of chitosan, yeasts extract or precursor feeding in elicitation of PLU in *Plumbago* roots cultures is popular [11,12,43,44]. Comparing these works to our results, elicited *D. muscipula* plants accumulate more PLU and other phenolic derivatives. This probably results from the specific acclimation strategies of carnivorous plants related to their ecophysiology and gives a basis for further studies on the metabolism of phenolic compounds in carnivorous plants.

3.3. Impact of Biotic Elicitation on Biological Properties of D. muscipula Plants

In our study we evaluated how the biotic elicitation with *C. sakazakii* lysate affects biological activity of extracts derived from *D. muscipula* tissue culture. For the first time antioxidant properties of elicited Venus flytrap tissue culture were evaluated using method based on scavenging of DPPH free radical. It was reported previously by Krolicka et al. [5], that carnivorous plant tissue from family Droseraceae is very potent antioxidant. In our research, the highest activity against DPPH free-radicals were noticed in plants with increased accumulation of CA and MYR, which is in agreement with research by Banasiuk et al. [45]. The reduction potential of plant-derived extract is strictly correlated with the quantity of phenolic compounds, especially FLA. Banasiuk et al. [45] have reported, that water extracts from carnivorous plants with the highest concentration of flavonoids gives the best results in

production of silver nanoparticles, while such feature is strictly related to their anti-oxidative potential. Similar findings have been shown by Ansari et al. [46], where transformed hairy root culture of *Ligularia fischeri* were characterized by increased synthesis of phenolic compounds and higher radical scavenging activity, than control plants. Moreover, *Cannabis sativa* cell suspension culture treated with jasmonates and some precursors of phenylpropanoid pathway accumulated more phenolic derivatives and had increased radical scavenging activity against DPPH [47].

Due to the rapid increase in human-pathogenic bacteria to antibiotic treatments, new sources of chemicals with strong antibacterial properties are needed [6]. Presented research was focused on potential use of extracts from elicited medical plant *D. muscipula* against antibiotic-resistant bacteria. In comparison to *D. muscipula* control culture growing without elicitation treatment, extracts from elicited plants had increased bactericidal activity against Gram-negative bacteria *E. coli*. Interestingly, regardless of exposure time and elicitor concentration antibacterial properties of examined plants increased 20%. It may be a consequence of lower sensitivity of Gram-negative bacteria to plant-derived metabolites, than Gram-positive bacteria [48], which is confirmed by the presented results, where MBC for Gram-positive bacteria *S. aureus* is dependent on duration of the elicitation. Krolicka et al. [1] showed, that elicitation with lysate of *A. rhizogenes* can stimulate antibacterial properties of *D. muscipula* tissue culture against Gram-negative bacteria *Klebsiella pneumonia*, while MBC for Gram-positive *S. aureus* was not changed. On the contrary, our elicitation strategy with lysate of *C. sakazakii* increased bactericidal properties of Venus flytrap tissue against this pathogen. Moreover, for the first time we can report that MBC of carnivorous plant tissue for *S. aureus* is dependent on elicitation treatment time. Furthermore, antibacterial activity of elicited plants does not correlate with the concentration of PLU, what can indicate, that not only accumulation of 1,4-naphtoquinones [5], but also quantity of others chemicals in carnivorous plants metabolic profile has crucial importance in potential healing properties of these plants.

4. Materials and Methods

4.1. Plant Material and Experiments Design

4.1.1. Plant Material

This study was conducted on a previously established in vitro culture of *D. muscipula* plants [1]. Plants were cultivated on $\frac{1}{2}$ strength MS medium [49] with no growth regulators, 3% sucrose and pH = 5.5 (adjusted prior autoclaving) and solidified with 0.8% agar. Plants were cultivated at temperature 23 ± 1 °C; in fluorescence light of 80 µmol × m^{-2} × s^{-1} photosynthetic photon flux density (PPFD); (photoperiod 16 h/8 h light/dark cycle) and subcultured at 30-daya intervals.

4.1.2. Experiment 1: Cultivation of Plants in Liquid Media with Rotary Shaking

About 1.5 g of plants cultured as described above were subcultured to flasks with solid medium (SM) or liquid medium (LM). Chemical composition of media was the same as above. Tissue cultures in LM were put on a rotary shaker (130 rpm × min^{-1}). After 30 days of cultivation, plants were subjected to growth parameters determination and biochemical analysis. There were 5 biological repetitions (flasks) of each variant (SM or LM) prepared, and the experiment was repeated in triplicate.

4.1.3. Experiment 2: Elicitation of Plants Growing in Liquid Media with Rotary Shaking

The elicitor in this experiment was lysate from human-pathogenic bacteria *Cronobacter sakazakii* ZOBR A741, and selected based on our preliminary data and available literature [39]. The elicitor was prepared according to Jesionek et al. [20]. Briefly, microbes were cultivated 24 h in Luria broth (LB) medium at 37 °C. Suspension cultures (15 × 10^{12} colony-forming units (CFU)/mL, according to McFarland scale) were treated with toluene (100:1 *v*/*v*), toluene was evaporated, and the elicitor was autoclaved. This prepared lysate was used for plant elicitation.

About 1.5 g of plant material was subcultured to flasks with LM (composition as described above) and placed on a rotary shaker (130 rpm × min^{-1}). Tissue cultures were cultivated in the same light and temperature conditions as above. After 21 days of cultivation, lysate of *C. sakazakii* was added to media up to final concentrations: 1.5; 2.5 and 5.0%. The exposure time for each treatment was 5, 6 or 7 days, according to the literature [20,23]. The control in the experiment was non-treated plants cultivated in the same conditions. Plant materials were examined for biometric and biochemical parameters. For experiment 5, biological repetitions (flasks) of the control and each treatment with the elicitor were prepared. The experiment was repeated in triplicate.

4.2. Growth Parameters Estimation

Growth Index (GI) and Dry Weight (DW) Content

Plants from experiment 1 and 2 were weighed immediately after harvesting. Growth index (GI) was calculated according to formula: GI [%] = (FW_2 − FW_1)/FW_2 × 100, where FW_1 is fresh weight of plants at the beginning of experiment and FW_2 is a final fresh weight. Next, plant material was freeze-dried for 72 h and weighed to determine content of dry weight (DW) using formula: DW [%] = DW_2 × 100/FW_2, where DW_2 is dry weight after freeze-drying. Freeze-dried plant tissue was homogenised and stored at −20 °C.

4.3. Biochemical Analysis

4.3.1. Spectrophotometric Estimation of Total Phenolic Content (TPC)

TPC was assessed using Folin-Ciocalteu's reagent [50], with modifications according to Makowski et al. [14]. In short, 10 mg of freeze-dried plant material was extracted in 1 mL of 80% methanol at 4 °C. Samples were centrifuged for 15 min (25,155 g, 4 °C). Of the diluted extract, 1 mL was mixed with 0.2 mL of Folin's reagent (Sigma-Aldrich Chemie, GmBH, Stein-heim, Germany), 1.6 mL of 5% Na_2CO_3 and incubated for 20 min at 40 °C. The absorbance of samples was measured at 740 nm, using a Double Beam spectrophotometer U-2900 (Hitachi High-Technologies Corporation). Chlorogenic acid (Sigma-Aldrich Chemie, GmBH, Steinheim, Germany) was used as a reference standard. Results were expressed as milligram of chlorogenic acid equivalents per 1 g of DW. Analyses were done in 5 replicates.

4.3.2. Spectrophotometric Estimation of Phenylpropanoids (PHE), Flavonoids (FLA) and Anthocyanins (ANT) Content

PHE, FLA and ANT accumulation were estimated using the method of Fukumoto and Mazza [51], with modifications [13]. Plant tissue was extracted like in the method for TPC estimation. Supernatant was mixed with 0.25 mL 0.1% HCl in 96% EtOH and 4.55 mL 2% HCl in H_2O. Samples were incubated at room temperature (darkness) for 20 min. Absorbance was measured at wavelengths of 320, 360 and 520 nm. Contents of PHE, FLA and ANT were calculated using calibration curves made for caffeic acid, quercetin and cyanidin (Sigma-Aldrich Chemie, GmBH, Steinheim, Germany), respectively. The results were expressed as milligram of standard equivalents per 1 g of DW. Analyses were done in 5 replicates.

4.3.3. High Pressure Liquid Chromatography (HPLC) Analysis of Phenolic Compounds

For analysis of PLU content, freeze-dried plant tissue was extracted in 0.5 mL of redistilled H_2O and 0.5 mL of tetrahydrofuran (THF) according to Tokarz et al. [52]. To extract other phenolic derivatives, like caffeic acid (CA), hyperoside (HYP), ellagic acid (EA), salicylic acid (SA), myricetin (MYR) and quercetin (QUE), 20 mg of dry tissue was homogenised in 2 mL of 100% methanol (4 °C) and sonicated for 30 min. Samples were centrifuged for 15 min (25,155 g, 4 °C). Supernatant was collected for chromatographic analysis (HPLC).

The chromatographic separation was carried out using Dionex UltiMate 3000 HPLC system equipped with a quaternary pump, autosampler, column oven and UV detector. For the stationary phase, an Agilent Zorbax SB-Phenyl (4.6 × 150 mm, 3.5 µm) was used. The flow rate used was 1 mL × min^{-1}. The sample injection volume was 10 µL. The mobile phase for the analysis consisted of 0.1% (*v/v*) trifluoroacetic acid in acetonitrile as eluent A and 0.1% (*v/v*) trifluoroacetic acid in water as eluent B. The separation gradient was 0 min (10% A)-> 5 min (10% A)-> 12 min (90% A)-> 20 min (90% A), followed by a 10-min column regeneration. Chromatographic separations were carried out at 25 °C. Typical compounds present in carnivorous plant tissues (plumbagin, hyperoside, ellagic acid, myricetin, quercetin, salicylic acid and caffeic acid) were used as standards to determine extract composition. A three-level standard curve was used for determining the concentration of the compounds 4-point. Monitoring was performed at 254 nm. All analyses were performed in triplicate.

4.4. Analysis of Biological Activity of Examined Plants

4.4.1. Spectrophotometric Estimation of Antioxidative Properties of Plant Extract Using DPPH Method

Scavenging of 2,2-diphenyl-1-picryl-hydrazyl (DPPH) free radical was measured using the methods of Sharma and Bhat [53] and Elshafie et al. [54], with modifications. Plant tissue was extracted like in the method for TPC estimation. 0.05 mL of diluted methanolic extract was mixed with 2.95 mL of 50 mM DPPH solution and incubated in the dark. After 30 min absorbance of samples were measured at 517 nm. The reduction of stable DPPH by plant extract was expressed as a gram of DPPH reduced by gram of DW tissue per one minute. Analyses were done in five replicates.

4.4.2. Antibacterial Activity

The antibacterial properties of the examined plants were evaluated using a minimal bactericidal concentration (MBC) method by Krolicka et al. [5]. MBC was determined against antibiotic-resistant bacteria: *Staphylococcus aureus* ATCC 25923 G (+) and *Escherichia coli* ATCC 25922 G (−), obtained from Intercollegiate Faculty of Biotechnology, University of Gdańsk and Medical University of Gdańsk, Poland. The bacteria were cultivated overnight on BHI medium at 37 °C, before the tests. Plant tissue (100 mg DW) was extracted in THF [13]. Extracts were evaporated and resuspended in methanol before application into wells of the 96-well plate. After application, extracts were evaporated to remove toxic for bacteria methanol. The residues were suspended in 100 µL liquid BHI medium for bacteria cultivation and aliquots of 10 µL of the bacterial suspension (10^5 CFU × ml^{-1}) in liquid medium was added into wells. Plates were incubated overnight. In order to establish the MBC value, 100 µL of the content of each well that were shown no visible growth of bacteria were plated out on an BHI agar plate, for 24 h incubation at 37 °C. The MBC was defined as the lowest concentration of the extract that reduced the inoculum by 99.9% within 24 h.

4.5. Statistical Analyses

Results from experiment 1 were subjected to Student's T-test with $p < 0.05$ level. In experiment 2, one-way analysis of variance (ANOVA) was used to determine significant differences between means (Tukey test at $p < 0.05$ level). STATISTICA 12.0 (StatSoft Inc., Tulsa, OK, USA) was used to carry out statistical analyses.

5. Conclusions

The presented research enabled us to study growth and accumulation of pharmacologically active phenolic compounds in a carnivorous *D. muscipula* plant tissue culture, using liquid medium with rotary shaking system and biotic elicitation. The results in experiment 1 proved that liquid media, with rotary shaking, are promising for bigger scale use in, not only the scientific, but also the industrial field. Due to the sensitivity of carnivorous plants to mechanical stimulation, hydromechanical stress in shaking cultures makes it possibility to increase the content of defense compounds. Furthermore,

we conclude that lysate from *C. sakazakii* can be a useful elicitor for some of phenolic compounds in *D. muscipula* tissue cultures and it increases biological activity of *D. muscipula* plants against ROS and highly antibiotic-resistant, human-pathogenic bacteria. We can conclude, that examined plant is strongly antioxidant potent and increasing of radical scavenging activity of Venus flytrap tissue is the most dependent on the concentration of compounds, like PHE and FLA. Also, elicitation with lysate of *C. sakazakii* turned out to be a useful tool for enhancing of antibacterial activity for both: Gram-positive and Gram-negative pathogens. These report gives bases for further investigations on carnivorous plants from family Droseraceae in medical plant biochemistry and pharmacology.

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Abbreviations

ANT	Anthocyanins
CA	Caffeic acid
CFU	Colony-forming unit
DPPH	2,2-diphenyl-1-picryl-hydrazyl
DW	Dry weight
EA	Ellagic acid
FLA	Flavonoids
GI	Growth index
HYP	Hyperoside
LM	Liquid medium
MBC	Minimal Bactericidal Concentration
MYR	Myricetin
PHE	Phenylpropanoids
PLU	Plumbagin
PPFD	Photosynthetic photon flux density
QUE	Quercetin
SA	Salicylic acid
SM	Solid medium
THF	Tetrahydrofuran
TPC	Total phenolic content

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APPLIED MICROBIAL AND CELL PHYSIOLOGY

Transformed tissue of *Dionaea muscipula* J. Ellis as a source of biologically active phenolic compounds with bactericidal properties

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Abstract

The Venus flytrap (*Dionaea muscipula* J. Ellis) is a carnivorous plant able to synthesize large amounts of phenolic compounds, such as phenylpropanoids, flavonoids, phenolic acids, and 1,4-naphtoquinones. In this study, the first genetic transformation of *D. muscipula* tissues is presented. Two wild-type *Rhizobium rhizogenes* strains (LBA 9402 and ATCC 15834) were suitable vector organisms in the transformation process. Transformation led to the formation of teratoma (transformed shoot) cultures with the bacterial *rol*B gene incorporated into the plant genome in a single copy. Using high-pressure liquid chromatography, we demonstrated that transgenic plants were characterized by an increased quantity of phenolic compounds, including 1,4-naphtoquinone derivative, plumbagin (up to 106.63 mg × g^{-1} DW), and phenolic acids (including salicylic, caffeic, and ellagic acid), in comparison to non-transformed plants. Moreover, *Rhizobium*-mediated transformation highly increased the bactericidal properties of teratoma-derived extracts. The antibacterial properties of transformed plants were increased up to 33% against *Staphylococcus aureus*, *Enterococcus faecalis*, and *Escherichia coli* and up to 7% against *Pseudomonas aeruginosa*. For the first time, we prove the possibility of *D. muscipula* transformation. Moreover, we propose that transformation may be a valuable tool for enhancing secondary metabolite production in *D. muscipula* tissue and to increase bactericidal properties against human antibiotic-resistant bacteria.

Key points

- *Rhizobium-mediated transformation created Dionaea muscipula teratomas.*
- *Transformed plants had highly increased synthesis of phenolic compounds.*
- *The MBC value was connected with plumbagin and phenolic acid concentrations.*

Keywords Phenolic acids · Plumbagin · *Rhizobium rhizogenes* · Teratomas · Venus flytrap

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Introduction

Rhizobium rhizogenes (former: *Agrobacterium rhizogenes*) is bacteria from the family Rhizobiaceae and is a "natural genetic engineer" because of the ability to transfer T-DNA (transfer DNA) and incorporate bacteria-derived genes into the plant genome (Georgiev et al. 2007). The natural features of this Gram-negative soil bacterium are used in the plant biotechnology for obtaining transformed organisms with new unique properties. During T-DNA transfer to plant cells, *R. rhizogenes* pass on the set of *rol* genes occurring in Ri plasmids (root-inducing plasmids). These genes encode specific proteins responsible for control over auxin and cytokinin synthesis in plant cells (Guillon et al. 2006). Usually, the consequence of *R. rhizogenes* infection is the change in plant hormone balance and rise of the hairy root phenotype. However, depending on the plant genotype, transformed shoots (teratomas) may occur instead of hairy roots (Królicka et al. 2010). Despite the infection mechanism of plants by *R. rhizogenes* being known for a few decades, the physiological consequences of such events related to plant species need to be studied.

Because of the increasing demand for plant-derived phytochemicals and the consumption of herbal medicines (Canter et al. 2005), genetic transformation found application in the field of medicinal plants (Niazian 2019). Hairy roots or teratomas of medicinal plants characterized by a fast growth rate and biochemical stability are promising sources of plant secondary metabolites in large-scale propagation (Georgiev et al. 2007). Moreover, they can serve as a scientific model for studies on secondary metabolism engineering and the overproduction of phytochemicals of interests in plant tissue culture (Tusevski et al. 2017). To the best of our knowledge, many *Rhizobium*-mediated transformations were successfully established in medicinal plants (Królicka et al. 2001; Gangopadhyay et al. 2010; Libik-Konieczny et al. 2020), while transformation protocol for carnivorous plant *Dionaea muscipula* J. Ellis (Venus flytrap) is still missing.

Carnivorous plants belonging to the family Droseraceae have been used in natural medicine for centuries (Królicka et al. 2010). The healing properties of these plants arise from the ability to produce large amounts of phenolic compounds with strong biological activity (Gaascht et al. 2013). Extracts from carnivorous plant tissues were proven to have antibacterial (Krolicka et al. 2008; Makowski et al. 2020), antioxidative (Królicka et al. 2009; Makowski et al. 2020), antifungal (Padhye et al. 2010), and anticancer properties (Kawiak et al. 2019). Moreover, previous phytochemical studies showed that the species most abundant in phenolic derivatives in the family Droseraceae is *D. muscipula* (Gaascht et al. 2013). The major 1,4-naphtoquinone derivative in the biochemical composition of the Venus flytrap is plumbagin. Furthermore, concentrations of this metabolite in *D. muscipula* tissue are higher than in other commonly used sources of plumbagin, like *Plumbago* plants (Makowski et al. 2020).

Since plants from the family Droseraceae are endangered species and exploitation of natural habitats is impossible, in vitro propagation protocols were established (Banasiuk et al. 2012). Therefore, the implementation of biotechnological tools for the enhancement of secondary metabolite production, like elicitation (Putalun et al. 2010; Boonsnongcheepa et al. 2019) or genetic transformation became possible (Królicka et al. 2010). Nevertheless, increasing valuable phytochemicals with elicitation and obtaining transformed plants is a difficult task because of carnivorous plant biology (Blehova et al. 2015; Makowski et al. 2019).

The main goals of the presented study were (i) transformation of the medicinal plant *D. muscipula* using wild strains of *R. rhizogenes* bacteria, (ii) with simultaneously increased synthesis of phenolic compounds, in particular plumbagin, and (iii) evaluation of bactericidal properties of extracts derived from transformed plants against clinical strains of pathogenic bacteria. We hypothesized that inoculation of the Venus flytrap with *Rhizobium* bacteria would incorporate *rol* genes into plant DNA. The purpose was to establish transformed clones characterized by fast growth and high productivity of valuable secondary metabolites with strong biological activity.

Materials and methods

Plant materials and bacterial strains used for transformation

D. muscipula plants were propagated in in vitro conditions, according to Makowski et al. (2019). Whole plant tissue cultures were cultivated on ½ MS medium (Murashige and Skoog 1962) with no growth regulators, 3% sucrose, and pH 5.5 (adjusted before autoclaving), solidified with 0.75% of agar. Conditions included a temperature of 23 ± 1 °C, fluorescence light of 80 μmol × m^{-2} × s^{-1} photosynthetic photon flux density (PPFD), and a photoperiod of 16 h/8 h light/dark cycle. Plants were subcultured in 30-day intervals.

Wild *R. rhizogenes* strains, including LBA 9402 (NCPPB 1855), ATCC 15834, and A4 (ATCC 31798) were obtained from the Laboratory of Biologically Active Compounds, University of Gdansk, Poland. Bacteria were grown on yeast extract beef (YEB) agar medium with 200 μM of acetosyringone (Sigma) at 26 °C in the dark. For plant transformation, 48-h bacterial cultures were used.

Transformation of *D. muscipula* plants

Young (4-week-old) Venus flytrap rhizomes (150 pieces) were inoculated for each *R. rhizogenes* bacteria strain.

Inoculation was performed with preparation needle, according to Królicka et al. (2010). After inoculation, rhizomes were subcultured to ½ MS medium supplemented with 3% sucrose and 0.8% of agar with pH 5.5 and grown for 3 days in the dark. Next, co-cultures were transferred to ½ MS medium supplemented with antibiotics, claforan and carbenicillin (500 mg × L^{-1} each), to eliminate *R. rhizogenes*. After 4 weeks of cultivation in the dark, new transformed shoots of *D. muscipula* were excised and placed on fresh MS medium with the same antibiotic concentrations listed above and grown in the dark for 8 weeks. After 7 subcultures, axenic cultures were established from a single shoot of transgenic tissue. Next, transformed clones were subcultured on fresh MS media without antibiotic growth regulators. Transformed clones of *D. muscipula* were propagated for 8 weeks in liquid media, as described by Makowski et al. (2020). During this time, observations of plant morphology, growth rate, and preliminary screening for phenolic compound quantity in comparison to non-transformed plants were performed. Based on these observations, four transformed clones of *D. muscipula* were chosen for further analysis.

Transformed cultures were also tested for the presence of live *R. rhizogenes* found in tissue. Transformed shoots were homogenized and the obtained suspensions were plated on YEB agar medium and grown for 5 days at 26 °C in the dark.

Molecular analysis

To confirm transformation on a molecular level, a PCR reaction for the detection of bacteria T-DNA fragments in the plant genome was performed. To estimate the number of bacterial genes copies in plant genome Southern hybridization was used. Total genomic DNA from transformed and non-transformed *D. muscipula* plants was isolated using the CTAB method by Bekesiova et al. (1999). This method yields high-quality DNA, free from secondary metabolites. As a positive control in PCR, plasmid DNA from bacteria cells was used. A culture of 24-h old *R. rhizogenes* (OD_{600} 4.0) was extracted using alkaline lysis as described by Maniatis et al. (1982). Oligonucleotide primers for the PCR detection of *rol*B (forward primer 5′-GCTCTTGCAGTGCTAGATTT-3′, reverse primer 5′-GAAGGTGCAAGCTACCTCTC-3′), *rol*C (forward primer 5′-CTCCTGACATCAAACTCGTC-3′, reverse primer 5′-TGCTTCGAGTTATGGGTACA-3′), and *vir*G (forward primer 5′-ACTGAATATCAGGCAACGCC-3′, reverse primer 5′-GCGTCAAAGAAATAGCCAGC-3′) were used (Królicka et al. 2010). PCR was performed in three biological replicates for each examined transformed clone and non-transformed plant.

Southern hybridization was performed to evaluate the number of *rol*B and *rol*C gene copies incorporated in the plant genome. The probes for hybridization specific for *rol*B and *rol*C genes were prepared from Ri Plasmid and directly labeled using PCR with biotin-dUTP. Plant genomic DNA was isolated as described in the previous section. Two micrograms DNA was digested overnight at 37 °C with 1 unit of *Bam*HI enzyme (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, the samples were electrophoretically separated overnight on 1.2% 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS)–borate–EDTA agarose gels, depurinated, denatured, and neutralized as described (Nowicka et al. 2020). Blotting was performed for 7 h on Hybond™ N+ nylon membrane (GE Healthcare, Chicago, IL, USA) with 20× SSC, washed in 2× SSC, dried, and crosslinked using UV Stratalinker (Agilent, Santa Clara, CA, USA). Pre-hybridization, overnight hybridization, and post-hybridization washes were performed as described in (Nowicka et al. 2020).

Hybridization points were detected using the Chemiluminescent Nucleic Acid Detection Module Kit (Thermo Fisher Scientific, Waltham, MA, USA). To visualize, resulted signals were used Medical X-Ray Film Blue (Agfa Healthcare, Mortsel, Belgium).

Determination of biometric parameters

To estimate the growth of examined plants, transformed clones and non-transformed plants were harvested and weighed immediately. A growth index (GI) was calculated according to the formula: $GI\ [\%] = (FW_2 - FW_1)/FW_2 \times 100$, where FW_1 was the fresh weight of plants at the beginning of the experiment and FW_2 was a final fresh weight. To determine dry weight (DW) accumulation, plants were freeze-dried for 72 h and weighed. DW content in plant tissue was calculated according to the formula: $DW\ [\%] = DW_2 \times 100/FW_2$, where DW_2 was dry weight after freeze-drying. Freeze-dried plant tissue was homogenized and stored at − 20 °C for further analysis.

Biochemical analysis

Spectrophotometric and HPLC analysis were performed to estimate level of phenolic compounds in transformed and non-transformed plants. Total phenolic content was estimated according to the method by Swain and Hillis (1959) with Folin-Ciocalteu's reagent, after modifications (Tokarz et al. 2018). Freeze-dried tissue (10 mg) was extracted in 80% MeOH at 4 °C. Samples were centrifuged for 15 min (25,155 g, 4 °C). One milliliter of diluted extract was mixed with 0.2 mL Folin's reagent (Sigma-Aldrich Chemie, GmBH, Steinheim, Germany) and 1.6 mL 5% Na_2CO_3 and incubated for 20 min at 40 °C. The absorbance of the mixture was measured at 740 nm using a Double Beam spectrophotometer U-2900 (Hitachi High-Technologies Corporation, Japan). Chlorogenic acid (Sigma-Aldrich Chemie, GmBH, Steinheim, Germany) was used as a reference standard.

Results were expressed as milligram of chlorogenic acid equivalents per 1 g of DW. Analyses were performed in five biological replicates.

To determine the accumulation of phenylpropanoids, flavonoids, and anthocyanins in plant tissue, a method described by Fukumoto and Mazza (2000) was used with modifications (Tokarz et al. 2020). Tissue was extracted as described in the method for total phenolic content estimation. The supernatant (0.25 mL) was mixed with 0.25 mL 0.1% HCl in 96% EtOH and 4.55 mL 2% HCl in H_2O. Test tubes with mixtures were incubated for 20 min in the dark. The absorbance of samples was measured at wavelengths of 320, 360, and 520 nm. Contents of phenylpropanoids, flavonoids, and anthocyanins were calculated using calibration curves made for caffeic acid, quercetin, and cyanidin (Sigma-Aldrich Chemie, GmBH, Steinheim, Germany), respectively. The results were expressed as milligrams of standard equivalents per 1 g of DW. Analyses were performed in five replicates.

To estimate plumbagin content, 10 mg of freeze-dried plant tissue was extracted in 0.6 mL of MQ water and 0.6 mL of tetrahydrofuran (THF) (Tokarz et al. 2018). For analysis of caffeic acid, hyperoside, ellagic acid, salicylic acid, myricetin, and quercetin dry tissue (20 mg) was extracted in 2 mL of 100% methanol and sonicated for 30 min (Makowski et al. 2020). Samples were centrifuged (15 min, 25,155 g, 4 °C) and filtered. Chromatographic separation was performed according to Makowski et al. (2020) using Dionex UltiMate 3000 HPLC system equipped with a quaternary pump, autosampler, column oven, and UV detector. For the stationary phase, Agilent Zorbax SB-Phenyl (4.6 × 150 mm, 3.5 μm) was used. The flow rate was 1 mL × min^{-1}. The sample injection volume was 10 μL. The mobile phase for the analysis consisted of 0.1% (v/v) trifluoroacetic acid in acetonitrile as eluent A and 0.1% (v/v) trifluoroacetic acid in water as eluent B. The separation gradient was 0 min (10% A) → 5 min (10% A) → 12 min (90% A) → 20 min (90% A), followed by 10-min column regeneration. Chromatographic separations were carried out at 25 °C. Compounds present in examined plant tissues (plumbagin, hyperoxide, ellagic acid, myricetin, quercetin, salicylic acid, and caffeic acid) were used as standards to determine extract composition. A three-level standard curve was used for determining the concentration of the compounds 4-point. Monitoring was performed at 254 nm. Each analysis was performed in three biological replicates.

Productivity of phenolic derivatives

The productivity (P) of each phenolic compound was calculated according to the formula: P [mg of phenolic compound/8 weeks/flask] = $A \times B$, where A was the content of the phenolic compound in plant tissue after 8 weeks of growth and B was the fold of weight gain of one tissue culture (one flask).

Antibacterial activity of plant-derived extracts

To evaluate the bactericidal properties of examined plants, minimal inhibition concentrations (MIC) and minimal bactericidal concentrations (MBC) methods were used (Królicka et al. 2009). MIC and MBC were evaluated against antibiotic-resistant, human-pathogenic bacteria: *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 19433, *Pseudomonas aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 25922, obtained from the IFB UG & MUG Poland. The bacteria were cultivated on BHI medium (overnight, 37 °C). Freeze-dried plant tissue (100 mg) was extracted in THF (Makowski et al. 2020). Extracts were evaporated and resuspended in methanol before application into wells of the 96-well plate. To remove toxic methanol, extracts were evaporated in the wells. The residues were suspended in 100 μL of liquid BHI media, and bacterial suspensions (10 μL, 10^5 CFU × mL^{-1}) in liquid media were added to wells. Plates were incubated overnight. The MIC value was defined as the lowest concentration of applied extract that inhibits bacteria growth in the well. To establish the MBC value, 100 μL from each well that showed no visible growth of bacteria were plated out on a BHI agar plate for 24 h of incubation at 37 °C. The MBC was defined as the lowest concentration of the extract that reduced the inoculum by 99.9% within 24 h.

Statistical analyses

One-way analysis of variance (ANOVA) was used to determine significant differences between means (Tukey test at $p < 0.05$ level). STATISTICA 12.0 (StatSoft Inc., Tulsa, OK, USA) was used to carry out statistical analyses.

Results

Obtaining transformed plants after inoculation with bacteria and molecular analysis

D. muscipula tissue was inoculated with three different strains of wild-type *R. rhizogenes*: LBA 9402, ATCC 15834, and A4. Only inoculation with LBA 9402 and ATCC 15834 resulted in the appearance of presumably transformed shoots (teratomas). Hairy root production was not observed in teratomas tissue culture. Teratomas appearing efficiency was 14% and 16% for *R. rhizogenes* LBA 9402 and ATCC 15834, respectively. Based on preliminary results from growth observations and screening for phenolic compound accumulation in teratomas tissue compared to non-transformed plants (NT plants), four transformed clones of the Venus flytrap were taken for further analysis: clones P, K, L, and E. Clones P and K were obtained after inoculation with *R. rhizogenes* LBA 9402, while L and E with ATCC 15834.

For molecular analysis of the incorporation of bacterial T-DNA in the plant genome, PCR detection of *rol*B and *rol*C genes was performed. Presence of the *rol*B gene was confirmed for each putatively transformed *D. muscipula* clone (P, K, L, and E), in each of three biological repetitions (samples from three independent tissue cultures), while the *rol*B gene was not detected in NT plants (Fig. 1). The presence of the *rol*C gene in transformed clones was not detected. For confirmation of *R. rhizogenes* elimination from tissue cultures of transformed plants, PCR for the *vir*G gene was performed. This gene was present in the Ri plasmid but beyond the transferred T-DNA and was not incorporated into the plant genome. *Vir*G was not found in transformed tissue cultures.

To estimate the number of *rol*B and *rol*C gene copies incorporated to the *D. muscipula* genome, Southern hybridization was performed. The obtained signals showed that each transformed clone of the Venus flytrap had a single copy of the searched *rol*B gene, visible as a single band in lanes 2, 3, 4, and 5 (Fig. 2), whereas the signals for *rol*C gene were not detected.

Growth index, dry weight accumulation, and morphology of transformed clones

After 8 weeks of cultivation of transformed clones and NT plants (control) in liquid media with rotary shaking, GI, DW content, and plant morphology were evaluated. Estimation of growth parameters showed that in comparison to NT plants, GI of clone K was significantly decreased by 29% (Fig. 3a) with simultaneously increased accumulation of DW by 15% (Fig. 3b). Clone L was characterized by 1.2-fold higher GI compared to NT plants (Fig. 3a) and changed morphology, including longer teratomas with bigger leave-traps (Fig. 3c).

Total phenolic, phenylpropanoid, flavonoid, and anthocyanin content in transformed clones

Next, we examined the synthesis of phenolic compounds in transformed clones of the Venus flytrap. Using spectrophotometric methods, total phenolic content and accumulation of selected groups of phenolic derivatives were evaluated. Transgenic *D. muscipula* plants had significantly increased accumulation of total phenolic content, phenylpropanoids, flavonoids, and anthocyanins in clone P, L, and E compared to NT plants (Fig. 4). Interestingly, clone K synthesized significantly less phenolic compounds

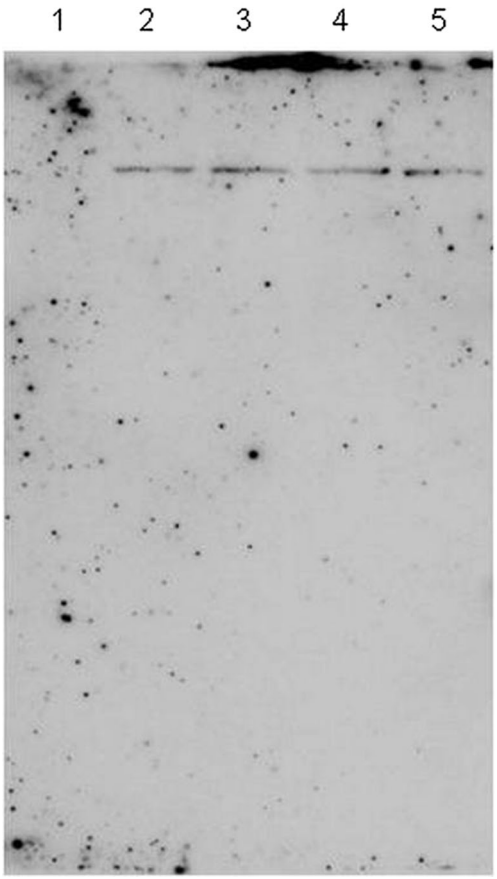

Fig. 2 Southern hybridization with the *rol*B probe: non-transformed tissue of *Dionaea muscipula* (lane 1) and transformed clones of *Dionaea muscipula*: clone P (lane 2), clone K (lane 3), clone L (lane 4), and clone E (lane 5). The number of bands in each lane show the number of *rol*B gene copies

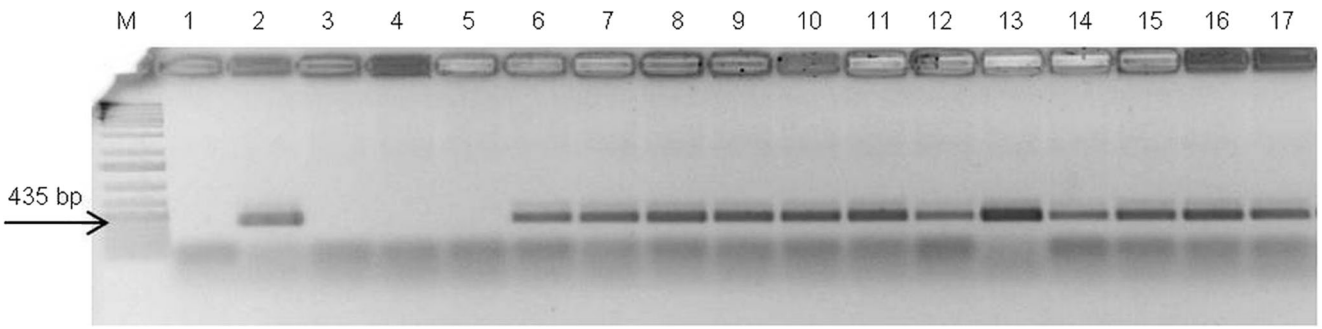

Fig. 1 PCR analysis of non-transformed tissue of *Dionaea muscipula* (lanes 3–5) and transformed clones of *Dionaea muscipula*: clone P (lanes 6–8), clone K (lanes 9–11), clone L (lanes 12–14), and clone E (lanes 15–17). Lane 1: negative control, lane 2: positive control (*Rhizobium rhizogenes* ATCC 15834 plasmidic DNA). GeneRuler™ 100 bp DNA ladder (lane M). Bands show amplified fragments of the *rol*B gene

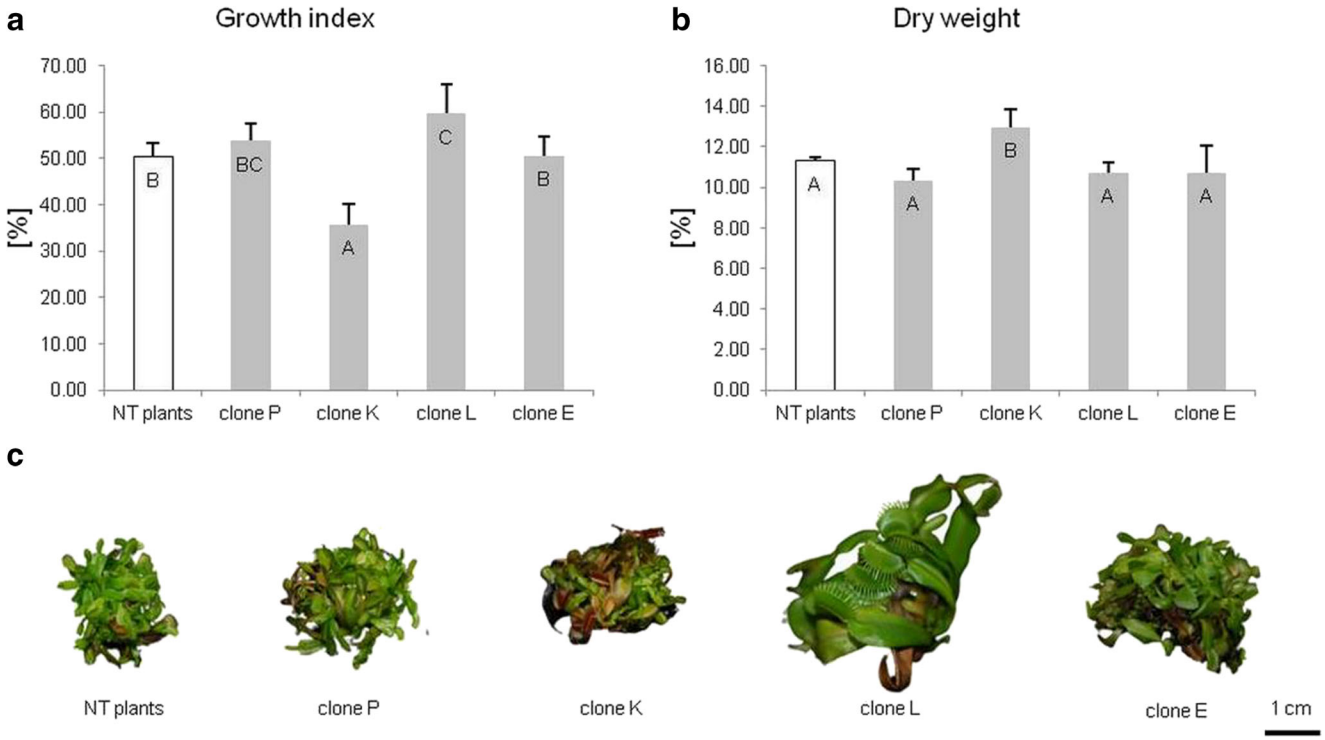

Fig. 3 **a** Growth index (%), **b** dry weight (%), and **c** morphology of non-transformed and transformed tissue of *Dionaea muscipula* clones

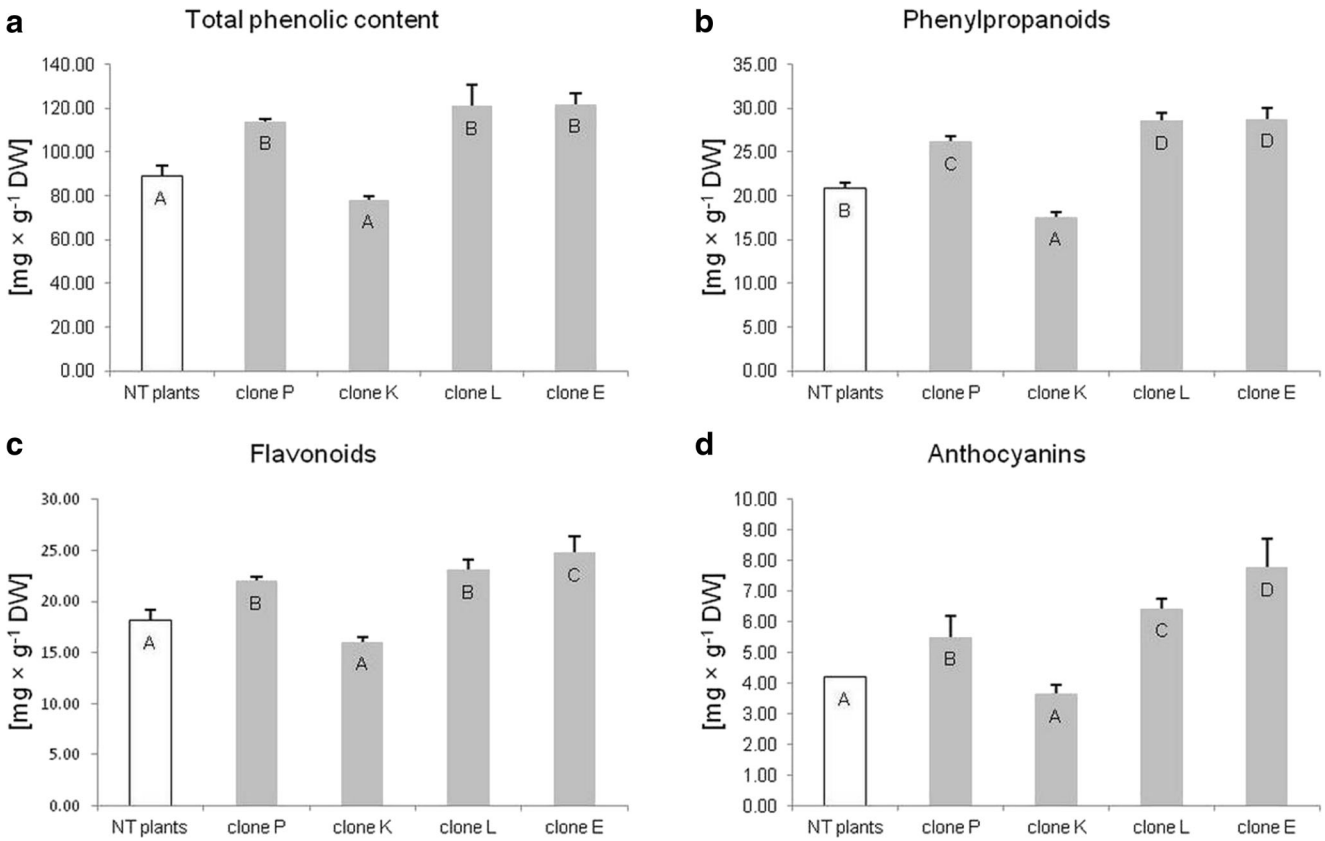

Fig. 4 Accumulation of phenolic compounds in non-transformed and transformed tissue of *Dionaea muscipula* clones: **a** total phenolic content, **b** phenylpropanoids, **c** flavonoids, and **d** anthocyanins. Different letters indicate significant differences between means at $p < 0.05$; bar: standard deviation

than control plants. Clone L was transformed with *R. rhizogenes* ATCC 15834 and accumulated significantly more phenylpropanoids and anthocyanins than those transformed with *R. rhizogenes* LBA 9402 (Fig. 4b and d).

Accumulation and productivity of phenolic compound derivatives in transgenic clones

To study how the transformation of *D. muscipula* with wild strains of *R. rhizogenes* bacteria affected the production of selected phenolic compounds, HPLC was used. In comparison to NT plants, phenolic compound metabolism in transformed clones was significantly affected (Table 1). Clones P, L, and E accumulated significantly increased amounts of plumbagin, salicylic acid, and ellagic acid, while among them, clone E reached the highest level of these metabolites (1.5-, 1.9-, and 1.8-fold higher than NT plants, respectively). The level of caffeic acid was increased in clones L and E by 68 and 42%, respectively. Accumulation of hyperoside was significantly decreased in all transformed clones compared to NT plants (Table 1).

The productivity of phenolic compounds in obtained transgenic clones was calculated. Presented results showed that clone L was characterized by the best productivity of plumbagin, myricetin, caffeic acid, and ellagic acid, which increased 1.7-, 1.2-, 2.0-, and 2.0-fold, respectively. Clones P, L, and E had increased productivity of plumbagin, salicylic acid, and ellagic acid in comparison to NT plants, respectively. Decreased productivity of hyperoside was observed in all examined plants when compared to the control. Moreover, clone K was characterized by decreased productivity of myricetin, quercetin, and salicylic acid (Table 2).

Bactericidal properties of extracts derived from transformed clones

This study focused on the synthesis of phenolic compounds in the transformed tissue of *D. muscipula* and their bactericidal properties. To estimate the antibacterial potential of transformed tissues, MIC and MBC were examined with four antibiotic-resistant, human-pathogenic bacteria. Extracts from clones P, L, and E had decreased MIC values for all tested pathogens. Similar to MBC, in the case of *P. aeruginosa*, only clones L and E were characterized by increased bactericidal properties. Antibacterial activity of clones P, L, and E against *S. aureus*, *E. faecalis*, and *E. coli* increased 33% compared to NT plants. Extracts from clones L and E had decreased MBC value against *P. aeruginosa* (7%) (Table 3).

Discussion

To the best of our knowledge, five articles about genetic transformation of carnivorous plants are available (Hirsikorpi et al. 2002; Królicka et al. 2010; Blehova et al. 2015; Miguel et al. 2019; Oropeza-Aburto et al. 2020), while only one is about transformation with wild strains of *R. rhizogenes* bacteria (Królicka et al. 2010). Hirsikorpi et al. (2002) and Oropeza-Aburto et al. (2020) used *Rhizobium tumefaciens* (former: *Agrobacterium tumefaciens*) as a vector organism, while in the work of Blehova et al. (2015), *R. rhizogenes* with *green fluorescent protein* gene served for *Drosera rotundifolia* L. transformation. Furthermore, Miguel et al. (2019) explored virus-based plant transformation to create transgenic sundew and pitcher plants, for the overproduction of recombinant proteins. In this study, for the first time, the successful transformation of *D. muscipula* is presented.

It was shown by Franklin et al. (2008) on the *Hypericum perforatum* L. model that the most limiting factor in successful genetic transformation of plants using *Rhizobium* bacteria is plant's recalcitrance. When plant cells are challenged with *Rhizobium,* the stress-induced defense response appears, based on induction of stress-involved gene expression patterns and up-regulation of enzymatic protein activity in the phenylpropanoid pathway, leading to increased synthesis of secondary metabolites (Franklin et al. 2009; Tusevski et al. 2019). Being a rich source of phenolic compounds with strong bactericidal properties, carnivorous plants from the family Droseraceae are hard to transform (Blehova et al. 2015), while transformation of such organisms gives the possibility to

Table 1 Accumulation of phenolic derivatives in non-transformed and transformed tissue of *Dionaea muscipula* clones. Different letters indicate significant differences between means at $p < 0.05$, SD - standard deviation

Phenolic derivatives		NT plants	Clone P	Clone K	Clone L	Clone E
Plumbagine	mg × g^{-1} DW	$69.57^{A} \pm 4.15$	$92.40^{B} \pm 4.41$	$74.78^{A} \pm 8.85$	$101.35^{BC} \pm 1.79$	$106.63^{C} \pm 3.47$
Hyperoside		$4.47^{B} \pm 0.68$	$2.06^{A} \pm 0.28$	$2.52^{A} \pm 0.15$	$2.04^{A} \pm 0.36$	$2.33^{A} \pm 0.45$
Myricetin		$3.17^{A} \pm 0.25$	$3.48^{A} \pm 0.05$	$3.37^{A} \pm 0.26$	$3.21^{A} \pm 0.20$	$3.67^{A} \pm 0.13$
Quercetin		$2.21^{AB} \pm 0.13$	$2.48^{B} \pm 0.02$	$1.64^{A} \pm 0.48$	$2.22^{AB} \pm 0.13$	$2.59^{B} \pm 0.08$
Caffeic acid		$1.16^{A} \pm 0.05$	$1.47^{AB} \pm 0.15$	$1.34^{AB} \pm 0.15$	$1.96^{C} \pm 0.24$	$1.65^{BC} \pm 0.11$
Salicylic acid		$340.83^{B} \pm 22.24$	$600.66^{D} \pm 12.02$	$281.04^{A} \pm 15.02$	$540.47^{C} \pm 27.10$	$649.93^{D} \pm 25.67$
Ellagic acid		$8.99^{A} \pm 0.39$	$14.43^{B} \pm 0.21$	$9.17^{A} \pm 0.86$	$15.06^{B} \pm 1.42$	$16.51^{B} \pm 0.64$

Table 2 The productivity of phenolic derivatives in non-transformed and transformed tissue of *Dionaea muscipula* clones. Different letters indicate significant differences between means at $p < 0.05$, *SD* standard deviation

Phenolic compounds		NT plants	Clone P	Clone K	Clone L	Clone E
Plumbagine	mg×8 weeks^{-1}×flask^{-1}±SD	142.5^{A}±8.51	194.0^{B}±9.27	118.9^{A}±14.08	248.0^{C}±4.37	216.9^{B}±3.16
Hyperoside		9.2^{B}±1.40	4.3^{A}±0.58	4.0^{A}±0.24	5.0^{A}±0.88	4.7^{A}±0.92
Myricetin		6.5^{B}±0.50	7.3^{BC}±0.11	5.4^{A}±0.41	7.9^{C}±0.49	7.5^{BC}±0.26
Quercetin		4.5^{B}±0.27	5.2^{B}±0.04	2.6^{A}±0.76	5.4^{B}±0.32	5.3^{B}±0.17
Caffeic acid		2.4^{AB}±0.10	3.1^{BC}±0.32	2.1^{A}±0.24	4.8^{D}±0.58	3.4^{C}±0.23
Salicylic acid		697.9^{B}±45.55	1261.2^{C}±25.24	447.1^{A}±23.90	1322.3^{C}±52.22	1322.3^{C}±52.22
Ellagic acid		18.4^{A}±0.80	30.3^{B}±0.45	14.6^{A}±1.36	36.9^{C}±3.48	33.6^{BC}±1.31

study pathways involved in the synthesis of valuable secondary metabolites and/or overproducing phytochemicals of interest (Gandhi et al. 2015). Moreover, effective transformation protocols for the Venus flytrap seem to be important tools in the field of physiological, ecological, and evolutionary research (Blehova et al. 2015).

During *Rhizobium*-mediated transformation, there are a few factors that affect the effectiveness of gene transfer from bacteria to the plant genome. One of them is the explant type (Alok et al. 2016; Hou et al. 2016). In our study, we chose rhizomes of the Venus flytrap as explants for transformation due to a lower concentration of secondary metabolites than that in leaves. The other crucial factor is the selection of the *Rhizobium* strain. Królicka et al. (2010) showed that during the transformation of *Drosera capensis* var. *alba, R. rhizogenes* ATCC 15834 strain was effective, while LBA 9402 and A4 were not conducive to transformation. Secondary metabolites contained in the leaves of medicinal plants can inhibit the growth of bacteria and decrease transformation efficiency (Królicka et al. 2010; Blehova et al. 2015). Additionally, Thiruvengadam et al. (2014a) observed differences in various *R. rhizogenes* strain effectiveness in the establishment of hairy root cultures of *Momordica charantia*. Wang et al. (2006) showed that induction of hairy roots in *Echinacea purpurea* was possible with *R. rhizogenes* A4, R1601, and R1000 strains but the performance of each strain was dependent on the type of plant explant. In contrast, the transformation rate of *Origanum vulgare* was similar for strains 15,834 and K599, while the type of medium affected hairy root appearance (Habibi et al. 2016). In our study, successful transformation of *D. muscipula* was possible with *R. rhizogenes* LBA 9402 and ATCC 15834, while the A4 strain did not caused T-DNA incorporation into plant genomic DNA. This can be the evidence that LBA 9402 and ATCC 15834 strains are less sensitive to secondary metabolites accumulated in Venus flytrap tissue, which are synthesized as defense compounds.

The presented results demonstrate that independent of the bacterial strain, obtained transformed clones of *D. muscipula* are characterized by the presence of only the *rol*B gene in plant DNA. Moreover, it was found that each clone (P, K, L, and E) had a single copy of the *rol*B gene combined in the plant genome. This result is in agreement with the findings of Królicka et al. (2010), where teratomas of transformed sundew had a single copy of the *rol*B gene. Similarly, in the research of Gangopadhyay et al. (2011), after the transformation of the medicinal plant *Plumbago indica*, obtained hairy root clones were characterized by a single copy of the *rol*B gene, which was confirmed with Southern hybridization. The type and copy number of *rol* genes (A, B, C, or D) transferred from bacteria to the plant genome during T-DNA delivery and combining, takes place accidentally, despite having a direct impact on transformed organism morphology and physiology (Ghimire et al. 2019; Ansari et al. 2019). Transforming a plant with *R. rhizogenes* usually results in hairy root culture creation but sometimes transformed cells can directly regenerate into whole plants (Blehova et al. 2015). *Rol* genes (the root loci) are

Table 3 Minimal inhibition concentration (MIC) and minimal bactericidal concentration (MBC) of *S. aureus*, *E. faecalis*, *E. coli*, and *P. aeruginosa* after treatment with extracts from non-transformed and transformed tissue of *Dionaea muscipula* clones

	Staphylococcus aureus ATCC 25923		*Enterococcus faecalis* ATCC 19433		*Escherichia coli* ATCC 25922		*Pseudomonas aeruginosa* ATCC 27853	
	μg DW×mL^{-1}							
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
NT plants	167	500	667	1250	500	1250	1250	1250
Clone P	83	333	500	833	417	833	1000	1250
Clone K	167	500	833	1250	500	1250	1250	1250
Clone L	83	333	500	833	417	833	1000	1166
Clone E	83	333	500	833	417	833	1000	1166

essential for hairy root creation, while the phenotype of transformed plants depends on the number of *rol* genes incorporated in plant DNA and their expression pattern (Vinterhalter et al. 2015). High expression of the *rol* gene family particularly stimulates hairy root creation and elongation (Tusevski et al. 2019). The transformation of *D. muscipula* did not affect hairy root formation. These findings can result from the incorporation of only a single copy of the *rol*B gene in each clone (P, K, L, and E) and can be evidence for low expression patterns of the *rol*B gene in *D. muscipula*. Conversely, the *rol*B gene can be a suppressor of cell division and growth (Bulgakov 2008). Królicka et al. (2010) and Blehova et al. (2015) also observed direct organogenesis into whole plants (creation of teratomas) after the transformation of plants from the family Droseraceae with *R. rhizogenes.* Habibi et al. (2016) showed that the hairy root culture of the medicinal plant *O. vulgare* could regenerate into whole plants with a callus phase intervening between them. Transformed cells differentiated into whole plants can conduct changes in leaf and shoot morphology, which is called "hairy root syndrome". Clone L was obtained after *D. muscipula* transformation and characterized by longer leaves and bigger leaf-traps than NT plants and other transformed clones, which could be the consequence of transformation.

Plant transformation with wild strains of the *R. rhizogenes* bacteria is a valuable biotechnology tool, allowing the creation of fast-growing, genetically stable organisms, with a high content of secondary metabolites (Gandhi et al. 2015). Since fast biomass accumulation in plant tissue cultures is one of the most important features in the industry field, research has been conducted to determine the growth parameters of hairy root cultures obtained after *R. rhizogenes* inoculation (Georgiev et al. 2007). Tusevski et al. (2019) showed great variation in the growth of *H. perforatum* hairy roots, although each clone was obtained using the same bacterial strain. Similar variability of growth parameters was observed by Tusevski et al. (2017). Additionally, hairy root lines of *Rehmannia elata* showed large differences in fresh weight growth rate and DW accumulation (Piątczak et al. 2019). *Polygonum multiflorum* Thunb. and *M. charantia* hairy roots reached nearly a 10-fold increase in the growth of fresh weight after 20 days (Thiruvengadam et al. 2014a, b). Binoy et al. (2016) reported a 12-fold increase in *Plumbago rosea* hairy roots. Nevertheless, little is known about the growth of teratomas. Królicka et al. (2010) demonstrated that teratomas of *D. capensis* had a three times higher growth index than non-transformed plants. In this study, for the first time, growth parameters of transformed *D. muscipula* plants were evaluated. The obtained results showed that clone L had increased GI in comparison to NT plants, while clone K accumulated significantly less biomass than control plants, with simultaneously increased DW content. In our study, *D. muscipula* transformed clones' growth characteristics had no connection with the bacteria strain. Differences between the obtained clones using the same bacteria strain (LBA 9402 or ATCC 15834) may have resulted from the fact that each clone originated from different transformation events. Heterogeneity among transgenic clones could depend on physiological conditions of the transformed organism, expression of *rol* genes, and copy numbers of genes from bacterial T-DNA inserted in the plant genome (Tusevski et al. 2019).

Franklin et al. (2009) studied the basis of plant recalcitrance under co-cultivation with *Rhizobium* bacteria. Inoculation of plant tissue conducted for the up-regulation of gene expression involved in defense response consequently led to increased secondary metabolism and affected accumulation of phytochemicals (Hou et al. 2016). In contrast, little is known about the metabolism of secondary compounds after the incorporation of bacterial T-DNA in the plant genome. Tusevski et al. (2019) reported that hairy root culture had the same, or an even greater, ability to produce valuable metabolites. It is postulated that the *rol* genes family can act as an endogenous elicitor and conduct changes in the phytochemical profile of medicinal plants (Tusevski et al. 2019). From the industrial point of view, modified organisms can be a low cost, environmentally friendly source of valuable chemicals (Gandhi et al. 2015), while the productivity of secondary compounds can be greatly improved (Królicka et al. 2010). Increased production of total phenolic content, total flavonoids, myricetin, quercetin, caffeic acid, and salicylic acid was shown to be a consequence of the up-regulation of the *rol*C gene in hairy roots of *Ligularia fischeri* (Ansari et al. 2019). Hairy roots of *H. perforatum* showed greater activity of enzymes involved in the phenylpropanoid pathway and in consequence, accumulated significantly more total phenolic and flavonoid content in comparison to non-transformed roots (Tusevski et al. 2017). Ghimire et al. (2019) reported the increased synthesis of total phenolic and flavonoid content with simultaneously higher production of selected phenolic derivatives in hairy root cultures of *Aster scaber* and postulated the vital role of *rol* genes in the greater synthesis of these compounds. These results could be based on the phenomenon of turning on the transcription of defense genes by the *rol* genes family (Thiruvengadam et al. 2014b). Increased synthesis of phenolic compounds in hairy root cultures compared to non-transformed plants were also reported (Thiruvengadam et al. 2014a). Transformation of *D. muscipula* plants also affected the metabolism of phenolic compounds in obtained teratomas, although only the *rol*B gene was confirmed to be incorporated in the plant genome. Three of the four selected clones (P, L, and E) had significantly higher total phenolic, phenylpropanoid, flavonoid, and anthocyanins content, than NT plants. It may be connected with the fact that the *rol*B gene was proved as the strongest inducer of secondary metabolites in the *rol* gene family (Bulgakov 2008).

Additionally, analysis of the use of HPLC showed great variation in phenolic derivatives accumulation in transformed tissues of the Venus Flytrap. The highest plumbagin

accumulation was observed in clone E after a 1.5-fold increase of this metabolite. Conversely, clone L was characterized by the greatest plumbagin productivity (1.7-fold increase compared to NT plants). Our observations agree with the results of Królicka et al. (2010), where teratomas of *D. capensis* had significantly increased accumulation and productivity of ramentaceone (1,4-naphtoquinone derivative) in comparison to non-transformed plants. Moreover, teratomas of *D. muscipula* accumulated over 20 times more plumbagin (106.63 mg × g^{-1} DW) than hairy roots of *P. indica* (4.9 mg × g^{-1} DW), as reported by Gangopadhyay et al. (2011). Furthermore, both accumulation and productivity of phenolic acids, like caffeic, salicylic, and ellagic acid, were increased as a consequence of transformation in some clones of *D. muscipula* created during the transformation process mediated by *R. rhizogenes* bacteria. These findings agree with previous findings (Ghimire et al. 2019; Ansari et al. 2019), where the production of phenolic acids in hairy roots of transformed plants was significantly increased in comparison to NT plants. In contrast to the results of Ansari et al. (2019), in clones of *D. muscipula*, metabolism of myricetin and quercetin was not affected by transformation, while the level of hyperoside in transformed plant tissue was decreased. In contrast to clones P, L, and E, synthesis of phenolic compounds in clone K was the same or decreased, compared to NT plants. Similar observations were reported by Wang et al. (2006) in the culture of hairy roots of *E. purpurea*. The expression pattern of *rol* genes in clone K is likely lower than in other obtained clones (Tusevski et al. 2017).

At the same time, medicinal plant tissue culture gives the possibility for producing valuable secondary metabolites and to quickly screen the biological properties of elite plant genotypes. Due to this, research on the healing properties of plant-derived extracts is available (Niazian 2019). Many studies were conducted to estimate the antimicrobial potential of medicinal plants because of the growing resistance of human pathogenic bacteria to available antibiotics (Krychowiak et al. 2014). In this article, we present for the first time, the bactericidal properties of transformed *D. muscipula* plants against Gram-positive and Gram-negative bacteria strains. It was reported by Krolicka et al. (2008) and Makowski et al. (2020) that extracts from carnivorous plants from the family Droseraceae are highly biologically active thanks to a high content of phenolic compounds, especially 1,4-naphtoquinone derivatives. According to this, biotechnological studies conducted for the improvement of healing properties in carnivorous plants seem to be needed. Ansari et al. (2019) showed that hairy roots of *L. fischeri* had greater antimicrobial activity than non-transformed tissue, against various clinical bacteria strains. Moreover, the bactericidal properties of hairy roots were improved for both Gram-positive and Gram-negative bacteria. More pronounced activity against pathogenic bacteria was also observed in hairy roots of *M. charantia* (Thiruvengadam et al. 2014a), *P. multiflorum* (Thiruvengadam et al. 2014b), and *H. perforatum* (Tusevski et al. 2017). This phenomenon is directly connected with the higher production of biologically active phytochemicals in this type of transformed tissue. In the presented study, we evaluated the antimicrobial potential of extracts derived from transformed clones of the Venus flytrap against two Gram-positive (*S. aureus* and *E. faecalis*) and two Gram-negative (*E. coli* and *P. aeruginosai*) bacteria. Clones P, L, and E showed improved bactericidal properties against *S. aureus*, *E. faecalis*, and *E. coli*, while only extracts from clones L and E had strong activity against *P. aeruginosa*. It may be connected to the increased level of salicylic acid in these clones. Our results agree with data presented by Wen et al. (2019), where *P. aeruginosa* was more resistant to *Orostachys cartilaginous*-derived extracts in comparison to the Gram-positive pathogen *Bacillus subtilis*. Except for plumbagin, which was proven to be biologically active and potent, phenolic acids have a crucial role in the antibacterial activity of medicinal plants. In this study, transformed clones of *D. muscipula* with increased bactericidal properties accumulated significantly more salicylic and ellagic acid. Gomes et al. (2018) showed that among the tested species, *Eucalyptus globules* had the highest antimicrobial activity, rich in ellagic acid glycoside. Clone K did not show improvement in antimicrobial potential, which agrees with results obtained from the estimation of secondary metabolite content. Interestingly, according to the obtained results from MBC tests, the Gram-positive pathogen *E. faecalis* had the same sensitivity to extracts derived from *D. muscipula* tissue as Gram-negative *E. coli.* This observation is in opposition to previous findings by Krolicka et al. (2008) and Makowski et al. (2020). Because Gram-positive bacteria do not have a lipopolysaccharide membrane surrounding the cell wall (Ansari et al. 2019) and permeability of antimicrobial compounds is increased (Tusevski et al. 2015), extracts from medicinal plants have stronger bactericidal properties against such pathogens. Nevertheless, the obtained results indicate that *D. muscipula* transformed clones had great bactericidal activity and can be used as a source of biologically active compounds in the pharmacological field.

Author contributions W.M., A.K., and K.M.T. designed the experiment, interpreted, and discussed the data. W.M. performed the statistical analysis, prepared the graphical part of the manuscript, and wrote the manuscript. W.M., A.K., and B.T. contributed to data acquisition and R.B. developed the analytical method for the determination of phenolic compounds using HPLC. A.K., K.M.T., B.T., A.N., J.Z., and A.P. checked and corrected the manuscript. W.M., AN, and JZ performed Southern hybridizations. A.P. consulted PCR and Southern hybridization experiments. All authors proofread the manuscript, agreed on its contents, and consented to its submission.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interests The authors declare that they have no conflict of interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Publikacja 3

BMC Plant Biology

RESEARCH

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Response of physiological parameters in *Dionaea muscipula* J. Ellis teratomas transformed with *rol*B oncogene

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Abstract

Background: Plant transformation with *rol* oncogenes derived from wild strains of *Rhizobium rhizogenes* is a popular biotechnology tool. Transformation effects depend on the type of *rol* gene, expression level, and the number of gene copies incorporated into the plant's genomic DNA. Although *rol* oncogenes are known as inducers of plant secondary metabolism, little is known about the physiological response of plants subjected to transformation.

Results: In this study, the physiological consequences of *rol*B oncogene incorporation into the DNA of *Dionaea muscipula* J. Ellis was evaluated at the level of primary and secondary metabolism. Examination of the teratoma (transformed shoots) cultures of two different clones (K and L) showed two different strategies for dealing with the presence of the *rol*B gene. Clone K showed an increased ratio of free fatty acids to lipids, superoxide dismutase activity, synthesis of the oxidised form of glutathione, and total pool of glutathione and carotenoids, in comparison to non-transformed plants (control). Clone L was characterised by increased accumulation of malondialdehyde, proline, activity of superoxide dismutase and catalase, total pool of glutathione, ratio of reduced form of glutathione to oxidised form, and accumulation of selected phenolic acids. Moreover, clone L had an enhanced ratio of total triglycerides to lipids and accumulated saccharose, fructose, glucose, and tyrosine.

Conclusions: This study showed that plant transformation with the *rol*B oncogene derived from *R. rhizogenes* induces a pleiotropic effect in plant tissue after transformation. Examination of *D. muscipula* plant in the context of transformation with wild strains of *R. rhizogenes* can be a new source of knowledge about primary and secondary metabolites in transgenic organisms.

Keywords: *Rhizobium rhizogenes*, Primary and secondary metabolism, Venus flytrap, *Rol* genes, Transformation

Background

Plant transformation with wild strains of *Rhizobium rhizogenes* (former: *Agrobacterium rhizogenes*) bacteria has been a popular tool used in biotechnology for decades [1]. This method is based on the natural ability of *R. rhizogenes* to pass a fragment of the Ri (root-inducing) plasmid and incorporate T-DNA (transfer DNA) into host genomic DNA [2]. Such an event leads to the formation of tumours, hairy roots, or teratomas (transformed shoots) because the expression of bacterial genes in the plant genome disturbs auxin and cytokinin synthesis

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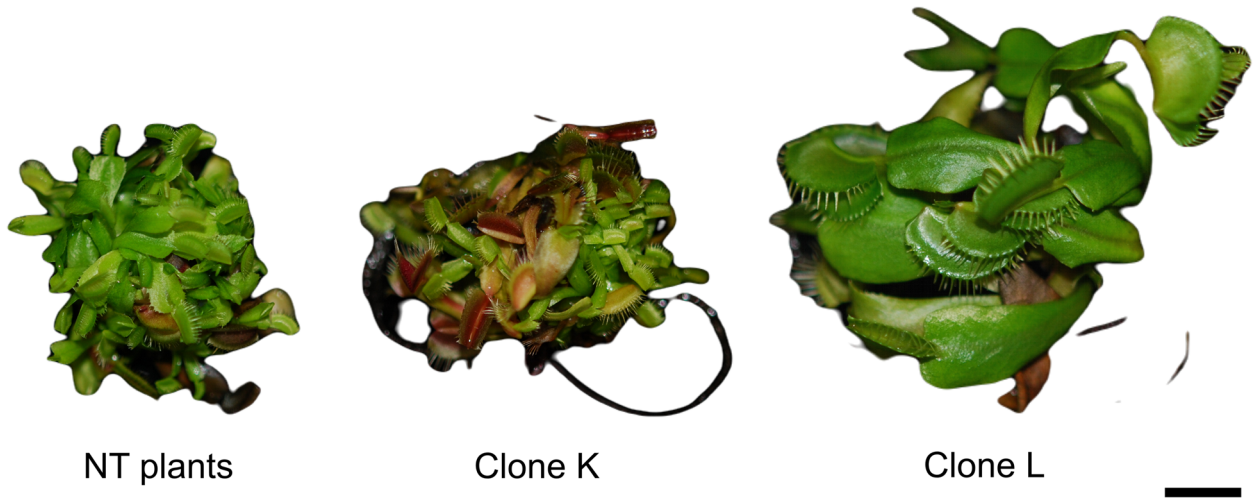

Fig. 1 Non-transformed (NT plants) and transformed clones (clones K and L) of *Dionaea muscipula* plants. Scale bar = 1 cm

pathways, which affects the hormonal balance in plant tissue [3]. Transformed plants are usually characterised by fast growth and long-term genetic and biochemical stability, which makes them a good model in the research and industrial field [2]. In terms of the transformation effects, the most important are oncogenes belonging to the *rol* family (*rol*A, *rol*B, *rol*C, *rol*D), which are part of bacterial T-DNA. Although *rol* genes have been studied for many years, clear knowledge of the complete mechanism of how these genes work is missing [1]. Until now, a few reports have shown possible scenarios of the action of *rol* genes in plant cells [4–8]. However, the whole picture is still missing.

One of the most popular and useful oncogenes in plant transformation is *rol*B [9]. This gene is the most powerful inducer of plant secondary metabolism, which is why *rol*B-transformed plants are models in the medical plant area, as well as in research concentrated on plant secondary metabolism [3]. Expression of the *rol*B oncogene can increase the level of secondary metabolites in plant tissue [10, 11], suppress reactive oxygen species (ROS) production [4], and modulate the antioxidant defence system [8]. Moreover, Veremeichik et al. [12] showed that *rol*B expression regulates the activity of NADPH oxidase, while Bulgakov et al. [5] reported the tyrosine phosphatase activity of the rolB protein. Nevertheless, the complex physiological response of medical plants transformed with the *rol*B oncogene has never been studied. To the best of our knowledge, this article is the first report about the response of carnivorous plants to the expression of the *rol*B gene studied primarily at the level of primary metabolism, and consequently secondary metabolism.

Dionaea muscipula J. Ellis (Venus flytrap), belonging to the Droseraceae family, is an interesting model for research in plant physiology and secondary metabolite production. This unique carnivorous plant is known as a rich source of phenolic compounds, particularly 1,4-naphthoquinone [13]. Because of a huge demand for plant material with a high concentration of phenolic compounds [14], plants from the Droseraceae family have become an important research model in modern plant biotechnology [15]. *D. muscipula* tissue has a strong biologically active potential [16, 17]. Additionally, Makowski et al. [11] reported the first genetic transformation of the Venus flytrap with wild strains of *R. rhizogenes* bacteria.

In the present study, we examined two clones (teratomas) of *D. muscipula* (clones K and L) selected from our previous research. At the molecular level, both plants were transformed with the *rol*B gene, and it was incorporated into plant genomic DNA in a single copy [11]. Independent of the *R. rhizogenes* strain, such transformation types led to the creation of teratoma (transformed shoots) cultures (Fig. 1). Nevertheless, the results of this research showed that plants differed from each other in terms of growth rate, accumulation of dry matter, and phenolic compound synthesis [11]. Therefore, to understand the differences between clones and to define their response to the *rol*B gene, it was necessary to examine some physiological parameters in *D. muscipula* plants.

The main goal of this study was to state the effect of *rol*B gene on the (I) lipid peroxidation level, (II) synthesis of proline, and (III) enzymatic and non-enzymatic antioxidant system activity in transformed clones of the Venus flytrap. Moreover, using Fourier transform infrared spectroscopy (FTIR), the lipid and sugar metabolism

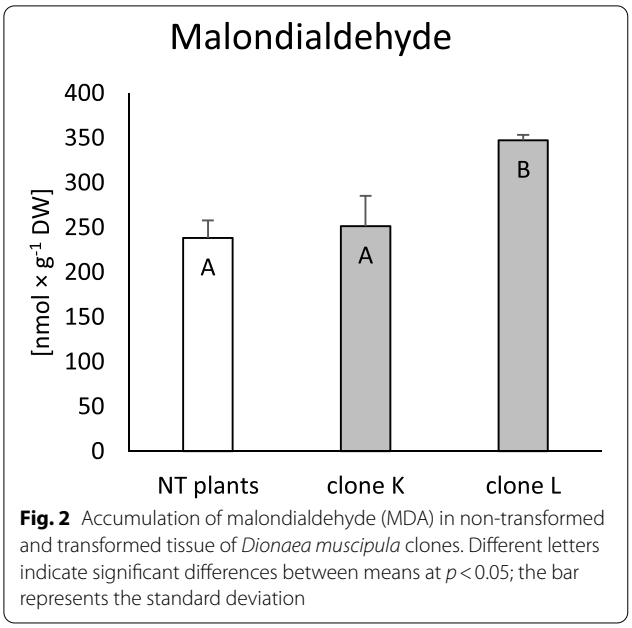

Fig. 2 Accumulation of malondialdehyde (MDA) in non-transformed and transformed tissue of *Dionaea muscipula* clones. Different letters indicate significant differences between means at $p < 0.05$; the bar represents the standard deviation

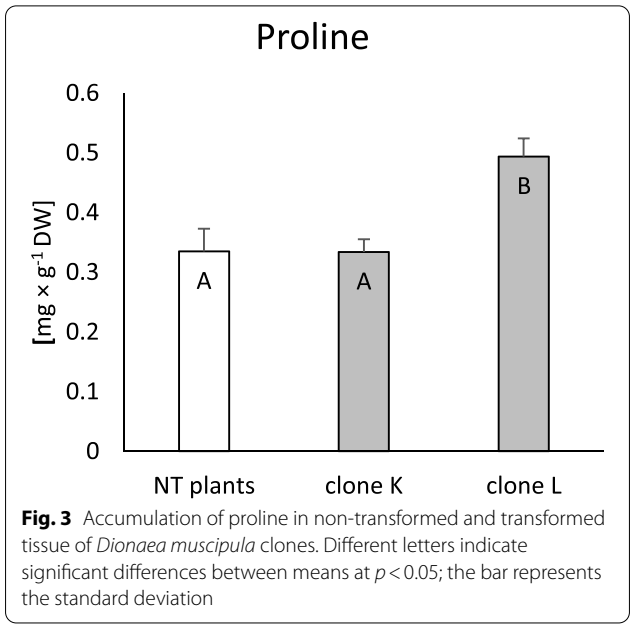

Fig. 3 Accumulation of proline in non-transformed and transformed tissue of *Dionaea muscipula* clones. Different letters indicate significant differences between means at $p < 0.05$; the bar represents the standard deviation

of transformed plants was evaluated for the first time in the context of genetic transformation. We hypothesised that transformation with the *rol*B oncogene would trigger the complex and pleiotropic effect manifested by changes in redox state, as well as primary and secondary metabolism of examined organisms.

Results

Accumulation of malondialdehyde (MDA) and proline

Membrane integrity was evaluated as the accumulation of MDA, a product of lipid oxidation ROS. Compared to non-transformed (NT) plants, only clone L accumulated significantly more MDA (46%), while in clone K, the level of MDA did not change (Fig. 2). Similarly, synthesis of the free amino acid proline only increased in clone L (47%; Fig. 3).

Enzymatic antioxidant system—activity of peroxidase (POD), catalase (CAT), and superoxide dismutase (SOD)

In the presented research, the total activity of three antioxidant enzymes was evaluated. Significantly decreased POD activity was found in transformed clone K tissue, compared to the control. In contrast, CAT was significantly more active (ab. 54%) in clone L. Moreover, the transformation of *D. muscipula* plants led to increased SOD activity in both examined clones in comparison to NT plants (Table 1).

Non-enzymatic antioxidant system

Total pool of glutathione (GSH + GSSG), reduced (GSH) and oxidised (GSSG) forms, and proportions of reduced and oxidised form (GSH/GSSG)

GSH + GSSG increased significantly in both transformed clones compared to NT plants (Fig. 4c), although individual analysis of GSH and GSSG showed that only the GSSG content increased significantly in clone K tissue (33%; Fig. 4a,b). The ratio between the reduced and oxidised forms of glutathione, calculated as the stress indicator, increased significantly in clone L compared to control plants (Fig. 4d).

Carotenoid content

One of the important elements of the non-enzymatic antioxidant system in plants is carotenoids. In the

Table 1 Activity of antioxidant enzymes: peroxidase (POD), catalase (CAT) and superoxide dismutase (SOD) in non-transformed and transformed tissue of *Dionaea muscipula* clones. Different letters in columns – significant differences between means at $p < 0.05$, SD – standard deviation

	POD [$U \times g^{-1}$ DW ± SD]	CAT [µmol $H_2O_2 \times min^{-1} \times g^{-1}$ DW ± SD]	SOD [$U \times g^{-1}$ DW ± SD]
NT plants	$24.32^{B} \pm 3.00$	$527.35^{A} \pm 151.01$	$111.22^{A} \pm 10.25$
clone K	$20.00^{A} \pm 0.83$	$428.76^{A} \pm 88.81$	$547.50^{B} \pm 78.21$
clone L	$21.85^{AB} \pm 0.61$	$811.56^{B} \pm 99.02$	$554.62^{B} \pm 24.19$

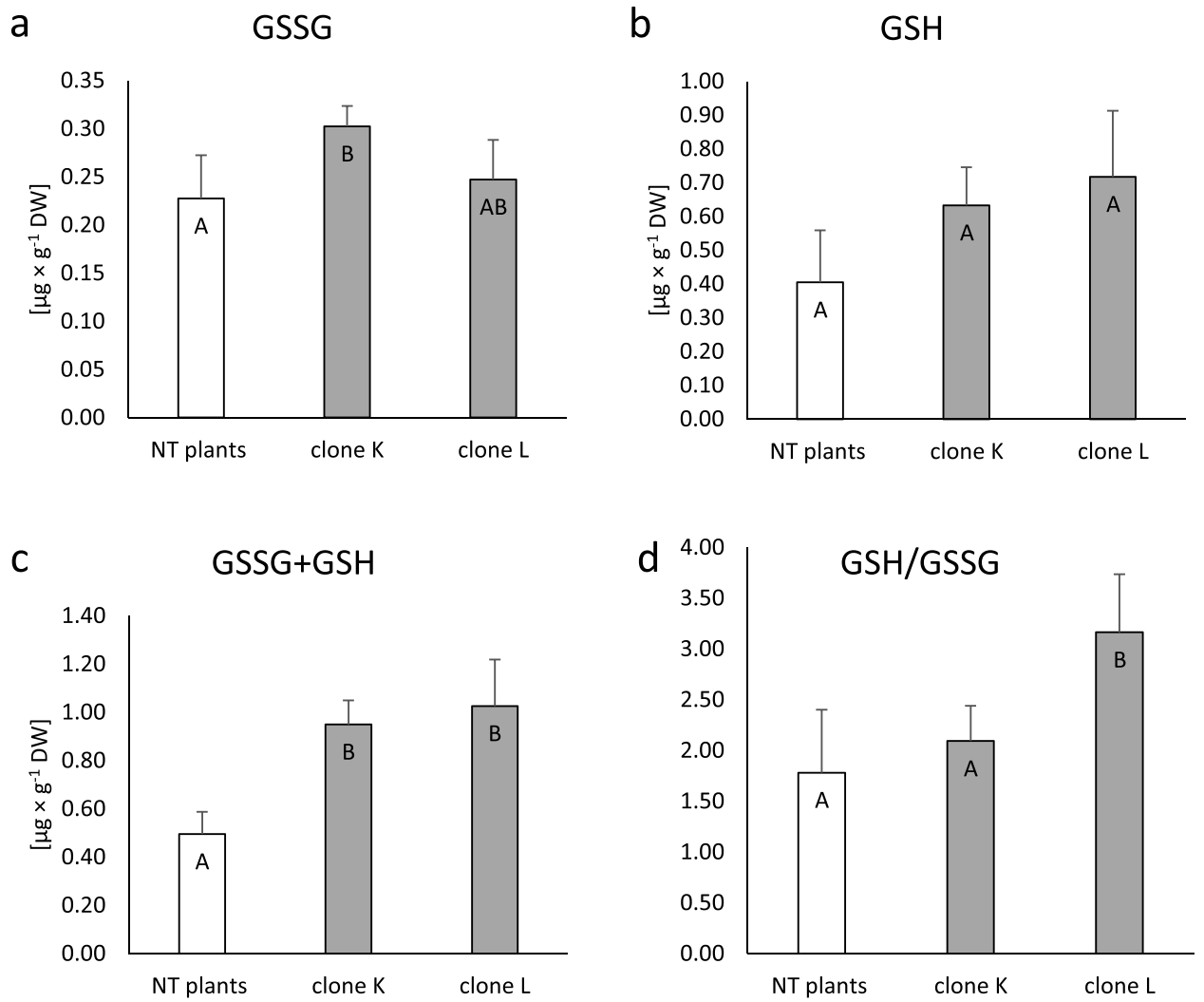

Fig. 4 Accumulation of glutathione in non-transformed and transformed tissue of *Dionaea muscipula* clones. **a** Oxidised glutathione (GSSG). **b** Reduced glutathione (GSH). **c** Total glutathione (GSH + GSSG). **d** Glutathione ratio (GSH/GSSG). Different letters indicate significant differences between means at $p < 0.05$; the bar represents the standard deviation

present study, estimation of these pigments showed that both transformed clones of the Venus flytrap synthesised significantly more carotenoids than NT plants. Moreover, clone K had a significantly higher carotenoid content (26%) than clone L (Fig. 5).

Phenolic compound accumulation

Transformation of *D. muscipula* plants significantly affected phenolic compound synthesis. The HPLC-DAD method was used to determine the content of five phenolic acids—chlorogenic acid, p-coumaric acid, ferulic acid, gallic acid, and protocatechuic acid—and one flavonoid—kaempferol—in extracts from transformed and non-transformed plants (Table 2).

The differences were found in the amounts of individual estimated compounds among NT plants and both clones. In NT plants and clone K, the major phenolic compound was kaempferol, except that its accumulation was twice as high in clone tissues (Table 2). In clone L tissues, the main phenolic compounds were gallic acid and ferulic acid (Table 2). In turn, in all examined plants, the minor phenol was p-coumaric acid, although its content differed significantly among tested plants (Table 2). Furthermore, accumulation of protocatechuic acid decreased in both clones in comparison to NT plants, and accumulation of chlorogenic acid increased only in clone L tissue (Table 2).

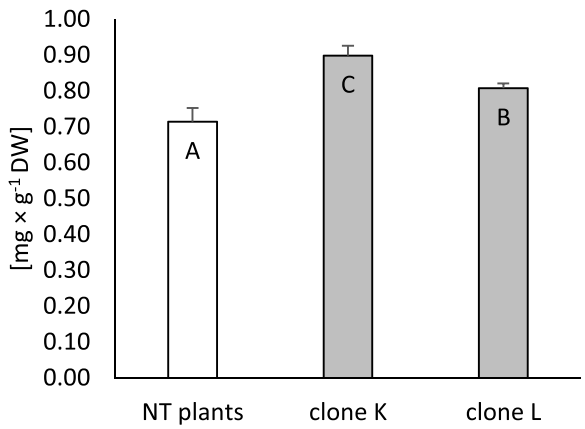

Fig. 5 Accumulation of carotenoids in non-transformed and transformed tissue of *Dionaea muscipula* clones. Different letters indicate significant differences between means at $p < 0.05$; the bar represents the standard deviation

Effect of plant transformation on lipids, sugars, phenolics, and tyrosine

Spectral lipidomics indicated different changes in the total lipid content (decreased in clone K, increased in clone L) and in the contribution of triacyclglycerols and free fatty acids to the lipidic composition (i.e., increased concentrations were determined for clone L and K; Fig. 6a, b, c). Numerous bands below 1200 cm^{-1} originated from sugars, and the spectral indicators of soluble and insoluble carbohydrates were selected based on reference FTIR spectra [18–20]. The soluble mono- (fructose and glucose) and disaccharides (saccharose) were overproduced only in clone L, in addition to the decomposition of insoluble starch. The control and clone K contained a similar content of all sugars (Fig. 6d, e, f). All absorbances assigned to the ring modes in the phenyl moieties indicated a significant increase of the phenolic compounds in clone L (showed for the 1611 cm^{-1} band only, Fig. 6g). In turn, the intensity of the band attributed to the tyrosine ring vibrations dropped down in the spectrum of clone K and increased for clone L compared to the non-transformed plants (Fig. 6h).

Discussion

D. muscipula is a rich source of phenolic compounds, e.g., flavonoids, phenolic acids, and 1,4-naphthoquinone. That is why this plant can be a suitable model for research on plant secondary metabolism [16]. For this reason, medical plant biotechnology uses genetic transformation with *R. rhizogenes* [2]. The *rol* gene family, derived from bacterial T-DNA and transferred to the plant genome, can act as an endogenous elicitor of secondary metabolites, allowing study of the production of valuable plant-derived chemicals in transformed organisms with stable phenotypes and genotypes [3]. Moreover, Veremeichik et al. [21] showed that the effect of *rol* gene action on plant metabolism is long-lasting and repeatable. However, little is known about the physiological response of plants to *rol* oncogene action.

In our previous study, transformation of the Venus flytrap using wild strains of *R. rhizogenes* led to the incorporation of a single copy of the *rol*B gene into plant DNA [11]. Such transformation occurs due to changes in the plant's growth rate, accumulation of DW, and synthesis of phenolic compounds. Clone K was characterised by a decreased growth rate with simultaneous increased DW content, while clone L grew significantly faster than clone K and non-transformed plants. Additionally, clone L synthesised an increased amount of various phenolic compounds, which positively affected the antibacterial properties of this clone [11].

The subject of this study was to define how the *rol*B gene affects *D. muscipula* physiology at the level of oxidative stress response, primary and secondary metabolism, in two clones, K and L. Tusevki et al. [8] hypothesised that transformation of *Hypericum perforatum* L. plants with wild strains of *R. rhizogenes* modifies cell redox status and consequently leads to an oxidative stress response. Furthermore, it was demonstrated that the *rol*B oncogene can suppress

Table 2 Accumulation of phenolic compounds in non-transformed and transformed tissue of *Dionaea muscipula* clones. Different letters in lines – significant differences between means at $p < 0.05$, SD – standard deviation

Phenolic compound [mg × 100 g^{-1} DW ± SD]	NT plants	clone K	clone L
gallic acid	$31.14^{A} \pm 0.48$	$28.89^{A} \pm 1.58$	$53.73^{B} \pm 0.63$
protocatechic acid	$29.12^{B} \pm 0.63$	$26.18^{A} \pm 2.70$	$24.20^{A} \pm 0.57$
chlorogenic acid	$20.60^{A} \pm 0.26$	$18.83^{A} \pm 2.54$	$28.02^{B} \pm 0.48$
p-coumaric acid	$4.19^{B} \pm 1.53$	$1.09^{A} \pm 0.14$	$8.42^{C} \pm 0.28$
ferulic acid	$16.07^{A} \pm 1.32$	$39.05^{B} \pm 0.87$	$61.49^{C} \pm 13.39$
kaempferol	$58.56^{B} \pm 1.65$	$127.04^{C} \pm 2.83$	$18.30^{A} \pm 0.37$

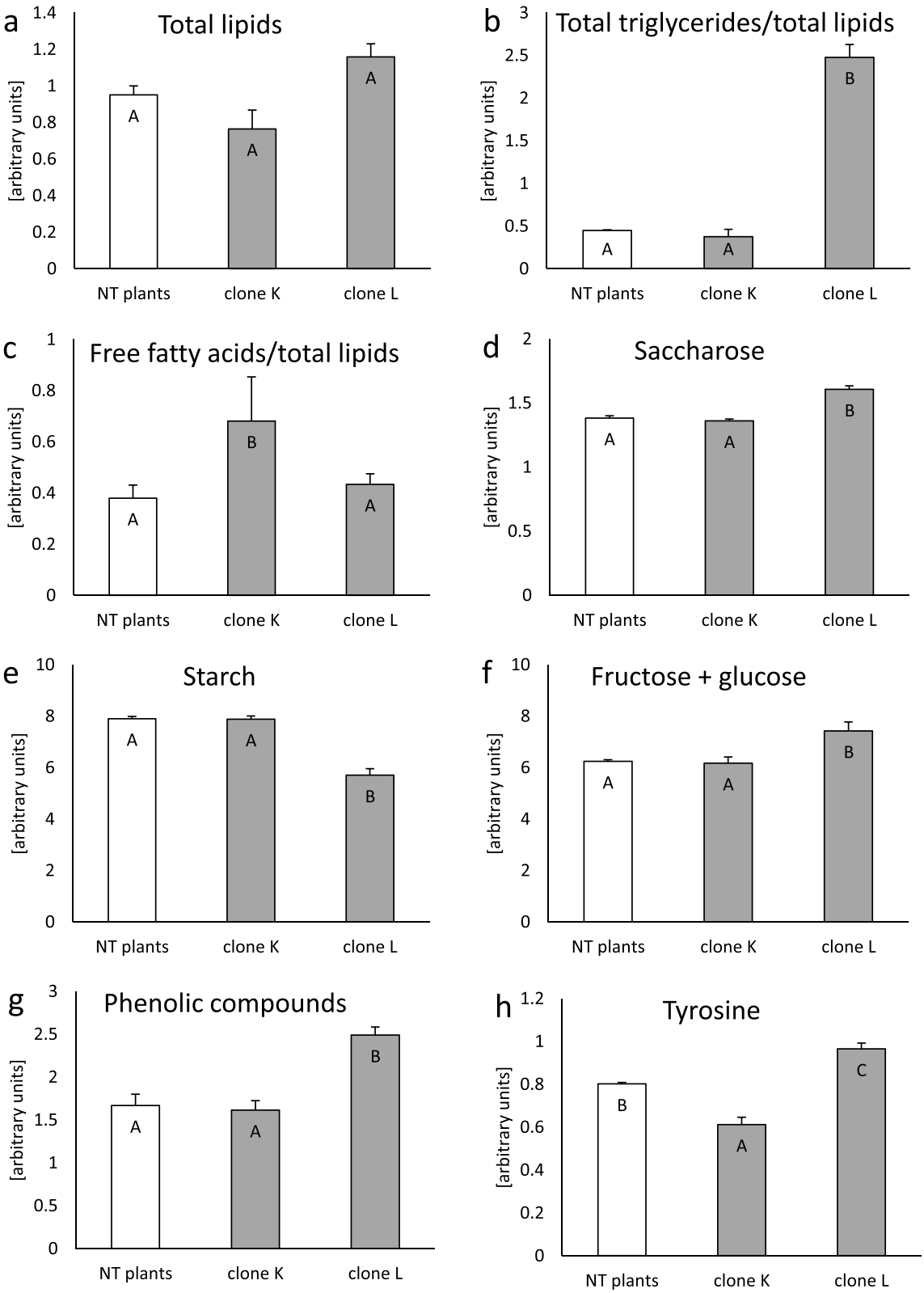

Fig. 6 Semi-quantification biocomponents in non-transformed and transformed tissue of *Dionaea muscipula* clones, identified in ATR-FTIR spectra. **a** Total lipids. **b** Total triglycerides/total lipids. **c** Free fatty acids/total lipids. **d** Saccharose. **e** Starch. **f** Fructose + glucose. **g** Phenolic compounds. **h** Tyrosine. Different letters indicate significant differences between means at $p < 0.05$; the bar represents the standard deviation

ROS production and activate secondary metabolism in transformed cells, while the mechanism of such an event is still poorly understood [12]. In the present research, MDA content was estimated in transformed plants as the oxidative stress marker. It is well known that an imbalance in ROS production can lead to cell membrane damage, where MDA is a product of lipid peroxidation [22]. Clone L had increased production of MDA, while clone K produced MDA on the same level as non-transformed plants (control). According to Franklin et al. [23], MDA synthesis decreased in cells of *H. perforatum* during treatment with *Agrobacterium tumefaciens*. In contrast, MDA content was increased in *H. perforatum* hairy root lines obtained after transformation with *R. rhizogenes*, compared to non-transformed plants [8]. The upregulation of MDA synthesis in transformed plants may be connected to fast growth in transformed organs and quick development, regardless of the increased activity of antioxidants [8]. Increased mitochondrial respiration during enhanced teratoma development may be a potential donor of huge amounts of ROS, which may lead to lipid-membrane peroxidation [24].

One of the most important elements of plant physiology is the amino acid proline, which is involved in a number of developmental processes, protein synthesis, and stress-related responses, acting as an osmolyte and non-enzymatic antioxidant [25]. Genetic transformation of wheat plants with upregulated proline synthesis and increased tolerance to salt stress was reported by Sawahel and Hassan [26]. Moreover, proline biosynthesis coupled to the pentose phosphate pathway can stimulate the production of phenolics [27]. Tovato et al. [25] discussed the role of proline in the formation of hairy root phenotypes in plants containing the *rol*D gene after *R. rhizogenes* infection. They postulated that apart from auxins, proline can play an important role in hairy root development and elongation. Transformation of *D. muscipula* with the *rol*B gene led to increased production of proline in clone L, while such transformation did not affect the creation of the hairy root phenotype. Accelerated proline synthesis in clone L could be the consequence of the fast growth and demand for primary metabolites, such as proteins in fast teratoma (transformed shoots) development [25]. However, it may be the result of increased oxidative stress in plant tissues, acting as a protectant metabolite in antioxidant defence mechanisms [28].

Oncogenes from *R. rhizogenes* make plant cells more resistant to environmental stress and can inhibit ROS accumulation [7]. The *rol*B gene can greatly activate secondary metabolism, including the activity of antioxidant proteins CAT, POD, or SOD [6, 21]. These enzymatic proteins neutralise ROS upon biotic or abiotic stress. Nevertheless, the mechanism by which the *rol*B gene stimulates the protein antioxidant system remains unknown [8]. Veremeichik et al. [12] showed that the *rol*B gene regulates the expression of NADPH oxidase in *Arabidopsis thaliana* and *Rubia cordifolia* transformed calli, while activity of this enzyme is one of the main sources of ROS production during plant-pathogen interactions. Moreover, it has been postulated that *rol* genes induce the reprogramming of transformed plant cells and provoke pleiotropic effects on primary and secondary metabolism, including enzymatic and non-enzymatic antioxidant systems [29]. Increased CAT, SOD, and ascorbic peroxidase (APX) activity was reported in transformed hairy roots of *H. perforatum* in comparison to non-transformed plants [8]. Kohsari et al. [30] also showed increased SOD and POD activity in hairy roots of *Trigonella foenum-graeceum* and *Trigonella monantha* compared to other organs of these species. Moreover, in *R. cordifolia* callus tissue transformed with the *rol*B gene, Shkryl et al. [31] demonstrated an increase in total POD activity and enhanced abundance in the transcripts of major POD genes. Interestingly, our study on transformed clones of *D. muscipula* demonstrated decreased POD activity in the tissue of clone K, with no changes in POD activity in clone L, in comparison to control plants. Furthermore, CAT activity increased in clone L, and SOD activity was enhanced in both examined clones. These findings may be interpreted as the consequence of increased oxidative stress levels in transformed *D. muscipula* clones. SOD is the first defence against ROS in plants, converting superoxide radicals to hydrogen peroxide [32]. Probably, oxidative stress in clone K does not require the increased action of POD and CAT, being on the same level as non-transformed plants. CAT catalyses the decomposition of hydrogen peroxide and only works actively in high hydrogen peroxide concentrations, while lower doses of hydrogen peroxide may be eliminated by POD [29]. The lack of increased POD activity in clone L could be compensation for SOD and CAT activity [8]. However, plant cells try to resist ROS by adjusting the available antioxidant machinery at the right place and time [4].

Another important element of the cellular redox state is a pool of GSSG and GSH, while the GSH/GSSG ratio is known as the oxidative stress indicator [33]. The results showed that clone K is characterised by an increased pool of GSSG and GSH + GSSG. In turn, clone L has an increased GSH/GSSG ratio and GSH + GSSG. Bulgakov et al. [4] reported that *R. cordifolia* plants transformed with the *rol*B gene had a slightly increased total pool of glutathione and GSH/GSSG ratio. Moreover, *A. thaliana*, after transformation, had an enhanced GSH/GSSG

and increased GSH accumulation. Our results confirmed that *rol*B oncogene expression may affect redox homeostasis in plant cells, which results not only in the activity of enzymatic antioxidants but also in the production of non-enzymatic antioxidants.

To support this hypothesis, the carotenoid and phenolic acid content of Venus flytrap teratomas was examined, which together with glutathione are an important element of the non-enzymatic antioxidant system in plants [34]. Both examined clones had an increased carotenoid content compared to the control. Moreover, clone K accumulated more carotenoids than clone L. Furthermore, transformation with the *rol*B oncogene led to changes in phenolic acid synthesis. Clone L synthesised more gallic, chlorogenic, and p-coumaric acid than clone K and control plants. Ferulic acid levels were increased in both clones. Clone K had the highest kaempferol synthesis, while protocatechuic acid synthesis decreased in both examined clones. These results are in agreement with findings by Makowski et al. [11], where clone L had an increased content of total phenolics and selected phenolic derivatives, while clone K accumulated phenolic compounds at the same level as control plants, or lower. Furthermore, other authors reported that plant transformation with wild strains of *R. rhizogenes* can lead to increased phenolic acid production [3, 35, 36]. This phenomenon may result from the fact that expression of the *rol*B gene enhances the activity of phenylalanine ammonia-lyase (PAL) [8]. PAL is a crucial protein in phenolic compound production, catalysing the reaction of *trans*-cinnamic acid synthesis in plant cells. *R. rhizogenes*-mediated transformation enhanced *PAL* expression and stimulated phenylpropanoid metabolism in *H. perforatum* [23]. Simultaneously enhanced growth and increased secondary compound production in response to transformation probably may occurred because of unlimited nutrition resources in vitro conditions. In situations when resources are limited and plants are under stress, a reduction in growth and development may be observed [27].

In the present study, for the first time, the ATR-FTIR technique was combined to estimate some metabolomic parameters in transformed clones of *D. muscipula*. This technique measured lipid, sugar, and protein metabolism changes, demonstrating how the *rol*B oncogene influences plants' physiology. Lipids are the major components of membranes and have a crucial role in stress signalling in plants [37]. Studying lipid membrane compatibility and composition may show the physiological status of plant cells under stress conditions [38]. Our results showed that plant transformation did not affect the total lipid concentration. Nevertheless, clone L was characterised by an increased triglyceride content in the total lipid content. Furthermore, clone K's response to *rol*B gene action was manifested by an increased level of free fatty acids. Such changes in lipid compositions following plant transformation may be the result of increased ROS action and lipid oxidation or/and changes in membrane permeability, which enables cell-cell communication and transport [39]. Walley et al. [40] reported that modulation of fatty acid metabolism is one of the elements in complex plants' response during the plant-pathogen interaction. Additionally, infection and transformation of plants with *R. rhizogenes* or *Rhizobium tumefaciens* (former: *A. tumefaciens*) affect sugar transport and metabolism, while the precise mechanism of action for *rol* oncogenes in sugar metabolism remains unclear [5]. Potato transformation with the *rol*C gene from *R. rhizogenes* changed the accumulation pattern of starch, glucose, and dry matter [41]. Grishchenko et al. [42] postulated that *rol* genes are involved in sugar metabolism through the regulation of enzyme activity, including by glycanases and esterases. These enzymes contribute to the structure of polysaccharides in plant cells. Furthermore, Grishchenko et al. [42] discussed that rather than the *rol*C gene, *rol*B expression can modulate the structure of saccharides in cell walls or plastids. Our examinations of transformed *D. muscipula* plants showed that sugar metabolism was affected by the *rol*B gene only in clone L. Transformation of this clone led to decreased accumulation of starch with simultaneously increased levels of soluble sugars: saccharose and the sum of fructose and glucose. This may be a consequence of faster primary metabolism in clone L. In our previous findings, this clone was characterised by an enhanced growth rate [11]. In such a scenario, the plant needs simple sugars for primary processes and development [43]. It can also be postulated that increased accumulation of simple sugars is connected with oxidative stress in clone L teratomas [44]. In addition to the role in plant growth and development, sugars play a crucial role in signalling cross-talk during the response to environmental stress [44].

Analysis of FTIR spectra also confirmed our findings about phenolic compound accumulation in clones K and L. In our previous paper, teratomas of clone L accumulated an increased quantity of phenolic compounds [11]. In this article, the same trend was observed for phenolic acids evaluated with HPLC. Using a very sensitive technique, FTIR, it was demonstrated that clone L accumulated more phenolic compounds than the control and clone K.

In the context of plant transformation, the synthesis of tyrosine is an important element. In contrast, this aromatic amino acid is the product of primary metabolism.

It is used as the precursor for phenolic compound synthesis in the first step of the phenylpropanoid pathway [28]. However, it is postulated that the *rol*B oncogene encodes proteins with tyrosine phosphatase activity, which is crucial in the oncogenesis process [9]. Clone L had an increased accumulation of tyrosine, while the level of this amino acid decreased in clone K. This corresponds with the hypothesis of Bulgakov [9] that the tyrosine phosphatase function of the *rol*B gene enhances secondary metabolism, while dephosphorylation of tyrosine in proteins is an element of the pleiotropic effect of the *rol*B oncogene in plant cells.

Conclusions

Transformed plants of *D. muscipula* are a new source of knowledge about the physiology of transgenic organisms. Examination of two clones, K and L, showed differences in the response of these plants to transformation events, although both were transformed with a single copy of the *rol*B gene. The pleiotropic effect of the *rol*B gene in transformed plants may be manifested by the regulation of primary and secondary metabolism. The example of clone L showed that transformation with *R. rhizogenes* may lead to enhanced primary and secondary metabolism, as well as promotion of the antioxidant system. Analysis of clone K showed that incorporation of the *rol*B gene in plant genomic DNA does not always cause significant physiological changes. Understanding the mechanisms involved in plant responses to transformation with the *rol*B gene needs further research.

Methods

Plant material

In this study, the plant materials were two transformed clones (teratomas) of *D. muscipula*. Plants were transformed with two wild *R. rhizogenes* strains: LBA 9402 (clone K) and ATCC 15834 (clone L; Fig. 1). Plant transformation and selection process, as well as molecular confirmation of transformation, were described by Makowski et al. [11].

For this research, non-transformed plants (NT plants) and transformed clones were cultivated using in vitro conditions [17]. Briefly, plants were grown in liquid ½ strength Murashige and Skoog medium (½ MS) [45] with no growth regulators, 3% sucrose, and pH = 5.5 (adjusted prior to autoclaving), with rotary shaking (130 rpm). Plants were cultivated at a temperature of 23 ± 1 °C, in fluorescence light at $80 \times mol \times m^2 \times s^1$ photosynthetic photon flux density (PPFD) and a photoperiod of 16 h/8 h light/dark cycle. NT plants and transformed teratomas of both clones were cultivated in 10 biological repetitions. Sixty-day-old tissue cultures were harvested, freeze-dried for 72 h, and homogenised for further analysis.

MDA content estimation

MDA levels were estimated according to Dhindsa et al. [46], with modifications [47]. Plant tissue was extracted in 1 ml of 0.1% trichloroacetic acid (TCA) solution at 4 °C and centrifuged for 15 min at 25155 × g. Subsequently, 0.2 ml of the obtained supernatant was mixed with 0.8 ml of 20% TCA and 0.5% thiobarbituric acid (TBA). Samples were incubated at 95 °C for 30 min and centrifuged for 10 min at 25155 × g. The absorbance of mixtures was measured at 532 and 600 nm. Each spectrophotometric analysis in this study was done using a double beam spectrophotometer U-2900 (Hitachi High-Technologies Corporation, Tokyo, Japan). The content of MDA was calculated using the absorbance coefficient for MDA ($\varepsilon = 155\,mM\,cm^{-1}$) after reduction of the value at 532 nm by the correction value at 600 nm. The results were expressed as nM MDA per 1 g of DW tissue.

Proline content

Accumulation of proline in plant tissue was measured according to Bates et al. [48], with modifications [49]. Dry plant tissue was homogenised in 1 mL 3% aqueous solution of sulfosalicylic acid at 4 °C. Extracts were centrifuged for 15 min, and 0.5 mL was mixed with 0.5 ml acid ninhydrin and 0.5 ml glacial acetic acid. Samples were incubated for 1 h at 100 °C, and the reaction was stopped on ice. Toluene (1 ml) was used to extract the reaction mixture. Absorbance was measured at 520 nm, and the proline concentration was determined from a calibration curve. Calibrations were made with L-proline (Sigma-Aldrich Chemie, GmBH, Steinheim, Germany) as the standard. The results were expressed as mg of proline per 1 g of DW tissue.

Antioxidant enzyme activity

To estimate the CAT, POD, and SOD activity, native proteins were extracted from dry plant material (20 mg) using 2 mL potassium phosphate buffer (0.05 M, pH = 7.00) at 4 °C. The samples were centrifuged for 15 min at 25155 × g (4 °C). The obtained supernatant was collected for protein content estimation and analysis of enzyme activity. All measurements were performed using a double beam spectrophotometer U-2900 (Hitachi High-Technologies Corporation, Tokyo, Japan).

The protein concentration in the extract was determined using the Bradford reagent and bovine serum albumin (BSA) as a standard [50]. CAT activity was determined using the method described by Aebi [51] with modifications by Tokarz et al. [52]. The supernatant (0.2 mL) was mixed with 1.8 mL phosphate buffer (pH 7.0)

and 1 mL H_2O_2 solution in phosphate buffer. The absorbance of H_2O_2 decomposed by the enzyme was measured at 240 nm for 4 min in 1-min intervals. The results were presented as the amount of enzyme that decomposed 1 µmol H_2O_2 in 1 min.

The POD activity was determined using the spectrophotometric method by Lück [53], with modifications by Tokarz et al. [52]. This method was based on the reaction of p-phenyldiamine oxidation to phenazine by the tested enzyme. Phosphate buffer (1.5 mL; pH 6.2), supernatant (0.5 mL), and 1%p-phenyldiamine solution (0.1 mL) was mixed with 0.1 mL 0.1% H_2O_2. The absorbance was measured at 485 nm (0.1 rise of absorbance correspond to one unit of POD activity).

SOD activity was measured according to Hwang et al. [54], with modifications [34]. The enzyme extract was mixed with methionine, nitro blue tetrazolium, and riboflavin. The mixture was incubated in light (two 18 W fluorescence lamps). Absorbance was measured at 560 nm after 5 and 10 min. A similar mixture without the enzyme was prepared as a control, in which the reaction efficiency reached 100%. One unit of enzyme activity was defined as 50% inhibition of the reaction.

Non-enzymatic antioxidants

Carotenoid accumulation

The dried sample (20 mg) was extracted three times in 1 mL 80% acetone with the addition of $MgCl_2$ to discolour plant tissue at 4 °C [55]. The samples were centrifuged for 15 min at 25155×g (4 °C). The absorbance of the diluted supernatant was measured at 470 nm using a double beam spectrophotometer U-2900 (Hitachi High-Technologies Corporation, Tokyo, Japan). The carotenoid content was calculated according to Wellburn [56].

Reduced and oxidised glutathione

The glutathione pool was measured according to Queval and Noctor [57], where 5,5-dithiobis(2-nitro-benzoic acid) (DTNB) is glutathione reductase (GR)-dependent reduced. DW tissue (20 mg) was extracted at 4 °C using 1 mL 0.2 N HCl. The samples were centrifuged at 25255×*g* for 10 min at 4 °C. The obtained supernatant (0.5 mL) was neutralised with 0.5 M NaOH in the presence of 50 µL 0.2 M NaH_2PO_4 (pH 5.6) to reach a final pH between 5 and 6. The method allowed the measurement of the total glutathione pool (reduced plus oxidised form: GSSG+GSH) and, after pre-treatment of the extract aliquots with 2-vinylpyridine (VPD), only GSSG was measured. To measure GSSG+GSH, aliquots of 30 µL neutralised extracts were added to 300 µL 0.2 M NaH_2PO_4 (pH 7.5), 30 µL 10 mM EDTA, 30 µL 10 mM NADPH, 30 µL 12 mM DTNB, and 180 µL distilled water. The reaction was started by the addition of 30 µL GR (20 U mL^{-1}), and the increase in the absorbance at 412 nm was monitored for 2 min. The GSSG fraction was measured using the same routine after incubation of 200 µL neutralised extract with 3 µL VPD for 30 min at room temperature to complex GSH. Calculations were made on the basis of standard curves plotted simultaneously for GSH and GSSG. The GSH/GSSG ratio was also calculated.

Phenolic compound estimation using DAD-HPLC

The phenolic compounds were estimated in methanolic extracts prepared from 200 mg DW tissue in 2.5 mL HPLC-grade methanol using sonication (two times for 30 min at 25 ± 2 °C) (Polsonic). Samples were centrifuged (25,255×*g* for 15 min at 4 °C). The obtained supernatant was filtered through syringe filters (0.22 µm Millex®GP, Millipore, Merck, Darmstadt, Germany) for analysis with high pressure liquid chromatography with a diode array detector (DAD-HPLC).

The quantitative analyses of phenolic compounds in the extracts were done by a validated method, using an apparatus from Merck-Hitachi (LaChrom Elite) with a DAD L-2455 detector and a Purospher RP-18 (250 × 4 mm; 5 µm, Merck, Germany) column [58, 59]. The flow rate was 1 mL × min^{-1}, and temperature was set at 25 °C; the injection volume was 10 µL. The detection wavelength was set at 254 nm. The mobile phase consisted of A—methanol, 0.5% acetic acid 1:4 and B—methanol (v/v). The gradient program was as follows: 0–20 min, 0% B, 20–35 min, 0–20% B, 35–45 min, 20–30% B, 45–55 min, 30–40% B, 55–60 min, 40–50% B, 60–65 min, 50–75% B, and 65–70 min, 75–100% B, with a hold time of 15 min. Identification was performed by comparison to retention times and UV spectra of standards (chlorogenic acid, p-coumaric acid, ferulic acid, gallic acid, protocatechuic acid, and kaempferol acquired from Sigma-Aldrich Co., Germany). The quantification was performed based on the calibration curves method. Samples were prepared and analysed in five replications. The results were expressed in mg × 100 g^{-1} DW ± SD.

ATR-FTIR measurements

Various plant organics absorb the mid-infrared light, giving a molecular fingerprint to the chemical composition when examined in the 4000–900 cm^{-1} region. In our study label-free and rapid FTIR spectroscopy with attenuated total reflection mode (ATR) was used to determine changes in plant tissue composition after transformation with wild *R. rhizogenes* bacteria. Analysis revealed the presence of various characteristic functional groups originating from lipids, phenolic compounds, and a plethora

of mono- and polysaccharides (see Additional file 1). Amide I and II bands of proteins were absent. There was an alternation of intensities in IR bands assigned to lipids (stretches of the CH_2 groups; 2850 cm^{-1}), triglycerides (stretches of the ester C=O groups; 1735 cm^{-1}), fatty acids (stretches of the acidic C=O groups 1718 cm^{-1}), sugars (stretches and deformations of the C-C and C-O groups; saccharose—866 cm^{-1}; starch—1154/1076 cm^{-1}; fructose and glucose—1104/1020 cm^{-1}), phenolic compounds (stretches of the C=C groups; 1611 cm^{-1}), and tyrosine residues (stretches of the C=C groups 1511 cm^{-1}). Their values are the estimate of the content of molecules [18, 19].

Ground, freeze-dried leaves and stems were deposited on an ATR crystal. ATR-FTIR spectra were recorded with a Bruker Alpha FTIR spectrometer with a single-bounce diamond ATR crystal. For each sample, at least three spectra were acquired with a spectral resolution of 4 cm^{-1} in the region of 4000 to 600 cm^{-1} by co-adding 64 scans. Spectra pre-processing and analysis were performed using OPUS software (Bruker Optics, Bullerica, MA, USA, Version 7.2.139.1294). First, the extended ATR correction was applied as implemented in the software. After vector normalisation in the region of 3700–600 cm^{-1}, the second derivative IR spectra were calculated with 9 smoothing points according to a Savitzky-Golay protocol. Second derivative/absorption spectra were used for the calculation of the integral intensity of various bands. For this purpose, a linear baseline was drawn through the peak edges, and the spectrum below this line was integrated over the wavenumber range of the band. For the comparison of spectral differences between studied groups, spectra from each measurement were averaged within the sample.

Statistical analyses

Statistical analyses were performed using STATISTICA 12.0 (StatSoft Inc., Tulsa, OK, USA). The results were subjected to one-way analysis of variance (ANOVA), and the significance of differences between the arithmetical means was determined by Tukey's post hoc test at $p \leq 0.05$.

Abbreviations

ROS: Reactive oxygen species; T-DNA: Transfer DNA; FTIR: Fourier transform infrared spectroscopy; DW: Dry weight; MDA: Malondialdehyde; POD: Peroxidase; CAT: Catalase; SOD: Superoxide dismutase; GSH: Reduced form of glutathione; GSSG: Oxidised form of glutathione; NT plants: Non-transformed plants; APX: Ascorbic peroxidase; PAL: Phenylalanine ammonia lyase; TCA : Trichloroacetic acid; TBA: Thiobarbituric acid; DTNB: 5,5-dithiobis(2-nitrobenzoic acid); BSA: Bovine serum albumin; PPFD: Photosynthetic photon flux density.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-021-03320-y.

Additional file 1: Averaged ATR-FTIR spectra (**a**) and their second derivatives (**b**) (±SD) of non-transformed and transformed tissue of *Dionaea muscipula* clones.

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Authors' contributions

WM, AKr, and KMT: conception and experiment design; WM: performed all spectrophotometric and statistical analysis, interpreted the data, prepared the graphical part of the manuscript, and wrote the manuscript; BT and KMi: contributed to data acquisition; AKo: developed the analytical method for estimation of the glutathione pool and superoxide dismutase activity; ŁP and KMa: performed ATR-FTIR analysis; ASz and HE: performed the HPLC analysis; WM, AKr, KMT, and BT: checked and corrected the manuscript. All authors proofread the manuscript, agreed on its contents, and consented to its submission. The author(s) read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

Our research did not involve any human or animal subjects, material, or data. The plant materials used in this study came from the collection of in vitro cultures of the Laboratory of Biologically Active Compounds, Intercollegiate Faculty of Biotechnology UG and MUG, University of Gdansk, Poland.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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- opracowaniu koncepcji badań i sformułowaniu hipotez badawczych,
- zaprojektowaniu doświadczeń,
- transformacji roślin, wykonaniu analiz PCR oraz Southern Blotting,
- przeprowadzeniu analiz biometrycznych, spektrofotometrycznych i mikrobiologicznych,
- analizie statystycznej uzyskanych wyników,
- interpretacji uzyskanych wyników,
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- pomocy w opracowaniu koncepcji badań i sformułowaniu hipotez badawczych,
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- pomocy w interpretacji wyników,
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- optymalizacji warunków hybrydyzacji typu Southern,
- pomocy w optymalizacji warunków reakcji PCR
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mój indywidualny udział polegał na:

- opracowaniu koncepcji badań i sformułowaniu hipotez badawczych,
- zaprojektowaniu doświadczeń,
- przeprowadzeniu analiz spektrofotometrycznych,
- analizie statystycznej uzyskanych wyników,
- interpretacji uzyskanych wyników,
- napisaniu pierwotnej wersji manuskryptu,
- odpowiedzi na uwagi recenzentów i poprawie manuskryptu.

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- pomocy w opracowaniu koncepcji badań i sformułowaniu hipotez badawczych,
- pomocy w interpretacji wyników,
- krytycznej ocenie manuskryptu przed złożeniem do Redakcji.

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mój indywidualny udział polegał na:

- pomocy w namnażaniu materiału roślinnego do doświadczenia,
- pomocy technicznej przy analizach biochemicznych.

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mój indywidualny udział polegał na:

- optymalizacji metod oznaczania aktywności dysmutazy ponadtlenkowej i zawartości glutationu,
- krytycznej ocenie manuskryptu przed złożeniem do Redakcji.

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mój indywidualny udział polegał na:

- wykonaniu pomiarów przy pomocy spektroskopii absorpcyjnej w podczerwieni oraz analiza widm.

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mój indywidualny udział polegał na:

- merytorycznej opiece nad wykonaniem pomiarów i ich analizy przy pomocy spektroskopii absorpcyjnej w podczerwieni,
- krytycznej ocenie manuskryptu przed złożeniem do Redakcji.

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mój indywidualny udział polegał na:

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mój indywidualny udział polegał na:

- wykonaniu analiz zawartości związków fenolowych przy użyciu wysokosprawnej chromatografii cieczowej,
- wykonaniu opisu badań chromatograficznych i krytycznej ocenie manuskryptu przed złożeniem do Redakcji.

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