Szkoła Główna Gospodarstwa Wiejskiego

w Warszawie

Instytut Biologii

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Funkcje reaktywnych form tlenu i azotu w pułapce dzbanecznika brzusznego (*Nepenthes x ventrata*) podczas rozwoju i trawienia pokarmu

Functions of reactive oxygen and nitrogen species in the trap of *Nepenthes x ventrata* during development and digestion

Rozprawa doktorska

Doctoral thesis

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Spis treści:

Spis rycin:

Spis tabel:

Streszczenie

Rośliny mięsożerne wabią ofiary, uśmiercają oraz absorbują składniki odżywcze z rozkładanych ciał, co definiuje syndrom mięsożerności. Dzbaneczniki (*Nepenthes*) należą do grupy roślin mięsożernych i wytwarzają pasywne pułapki w postaci dzbanków wypełnionych cieczą trawienną. W skład cieczy wchodzą białka (w tym enzymy hydrolityczne), związki niskocząsteczkowe (związki fenolowe) oraz składniki mineralne. W cieczy trawiennej dzbanecznika potwierdzono również obecność reaktywnych form tlenu (ROS). Dotychczas brakowało informacji dotyczących reaktywnych form azotu (RNS) w tym tlenku azotu (NO) w cieczy trawiennej dzbaneczników, choć interakcje między ROS i RNS (RONS) odgrywają istotną rolę w procesach fizjologicznych roślin.

Celem pracy było potwierdzenie obecności RNS i określenie funkcji RONS podczas trawienia pokarmu przeprowadzanego w pułapkach dzbanecznika brzusznego (*Nepenthes x ventrata*) na różnych etapach pozyskiwania składników pokarmowych i w zależności od fazy rozwojowej organu.

Badania zmian zachodzących w cieczy podczas trawienia pokarmu, bez lub z jednocześnie wprowadzanym donorem tlenków azotu (NO_x), mogą przyczynić się do lepszego zrozumienia roli RONS w rozwoju pułapek oraz w regulacji procesów pozyskiwania składników pokarmowych zachodzących w tych organach u roślin mięsożernych.

Dążąc do zrozumienia mechanizmów działania RONS w trawieniu, sformułowano i zweryfikowano sześć hipotez badawczych, stanowiących podstawę prowadzonych eksperymentów.

1. RNS występują w cieczy trawiennej *N. x ventrata*, niezależnie od etapu rozwoju pułapki.
2. NO_x stymulują ekspresję genów, na poziomie RNA kodujących enzymy „trawienne” w tkance pułapki oraz zwiększają aktywność tych enzymów w cieczy *N. x ventrata* w czasie trawienia.
3. NO_x modyfikują ilość i jakość składników cieczy trawiennej *N. x ventrata* podczas pozyskiwania składników odżywczych z wprowadzanego do pułapek pokarmu.
4. NO_x modulują zawartość ROS poprzez zmiany aktywności systemu antyoksydacyjnego w cieczy trawiennej *N. x ventrata*.
5. NO_x sprzyjają utlenianiu białek, ułatwiają ich degradację i pozyskiwanie składników odżywczych z wprowadzanego do pułapek *N. x ventrata* pokarmu .
6. Białka w cieczy trawiennej *N. x ventrata* ulegają nitrowaniu i/lub karbonylacji.

Materiałem badawczym była ciecz trawienna oraz tkanka strefy gruczołowej pułapki dzbanecznika brzusznego (*N. x ventrata*). Kultura dzbaneczników była prowadzona w Szklarni SGGW w kontrolowanych warunkach temperatury i wilgotności. Do zweryfikowania postawionych hipotez badawczych wykorzystane zostały dwa modele doświadczalne: „rozwojowy" oraz „z donorem NO_x". W modelu „rozwojowym" ciecz trawienna była pobierana z pułapek na różnych etapach ich rozwoju: pułapek zamkniętych (niedojrzałe), pułapek otwartych (niedojrzałych, 16 godzin po otwarciu), pułapek dojrzałych (niekarmionych), dojrzałych po pierwszym dniu od podania pokarmu oraz pułapek z widocznymi oznakami starzenia. W modelu „z donorem NO_x" ciecz oraz tkankę strefy gruczołowej, pobierano z pułapek otwartych (pułapki dojrzałe) niekarmionych, pułapek karmionych roztworem białka jaja kurzego (40 µl dzbanek^{-1}) oraz roztworem białka jaja kurzego wraz z donorem NO_x (zakwaszony azotyn sodu). Ciecz trawienną oraz tkankę ze strefy gruczołowej, pobierano z pułapek niekarmionych oraz po pierwszym, drugim oraz czwartym dniu od karmienia lub karmienia z dodatkiem donora NO_x.

Obecność RNS u roślin mięsożernych po raz pierwszy potwierdzono w cieczy trawiennej *N. x ventrata,* w czasie badań przeprowadzonych w toku realizacji tej pracy doktorskiej. Udało się wykazać, że zawartość RNS zamienia się w zależności od etapów rozwoju pułapki jak i fazy dekompozycji składników pokarmu (trawienia). Najwyższe stężenia RNS w cieczy trawiennej odnotowano w dojrzałych pułapkach jeszcze przed wprowadzeniem pokarmu, co sugeruje ich potencjalną rolę w ograniczaniu nadmiernej kolonizacji przez mikroorganizmy. Z kolei najwyższą zawartość ROS zaobserwowano w cieczy zamkniętych pułapek, co może być związane z intensywnym wzrostem pułapki, zatem funkcją regulacyjną ROS. Wprowadzanie pokarmu do pułapek prowadziło do obniżania zawartości RONS, oraz do wzrostu całkowitej zdolności antyoksydacyjnej cieczy. Jednocześnie towarzyszyło temu obniżanie stężenia związków fenolowych, które prawdopodobnie w tym przypadku służą do neutralizacji wolnych rodników. Szybszy powrót do początkowego stężenia flawonoidów i związków fenolowych po podaniu pokarmu z NO_x wskazuje na udział RNS w regulacji zawartości ROS w cieczy trawiennej.

Przeprowadzone badania potwierdziły, że wprowadzenie donora NO_x do cieczy trawiennej wraz z pokarmem przyspiesza procesy trawienne, zwiększając aktywność enzymów proteolitycznych oraz ekspresję genów kodujących proteazy, nepentezyny (Nep I i II). Było to szczególnie widoczne w początkowej fazie trawienia (po pierwszym

dniu po karmieniu). NO_x sprzyjały również zwiększeniu zawartości fosforu i jonów amonowych w cieczy, co sugeruje większą dostępność składników odżywczych.

Ponadto, NO_x wpływał na homeostazę redoks poprzez obniżenie zawartości ROS oraz regulację aktywności peroksydaz klasy III (prawdopodobnie za pośrednictwem modyfikacji potranslacyjnych (PTM)). Stwierdzono również, że białka obecne w cieczy trawiennej podlegają modyfikacjom PTM, takim jak nitrowanie tyrozyny i/lub karbonylacja, niezależnie od obecności pokarmu i donora NO_x. Zmiany te mogą wpływać na stabilność i aktywność enzymów hydrolitycznych, jeszcze przed rozpoczęciem trawienia.

Uzyskane wyniki wskazują, że NO_x odgrywają istotną rolę w regulacji trawienia u *N. x ventrata*, wspomagając dekompozycję składników pokarmowych, ich przyswajanie oraz utrzymanie równowagi oksydoredukcyjnej cieczy trawiennej.

Słowa klucze: dzbaneczniki, RONS, modyfikacje potranslacyjne białek, enzymy hydrolityczne

Summary

Carnivorous plants attract prey, kill it, and absorb nutrients from decomposed bodies, which defines the carnivory syndrome. Pitcher plants (*Nepenthes*), belonging to this group of carnivorous plants, produce passive traps in the form of pitchers filled with digestive fluid. The fluid contains proteins (including hydrolytic enzymes), low-molecular-weight compounds (such as phenolics), and mineral components. The presence of reactive oxygen species (ROS) has also been confirmed in the digestive fluid of pitcher plants. To date, no information has been available regarding reactive nitrogen species (RNS), including nitric oxide (NO), in the digestive fluid of pitcher plants, although interactions between ROS and RNS (RONS) play an important role in physiological processes in plants.

The aim of this study was to confirm the presence of RNS and to determine functions of RONS during food digestion in the traps of the pitcher plant *Nepenthes x ventrata*, at different stages of nutrient uptake and depending on the developmental phase of the pitcher.

Analyses of changes occurring in the digestive fluid during digestion of food without or with a nitric oxide donor (NO_x) may contribute to a better understanding of the role of RONS in trap development and in the regulation of digestive processes in these organs of carnivorous plants.

In order to investigate the mechanisms of RONS involvement in digestion, six research hypotheses were formulated and verified, forming the basis of the conducted experiments:

1. RNS are present in the digestive fluid of *N. x ventrata*, regardless of the trap developmental stage.
2. NO_x stimulate the expression of genes, at the RNA level, encoding “digestive enzymes” in trap tissue and increase the activity of these enzymes in the digestive fluid of *N. x ventrata* during digestion.
3. NO_x modify the quantity and quality of digestive fluid components in *N. x ventrata* during nutrient acquisition from prey introduced into the traps.
4. NO_x modulate ROS content through changes in the activity of the antioxidant system in the digestive fluid of *N. x ventrata*.
5. NO_x promote protein oxidation, facilitating their degradation and the acquisition of nutrients from prey introduced into the traps *N. x ventrata*.

6. Proteins in the digestive fluid of *N. x ventrata* are nitrated and/or carbonylated.

The research materials were digestive fluid and glandular zone tissue from the traps of the pitcher plant *N. x ventrata*. The culture of pitcher plants was carried out in the greenhouse of the Warsaw University of Life Sciences (SGGW) under controlled temperature and humidity. To verify the formulated research hypotheses, two experimental models were applied: the "developmental" and the "NO_x donor". In the "developmental" model, digestive fluid was collected from traps at different stages of development: closed traps (immature), open traps (immature, 16 hours after opening), mature traps (unfed), traps one day after food addition, and traps showing visible signs of senescence. In the "NO_x donor" model, digestive fluid and glandular zone tissue were collected from open (mature) traps: unfed, fed with egg white protein solution (40 µl pitcher $^{-1}$), or fed with egg white protein solution supplemented with a NO_x donor (acidified sodium nitrite). Digestive fluid and glandular zone tissue were sampled from unfed traps, as well as one, two, and four days after feeding or feeding with the addition of a NO_x donor.

The presence of RNS in carnivorous plants was confirmed for the first time in the digestive fluid of *N. x ventrata* as part of this doctoral research. It was shown that the RNS content varies according to the developmental stage of the trap as well as the phase of decomposition of the food components (digestion). The highest RNS concentrations in the digestive fluid were noted in mature traps before the food was introduced, suggesting a potential role in limiting overcolonisation by microorganisms. By contrast, the highest ROS levels were observed in the digestive fluid of closed traps, which may be associated with intensive trap growth and, consequently, a regulatory function of ROS. The introduction of food into the traps led to a reduction in RONS content and an increase in the total antioxidant capacity of the fluid. This was combined with a decrease in the levels of phenolic compounds, which in this context most likely acted to neutralise free radicals. A faster return to the initial levels of flavonoids and phenolic compounds following the addition of food supplemented with NO_x indicates the involvement of RNS in the regulation of ROS content in the digestive fluid. A faster return to the initial levels of flavonoids and phenolic compounds following the administration of food supplemented with NO_x indicates the involvement of RNS in regulating ROS content in the digestive fluid.

The conducted study confirmed that the introduction of a NO_x donor into the digestive fluid, together with food, accelerates digestive processes by increasing the activity of proteolytic enzymes and the expression of genes encoding proteases, nepenthesins (Nep I and II). This effect was particularly evident during the early phase of digestion (on the first day after feeding). NO_x also promoted an increase in the content of phosphorus and ammonium ions in the fluid, suggesting greater nutrient availability.

Furthermore, NO_x influenced redox homeostasis by lowering ROS levels and regulating the activity of class III peroxidases (probably through post-translational modifications, PTMs). It was also found that proteins present in the digestive fluid undergo PTMs such as nitration and/or carbonylation, regardless of the presence of food or a NO_x donor. These changes may affect the stability and activity of hydrolytic enzymes even before the onset of digestion.

The results obtained indicate that NO_x play an important role in regulating digestion in *N. x ventrata*, supporting the decomposition of food components, their assimilation, and the maintenance of redox balance in the digestive fluid.

Keywords: pitcher plants, RONS, post-translational protein modifications, hydrolytic enzymes

Wykaz publikacji stanowiących rozprawę doktorską:
„Funkcje reaktywnych form tlenu i azotu w pułapce dzbanecznika brzusznego (*Nepenthes x ventrata*) podczas rozwoju i trawienia pokarmu"

Publikacja 1

ROS and RNS alterations in the digestive fluid of *Nepenthes x ventrata* trap at different developmental stages. **Wal A.**, Staszek P., Pakula B., Paradowska M., Krasuska U. Plants 2022, 11(23), 3304. https://doi:10.3390/plants11233304
(IF: 4,5 ; MNiSW: 70)

Publikacja 2

Do reactive oxygen and nitrogen species have a similar effect on digestive processes in carnivorous *Nepenthes* plants and humans? Krasuska U., **Wal A.**, Staszek P., Ciacka K., Gniazdowska A. Biology 2023, 12(10), 1356. https://doi.org/10.3390/biology1210135
(IF: 4,0; MNiSW: 100)

Publikacja 3

Nitric oxide action in the digestive fluid of *Nepenthes* × *ventrata* is linked to the modulation of ROS level. **Wal A.**, Piekarniak M., Staszek P., Chodór K., Bieniek J., Gniazdowska A., Krasuska U. Plant Physiology and Biochemistry 2024, 216, 109088. https://doi.org/10.1016/j.plaphy.2024.109088
(IF: 6,4; MNiSW: 70)

Publikacja 4

Nitric oxide stimulates digestion modifying the nutrient composition of the traps' fluid of *Nepenthes x ventrata*. **Wal A.**, Staszek P., Gniazdowska A., Chrastný V., Šípková A., Bieniek J., Krasuska U. Plant Science 2025,
https://doi.org/10.1016/j.plantsci.2025.112558
(IF: 4,7; MNiSW: 100)

Łączny IF publikacji stanowiących rozprawę doktorską: 19,6 , łączna liczba punktów

ministerialnych: 340

Punktacja podana według listy czasopism punktowanych z 2024 r.,
Impact Factor (IF) z ostatnich 5 lat.

Wykaz skrótów:

$^{\bullet}NO_2$ – rodnik dwutlenku azotu

$^{\bullet}OH$ - rodnik hydroksylowy

3-NT – 3-nitrotyrozyna

ABA – kwas abscysynowy

ACC – kwas 1-aminocyklopropano-1-karboksylowy

APF – fluorofor 3′-(p-aminofenylu)fluoresceiny

Arg – arginina

ASA – kwas askorbinowy

BCIP – fosforan 5-bromo-4-chloro-3-indoilowy

Br^- – jon bromkowy

BSA – ang. bovine serum albumin, albumina surowicy bydlęcej

CAT – katalaza

Cl^- – jon chlorkowy

Cys – cysteina

DAF-FM DA – dwuoctan fluoroforu 4-amino-5-metyloamino-2',7'-difluorofluoresceiny

DETA/NO – diazeniumdiolan dietylotriaminy

DPPH – rodnik 2,2-difenylo-1-pikrylohydrazylowy

DTNB – kwas 5,5′-ditiobis(2-nitrobenzoesowy)

ET – etylen

FRSC – ang. free radical scavenging capacity, całkowita zdolność do neutralizacji wolnych rodników

GA – giberelina

GR – reduktaza glutationowa

GSH – glutation, forma zredukowana

GSNO – *S*-nitrozoglutation

GSNOR – reduktaza *S*-nitrozoglutationu

GSSG – glutation, forma utleniona

H_2O_2 – nadtlenek wodoru

HNO – nitorksyl

IAA – kwas indolilo-3-octowy

IBA – kwas indolilomasłowy

LMWA – ang. free radical-reducing capacity of low molecular weight antioxidants, zdolność niskocząsteczkowych antyoksydantów do redukcji wolnych rodników

MDA – dialdehyd malonowy

NADPH – ang. nicotinamide adenine dinucleotide phosphate, zredukowana forma fosforanu dinukleotydu nikotynoamidoadeninowego

Nep - nepentezyna

NH_4^+ - jon amonowy

NiR – ang. nitrite reductase, reduktaza azotynowa

NO^- – anion nitroksylowy

-NO – grupa nitrozowa

NO – tlenek azotu (II)

NO^+ – kation nitrozoniowy

$-NO_2$– grupa nitrowa

NO_2^- – jon azotynowy

NO_3^- – jon azotanowy

N_2O_3 – trójtlenek diazotu

NOG – ang. nitric oxide–generating enzyme, enzym generujący NO

NOS – ang. nitric oxide synthase, syntaza NO

NR – ang. nitrate reductase, reduktaza azotanowa

O_2 – tlen cząsteczkowy

$O_2^{\bullet-}$ – anionorodnik ponadtlenkowy

$ONOO^-$ – nadtlenoazotyn

ONOOH – uprotonowana forma nadtlenoazotynu

Pap – ang. acid phosphatases, fosfatazy kwaśne

POx – peroksydazy klasy III

PTM – ang. post-translational modification, potranslacyjna modyfikacja białek

Rboh - ang. respiratory burst oxidase homolog, homolog oksydazy wybuchu tlenowego

RCS – ang. reactive carbonyl species, reaktywne formy karbonylowe

RNS – ang. reactive nitrogen species, reaktywne formy azotu

RONS – ROS i RNS

ROS – ang. reactive oxygen species, reaktywne formy tlenu

Rubisco – karboksylaza/oksygenaza rybulozo-1,5-bisfosforanu

-SH – grupy tiolowe

SNP – nitroprusydek sodu

SO_4^{2-} – jon siarczanowy

SOD – ang. superoxide dismutase, dysmutaza ponadtlenkowa

TLP – ang. thaumatin-like proteins, białka podobne do taumatyny

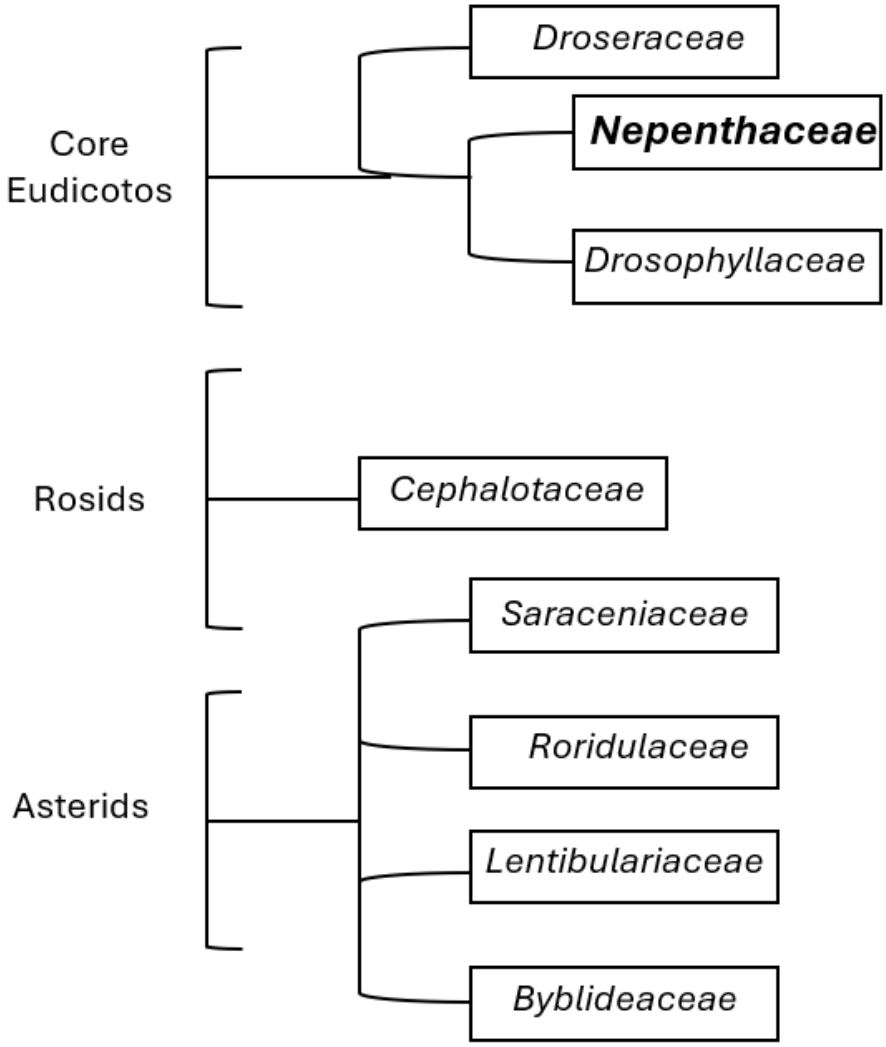
Trp – tryptofan

Tyr – tyrozyna

Wprowadzenie

Rośliny mięsożerne

Rośliny mięsożerne (*Plantae carnivorae*) stanowią unikalną grupę organizmów autotroficznych zdolnych do wabienia, zabijania i trawienia ofiar oraz wchłaniania składników odżywczych pochodzących z ich ciał (syndrom mięsożerności) (Krasuska i in., 2015). Jest to bardzo zróżnicowana grupa obejmująca około 860 gatunków (Ryc. 1), w tym 169 gatunków dzbaneczników (*Nepenthes* spp.), należących do monotypowej rodziny dzbanecznikowatych (Nepenthaceae) (Ryc. 1) (Cross i in., 2020).



Ryc. 1. Uproszczony schemat przedstawiający umieszczenie rodzin roślin mięsożernych w filogenezie roślin okrytozalążkowych (Ellison i Gotelli, 2009,zmodyfikowane).

Wykształcenie syndromu mięsożerności stanowiło konsekwencję ograniczonego dostępu do składników mineralnych dla roślin w środowiskach, takich jak bagna

i torfowiska. Podmokłe i kwaśne gleby utrudniają pobieranie przez korzenie niezbędnych makroskładników, głównie azotu (N) i fosforu (P) (Krasuska i in., 2015, 2023).

Dzbaneczniki

Dzbaneczniki występują głównie w Azji Południowo-Wschodniej (Bauer i in., 2011). Dzbanecznik brzuszny (*Nepenthes x ventrata*) jest naturalną hybrydą: *N. ventricosa* Blanco i *N. alata* Blanco. *N. x ventrata* oraz jego gatunki rodzicielskie są endemitami lasów na Filipinach (Ryc. 2).

Ryc. 2. Naturalny obszar występowania dzbanecznika brzusznego (*Nepenthes x ventrata*), *N. ventricosa* i *N. alata*. Zmodyfikowane na podstawie **Publikacja 2, Fig.1**.

W literaturze dotyczącej roślin mięsożernych stosowany jest termin „trawienie" (ang. digestion) w odniesieniu do pozyskiwania przez rośliny składników odżywczych z ciał ofiar (**Publikacja 2**, Adamec i in., 2021; Adlassnig i in., 2012). Termin ten jest używany również w dalszej części niniejszej pracy. Różnorodność źródeł pokarmu/składników odżywczych zależy od morfologii pułapek oraz miejsca występowania roślin. Typowymi ofiarami *N. x ventrata* są: muchówki, mrówki, termity oraz chrząszcze (Capó-Bauçà i in., 2020; Thorogood i Bauer, 2020).

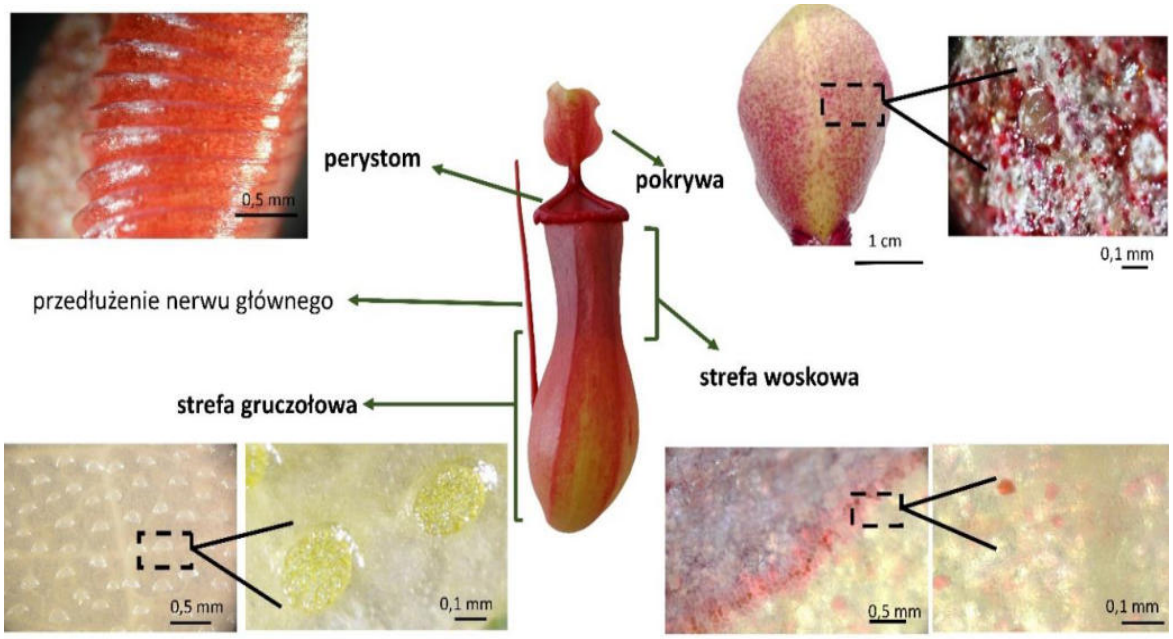
Budowa pułapki dzbanecznika

Dzbaneczniki charakteryzują się pasywnymi pułapkami wykształconymi na końcu nerwu głównego blaszki liściowej. Pułapka w ujęciu funkcjonalnym składa się z tkanek budujących pułapkę z oraz cieczy trawiennej, które wspólnie tworzą organ zdolny do chwytania i trawienia ofiar. Pułapki dzbanecznika są zmodyfikowanymi liśćmi, w

których powierzchnia adaksjalna (górna) zawija się i zrasta, tworząc wewnętrzną ścianę dzbanka (Juniper i in., 1989). Tworzenie pułapki rozpoczyna się od niewielkiej struktury na końcu nerwu oddzielającego dzbanek od podstawy liścia, która stopniowo ulega powiększeniu (Ryc. 3). Następnie dochodzi do wydłużania kształtu pułapki, częściowego wywinięcia perystomu i odchylenia pokryw (Ryc. 3) (Owen i Lennon, 1999). W kolejnym etapie rozwoju następują zmiany zabarwienia pułapki, szczególnie w strefie woskowiej, na całkowicie wywiniętym już perystomie i pokrywie (Ryc. 4) (Dkhar i Pareek, 2019). Pułapka osiąga pełną dojrzałość i funkcjonalność po około sześciu dniach od otwarcia pokrywy (Bauer i in., 2009). Tempo rozwoju pułapki jest zależne od gatunku (Dkhar i Pareek, 2019; Owen i Lennon, 1999).

Ryc. 3. Schemat rozwoju pułapki *N. x ventrata* (Fot. M. Piekarniak).

W pułapce dzbanecznika, można wyróżnić cztery strefy (Ryc. 4). Najwyżej umieszczoną częścią pułapki jest pokrywa, która chroni ciecz trawienną przed rozcieńczeniem opadami deszczu. Perystom to żeberkowana krawędź górnej części pułapki. Na pokrywie oraz na perystomie znajdują się gruczoły wytwarzające nektar i lotne związki wabiące zwierzęta. Wnętrze dzbanka dzieli się na dwie strefy. Funkcją strefy woskowej jest utrudnienie ucieczki ofierze, która znalazła się już w pułapce. W strefie gruczołowej pułapki wydzielane są składniki cieczy trawiennej.



Ryc. 4. Schemat budowy pułapki *N. x ventrata* z wyróżnionymi czterema strefami: pokrywą, perystomem, strefą woskową i strefą gruczołową. Wyróżniono zbliżenie (sześciokrotne i trzynastokrotne) strefy gruczołowej pułapki. Zdjęcia wykonano za pomocą mikroskopu stereoskopowego z wbudowanym aparatem Nikon H550S (ISO, 2000; przysłona f/3, czas ekspozycji 1/10 s). Zmodyfikowane na podstawie **Publikacji 2**.

Charakterystyka cieczy trawiennej dzbaneczników

W skład cieczy trawiennej dzbaneczników wchodzą różne białka, w tym enzymy hydrolityczne takie jak proteazy (nepentezyny I i II) fosfatazy, RNazy, chitynazy i esterazy.

- Nepentezyny (Nep) (EC 3.4.23.12) to proteazy aspartylowe, charakterystyczne dla roślin mięsożernych, wykazują najwyższą aktywność w niskim pH. Cechują się wysoką stabilnością w szerokim zakresie pH i temperatur, co stanowi przystosowanie do warunków panujących wewnątrz pułapki (Athauda i in., 2004; Takahashi i in., 2005).
- Fosfatazy (EC 3.1.3), głównie o charakterze kwaśnym (Pap), uczestniczą w mobilizacji P. Odpowiadają za pozyskanie P przez rośliny, poprzez hydrolizę związków organicznych zawierających reszty fosforanowe, do których należą m.in.: fosfomonoestry – takie jak glukozo-6-fosforan czy rybozo-5-fosforan; związki fosfodiestrowe, w tym kwasy nukleinowe i fosfolipidy (Sharma i in., 2023). Obecność Pap potwierdzono w gruczołach trawiennych *N. tobaica* i *N. x mixta*,

natomiast ich aktywność wzrasta w czasie trawienia (Płachno i in., 2006; Saganová i in., 2018).

- RNazy (EC 3.1.27) to grupa hydrolaz, które katalizują degradację RNA poprzez hydrolizę wiązań fosfodiestrowych. W wyniku ich aktywności uwalniane są oligonukleotydy oraz wolne mononukleotydy zawierające reszty fosforanowe (Han i in., 2022).
- Chitynazy (EC 3.2.1.14), szczególnie klasy III i IV, rozkładają chitynę (główny składnik egzoszkieletów owadów) na N–acetyloglukozaminę, wspomagając tym samym trawienie ofiar. Ich aktywność jest indukowana obecnością zdobyczy (Renner i Specht, 2013).
- Esterazy (EC 3.1.1) uczestniczą w hydrolizie estrów lipidowych uwalnianych z ciał ofiar (Buch i in., 2013). Podczas zakwaszania środowiska wewnątrz pułapki, esterazy biorą udział w tworzeniu hydrofobowych powłok ochronnych na wewnętrznych powierzchniach dzbanka, chroniąc tkanki rośliny przed szkodliwym działaniem enzymów trawiennych i niskiego pH (Duong i in., 2018).

Ciecz trawienna dzbaneczników zawiera nie tylko enzymy, lecz także inne związki o aktywności biologicznej. W cieczy obecne są również pierwiastki m. in. wapń (Ca), potas (K), magnez (Mg), sód (Na), żelazo (Fe) oraz jony chlorkowe (Cl^-), bromkowe (Br^-) i siarczanowe (SO_4^{2-}) (Buch i in., 2013). Ich rola nie jest do końca wyjaśniona w trawieniu przeprowadzanym w pułapkach, jednak przypuszcza się, że mogą uczestniczyć w regulacji pH oraz potencjału osmotycznego cieczy, wpływać na stabilność i aktywność enzymów trawiennych. Wskazuje się również, że jony takie jak Fe i Mn mogą być aktywnie pobierane przez tkanki pułapek i uczestniczyć w asymilacji składników mineralnych, ponieważ pełnią rolę niezbędnych kofaktorów enzymatycznych, warunkujących prawidłowe funkcjonowanie oraz aktywację enzymów trawiennych (Adlassnig i in., 2009; Moran i in., 2010). Wykazano, że stymulacja pułapki poprzez podanie BSA (ang. Bovine Serum Albumin, BSA, albumina surowicy bydlęcej), owadów (muszek owocówek) lub chityny ma wpływ na skład cieczy trawiennej dzbaneczników. Zarówno wprowadzanie owadów jak i BSA do pułapki (*N. x mixta*) ponad czterokrotnie zwiększało aktywność proteolityczną mierzoną dla cieczy (Saganová i in., 2018). Aktywność Pap cieczy pułapek, do których podano owady była czterdziestokrotnie wyższa niż cieczy pobranej z pułapek niekarmionych, natomiast podanie do pułapki BSA prowadziło do zwiększenia tej aktywności dziesięciokrotnie (Saganová i in., 2018).

W cieczy *N. gracilis* wykazano obecność rodnika hydroksylowego (•OH) należącego do reaktywnych form tlenu (ROS) (Chia i in., 2004). Uważa się, że •OH ułatwia rozkład ofiar poprzez utlenianie. Dodatkowo ciecz trawienna zawiera związki bioaktywne, w tym fenole i flawonoidy. Naftochinony, które zidentyfikowano w cieczy pułapek to m. in. plumbagina i 7-metylo-juglon (Buch i in., 2013) oraz droseron i 5-O-metylodroseron (Eilenberg i in., 2010). Prawdopodobnie pełnią funkcje ochronne, wykazując właściwości antybakteryjne i antygrzybiczne (Buch i in., 2013; Eilenberg i in., 2010). W cieczy trawiennej, w której były owady wykazano obecność mRNA kodującego białka podobne do taumatyny (ang. thaumatin-like proteins, TLP) mogącewykazywać analogicznie zdolności przeciwdrobnoustrojowe (Buch i in., 2013; Mithöfer, 2011).

Reaktywne formy tlenu u roślin

ROS to cząsteczki powstające w wyniku niepełnej redukcji lub wzbudzenia tlenu (O_2). Zalicza się do nich m. in. anionorodnik ponadtlenkowy ($O_2^{\bullet-}$), •OH oraz nadtlenek wodoru (H_2O_2) (Corpas i in., 2015). U roślin ROS są głównie wytwarzane w chloroplastach, mitochondriach, peroksysomach oraz w przestrzeni apoplastycznej. W apoplaście powstawanie ROS jest związane m. in. z aktywnością oksydazy NADPH (ang. nicotinamide adenine dinucleotide phosphate, zredukowana forma fosforanu dinukleotydu nikotynoamidoadeninowego) (EC 1.6.3.1) znanej również jako Rboh (ang. respiratory burst oxidase homolog), zlokalizowanej w błonie plazmatycznej. Enzym ten, redukuje O_2 do $O_2^{\bullet-}$, wykorzystując NADPH jako donor elektronów (Kumar i in., 2015). Na terenie komórki jednymi z mechanizmów powstawania $O_2^{\bullet-}$ są reakcje Fentona i Habera-Weissa (Mittler, 2017; Thiel i in., 2011). Reaktywność ROS zależy od ich chemicznego charakteru: wolne rodniki, takie jak •OH, mają bardzo krótki czas półtrwania (ok. 1 μs) i reagują z cząsteczkami zlokalizowanymi w najbliższym sąsiedztwie, natomiast H_2O_2 charakteryzuje się dłuższym okresem półtrwania (ok. 1 ms) i większą mobilnością (Bhattacharjee, 2012), co umożliwia mu pełnienie funkcji sygnałowej. Biologiczna funkcja ROS zależy od ich zawartości w komórce. W niskim stężeniu pełnią funkcję sygnałową, natomiast ich wysokie stężenie potencjalnie prowadzi do śmierci komórkowej (Mittler i in., 2022). ROS wchodzą w reakcje ze składnikami komórek, takimi jak białka, lipidy i kwasy nukleinowe (Valavanidis i in., 2009). Modyfikują reszty aminokwasowe w białkach, prowadząc do takich modyfikacji potranslacyjnych (PTM) jak sulfonylacja czy karbonylacja. W wyniku tych nieodwracalnych modyfikacji białka stają się bardziej podatne na degradację. Reaktywne

formy karbonylowe (RCS) to nienasycone związki karbonylowe, powstające na skutek degradacji nadtlenków lipidów. RCS oddziałują z białkami poprzez tworzenie kowalencyjnych wiązań, co zmienia ich funkcję. Cząsteczki te mogą modulować odpowiedź komórkową na stres oksydacyjny (Mano i in., 2019). Grupy karbonylowe wprowadzane do reszt argininy (Arg), proliny, treoniny lub lizyny modyfikują aktywność biologiczną białek, co sprzyja ich agregacji (Ciacka i in., 2020). ROS reagują z wielonienasyconymi kwasami tłuszczowymi w lipidach, prowadząc do ich peroksydacji. W wyniku tej reakcji powstają wodoronadtlenki lipidów oraz różne aldehydy, w tym dialdehyd malonowy (MDA) i propionaldehyd. Cząsteczki te mogą pełnić funkcje sygnałowe, aktywując mechanizmy obronne (antyoksydacyjne) w komórce. Jednak ich nadmierne stężenie prowadzi do autofagi, apoptozy lub nekrotycznej śmierci komórek (Endale i in., 2023; Schaur i in., 2015). ROS uszkadzają DNA, prowadząc do modyfikacji zasad azotowych oraz degradacji reszt cukrowych (Tripathi i in., 2019). Mogą być przyczyną występowania mutacji wpływających na transkrypcję i translację (Roldán-Arjona i Ariza, 2009).

System antyoksydacyjny w roślinach

Za utrzymanie homeostazy ROS w komórce roślinnej odpowiada system antyoksydacyjny: enzymatyczny i nieenzymatyczny. Enzymy neutralizujące ROS to: dysmutaza ponadtlenkowa (SOD EC 1.15.1.1), katalaza (CAT, EC 1.11.1.6), peroksydazy klasy I (np. peroksydaza askorbinianowa EC 1.11.1.11 i peroksydaza glutationowa EC 1.11.1.9), peroksydazy klasy III (POx) (EC 1.11.1.7) oraz reduktaza glutationowa (GR, EC 1.8.1.7) (Mishra i in., 2023). Wśród antyoksydantów nieenzymatycznych wyróżnia się kwas askorbinowy (ASA), glutation w formie zredukowanej (GSH), karotenoidy i tokoferole (Rudenko i in, 2023). POx będące hemoproteinami charakterystycznymi dla roślin, uczestniczą zarówno w usuwaniu, jak i wytwarzaniu ROS (Takahama i Oniki, 2000). Odgrywają kluczową rolę w detoksykacji H_2O_2 redukując go do wody z udziałem różnych donorów elektronów będących często nieenzymatycznymi antyoksydantami (Almagro i in., 2009). W reakcji utleniania tioli lub w obecności kwasu salicylowego, POx generują rodniki, które reagują z O_2, tworząc m. in. $O_2^{\bullet -}$ oraz $^{\bullet}OH$ (Takahama i Oniki, 2000). Istotną rolę obronną (antyoksydacyjną) pełnią różne związki zawierające grupy tiolowe (-SH), które stanowią dominującą formę zredukowanej siarki w roślinach. Występują one w postaci niskocząsteczkowych peptydów i białek z grupami -SH (Corpas i in. 2022b; Tausz, 2003). Biorąc udział w reakcjach redoks,

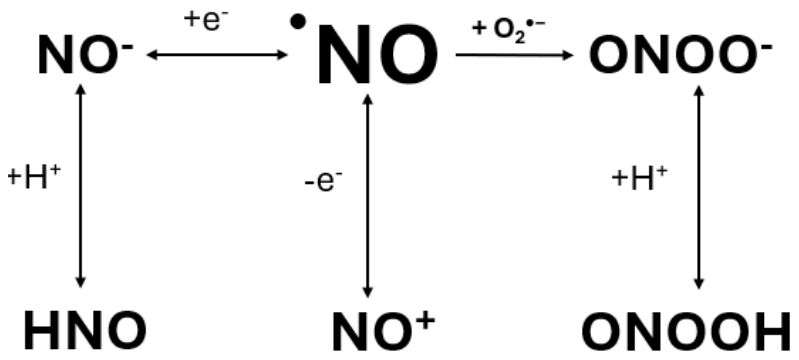
(np. odwracalne tworzenie mostków disiarczkowych) uczestniczą nie tylko w neutralizacji ROS, ale także działają jako cząsteczki sygnałowe (Noctor i Foyer, 2016). Zmiany potencjału redoks mogą aktywować bądź hamować szlaki sygnałowe związane z reakcją na stres, podziały komórkowe czy różnicowanie tkanek (Meyer i in., 2009). Podsumowując, różne związki z grupami -SH pełnią podwójną funkcję: ochronną i regulacyjną, przyczyniając się do przystosowania roślin do zmiennych warunków środowiskowych (Corpas i in., 2022b; Noctor i Foyer, 2016).

Stres oksydacyjny w roślinach mięsożernych

Nadmierne wytwarzanie ROS wraz z niewystarczającym ich usuwaniem przez systemem antyoksydacyjny prowadzą do stresu oksydacyjnego (Mittler i in., 2022). Badania prowadzone na liściach *Dionaea muscipula* (Galek i in., 1990) oraz cieczy trawiennej *N. gracilis*, *N. rafflesiana* i *N. ampullaria*, wykazały, że ROS są wytwarzane na samym początku trawienia (Chia i in., 2004). Przypuszcza się, że pierwszą formą ROS w cieczy jest $O_2^{\bullet-}$. Następnie podczas dysmutacji $O_2^{\bullet-}$ powstaje H_2O_2. Redukcja H_2O_2 jest katalizowana z udziałem jonów metali przejściowych, takich jak Fe^{2+} lub Cu^{+} i prowadzi do wytwarzania $^{\bullet}OH$. W cieczy trawiennej dochodzi do utleniania i denaturacji białek, w których uczestniczy $^{\bullet}OH$. Białka poddane modyfikacjom oksydacyjnym wykazują zwiększoną podatność na działanie enzymów proteolitycznych (Chia i in., 2004; Galek i in., 1990, **Publikacja 2**).

Reaktywne formy azotu (RNS)

Do RNS należą: tlenek azotu (NO) oraz produkty jego reakcji z O_2 lub $O_2^{\bullet-}$. W zależności od pH środowiska, powstają m. in. anion nitroksylowy (NO^{-}), kation nitrozoniowy (NO^{+}), nitroksyl (HNO) oraz nadtlenoazotyn ($ONOO^{-}$) i jego uprotonowana forma (ONOOH) (Ryc. 5) (Arasimowicz-Jelonek i in., 2022; Durzan i Pedroso, 2002). RNS są wytwarzane w różnych przedziałach komórkowych roślin i zwierząt. Ich funkcja podobnie jak ROS zależy od stężenia i lokalizacji, w niskich stężeniach pełnią rolę sygnałową i regulacyjną, natomiast w wysokich mogą działać cytotoksycznie (Corpas i in., 2022a; Kolbert i in., 2019).



Ryc. 5. Uproszczony schemat przemian tlenku azotu (•NO) do reaktywnych form azotu (RNS) w zależności od transferu protonów ($+H^+$ oznacza przyłączenie protonu) i elektronów (e^- przyłączenie/odłączenie elektronu), anion nitroksylowy (NO^-), kation nitrozoniowy (NO^+), nitroksyl (HNO) oraz nadtlenoazotyn ($ONOO^-$) i jego uprotonowana forma (ONOOH).

W komórkach zwierzęcych głównym enzymem odpowiadającym za syntezę NO jest syntaza NO (NOS). W reakcji katalizowanej przez NOS, Arg jest utleniana do cytruliny z jednoczesnym uwalnianiem NO (Lind i in., 2017, Zhang i in., 2023). Jednym z autotroficznych organizmów, u którego potwierdzono obecność białka homologicznego do ssaczej NOS, jest zielenica *Ostreococcus tauri* (Foresi i in., 2010). U roślin wyższych, do tej pory, nie udało się jednoznacznie potwierdzić na poziomie transkryptomicznym i proteomicznym obecności enzymu homologicznego do ssaczej NOS. Z drugiej strony, stosując materiał roślinny, wielokrotnie wykazano aktywność NOS-podobną z udziałem jak dotąd niezidentyfikowanych białek wymagających tych samych kofaktorów, substratów i jednocześnie wrażliwych na inhibitory ssaczego odpowiednika enzymu (Hancock i Neill, 2019). W związku z tym niektórzy autorzy sugerują używanie nowej nazwy „enzym generujący NO" (NOG) zamiast enzymu NOS-podobnego (Hancock i Neill, 2019, **Publikacja 2**).

Reduktaza azotanowa (NR, EC 1.7.1.) jest enzymem cytoplazmatycznym, który katalizuje redukcję azotanów (NO_3^-) do azotynów (NO_2^-), wykorzystując elektrony z NAD(P)H. W warunkach hipoksji dochodzi do zakwaszenia cytoplazmy na skutek intensyfikacji fermentacji oraz zahamowania aktywności reduktazy azotynowej (NiR), odpowiedzialnej za dalszą redukcję NO_2^- do amoniaku. Skutkuje to nagromadzeniem NO_2^- które są przekształcane przez NR do NO, co potwierdzono dla roślin m. in. rzodkiewnika

pospolitego (*Arabidopsis thaliana* (L.) Heynh.) (Bright i in., 2006; Kaiser i Brendle-Behnisch, 1995; Kolbert i in., 2019; Mohn i in., 2019).

NO jest spontanicznie uwalniany z NO_2^-, jednak w warunkach neutralnego pH reakcja ta przebiega powoli. Dodanie związku o właściwościach redukujących np. kwasu askorbinowego lub zakwaszenie środowiska reakcji przyspieszają uwalnianie RNS w tym NO z NO_2^- (Ryc. 6) (Yamasaki, 2000, **Publikacja 2**).

$$NO_2^- + H^+ \longleftrightarrow HNO_2$$

$$HNO_2 \longleftrightarrow N_2O_3 + H_2O$$

$$N_2O_3 \longleftrightarrow \mathbf{NO} + NO_2$$

$$2NO + O_2 \longleftrightarrow 2NO_2$$

Ryc. 6. Przebieg reakcji chemicznej uwalniania tlenku azotu (NO) i tlenku azotu (III) (NO_2) z azotynów (NO_2^-), z produktami pośrednimi: kwasem azotowym (III) (HNO_2) i udziałem tlenu (O_2) . Na podstawie Yamasaki, 2000.

Donory RNS

W badaniach dotyczących roślin najczęściej stosowanymi donorami są SNAP (*S*-nitroso-N-acetylopenicylamina), GSNO (*S*-nitrozoglutation), nitroprusydek sodu (SNP), diazeniumdiolan dietylotriaminy (DETA/NO) oraz zakwaszony azotyn sodu ($NaNO_2$) (Gupta i in., 2020).

SNAP jest związkiem należącym do grupy *S*-nitrozotioli, grupa nitrozylowa (–SNO) jest przyłączona do atomu siarki w -SH . Uwalnianie NO z SNAP może być katalizowane światłem niebieskim (450–470 nm), w obecności jonów metali przejściowych (Cu^+/Cu^{2+}, Fe^{2+}) lub podwyższoną temperaturą(powyżej 30–37°C) (Arasimowicz-Jelonek i in., 2011; Gupta i in., 2020). W procesie tym, oprócz NO, powstają także inne RNS, w tym rodnik dwutlenku azotu ($^{\bullet}NO_2$), trójtlenek diazotu (N_2O_3), NO^+, a w obecności $O_2^{\bullet-}$ również $ONOO^-$ (Blough i Zafiriou, 1985).

GSNO również należy do grupy *S*-nitrozotioli, jest *S*-nitrozowaną pochodną GSH. Denitrozacja GSNO prowadzi do uwolnienia NO, następuje na drodze nieenzymatycznej przy udziale jonów metali przejściowych, światła UV oraz niskocząsteczkowych reduktantów takich jak kwas askorbinowy i GSH (Corpas i in., 2013; Leterrier i in., 2011). W takich warunkach dochodzi do rozerwania wiązania S–N i powstania rodnika tlenku azotu ($^{\bullet}NO$). Ulega on szybkim przemianom prowadzącym do powstania RNS, takich jak $^{\bullet}NO_2$, N_2O_3 oraz $ONOO^-$/ONOOH (Coraps i in., 2013).

SNP należy do grupy donorów NO określanych jako kompleksy nitrożelazowe, w których cząsteczka NO wiąże się koordynacyjnie z atomem żelaza poprzez atom azotu. Pod wpływem światła z zakresu 400–500 nm dochodzi do rozpadu kompleksu $[Fe(CN)_5NO]^{2-}$ w cząsteczce SNP, co prowadzi do uwalniania NO (i/lub NO^+) (Lynch i in., 2011). Tej reakcji może towarzyszyć uwalnianie produktów ubocznych pochodzących od fragmentów cyjankowych kompleksu (np. anionu cyjankowego (CN^-) lub kwasu cyjanowodorowego (HCN)) oraz powstawanie innych RNS ($^{\bullet}NO_2$, N_2O_3) (Bethke i in., 2006; Mur i in., 2013).

DETA/NO należy grupy diazeniumdiolanów, są to związki powstające w wyniku przyłączenia NO do amin drugorzędowych pod wysokim ciśnieniem. Cechą charakterystyczną tych związków jest to, że w środowisku wodnym ulegają one powolnej, hydrolizie prowadzącej uwalniania do NO (Floryszak-Wieczorek i in., 2006, Mur i in., 2013). Produktami ubocznymi tej reakcji są: amina donorowa (dietylenotriamina), a w warunkach tlenowych również NO_2^- i NO_3^- (Keefer, 2003).

Donorem NO działającym na zasadzie reakcji dysproporcjonowania jest $NaNO_2$. W środowisku kwaśnym azotyn ulega protonacji do HNO_2, który następnie dysproporcjonuje, prowadząc do powstawania RNS: NO, NO^+ oraz N_2O_3 powstającego z połączenia NO i $^{\bullet}NO_2$ (Ryc.6) (Yamasaki, 2000). W warunkach fizjologicznych u roślin azotyn może być dodatkowo redukowany do NO enzymatycznie przez NR i NiR, zwłaszcza w warunkach hipoksji (Corpas et al., 2013; Gupta et al., 2020)

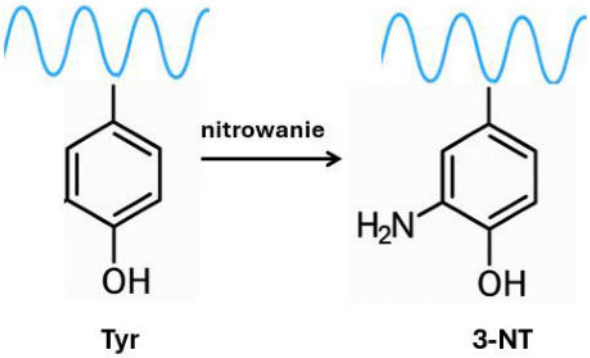
Donory NO różnią się kinetyką jego uwalniania, w roztworach wodnych DETA/NO i SNAP indukują krótkotrwałą emisję NO, trwającą od kilku sekund do minut, natomiast SNP i GSNO charakteryzują się wydłużonym, nawet kilkugodzinnym uwalnianiem tego związku (Floryszak-Wieczorek i in., 2006). Należy podkreślić, że większość związków

określanych jako donory NO prowadzi nie tylko do emisji NO, ale także do powstawania różnych RNS, w tym innych tlenków azotu (NO_x).

Donory takie jak SNAP, SNP czy GSNO wymagają dodatkowych czynników aktywujących, takich jak światło lub obecność reduktorów, dlatego w niniejszych badaniach zdecydowano się na zastosowanie $NaNO_2$.

Modyfikacje potranslacyjne inicjowane RNS

RNS, podobnie jak ROS, modyfikują kwasy nukleinowe, kwasy tłuszczowe i białka (del Río, 2015). Nitrowanie białek jest PTM, która wiąże się z przyłączeniem grupy nitrowej ($-NO_2$) do aminokwasów, takich jak tyrozyna (Tyr) czy tryptofan (Trp). Wprowadzenie grupy $-NO_2$ do reszt Tyr lub Trp powoduje zmianę funkcji zmodyfikowanego białka, wpływając na jego aktywność oraz zdolność do oddziaływań z innymi białkami. Nitrowanie Tyr w wyniku którego powstaje 3-nitrotyrozyna (3-NT) (Ryc.7), w warunkach fizjologicznych, jest procesem nieodwracalnym. Jednakże, pojawiają się doniesienia, dotychczas dotyczące tylko modeli zwierzęcych, że proces ten może być odwracalny w wyniku aktywności denitrazowej. Usuwanie $-NO_2$ z Tyr385 cyklooksygenazy-1 obserwowano w komórkach śródbłonka i makrofagach mysich oraz ludzkich, co sugeruje, że nitrowanie Try nie musi być procesem trwałym (Deeb i in., 2013). U ssaków wykazano aktywność o charakterze denitrazacyjnym, jednak dotychczas nie udało się zidentyfikować konkretnego enzymu, który można by jednoznacznie określić jako denitraza (Deeb i in., 2013). U roślin obecności denitrazy nie potwierdzono. Nitrowanie białek może przebiegać z udziałem $ONOO^-$ i wolnego rodnika dwutlenku azotu ($^{\bullet}NO_2$) oraz H_2O_2 z NO_2^-. Nitrowanie białek zachodzi w warunkach fizjologicznych, m.in. podczas rozwoju roślin (Airaki i in., 2015; Lozano-Juste i León, 2010), czy dojrzewania owoców (Chaki i in., 2015; Zuccarelli i in., 2021). Jednak wzrost zawartości 3-NT w białkach obserwowany jest również podczas stresu oksydacyjnego. W takich warunkach dochodzi do nadmiernej produkcji ROS, co zaburza równowagę wytwarzania RNS i w konsekwencji wzrasta zawartość $ONOO^-$. Białka nitrowane podobnie jak karbonylowane mogą być szybciej degradowane w wyniku ich proteolizy (Krasuska i in., 2016a; León, 2022).



Ryc. 7. Uproszczony schemat przedstawiający nitrowanie reszt Tyr w białkach i powstawanie 3-NT.

S-nitrozacja (dawniej *S*-nitrozylacja), jest to odwracalna PTM polegająca na przyłączeniu grupy nitrozowej (-NO) do -SH cysteiny (Cys). Odgrywa ona kluczową rolę w sygnalizacji NO zarówno w procesach rozwojowych jak i w odpowiedzi roślin na stres (Jedelská i in., 2021; Lindermayr i in., 2005). *S*-nitrozacja białek prowadzi do zmian ich konformacji i aktywności. Modyfikacja ta hamuje m.in. aktywność Rboh, a tym samym zmniejsza wytwarzanie $O_2^{\bullet-}$. W konsekwencji prowadzi to do obniżenia stężenia $^{\bullet}OH$ i $ONOO^-$. W ten sposób zwiększa się ilość wolnego NO, który może wchodzić w reakcję z GSH, prowadząc do powstania GSNO. GSNO to najlepiej poznany *S*-nitrotiol o niskiej masie cząsteczkowej w roślinach. Stężenie tego metabolitu jest regulowane zmienną aktywnością reduktazy GSNO (GSNOR, EC 1.1.1.284) (Begara-Morales i in., 2013; Lee i in., 2008; Tichá i in., 2017). GSNOR katalizuje przekształcenie GSNO do utlenionej formy glutationu (GSSG). Pośrednio wpływa to na zwartość *S*-nitrozotioli białkowych, które mogą powstawać w mechanizmie trans-nitrozacji między GSNO a resztami Cys. GSNOR zawiera liczne reszty Cys, które również są podatne na *S*-nitrozację, która ma wpływ na aktywność tego enzymu (Borrowman i in., 2023).

Wszechstronne działanie NO w świecie roślin - przykłady

W roślinach NO pełni istotne funkcje sygnałowe, uczestnicząc w regulacji procesów życiowych, takich jak kiełkowanie nasion, rozwój roślin czy reakcje na stresy (Kolbert i in., 2019; Sedlářová i in., 2025). Mechanizmy te pozostają jednak nieopisane u roślin mięsożernych.

NO odgrywa również istotną rolę w procesach związanych z cyklem życiowym nasion, w tym w regulacji ich spoczynku, inicjacji kiełkowania oraz wczesnych etapów rozwoju siewek. Badania wykazały, że krótkotrwałe traktowanie zarodków jabłoni (*Malus domestica* Borkh.) donorami NO, znosi ich głęboki spoczynek, stymuluje kiełkowanie i zmniejsza anomalie morfologiczne siewek (Gniazdowska i in., 2007). NO współdziałając z fitohormonami m. in. kwasem abscysynowym (ABA), giberelinami (GA) i etylenem (ET) modyfikuje stan spoczynku oraz kiełkowanie nasion (Aranda-Caño i in., 2023; Bethke i in., 2007; Gniazdowska i in., 2010). Wzrost stężenia NO w nasionach sprzyja ich kiełkowaniu poprzez obniżenie zawartości ABA, który odgrywa kluczową rolę w indukcji i utrzymaniu spoczynku (Albertos i in., 2015). Ponadto, NO wpływa na metabolizm GA i ET (Bethke i in., 2007; Gniazdowska i in., 2010).

NO jako cząsteczka sygnałowa reguluje szlaki metaboliczne związane ze wzrostem, rozwojem i reakcją roślin na czynniki środowiskowe. NO reguluje fotosyntezę poprzez *S*-nitrozację karboksylazy/oksygenazy rybulozo-1,5-bisfosforanu (Rubisco), co obniża aktywność enzymu, a tym samym wydajność fotosyntezy (Abat i in., 2008). NO uczestniczy w procesie zamykania aparatów szparkowych, powodując wzrost pH cytoplazmy i zwiększając zawartość ROS w komórkach. Jednocześnie sprzyja nagromadzeniu Ca^{2+}, które aktywuje kanały jonowe odpowiedzialne za wypływ kationów i anionów z komórek szparkowych (Wilson i in., 2009).

RNS biorą udział także w kształtowaniu budowy korzeni poprzez udział w tworzeniu korzeni bocznych oraz włośników (Corpas i Barroso, 2015; Fernández-Marcos i in., 2012; Lanteri i in., 2008). Bierze udział w odpowiedzi rośliny na auksyny podczas tworzenia korzeni przybyszowych. Przekształcanie kwasu indolilomasłowego (IBA) w kwas indolilo-3-octowy (IAA) w peroksysomach komórek korzeni wiąże się z produkcją NO. Wskazuje to na współdziałanie RNS w organogenezie indukowanej auksynami (Schlicht i in., 2013). NO wpływa na ekspresję genów regulujących cykl komórkowy, syntezę celulozy oraz skład ligniny (Correa-Aragunde i in., 2006; 2008).

NO pełni również istotną funkcję podczas starzenia roślin. Jego rola zależy od typu tkanki i gatunku, może wykazywać działanie zarówno opóźniające, jak i przyspieszające ten proces (Bruand i Meilhoc, 2019). Wykazano, że zawartość NO obniża się w miarę dojrzewania organów roślinnych, a niższe stężenie tego gazu wiąże się z przyspieszonym

starzeniem. Traktowanie liści NO spowalnia tempo degradacji chlorofilu. Liście bawełny (*Gossypium hirsutum* L.) opryskiwane roztworem SNP, wykazywały opóźnione starzenie indukowane stresem solnym. Wiązało się to z utrzymaniem wyższej zawartości chlorofilu i zwiększoną ekspresją genów regulujących fotosyntezę (Kong i in., 2016). Podobnie jak u bawełny, u rzodkiewnika pospolitego zastosowanie donora NO hamowało degradację chlorofilu oraz destabilizację błon tylakoidów, typowych objawów starzenia (Liu i Guo, 2013). NO może także pełnić funkcję regulatora procesów starzenia nasion jabłoni, poprawiając ich żywotność i wigor działając na poziomie molekularnym i fizjologicznym. Zastosowanie NO po laboratoryjnym przyspieszonym starzeniu nasion zwiększało zdolność do kiełkowania zarodków oraz stymulowało aktywność enzymów antyoksydacyjnych takich jak SOD i CAT (Ciacka i in., 2021).

ROS i RNS jako cząsteczki regulatorowe u roślin mięsożernych

Rola RNS w tym NO w rozwoju i funkcjonowaniu roślin jest przedmiotem badań prowadzonych od wielu lat. Poznawane są mechanizmy współdziałania ROS i RNS (RONS) (Krasuska i in., 2023) jako cząsteczek sygnałowych (od kiełkowania nasion po dojrzewanie owoców). Daje możliwość spekulacji, że RONS mogą pełnić zbliżone funkcje jako cząsteczki modulatorowe także u roślin mięsożernych.

Dotychczasowe badania dotyczące roślin mięsożernych wskazywały jedynie na obecność •OH w cieczy trawiennej *N. gracilis*. Jednak dokładne działanie ROS podczas rozwoju pułapki i trawienia przeprowadzanego przez dzbaneczniki nie zostały dokładnie scharakteryzowane. Do 2022 roku, obecność RNS w cieczy trawiennej roślin mięsożernych, w tym dzbaneczników, nie została opisana w literaturze. Również ich udział w procesie rozwoju pułapek oraz w pozyskiwaniu składników mineralnych przez te rośliny nie był znany.

Rola NO w przewodzie pokarmowym zwierząt

U zwierząt, w tym u ludzi, NO odgrywa istotną rolę w regulacji wielu funkcji przewodu pokarmowego, m. in. w żołądku oraz jelicie cienkim i grubym, zarówno w warunkach fizjologicznych, jak i patologicznych. NO uczestniczy w utrzymaniu integralności błony śluzowej poprzez modulację mechanizmów obronnych, regulację funkcji mięśni gładkich żołądka i jelit oraz wydzielania śluzu i wodorowęglanów, co znacząco zwiększa odporność nabłonka na uszkodzenia (Wallace, 2019). Dzięki temu

NO przyczynia się do utrzymania odpowiedniego lokalnego środowiska o neutralnym odczynie, które sprzyja regeneracji nabłonka w przewodzie pokarmowym, mimo ogólnego bardzo niskiego pH (około 2), charakterystycznego dla soku żołądkowego (Brown i in., 1992). NO bierze udział w interakcjach pomiędzy gospodarzem a mikrobiotą przewodu pokarmowego. Z drugiej strony niektóre bakterie patogenne np. *Staphylococcus aureus* potrafią wykorzystać ten gazotransmiter do ochrony przed stresem oksydacyjnym (Tran i in., 2020). NO ma właściwości o charakterze przeciwdrobnoustrojowym. Makrofagi znajdujące się w pobliżu bariery jelitowej oraz komórki nabłonka mogą wydzielać NO do krypt jelitowych, skąd dyfunduje do światła jelita. NO w warunkach fizjologicznych pomaga kontrolować populacje komensalnych mikroorganizmów i utrzymywać integralność bariery jelitowej. Jednak zbyt wysokie stężenie tego gazu (stu krotnie wyższe niż u osób zdrowych) jest odnotowywane w stanach zapalnych np. we wrzodziejącym zapaleniu jelit (Leclerc i in., 2021; Roberts i in., 2024).

Cel pracy

Dzbaneczniki (*Nepenthes*) należące do roślin mięsożernych, wykształciły pasywne pułapki w kształcie dzbanka, wypełnione cieczą trawienną. Pobieranie składników odżywczych jest procesem uniwersalnym dla organizmów żywych, pułapki dzbaneczników są porównywane do przewodu pokarmowego zwierząt (Freund i in., 2022). Znaczenie NO u roślin mięsożernych podczas trawienia pokarmu jak i jego działanie na mikrobiom jest dopiero poznawane. Zmiany na poziomie molekularnym zachodzące podczas trawienia w cieczy oraz w tkankach pułapki dzbaneczników pozostają słabo poznane. W cieczy trawiennej potwierdzono obecność $^{\bullet}OH$ (Chia i in., 2004). RNS, w tym NO_x, modyfikują ilość i jakość ROS w komórkach (Hancock i Neill, 2019). Inspiracją do badań był fakt, że dotychczas nie było żadnych informacji dotyczących obecności RNS w cieczy trawiennej roślin mięsożernych, w tym dzbaneczników. Ponadto, funkcje RNS w czasie rozwoju pułapek oraz pozyskiwania składników odżywczych przez te rośliny nie zostały opisane. Badanie zmian zachodzących w cieczy podczas trawienia pokarmu bez lub z jednocześnie wprowadzanym donorem NO_x mogą przyczynić się do lepszego zrozumienia roli RONS w rozwoju pułapek oraz w procesach zachodzących w pułapkach roślin mięsożernych.

Celem pracy było potwierdzenie występowania RNS i określenie ich funkcji podczas trawienia pokarmu w pułapkach dzbanecznika brzusznego (*N. x ventrata*). Biorąc pod uwagę wieloaspektowy charakter działaniadziałanie RONS, założono, że podawanie pokarmu wraz z donorem NO_x będzie modyfikowało homeostazę tych reaktywnych cząsteczek w pułapkach.

Efekt działania RONS podczas trawienia będzie widoczny na poziomach:

- molekularnym,

- metabolicznym,

-proteomicznym.

Realizacja celu pracy doktorskiej objęła:

- Analizę zmian zawartość RONS w tym: $O_2^{\bullet-}$ i NO_x w cieczy dzbanecznika niezależnie od etapu rozwoju pułapki oraz fazy przeprowadzanego trawienia.
- Określenie wpływu NO_x na tempo degradacji białek, przez analizę aktywność enzymów proteolitycznych i profil białek wyizolowanych z cieczy pułapki dzbanecznika na różnych etapach trawienia.
- Zbadanie oddziaływania NO_x na ekspresję genów kodujących enzymy hydrolityczne (Nep I i II, Pap oraz RNazy) oraz aktywność fosfataz kwaśnych i esteraz w czasie trawienia zachodzącego w cieczy *N. ventrata*.
- Analizę składu pierwiastków (Ca, Cu, Fe K, Mg, Mn, Na, P, S i Zn) w cieczy pułapek do których wprowadzano pokarm bez donora oraz z donorem NO_x.
- Analizę ilościową i jakościową składowych systemu antyoksydacyjnego w cieczy pułapek podczas trawienia pokarmu bez i z NO_x. Zbadano całkowitą zdolność antyoksydacyjną cieczy, zawartość fenoli, flawonoidów, związków z grupami -SH oraz aktywność POx.
- Potwierdzenie obecności i zamian zawartości białek podlegającym RONS zależnym nieodwracalnym PTM białek (karbonylacji i nitrowania tyrozyny) izolowanych z cieczy trawiennej w obecności NO_x wprowadzanych z pokarmem podczas trawienia.

Hipotezy badawcze

1. RNS występują w cieczy trawiennej *N. x ventrata*, niezależnie od etapu rozwoju pułapki.
2. NO_x stymulują ekspresję genów, na poziomie RNA, kodujących enzymy „trawienne” w tkance pułapki oraz zwiększają aktywność tych enzymów w cieczy *N. x ventrata* w czasie trawienia.
3. NO_x modyfikują ilość i jakość składników cieczy trawiennej *N. x ventrata* podczas pozyskiwania składników odżywczych z wprowadzanego do pułapek pokarmu.
4. NO_x modulują zawartość ROS poprzez zmiany aktywności systemu antyoksydacyjnego w cieczy trawiennej *N. x ventrata*.
5. NO_x sprzyjają utlenianiu białek, ułatwiają ich degradację i pozyskiwanie składników odżywczych z wprowadzanego do pułapek *N. x ventrata* pokarmu.
6. Białka w cieczy trawiennej *N. x ventrata* ulegają nitrowaniu i/lub karbonylacji.

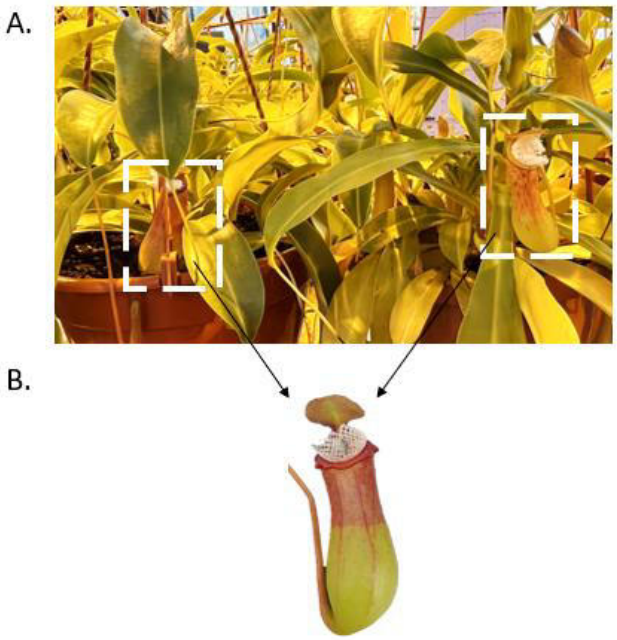
Założono, że podczas pozyskiwania składników odżywczych z wprowadzanego do pułapek pokarmu może dochodzić do obniżenia zdolności antyoksydacyjnej cieczy i zmian w zawartości ROS, które biorą udział w tym procesie.

Materiał badawczy i modele doświadczalne

Materiałem badawczym była ciecz trawienna oraz tkanka strefy gruczołowej pułapki dzbanecznika brzusznego *Nepenthes x ventrata* Hort. ex Fleming (=(*Nepenthes ventricosa* Blanco x *Nepenthes alata* Blanco)) (Ryc. 3).

Kultura dzbaneczników brzusznych (Ryc. 8) była prowadzona w Szklarniowym Obiekcie Doświadczalnym SGGW. Rośliny rosły w doniczkach wypełnionych torfem kwaśnym wymieszanym z perlitem (9:1) oraz mchem torfowcem *Sphagnum*, zawieszonych nad zbiornikami wypełnionymi wodą. Warunki kultury:

- wilgotność (60%) utrzymywano poprzez podlewanie roślin co drugi dzień wodą deszczową,
- temperatura ok 26-30°C,
- światło fotoperiod 16/8 godzin (dzień/noc), w okresie letnio-wiosennym cieniowane (średnie naświetlenie: 250 µE m^{-2} s^{-1}), a w okresie jesienno-zimowym rośliny były doświetlane lampami sodowo-potasowymi (średnie naświetlenie: 336 µE m^{-2} s^{-1}).



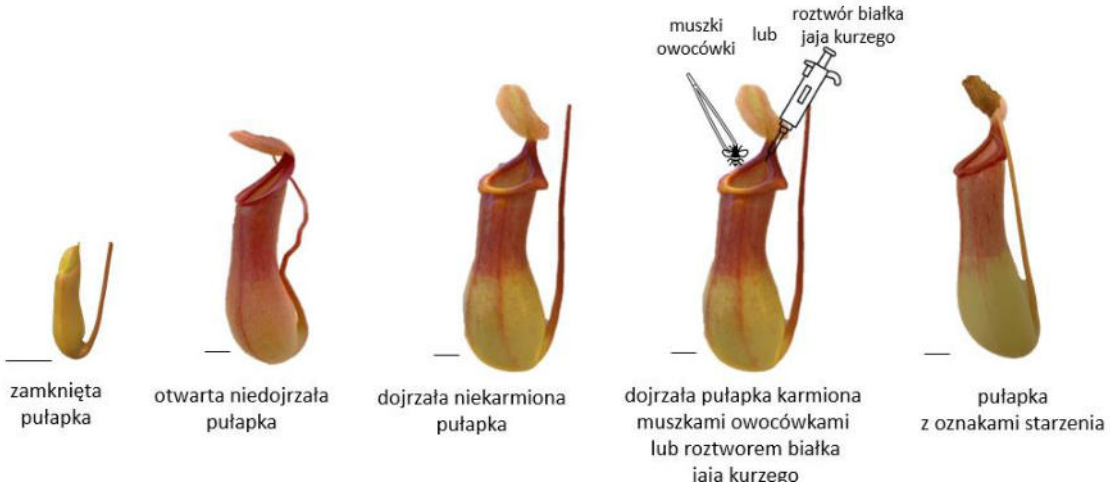
Ryc. 8. Kultura dzbaneczników brzusznych w Szklarniowym Obiekcie Doświadczalnym SGGW (A.). Zdjęcie pułapki z otworem zasłoniętym gazą (B.) (zmodyfikowane na podstawie **Publikacji 3).**

W publikacjach opisano dwa modele doświadczalne. Model tzw. „rozwojowy" (**Publikacja 1**), w którym ciecz trawienna była pobierana z pułapek na różnych etapach ich rozwoju: pułapki zamknięte niedojrzałe, pułapki otwarte niedojrzałe (16 godzin po otwarciu), pułapki dojrzałe (niekarmione), pułapki pierwszy dzień po karmieniu oraz pułapki z widocznymi oznakami starzenia (Ryc. 9).

Termin „karmienie" oznacza wprowadzanie do pułapek pokarmu: roztworu białka jaja kurzego lub całych żywych muszek owocówek (*Drosophilla melanogaster*). Roztwór białka jaja kurzego (1µg białka μl^{-1}) dodawano pipetą w ilości 40 µl $dzbanek^{-1}$ bezpośrednio do cieczy pułapek. Do eksperymentów wybierane były pułapki, o wysokości około 8 cm i o średniej objętości cieczy około 6 ml.

Kryteria doboru białka jaja kurzego jako pokarmu

Białko jaja kurzego jest to mieszanina białek m. in.: owoalbuminy, owotransferryny, owomukoidu, lizozymu, owomucyny i awidyny oraz różnych mikroskładników (Li i in., 2022). Zawiera również ryboflawinę, nikotynamid oraz ergokalcyferol (Bagheri i in., 2020; Kudre i in., 2018). Skład ten sprawia, że białko jaja kurzego jest bardziej fizjologicznym pokarmem niż np. BSA lub chityna. Jednocześnie jest bezbarwne, co umożliwia wykonywanie pomiarów spektrofotometrycznych. Kolejną zaletą tego pokarmu jest stosunkowo krótki czas absorbcji składników, który trwa około cztery dni. W przygotowaniu materiału do oznaczania emisji NO z zastosowaniem dwuoctanu 4-amino-5-metyloamino-20,70-difluorofluoresceiny (DAF-FM DA) pokarm stanowiły dwie żywe muszki owocówki wprowadzane pęsetą do cieczy trawiennej. Zmiana rodzaju pokarmu była w tym przypadku spowodowana koniecznością wyeliminowania autofluorescencji roztworu białka jaja kurzego, wynikającej najprawdopodobniej z obecności ryboflawiny.

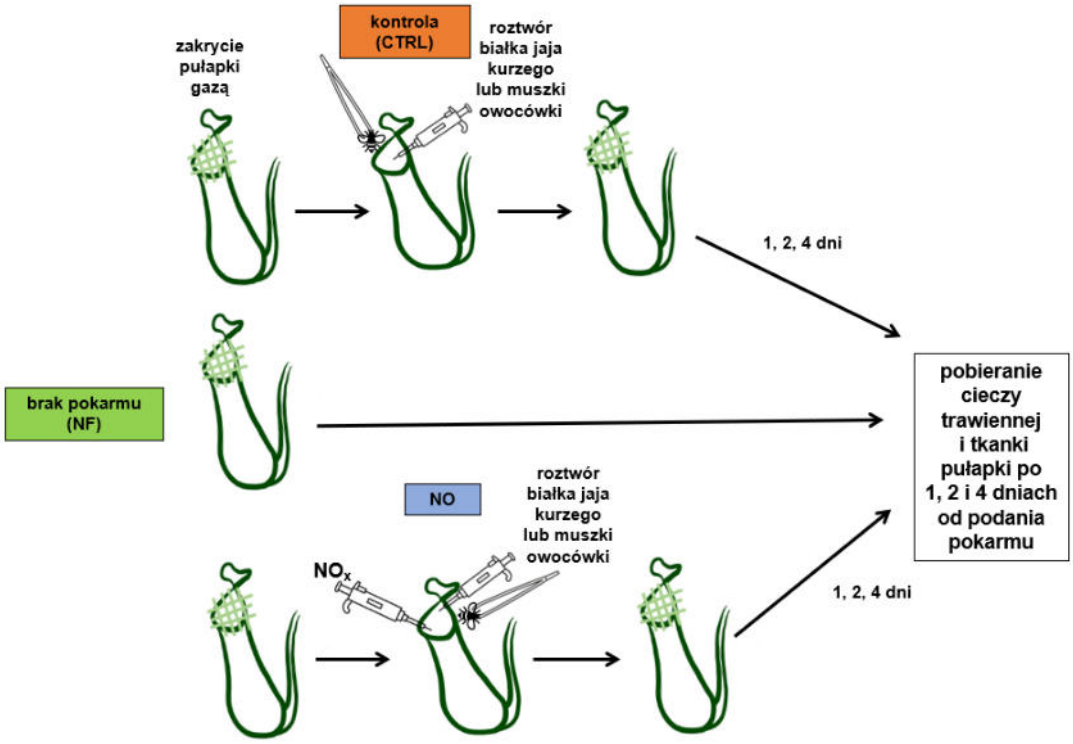


Ryc. 9. Pułapki dzbanecznika brzusznego na różnych etapach rozwoju – wykorzystywane w tzw. modelu „ rozwojowym". Od lewej: pułapka zamknięta, otwarta niedojrzała pułapka, dojrzała pułapka niekarmiona, dojrzała pułapka karmiona dwiema muszkami owocówkami lub roztworem białka jaja kurzego oraz pułapka z oznakami starzenia (widoczna zasychająca pokrywa pułapki). Odcinek skalujący = 1cm, (zmodyfikowano na podstawie **Publikacji 1**).

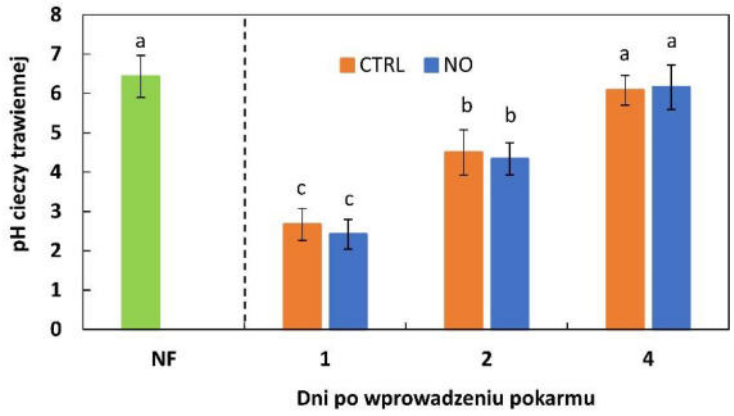
W **Publikacji 3** i **Publikacji 4** zastosowano tzw. model „z donorem NO_x", wykorzystano ciecz oraz tkankę strefy gruczołowej, które pobierano z pułapek otwartych (pułapki dojrzałe) niekarmionych, pułapek karmionych roztworem białka jaja kurzego (jak opisano powyżej) oraz roztworem białka jaja kurzego wraz z donorem tlenków azotu (NO_x). Materiał badawczy, który stanowiła ciecz trawienna oraz tkanka ze strefy gruczołowej pułapki, pobierano z pułapek niekarmionych oraz po pierwszym, drugim oraz czwartym dniu od karmienia lub karmienia z jednoczesnym podaniem donora NO_x (Ryc. 10). Donorem NO_x był zakwaszony azotyn sodu ($NaNO_2$). Do cieczy wprowadzano 1 mM $NaNO_2$ (49 µl) i zakwaszano 1 mM HCl (1µl). Ilość dodawanego pokarmu i donora NO_x została zweryfikowana eksperymentalnie (dane nieopublikowane).

Otwory wlotowe wszystkich pułapek roślin wykorzystywanych do badań zasłaniano gazą, w celu ograniczenia niekontrolowanego dostępu do pokarmu (Ryc. 8). Pułapki do eksperymentów były wybierane losowo, do pomiarów była brana jedna pułapka z jednej rośliny. Przed wprowadzeniem pokarmu lub pobraniem materiału badawczego, sprawdzano pH cieczy trawiennej (z zastosowaniem papierków lakmusowych). Odczyn

cieczy trawiennej zmienia się w zależności od etapu trawienia (Ryc. 11). Pokarm był wprowadzony do pułapek, których ciecz miała pH zbliżone do neutralnego około (6,5).



Ryc. 10. Schemat modelu eksperymentalnego „z donorem NO_x" przedstawiający pobieranie próbek pierwszego, drugiego i czwartego dnia (ciecz trawienna i/lub tkanka pułapki ze strefy gruczołowej *N. x ventrata*) od wprowadzenia pokarmu do cieczy trawiennej pułapki. NF (brak pokarmu) - materiał pobrany bez wprowadzenia pokarmu. Kontrola (CTRL) - materiał pobrany po podaniu pokarmu, NO - materiał pobrany po jednoczesnym podaniu pokarmu i donora NO_x (zmodyfikowano na podstawie **Publikacji 3**).



Ryc. 11. Odczyn cieczy trawiennej w pułapkach *N. x ventrata* niekarmionych oraz w pułapkach po pierwszym, drugim i czwartym dniu od wprowadzenia pokarmu oraz pokarmu wraz z donorem NO_x. NF (brak pokarmu) - materiał pobrany bez podania pokarmu: roztworu białka jaja kurzego. Kontrola (CTRL) - materiał pobrany po podaniu pokarmu, NO - materiał pobrany po jednoczesnym podaniu pokarmu i donora NO_x. Wartości są średnią z 5 powtórzeń. Litery (a-c) oznaczają jednorodne grupy określone z użyciem ANOVA i testu post hoc NIR Fishera przy $p \leq 0,05$.

Metody badawcze zastosowane w przeprowadzonych doświadczeniach

Techniki immunoenzymatyczne:

Profilowanie białek nitrowanych oraz białek karbonylowanych izolowanych z cieczy trawiennej dzbaneczników, przeprowadzono z zastosowaniem techniki elektroforezy w żelu poliakryloamidowym SDS-PAGE (Laemmli, 1970) z elektrotransferem na membranę nitrocelulozową (Towbin i in., 1979) i detekcją przy użyciu przeciwciał (Western blot). Ilościowe oznaczenie białek zawierających 3-NT wykonano z wykorzystaniem immunoenzymatycznego testu ELISA (Krasuska i in., 2016b z modyfikacjami). Dokładny opis **Publikacja 3 i 4**.

Metody amperometryczne:

Pomiar stężenia NO w cieczy trawiennej przeprowadzono z wykorzystaniem systemu pomiarowego Nitric Oxide Measuring System (inNO-T inNO, Nitric Oxide Measuring System, Innovative Instruments), wyposażonego w elektrodę amperometryczną (amiNO-700) (Foresi i in., 2016). Dokładny opis **Publikacja 1** i **3**.

Oznaczenie stężenia O_2 w cieczy trawiennej przeprowadzono z wykorzystaniem systemu pomiarowego z elektrodą typu Clarka w fazie ciekłej (Hansatech Instruments Ltd, Oxygen Electrode Measurement System). Dokładny opis **Publikacja 4**.

Techniki spektroskopii emisyjnej:

Zawartość pierwiastków: Ca, Cu, Fe, K, Mg, Mn, Na, P, S oraz Zn w cieczy trawiennej oznaczono przy użyciu spektrometrii emisyjnej ze wzbudzeniem w plazmie indukcyjnie sprzężonej (ICP-OES, iCAP 7000, Thermo Fisher Scientific). Dokładny opis **Publikacja 4**.

Oznaczenie wykonane w czasie stażu zagranicznego w ramach Grantu mobilnościowego CEEPUS – Freemover, w ramach współpracy z prof. Vladislavem Chrastným i dr Adélą Šípkovą w Czeskim Uniwersytecie Przyrodniczy w Pradze (CZU), na Wydziale Nauk o Środowisku, Laboratorium Geochemii Środowiska II.

Reakcja łańcuchowej polimerazy w czasie rzeczywistym (RT-qPCR):

Analizę ekspresji genów kodujących: Nep I i II, Pap oraz rybonukleazy typu S (RNazy) w tkance pułapki strefy gruczołowej przeprowadzono z wykorzystaniem techniki RT-qPCR. Dokładny opis **Publikacja 4**.

Techniki wizualizacji *in situ*:

Lokalizację $O_2^{\bullet -}$ w tkance dzbaneczka przeprowadzono z użyciem chlorku nitrotetrazolowego (NBT) (Bournonville i Díaz-Ricci, 2011) w obecności lub braku inhibitora aktywności Rboh chlorku diphenyleneiodonium (DPI) (Jiménez-Quesada i in., 2022). Dokładny opis **Publikacja 3**.

Lokalizację aktywności fosfataz kwaśnych w strefie gruczołowej pułapki dzbanecznika przeprowadzono z użyciem fosforanu 5-bromo-4-chloro-3-indoilowego (BCIP) (Wang i Liu, 2017). Dokładny opis **Publikacja 4**.

Metody spektrofluorymetryczne:

Detekcję NO w cieczy trawiennej oznaczano wykorzystując emisję pochodnych DAF-FM DA. Dokładny opis **Publikacja 1**.

Detekcję $ONOO^-$ w cieczy trawiennej oceniano z wykorzystaniem znacznika 3′-(p-aminofenyl)fluoresceiny (APF) (Halliwell i Whiteman, 2004). Dokładny opis **Publikacja 1**.

Techniki spektrofotometryczne :

Oznaczenie aktywności enzymów: proteolitycznych, fosfataz kwaśnych (Saganová i in., 2018), esteraz (Morohoshi i in., 2011) oraz POx (Saunders i in., 1964) w cieczy trawiennej wykonano z zastosowaniem odpowiednich substratów: azokazeiny, fosforanu 4-nitrofenylu, maślanu 4-nitrofenylu oraz trihydroksybenzenu. Dokładny opis **Publikacje 3** i **4**.

Powstawanie ROS w cieczy trawiennej dzbaneczników: produkcja $O_2^{\bullet -}$ oznaczona metodą utleniania epinefryny (Misra i Fridovich, 1972) i zawartość H_2O_2 zmierzona metodą z użyciem jodku potasu (Junglee i in., 2014). Dokładny opis **Publikacje 1** i **3**.

Całkowitą zdolność cieczy trawiennej do neutralizacji wolnych rodników (FRSC) oraz zdolność niskocząsteczkowych antyoksydantów do redukcji wolnych rodników (LMWA) oznaczono przy użyciu rodnika 2,2-difenylo-1-pikrylohydrazylowego (DPPH) (Molyneux, 2004), natomiast całkowitą zdolność redukcyjną cieczy trawiennej określono metodą z zastosowaniem heksacyjanożelazianu (III) potasu (Ferreira i in., 2007). Dokładny opis **Publikacje 1**, **3** i **4**.

Zawartość nieenzymatycznych antyoksydantów w cieczy trawiennej dzbaneczników oznaczono: całkowitą zawartość związków zawierających -SH z użyciem kwasu 5,5′-ditiobis(2-nitrobenzoesowego) (DTNB) (Chan i Wasserman, 1993), całkowitą zawartość związków fenolowych z odczynnikiem Folina-Ciocalteu (Staszek i in., 2020) oraz zawartość flawonoidów metodą z wykorzystaniem chlorku glinu (Silva-Beltrán i in., 2015). Dokładny opis **Publikacje1**, **3** i **4**.

Zawartość nieorganicznych form azotu w cieczy trawiennej oznaczono: NH_4^+ metodą z odczynnikiem Berthelota (Witte i Medina-Escobar, 2001), a NO_2^- przy użyciu zmodyfikowanego odczynnika Griessa (Sigma G4410). Dokładny opis **Publikacje 1** i **4**.

Opis najważniejszych wyników wraz z elementami dyskusji

Zawartość ROS i RNS zmienia się podczas rozwoju pułapki *N. x ventrata*

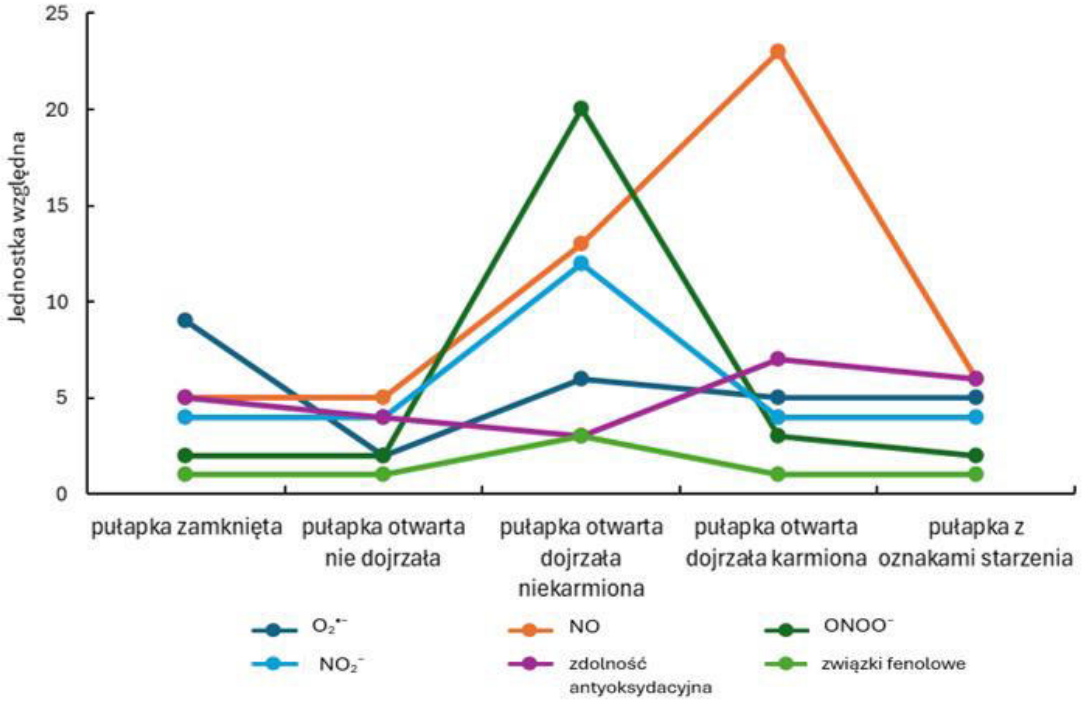
Stosując model „rozwojowy" potwierdzono obecność ROS w cieczy trawiennej roślin mięsożernych na różnych etapach ich rozwoju. Ciecz trawienna *N. x ventrata* pobrana z pułapek zamkniętych charakteryzowała się najwyższą produkcją $O_2^{\bullet-}$ w porównaniu z cieczą pobraną z pułapek w innych fazach rozwojowych **(Publikacja 1, Fig. 1**). Uzyskane wyniki można powiązać z obserwacjami Pavlovič i Kocáb (2022), którzy wykazali obecność oksydazy alternatywnej (AOX) w białkach wyizolowanych z pułapek *N. x ventrata*. Ilość tego białka zmieniała się w zależności od etapu rozwoju pułapek. Najwyższa zawartość AOX została zaobserwowana w pułapkach rozwijających się i w pełni dojrzałych, a znacząco niższa w pułapkach z oznakami starzenia (Pavlovič i Kocáb, 2022). Najwyższa produkcja $O_2^{\bullet-}$ w pułapkach zamkniętych może być związana z intensywnymi procesami metabolicznymi i dynamicznym wzrostem, natomiast w cieczy z pułapek 16 godzin po otwarciu była najniższa. W pułapkach na tym etapie rozwoju była odnotowana wysoka zawartość AOX i mogła ona stabilizować równowagę redoks w cieczy trawiennej dzbaneczników. W pułapkach z oznakami starzenia produkcja $O_2^{\bullet-}$, jak i zawartość AOX były niższe, może to sugerować zaburzenia w utrzymywaniu równowagi redoks w późniejszych fazach rozwoju. Wzmożona produkcja tej formy ROS w zamkniętej pułapce może wiązać się z procesami wzrostowymi tego organu lub z modyfikacją aktywności białek, w tym enzymów trawiennych. Buch i in. (2013) wykazali, że w cieczach pobieranych z zamkniętych pułapek niektórych gatunkach dzbaneczników (*N. alata, N. fusca, N. mirabilis, N. superba, N. thorelii, N. ventricosa*) nie ma bakteryjnego 16s-rDNA, co z kolei może wskazywać na przeciwbakteryjne działanie ROS. Zatem wolne rodniki na tym etapie mogą regulować liczebność mikroorganizmów, których obecność przed zdobyciem ofiary nie jest korzystna dla rośliny. Potwierdza to poniekąd ponad czterokrotne obniżenie produkcji $O_2^{\bullet-}$ po 16 godzinach od otwarcia pułapki. Była to najniższa wartość $O_2^{\bullet-}$ stwierdzona dla tego modelu doświadczalnego **(Publikacja 1, Fig. 1**). Tak znaczące obniżenie produkcji wolnych rodników sugeruje ponownie udział ROS w regulacji mikrobiomu w cieczy rozwijających się pułapek (Chou i in., 2014; Kanokratana i in., 2016). Zwiększona produkcja ROS w cieczy pułapek dojrzałych, ale niekarmionych w stosunku do odnotowanej w cieczy pułapek dojrzałych karmionych może być wynikiem wyższego pH tego środowiska (około 6,5) przed wprowadzeniem pokarmu (Ryc. 11). Najprawdopodobniej źródłem $O_2^{\bullet-}$ są nie tylko reakcje przebiegające w cieczy,

ale również w tkankach pułapki, wynikające z aktywności metabolicznej. Podwyższone pH środowiska komórki sprzyja wytwarzaniu ROS, co wykazano dla mitochondriów izolowanych z krwi obwodowej człowieka. Autorzy potwierdzili, że alkalizacja otoczenia komórek prowadziła do około sześciokrotnego zwiększenia generowania ROS już po 120 minutach od zmiany pH środowiska (Naffah de Souza i in., 2018). Trawieniu pokarmu towarzyszyło obniżanie pH cieczy *N. x ventrata* (do około 2,5) oraz jednoczesne ograniczanie powstawania $O_2^{\bullet-}$ (**Publikacja 1, Fig.1**).

W **Publikacji 1** wchodzącej w skład niniejszej pracy doktorskiej, po raz pierwszy przedstawiono dane potwierdzające obecność RNS w cieczy trawiennej dzbaneczników, na przykładzie *N. x ventrata* (**Publikacja 1, Fig. 4 i 5**). Jest to jednocześnie pierwsze doniesienie o występowaniu RNS u roślin mięsożernych. Najniższą produkcję NO oznaczono w cieczy pułapek zamkniętych, po 16 godzinach od otwarcia oraz wykazujących widoczne oznaki starzenia. Najwyższa zawartość NO została odnotowana w cieczy dojrzałych, otwartych pułapek niezależnie, od tego czy były one karmione, czy nie (**Publikacja 1, Fig. 4)**. Najwyższą produkcję $ONOO^-$ odnotowano w cieczy trawiennej pułapek dojrzałych, ale niekarmionych i była ona prawie dziesięciokrotnie wyższa w porównaniu do wytwarzania tej cząsteczki zmierzonego w cieczy pobieranej z pułapek na innych etapach rozwoju (**Publikacja 1, Fig. 5**). Może być to konsekwencją jednoczesnej wysokiej zawartości $O_2^{\bullet-}$ **(Publikacja 1, Fig. 1**). Po wprowadzeniu pokarmu do pułapek nastąpiło obniżenie produkcji $O_2^{\bullet-}$oraz powstawania $ONOO^-$, mimo obecności NO w cieczy. Podwyższenie wytwarzania $ONOO^-$ (**Publikacja 1, Fig. 5**) w cieczy pułapek dojrzałych, niekarmionych wskazuje na potencjalnie „ochronny" charakter tej cząsteczki, poprzez ograniczenie nadmiernej kolonizacji pułapki przez mikroorganizmy. Natomiast obniżenie produkcji $ONOO^-$ po wprowadzeniu pokarmu do pułapki, może wiązać się z mechanizmami adaptacyjnymi polegającymi na optymalizacji środowiska cieczy dla aktywności enzymów hydrolitycznych. Bazując na tych wynikach można przypuszczać, że RNS pełnią istotną w rolę w funkcjonowaniu pułapki. Zwiększona zawartości RNS w cieczy pochodzącej z pułapek dojrzałych, niekarmionych oraz karmionych sugeruje, iż te cząsteczki są zaangażowane w mechanizm pozyskiwania składników odżywczych z wprowadzanego do cieczy pułapek pokarmu. RNS poprzez działanie sygnałowe, mogą modulować zawartość ROS, zatem oddziaływać na równowagę RONS w cieczy trawiennej.

Zmiany stężenia NO_2^- w cieczy trawiennej charakteryzowały się podobnym trendem podczas rozwoju pułapek jak zmiany zawartości RNS (Ryc. 12). Uzyskane wyniki potwierdziły niską zawartość NO_2^- w cieczy pułapek zamkniętych i otwartych, niedojrzałych (16 godzin po otwarciu) (**Publikacja 1, Fig. 6**). Najwyższą zawartość NO_2^- oznaczono w cieczy pułapek otwartych, dojrzałych i niekarmionych. W środowisku wodnym NO_2^- może ulegać protonacji i przekształceniom w reaktywne pochodne, takie jak N_2O_3 czy $^{\bullet}NO_2$, które w wyniku reakcji z $O_2^{\bullet-}$, ulegają przekształceniu do $ONOO^-$. Podanie pokarmu prowadziło do obniżania stężenia NO_2^- przy jednoczesnym zwiększeniu zawartości NO w badanej cieczy (Ryc. 12). Analizując te wyniki można przypuszczać, że w cieczy trawiennej o kwaśnym pH, charakterystycznym dla etapu pozyskiwania składników odżywczych, NO_2^- stanowi substrat w syntezie NO (Yamasaki, 2000).

Całkowita zdolność antyoksydacyjna cieczy zmieniała się w czasie rozwoju pułapek (**Publikacja 1, Fig. 2**), a jej najwyższą wartość odnotowano po wprowadzeniu pokarmu. Wzrost całkowitej zdolności antyoksydacyjnej cieczy trawiennej po karmieniu prowadzi do obniżenia zawartości ROS (Ryc. 12). System antyoksydacyjny przeciwdziała nadmiernemu nagromadzeniu tlenowych pochodnych, których produkcja jest stymulowana bodźcami mechanicznymi i biochemicznymi związanymi z wprowadzaniem pokarmu do cieczy. Stężenie ROS w cieczy trawiennej dzbaneczników jest regulowane przez m. in. związki fenolowe. Zawartość tych związków zmniejszała się w cieczy pułapek karmionych w stosunku do pułapek dojrzałych, niekarmionych (**Publikacja 1, Fig 3**). Może to świadczyć o ich wykorzystaniu do neutralizacji ROS. Związki fenolowe działają przeciwutleniająco bezpośrednio oraz mogą być wykorzystywane jako donory elektronów przez enzymy antyoksydacyjne m. in. POx (Almagro i in., 2009; O'Brien i in., 2012). Związki fenolowe występują zarówno w liściach i pułapkach roślin mięsożernych: rosiczek (np. *Drosera capensis*), muchołówek (np. *Dionaea muscipula*) oraz dzbaneczników (np. *N. anamensis*) (Kováčik i in., 2012).



Ryc. 12. Zmiany zawartości RNS i ROS, zdolności antyoksydacyjnej, zawartości związków fenolowych oraz stężenie NO_2^- w cieczy trawiennej pułapek *N. x ventrata* na różnych etapach rozwoju pułapki.

Podanie donora NO_x wraz z pokarmem do cieczy trawiennej *N. x ventrata* przyspiesza degradację białek wchodzących w skład pokarmu

Analiza profilu białkowego po rozdziale elektroforetycznym w warunkach denaturujących (SDS-PAGE) dowodzi, że dodanie donora NO_x do cieczy trawiennej przyspiesza degradację białek pochodzących z pokarmu. Wskazuje na to większa liczba wąskich prążków, (o masie odpowiadającej tym, które uzyskano po rozdziale białka jaja kurzego), w ścieżce uzyskanej dla rozdzielonych białek cieczy trawiennej z pułapek po pierwszym dniu od karmienia. Ponadto w próbkach pobranych z pułapek czwartego dnia po karmieniu z NO_x zaobserwowano mniej prążków białkowych w profilu w porównaniu do liczby prążków po rozdziale białek cieczy, do której dodany był tylko roztwór białka jaja kurzego (**Publikacja 3, Fig. 2A**). Zaobserwowano również, że podanie NO_x wraz z pokarmem zwiększało aktywność proteolityczną cieczy trawiennej pierwszego dnia po karmieniu w stosunku do aktywności oznaczonej w cieczy po karmieniu bez NO_x (**Publikacja 3, Fig. 2B**). Odnotowana po rozdziale elektroforetycznym większa liczba wąskich prążków białkowych oraz wyższa aktywność proteolityczna cieczy po karmieniu

z NO_x były powiązane z wysokim stężeniem NO w cieczy trawiennej w tym punkcie pomiarowym (**Publikacja 3, Fig. 3 A i B**).

Analiza poziomu transkryptów genów kodujących proteazy aspartylowe *Nep I* i *Nep II* w tkance strefy gruczołowej pułapki wykazała, że podanie pokarmu wraz z NO_x skutkuje ich wyższą ekspresją (**Publikacja 4, Fig. 7 A i B**). Najwyższy poziom transkryptów genów kodujących Nep I odnotowano pierwszego dnia po karmieniu z NO_x, natomiast Nep II – drugiego dnia po karmieniu również z NO_x. Poziom transkryptów *Nep II* był na granicy wykrywalności zarówno przed karmieniem, jak i czwartego dnia po karmieniu, niezależnie od obecności NO_x. Wysoki poziom transkryptów *Nep I*, odnotowany pierwszego dnia po karmieniu wraz z NO_x, jest zgodny z wynikami wykazującymi zwiększoną aktywność proteolityczną w tym samym punkcie czasowym. Uzyskane wyniki są spójne z danymi opublikowanymi przez Takahashi i in. (2005), które wykazały, że Nep II jest aktywna jedynie w środowisku o obniżonym pH (około 3). Zakwaszenie cieczy *N. x ventrata* odnotowano pierwszego dnia po podaniu pokarmu (Ryc. 11).

Za główne źródło N wykorzystywanego przez dzbaneczniki uznawane są NH_4^+ (Schulze i in., 1997). W pułapkach dzbanecznika brzusznego zaobserwowano wzrost stężenia NH_4^+ w cieczy trawiennej pierwszego dnia po karmieniu z NO_x, a już po dwóch dniach od karmienia nastąpiło ich obniżanie do wartości odnotowanej dla cieczy pułapek niekarmionych (**Publikacja 4, Fig. 6**). Uzyskane wyniki wskazują, że NO_x bierze udział w przyspieszeniu pozyskiwania składników odżywczych, w tym NH_4^+. Potwierdzają tę hipotezę również wyniki uzyskane dla cieczy pułapek karmionych wyłącznie roztworem białka jaja kurzego, w których obniżenie stężenia NH_4^+ nastąpiło dopiero po czterech dniach
po karmieniu. Można przypuszczać, że jest to sytuacja analogiczna do opisanej dla korzeni ryżu (*Oryza sativa* L.), kiedy zastosowanie SNP zwiększało ekspresję genów kodujących transportery NH_4^+ (AMT1.2 i AMT1.3) a tym samym podwyższało pobieranie NH_4^+ przez badane rośliny (Sun i in., 2015).

Dane uzyskane z oznaczenia zawartości P w cieczy trawiennej z pułapek niekarmionych, były zgodne z wynikami uzyskanymi przez Buch i in. (2013), gdyż w obu eksperymentach zawartość P była bardzo niska (poniżej progu detekcji). Dodanie NO_x do cieczy *N. x ventrata* wraz z pokarmem zwiększało zawartość całkowitego P

(**Publikacja 4, Tab. 1**). Wzrost dostępność tego pierwiastka, który należy do głównych czynników ograniczających wzrost roślin mięsożernych (Winkelmann i in., 2023), może być przejawem przystosowania umożliwiającego lepsze wykorzystanie składników mineralnych. NO stymulował poziom transkryptów genu *Pap* w tkance pułapek we wszystkich analizowanych punktach czasowych. Pierwszego dnia po karmieniu z NO_x odnotowano najwyższy poziom transkryptów niemal dwukrotnie wyższy niż w pułapkach karmionych jedynie roztworem białka jaja kurzego (**Publikacja 4, Fig. 7D**) (Tab. 1). Uzyskane dane wskazują na korzystne działanie NO_x na aktywność trawienną. Równolegle ze wzrostem zawartości P i poziomu traksrtyptów genów kodujących Pap odnotowano zwiększenie aktywności Pap w cieczy trawiennej *N. x ventrata* po dodaniu NO_x, niezależnie od czasu po karmieniu (**Publikacja 4, Fig. 8A**). Zatem można stwierdzić, że NO_x, poprzez stymulację aktywności Pap oraz zwiększenie poziomu transkryptów kodujących te enzymy, przyczynia się do bardziej efektywnego pozyskiwania P przez dzbaneczniki. Płachno i in. (2006) wykazali aktywność Pap w strukturach gruczołowych wielu gatunków roślin mięsożernych, w tym *N. tobaica*, *Aldrovanda vesiculosa, Pinguicula gypsicola*, *Cephalotus follicularis* oraz *Utricularia pubescens*. Ponadto aktywność Pap była stymulowana obecnością ofiary u *D. lusitanicum*, natomiast u *C. follicularis* obecność tych enzymów była konstytutywna (Pavlovič i in. 2024). Aktywność Pap w różnych typach gruczołów oraz ich wydzielanie niezależne od obecności ofiary sugerują, że enzymy te odgrywają istotną rolę w procesach trawiennych roślin mięsożernych. Ilość P wprowadzana wraz z pokarmem do pułapki dzbanecznika wynosiła około 0,06 mg L^{-1}, natomiast stężenie oznaczone po pierwszym dniu od podania pokarmu wraz z NO_x wynosiło około 2, 01 mg L^{-1}. Można przypuszczać, że część zawartości P w cieczy mogła pochodzić z pokarmu (około 0,057 mg L^{-1}), natomiast większość miała nieznane pochodzenie. Dodatkowo, po zastosowaniu pokarmu oraz pokarmu z donorem NO_x zaobserwowano wzrost poziomu transkryptów kodujących RNazy (**Publikacja4, Fig. 7C**), co sugeruje ich zwiększoną aktywność w procesie degradacji związków posiadających wiązania fosfodiestrowe, mimo, że wprowadzany do pułapek pokarm nie zawiera kwasów nukleinowych. Dkhar i in. (2020) oznaczyli ekspresję genów kodujących RNazy u *N. khasiana* w pułapkach zamkniętych oraz otwartych. Uzyskane przez nich wyniki sugerują, że geny kodujące RNazy mogą stale podlegać ekspresji w pułapkach dzbaneczników (Dkhar i in., 2020).

Zawartość RNS i ROS zmienia się podczas trawienia pokarmu oraz pokarmu z donorem NO_x w cieczy trawiennej *N. x ventrata*

Wyniki uzyskane w modelu „z donorem NO" dotyczące cieczy trawiennej *N. x ventrata* potwierdziły zmiany stężenia tej reaktywnej cząsteczki w czasie trawienia pokarmu (**Publikacja 3, Fig 3 A i B**). Pierwszego dnia po karmieniu stężenie NO w cieczy trawiennej pułapek, do których dodano donor NO_x, było wyższe o około 30% w stosunku do zawartości oznaczonej w pułapkach karmionych wyłącznie pokarmem. Najwyższe stężenie NO oznaczone pierwszego dnia po karmieniu w cieczy pułapek, do których dodano NO_x, może przyczyniać się do przyspieszonego rozkładu białek, który był szczególnie widoczny w profilach białkowych po rozdziale elektroforetycznym w postaci większej ilości prążków odpowiadającym białkom o niskiej masie cząsteczkowej (**Publikacja 3, Fig. 2A**). W kolejnych dniach (drugim i czwartym) poziom NO w cieczy trawiennej stopniowo się obniżał, podczas gdy w cieczy z pułapek karmionych jedynie pokarmem wzrost stężenia NO obserwowano jedynie pierwszego i czwartego dnia po karmieniu, w porównaniu do pułapek niekarmionych.

Podanie pokarmu bez donora NO_x prawie trzykrotnie zwiększało powstawanie $O_2^{\bullet-}$ w stosunku do wyników uzyskanych dla cieczy pułapek niekarmionych (**Publikacja 3, Fig. 3C**) (Ryc. 13). NO_x obniżał wytwarzanie tego wolnego rodnika w cieczy *N. x ventrata*. Lokalizacja $O_2^{\bullet-}$ w tkance pułapek dzbanecznika z zastosowaniem inhibitora Rboh (DPI) potwierdziła, że $O_2^{\bullet-}$ powstaje w wyniku aktywności tego enzymu (**Publikacja 3, Fig. 4)**. Nie ma danych literaturowych dotyczących aktywności Rboh u roślin mięsożernych. Jednak u innych gatunków roślin, ekspresja genów kodujących Rboh jest stymulowana w odpowiedzi na atak roślinożerców. U *Panicum virgatum* L. piątego dnia po ataku mszycy (*Schizaphis graminum*) ekspresja genów kodujących Rboh zwiększyła się ponad dwukrotnie, natomiast już dziesiątego dnia prawie czterokrotnie w stosunku do roślin niezaatakowanych. Podobne tendencje zmian obserwowano dla genów kodujących POx, których ekspresja wzrosła trzykrotnie dziesiątego dnia po ataku mszyc (Donze-Reiner i in., 2017). Geny kodujące POx zostały zidentyfikowane u *C. follicularis* na podstawie sekwencjonowania genomu, a ich aktywność potwierdzono poprzez oznaczanie poziomu transkryptów w liściach o różnej morfologii: pułapkowych i asymilacyjnych (Fukushima i in., 2017). Natomiast u *D. capensis* i *D. muscipula* zaobserwowano zwiększoną ekspresję genów kodujących POx w odpowiedzi na obecność

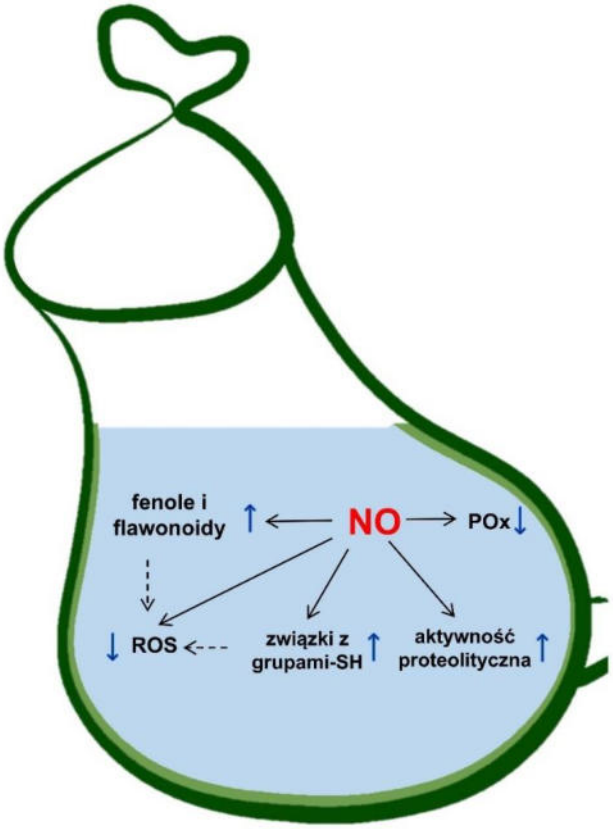
ofiary (Pavlovič, 2025). Wyniki te są tożsame z wynikami uzyskanymi dla *N. x ventrata* u których aktywność POx w cieczy trawiennej zwiększała się pierwszego dnia po karmieniu, szczególnie w próbkach bez NO_x, natomiast dodanie NO_x hamowało aktywność tego enzymu (**Publikacja 3, Fig. 5D**). Może to być wynikiem modyfikacji potranslacyjnych (*S*-nitrozacji lub nitrowania), które przeważnie zmniejszają aktywność enzymów. Takie działanie NO na aktywność POx zaobserwowano m. in. u papryki (*Capsicum annuum* L.) (González-Gordo i in., 2023). Autorzy wykazali, że aktywność POx obniża się o 10% lub dochodzi do całkowitego zahamowania aktywności enzymu w obecności NO_x, w zależności od badanej izoformy tego enzymu (González-Gordo i in., 2023). Autorzy zaproponowali, że PTM np. *S*-nitrozacja mogą przyczyniać się do obniżenia aktywności POx, a tym samym łagodzić skutki stresu oksydacyjnego poprzez zapobieganie zbyt wysokiemu stężeniu ROS w komórkach. Podobnie jak w badaniach z wykorzystaniem papryki, podanie NO_x prowadziło do utrzymywania na stałym poziomie aktywność POx w cieczy trawiennej *N. x ventrata* od pierwszego, aż do czwartego dnia po karmieniu. Potwierdza to funkcję ochronną NO, w ograniczaniu nadmiernej produkcji ROS, które mogłyby być generowane przez POx.

Zawartość grup -SH w cieczy dzbaneczników podczas trawienia nie ulegała większym zmianom, niezależnie od punktu pomiarowego oraz wprowadzenia NO_x (**Publikacja 3, Fig. 5C**) (Ryc. 13). Scherzer i in. (2017) wykazali obecność GSH w cieczy trawiennej *Dionaea muscipula* (J. Ellis). To sugeruje, że związki z grupami -SH które podlegały detekcji w cieczy *N. x ventrata* mogą również być GSH. Uważa się, że utrzymywanie równowagi ROS jest zapewniane przez zachowanie stabilności związków przeciwutleniających w niskim pH cieczy trawiennej, a to zapewnia aktywność enzymów hydrolitycznych zaangażowanych w trawienie (Scherzer i in., 2017).

Związki fenolowe, które zostały zidentyfikowane w cieczach trawiennych różnych gatunków dzbaneczników (*N. khasiana* i *N. ventricosa*) wykazywały właściwości przeciwdrobnoustrojowe (Buch i in., 2013; Eilenberg i in., 2010). Całkowita zawartość związków fenolowych i flawonoidów oznaczona w cieczy trawiennej *N. x ventrata* była zmienna w czasie trwania eksperymentu niezależnie od podawania NO_x (**Publikacja 3, Fig. 5 E i F**) (Ryc. 13). Pierwszego dnia po podaniu pokarmu bez oraz z NO_x w cieczy trawiennejodnotowano najwyższe stężenie fenoli (**Publikacja 3, Fig. 5E**). Już drugiego dnia zaobserwowano około czterokrotne obniżenie całkowitej zawartości

fenoli. Wyniki uzyskane dla *N. x ventrata* różniły się od otrzymanych przez Kováčik i in. (2012) dotyczących liści *D. capensis*, w których nie zmieniała się zawartość fenoli i flawonoidów trzy oraz dwadzieścia jeden dni po podaniu pokarmu (mrówek). W cieczy z pułapek *N. x ventrata* karmionych pokarmem bez NO_x, pierwszego dnia po karmieniu zaobserwowano obniżanie stężenia flawonoidów. Może to wskazywać na wykorzystanie tych związków podczas reakcji katalizowanej przez POx, gdyż aktywność tego enzymu wzrosła właśnie pierwszego dnia po karmieniu (**Publikacja 3, Fig. 5F**) (Ryc. 13). Obniżenie stężenia flawonoidów oznaczono drugiego dnia po karmieniu w cieczy z pułapek z dodatkiem NO_x. Czwartego dnia w cieczy z pułapek karmionych bez dodatku NO_x odnotowano takie stężenie flawonoidów jak w cieczy z pułapek karmionych pokarmem z NO_x drugiego dnia od karmienia.

W cieczy trawiennej pułapek karmionych z donorem NO_x zawartość ROS powracała szybciej do poziomu odnotowanego w cieczy z pułapek przed karmieniem, niż miało to miejsce w cieczy pułapek karmionych tylko białkiem jaja kurzego. Można przypuszczać, że NO_x działa jako cząsteczka regulująca stężenie flawonoidów, a tym samym może wpływać na homeostazę ROS w cieczy trawiennej *N. x vetrata*.



Ryc. 13. Schemat przedstawiający rolę jaką pełni NO (strzałki ciągłe) oraz przypuszczalne mechanizmy działania (strzałki przerywane) tej cząsteczki podanej wraz pokarmem do cieczy trawiennej *N. x ventrata*. Przedstawione zmiany dotyczą cieczy pierwszego dnia po podaniu pokarmu z donorem NO_x i obejmują: zawartość ROS, związków antyoksydacyjnych: fenoli i flawonoidów, związków z grupą -SH, aktywność POx oraz aktywność proteolityczną. po karmieniu pokarmem wraz z donorem NO (zmodyfikowano na podstawie **Publikacji 3**).

Białka cieczy trawiennej dzbaneczników ulegają potranslacyjnym modyfikacjom

Po raz pierwszy u roślin mięsożernych (dzbaneczników) potwierdzono obecność białek zawierających 3-NT (**Publikacja 3, Fig. 6**) oraz białek z grupami karbonylowanymi (**Publikacja 4, Fig. 5)** w cieczy trawiennej. Po wyznakowaniu białek przeciwciałami anty 3-NT najintensywniejsze prążki na membranie po rozdziale dotyczyły białek wyizolowanych z cieczy pułapek roślin niekarmionych (**Publikacja 3, Fig. 6**). Wyniki te sugerują, że RNS mogą oddziaływać bezpośrednio na enzymy proteolityczne cieczy trawiennej pułapek, najprawdopodobniej na proteazy aspartylowe, takie jak Nep jeszcze przed rozpoczęciem trawienia. Nitrowanie białek może prowadzić do zmian

ich aktywności, jak to opisali Zaragozá i in. (2009). Autorzy wykazali, że nitrowanie Tyr w miejscu aktywnym katepsyny prowadziła do zwiększania aktywności proteolitycznej. Z drugiej strony, w badaniach *in vitro* odnotowano, iż nitrowany pepsynogen, w niskim pH odpowiadającym środowisku żołądka, wykazywał niższą aktywność enzymatyczną niż jego forma niezmodyfikowana (Rocha i in., 2013, 2016). Uzyskane wyniki sugerują, że w cieczy trawiennej dzbaneczników ta PTM białek pełni rolę regulacyjną i podobną do „gastroprotekcyjnej" obserwowanej u ludzi w warunkach braku pokarmu.

Roztwór białka jaja kurzego, który był podawany jako pokarm do cieczy trawiennej dzbaneczników zawierał białka karbonylowane (**Publikacja 4, Fig. 5**). Od pierwszego dnia po karmieniu w profilach białek wyizolowanych z cieczy obserwowano zmniejszającą się liczbę i intensywność prążków odpowiadających białkom z grupami karbonylowanymi wprowadzanymi z pokarmem do cieczy trawiennej. Tak zmodyfikowane białka mogą również stymulować aktywność enzymów np. proteolitycznych, gdyż ich aktywność wzrastała już od pierwszego dnia po karmieniu. Obecność białek charakteryzujących się tą PTM pochodzących z pokarmu lub z rośliny w cieczy pułapek niekarmionych może wskazywać na ich funkcję regulacyjną. Można przypuszczać, że karbonylowane peptydy lub aminokwasy z grupą karbonylową mogą być pobierane przez tkanki dzbanków. Møller i in. (2017) wskazują, że białka/peptydy karbonylowane mogą pełnić funkcję sygnałową oddziałując z innymi komponentami sygnalizacji redoks. Ciacka i in. (2020) podkreślają, że tak zmodyfikowane białka mogą uczestniczyć w lokalnym i systemowym przekazywaniu sygnałów związanych ze stresem oksydacyjnym. Wprowadzenie do cieczy trawiennej białek karbonylowanych wraz z pokarmem może być sygnałem m in. do rozpoczęcia procesów trawienia.

Podsumowanie

Wyniki uzyskane w modelu „rozwojowym" potwierdzają, że zarówno ROS, jak i RNS odgrywają istotną rolę w funkcjonowaniu i rozwoju pułapek *N. x ventrata*. Najwyższa zawartość $O_2^{\bullet-}$ w zamkniętych pułapkach może być związana z intensywnymi procesami wzrostowymi oraz potencjalnym działaniem przeciwdrobnoustrojowym, natomiast jej obniżenie po otwarciu pułapki wskazuje na potencjalny udział ROS w regulacji mikrobiomu cieczy. Zwiększenie zawartości RNS, w tym NO i $ONOO^-$, w pułapkach dojrzałych, zwłaszcza niekarmionych, może sugerować rolę tych cząsteczek w ograniczaniu nadmiernej kolonizacji cieczy przez mikroorganizmy. Wprowadzenie pokarmu do pułapek prowadziło do ograniczenia produkcji RONS. Zjawisku temu towarzyszył wzrost całkowitej zdolności antyoksydacyjnej oraz obniżenie zawartości związków fenolowych, co może świadczyć o ich wykorzystaniu w procesach neutralizacji wolnych rodników. Uzyskane wyniki wskazują również, że zdolność do utrzymywania homeostazy ROS zmienia się wraz z rozwojem pułapek. Najwyższą produkcję $O_2^{\bullet-}$ odnotowano w pułapkach zamkniętych, co można wiązać z intensywnymi procesami metabolicznymi w fazie wzrostu. Uzyskane wyniki sugerują, że w trakcie rozwoju pułapek aktywacji ulegają mechanizmy umożliwiające utrzymanie równowagi redoks, o czym mogą świadczyć zmiany produkcji $O_2^{\bullet-}$. Wskazuje na to również, równowaga między ROS a RNS w cieczy trawiennej pułapek *N. x ventrata*, która jest ściśle regulowana i zależna od etapu rozwoju tego organu oraz obecności pokarmu. Najprawdopodobniej ma to istotne znaczenie dla procesów trawiennych.

Wyniki uzyskane w modelu „z donorem NO" potwierdzają, że wprowadzenie donora NO_x do cieczy trawiennej *N. x ventrata* wraz z pokarmem wpływa korzystnie na przebieg trawienia, przyspieszając degradację białek i stymulując aktywność enzymów proteolitycznych, co potwierdzają wyniki po rozdziale elektroforetycznym białek i wyniki oznaczeń aktywności enzymatycznej. Obecność NO_x prowadzi do zwiększenia ekspresji genów kodujących proteazy, takich jak Nep I i Nep II, szczególnie w pierwszych dniach po karmieniu, co jest zgodne z wyższą zawartością NO (Tab. 1) i obniżonym pH cieczy. Wprowadzenie NO_x przyczynia się również do szybszego wzrostu stężenia NH_4^+, co może wskazywać na udział RNS w efektywniejszym pozyskiwaniu składników odżywczych z wprowadzanego do pułapek pokarmu. Zaobserwowany wzrost poziomu transkryptów genów kodujących Pap oraz wyższa aktywność tych enzymów w cieczy trawiennej sugerują istotną rolę NO_x w mobilizacji P. NO_x dodane do cieczy trawiennej wraz

z pokarmem zmieniają zawartość RONS, ograniczając nadmierną produkcję wolnych rodników, takich jak $O_2^{\bullet-}$, poprzez obniżanie aktywności POx, (prawdopodobnie poprzez PTM), przy czym jednocześnie nie odnotowano zmian w aktywności Rboh w tkankach gruczołowych (**Publikacja 3, Fig. 4**). Wpływa to na zachowanie równowagi oksydacyjnej i sprzyja optymalnemu działaniu enzymów trawiennych. Wprowadzenie pokarmu z donorem NO_x powodowało także szybsze przywrócenie stężenia flawonoidów i związków fenolowych do wartości odnotowanych przed karmieniem, co może świadczyć o regulacyjnej roli NO w homeostazie ROS. Mimo, że zawartość grup -SH w cieczy pozostawała stabilna, ich obecność – prawdopodobnie w postaci GSH, może pełnić funkcję ochronną wobec enzymów w kwaśnym odczynie tego środowiska. Wykazano również, że białka obecne w cieczy trawiennej dzbaneczników ulegają PTM, takim jak nitrowanie Tyr i karbonylacja, niezależnie od obecności pokarmu. Występowanie tych modyfikacji sugeruje, że RONS mogą regulować aktywność enzymów trawiennych, już przed rozpoczęciem procesu trawienia, wpływając na ich stabilność. Zmodyfikowane białka, mogą pełnić funkcje sygnałowe, inicjując trawienie. Wszystkie uzyskane wyniki wskazują, że NO_x odgrywają wieloaspektową rolę w regulacji procesów trawiennych dzbaneczników, wspomagając zarówno rozkład składników pokarmowych, jak i ich efektywne przyswajanie, łącznie z ochroną przed nadmiernym stężeniem ROS.

Tab. 1 Zmiany parametrów związanych z trawieniem przeprowadzanym w pułapkach dzbaneczników po dodaniu pokarmu wraz z donorem NO_x

W tabeli zaznaczono różnice w stosunku do pułapek karmionych tylko roztworem białka jaja kurzego: ↑ podwyższenie, ↓ obniżenie, ~ brak istotnych różnic

	dzień po karmieniu z NO_x	**dwa dni po karmieniu z NO_x**	**cztery dni po karmieniu z NO_x**
Aktywność proteolityczna w cieczy trawiennej	↑	~	~
NH_4^+ w cieczy trawiennej	↑	↓	~
***Nep I* w tkance pułapki**	↑	~	↑
***Nep II* w tkance pułapki**	↑	↑	~
P w cieczy trawiennej	↑	~	~
Aktywność Pap w cieczy trawiennej	↑	↑	↑
***Pap I* w tkance pułapki**	↑	↑	↑

Wnioski i weryfikacja hipotez:

1. RNS występują w cieczy trawiennej *N. x ventrata*, niezależnie od etapu rozwoju pułapki.

   Hipoteza zweryfikowana pozytywnie.
2. NO_x stymulują ekspresję genów, na poziomie RNA, kodujących enzymy „trawienne” w tkance pułapki oraz zwiększają aktywność tych enzymów w cieczy *N. x ventrata* w czasie trawienia.

   Hipoteza zweryfikowana pozytywnie.
3. NO_x modyfikują ilość i jakość składników cieczy trawiennej *N. x ventrata* podczas procesu pozyskiwania składników odżywczych.

   Hipoteza zweryfikowana pozytywnie.
4. NO_x modulują zawartość ROS poprzez zmiany aktywność systemu antyoksydacyjnego w cieczy trawiennej *N. x ventrata*.

   Hipoteza zweryfikowana pozytywnie.
5. NO_x sprzyjają utlenianiu białek, ułatwiają ich degradację i pozyskiwanie składników odżywczych z wprowadzanego do pułapek *N. x ventrata* pokarmu.

   Hipoteza zweryfikowana pozytywnie.
7. RONS biorą udział w nitrowaniu i karbonylowaniu białek w cieczy trawiennej *N. x ventrata*.

   Hipoteza zweryfikowana pozytywnie.

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ROS and RNS Alterations in the Digestive Fluid of *Nepenthes* × *ventrata* Trap at Different Developmental Stages

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Abstract: The carnivorous pitcher plant, *Nepenthes* × *ventrata* (Hort. ex Fleming = *N. ventricosa* Blanco × *N. alata* Blanco), produces passive traps containing digestive fluid. Although reactive oxygen species (ROS) in the fluid were detected in some pitcher plants, the participation of reactive nitrogen species (RNS) in the digestion process has not yet been examined. The aim of this work was to investigate the production of superoxide anion ($O_2^{\bullet-}$), nitric oxide (NO) and peroxynitrite ($ONOO^-$) levels in the digestive fluid of traps throughout organ development. We revealed the ROS and RNS occurrence in the digestive fluid, linked to the ROS-scavenging capacity and total phenolics content. In digestive fluid from the fed traps, NO emission was higher than in the fluid from the developed unfed pitcher. The concentration of nitrite (NO_2^-) decreased in the fluid from the fed traps in comparison to the unfed ones, pointing at NO_2^- as the key source of NO. The enhanced emission of NO was associated with lowered content of $ONOO^-$ in the fluid, probably due to lower production of $O_2^{\bullet-}$. At the same time, despite a decline in total phenolics, the maximum ROS scavenging capacity was detected. In addition, ROS and RNS were noted even in closed traps, suggesting their involvement not only in digestion per se but also their action as signaling agents in trap ontogeny.

Keywords: nitric oxide; superoxide anion; carnivorous plants; pitcher plants; plant digestion

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1. Introduction

Carnivorous plants (*Plantae carnivorae*) are able to attract prey, kill it, digest the body, and absorb released nutrients. This is a relatively unusual method of nourishment for autotrophs, described as the "carnivorous syndrome". This system of obtaining nutrients from other organisms has made these creatures known as "the most wonderful plants in the world" [1,2]. Carnivorous plants do not have one ancestor and evolved independently, several times, in different parts of the world [2], and they are "the products" of an imposed adaptation to a particular environment. Carnivorous plants occur in specific habitats, mostly with moist conditions and low nutrient availability, such as swamps or peat bogs with waterlogged, poorly aerated and acidic soil [3,4]. The traps of carnivorous plants are metabolically active organs that evolve from "typical" leaves. Due to their ability to perform certain movements, traps are divided into categories: sticky, adhesive traps (fly-traps), pitcher-shaped containers, moveable snap traps, and suction bladders or eel traps [5]. The modified leaves have several features facilitating the attraction of potential prey: specific shapes, particular colors, and UV-visible patterns. Moreover, they can liberate volatiles and produce nectar [6–8].

Pitcher plants that form pitfall-shaped traps include: Sarraceniaceae, Cephalotaceae, and Nepenthaceae [9]. *Nepenthes* (Nepenthaceae) converted leaves into jug-shaped containers to lure and trap arthropods and other small animals [10]. It is currently estimated that the genus *Nepenthes* contains over 140 species [11]. These tropical plants are found mainly in Indonesia. Some species also occur in India (*N. khasiana* Hook. f.), Sri Lanka

(*N. distillatoria* L.), Seychelles (*N. pervillei* Blume), and Madagascar (*N. madagascarensis* Poir. and *N. masoalensis* Schmid-Hollinger) [12,13]. Their habitats are mostly heath forests (*kerangas*), peat-swamp forests, and mountainous forests [14]. *Nepenthes*' growth and development are usually related to the formation of dimorphic traps on the tips of their leaves: "terrestrial" and "aerial" pitchers [14,15]. The first type of trap is present mostly in young plants, which form compact rosettes and straight tendrils tipped with ovary pitchers. Mature plants, characterized by climbing stems with long internodes and curled tendrils, form "aerial" pitchers [14]. Recently, a new type of pitcher was discovered for *N. pudica*—underground traps [16]. The typical *Nepenthes* trap is divided into three zones [17], which include: a peristome (ribbed upper rim of the pitcher), a waxy zone [18], and a digestive zone [19]. A lid is considered as part of the trap, which mainly protects the trap against rain droplets [18,20].

Digestion occurs in the presence of different proteins, including Nepenthesins 1 and 2, specific aspartic proteases with broad cleavage specificity, high thermal stability, and optimal activities at acidic pH [21]. Other proteins that participate in digestion are phosphatases, chitinases, and esterases or peroxidases of class III (POx). Apart from the main function of the hydrolysis of organic compounds, some of these proteins take part in the host defense against microorganisms, their competitors for released nutrients. This function is attributed to POx, which generates reactive oxygen species (ROS). By contrast, this enzyme may also function as an antioxidant (through the reduction of hydrogen peroxide (H_2O_2) using electrons from different donors, including phenolic compounds (PCs) [22]. The nutrient absorption is achieved at the bottom of the trap equipped with a permeable cuticle and glands, which produce digestive enzymes. The transporters located across the pitcher wall are responsible for the active transport of liberated nitrogen into plant tissues [23]. The presence of thaumatin-like proteins belonging to pathogenesis-related proteins and POx has been demonstrated in *Nephenthes* traps. Additionally, some other compounds have been noted in digestive fluid, including naphthoquinones [24] and flavonols [25]. More than 15 years ago, there was a report regarding the first step of digestion in the pitcher fluid of *N. gracilis* Korth., which is accompanied by the generation of free radicals, mostly hydroxyl radicals ($^{\bullet}OH$) [26].

The incomplete reduction or excitation of oxygen leads to ROS generation [27]. The physiological activity of these compounds is dependent on their concentration. In non-stress conditions, the functionality of organisms is dependent on a low level of ROS (acting as signaling components) [28]. Furthermore, ROS participate in the removal of pathogenic microbiomes [29]. Uncontrolled ROS production or inefficient antioxidant-system activity lead to the generation of ROS at toxic levels. This increased ROS content is related to oxidative stress [30]. Commonly, the ROS family includes superoxide anion ($O_2^{\bullet -}$), as well as $^{\bullet}OH$ and H_2O_2, which are produced in plant tissues, mainly in chloroplasts, mitochondria, peroxisomes, and in the apoplast [31]. The reactivity of ROS depends on its radical/non-radical nature [32–34]. ROS react with the basic cellular molecules, including proteins and nucleic acids [28,32]. They modify amino-acid residues in peptides/proteins, leading to the modification of protein structure and function. The irreversible oxidation (e.g., carbonylation) of peptides makes them prone to faster degradation [28]. The increase in the activity of proteases was noted after the reaction of free radicals with proteins [35]. Cellular ROS balance is under the regulation of the ROS antioxidant system, which is divided into enzymatic and non-enzymatic. The enzymes that modulate ROS levels included superoxide dismutase (SOD), catalase (CAT), different peroxidases, and glutathione reductase (GR). The best-known low-molecule antioxidants are the reduced form of ascorbic acid (ASA) and the reduced form of glutathione (GSH), [32,36,37]. POxs can be exported to the extracellular space and are involved in ROS metabolism (formation or elimination) [22]. Furthermore, many of the known PCs can directly or indirectly interact with ROS. These endogenous growth regulators can chelate copper and iron ions and, thus, they are able to reduce free-radical formation due to the Fenton reaction [38].

Reactive nitrogen species (RNS) are products of nitric oxide (NO) and their reaction with oxygen or $O_2^{\bullet -}$. NO's reaction with the latter molecule leads to the formation of per-

oxynitrite ($ONOO^-$), a potent oxidizing and nitrating agent. The best-known RNS are: NO radical form ($^{\bullet}NO$), nitrosonium cation (NO^+), nitroxyl anion (NO^-), and $ONOO^-$ [39,40]. RNS are produced in various cellular compartments, including apoplastic space] [39,41,42]. Similarly to ROS, their action depends on concentration and location; they serve as regulatory or signaling molecules but are also cytotoxic (high level of RNS) [30,40,41]. In plants, NO synthesis depends on the reduction or oxidation pathways, and can be formed by enzymatic and non-enzymatic reactions. Nitrite (NO_2^-) is the key donor of various RNS. Under low pH, the protonation of NO_2^- leads to nitrous acid (HNO_2) formation; subsequently, the formation of different nitrogen oxides (NOx) depends on the actual redox state of the local environment and the presence of molecular oxygen or ROS [43,44]. Reductants increase the rate of NO generation from NO_2^- [44]. RNS react with nucleic acids, fatty acids or proteins [45]. Nitrated proteins (molecules with irreversible modified tyrosine's or tryptophane's residues) are also believed to be degraded faster, as is observed for carbonylated proteins [46,47].

It is known that ROS may be liberated outside plant tissues, including the digestive fluids of pitcher plants [26,48]. On the other hand, the emission of NO from autotrophs was studied in nitrogen-supplied plants. NO_2^- application in the growing medium of examined plants stimulated NO liberation even in darkness [49]. No data indicate the presence of NO in the digestive fluid of pitcher plants. Furthermore, little is known about the content of NO_2^-, which may be the main source of various RNS at acidic pH during digestion; the process carried out in the trap.

The aim of this work was to determine the alterations in ROS levels and RNS production in the digestive fluid of *Nepenthes* × *ventrata* throughout their organ development until their aging.

We hypothesize that ROS and RNS act as signaling molecules that regulate development of the trap and digestion process. We propose the presence of ROS–RNS crosstalk in digestion fluid, from the trap's formation until the trap's ageing.

2. Results

2.1. $O_2^{\bullet-}$ Generation in Digestive Fluid

The highest level of $O_2^{\bullet-}$ production was in the digestive fluid of the closed traps (9 nmol min^{-1} mL^{-1}) (Figure 1). In the developed traps, the $O_2^{\bullet-}$production was 6 nmol min^{-1} mL^{-1}. In the fed traps and in the aged traps, the $O_2^{\bullet-}$ production in fluid was at similar levels. In the digestive fluid of the just-opened traps, the $O_2^{\bullet-}$ generation was the lowest.

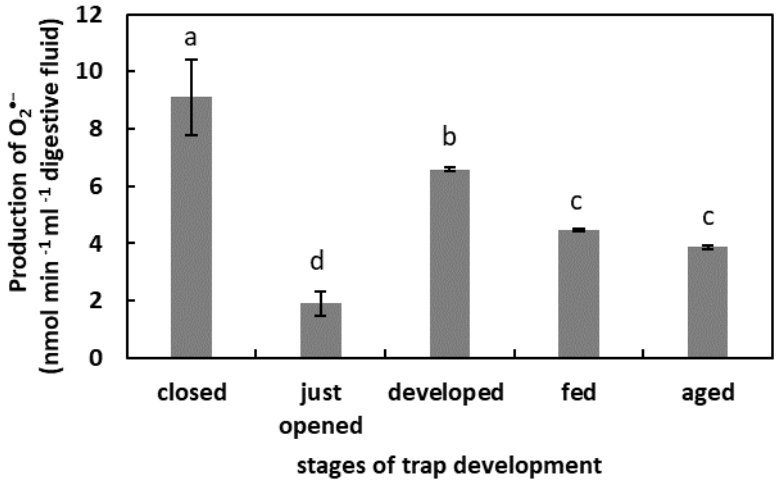

Figure 1. Superoxide anion ($O_2^{\bullet-}$) production in digestive fluid in the traps at different developmental stages: closed, just opened, developed, fed (one day after feeding), and aged. Values are average ± SD of 3 repetitions. Letters (a–d) indicate homogenous groups determined after ANOVA and post hoc Fisher's LSD test at $p \leq 0.05$.

2.2. The Free-Radical-Scavenging Capacity in Digestive Fluid

In the unfed, developed traps, the total capacity of the free-radical reduction was the lowest. One day after feeding, the total free-radical-scavenging capacity was the highest in the studied model. In the digestive fluid of the closed traps, just-opened traps, and aged traps, the total capacity of the free-radical reduction was at a similar level, of about 5% (Figure 2).

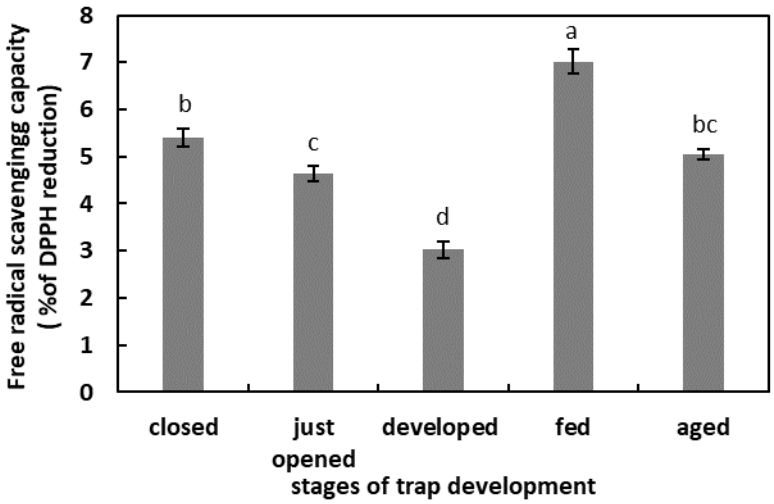

Figure 2. Total capacity of the free-radical reduction in digestive fluid in the traps at different developmental stages: closed, just opened, developed, fed (one day after feeding), and aged. Values are average ± SD of 3 repetitions. Letters (a–d) indicate homogenous groups determined after ANOVA and post hoc Fisher's LSD test at $p \leq 0.05$.

2.3. The Phenolic Compounds (PCs) Content in Digestive Fluid

In the digestive fluid of the closed and just-opened traps, the PCs content was at similar levels (below 1.4 μg mL^{-1} digestive fluid). An increase in the PCs content was observed in the digestive fluid taken from the unfed developed traps. This increase was more than 2.5 times that observed in the closed traps. The total PCs content in the digestive fluid of the fed traps and aged traps was at similar levels, about 1.8 μg mL^{-1} digestive fluid (Figure 3).

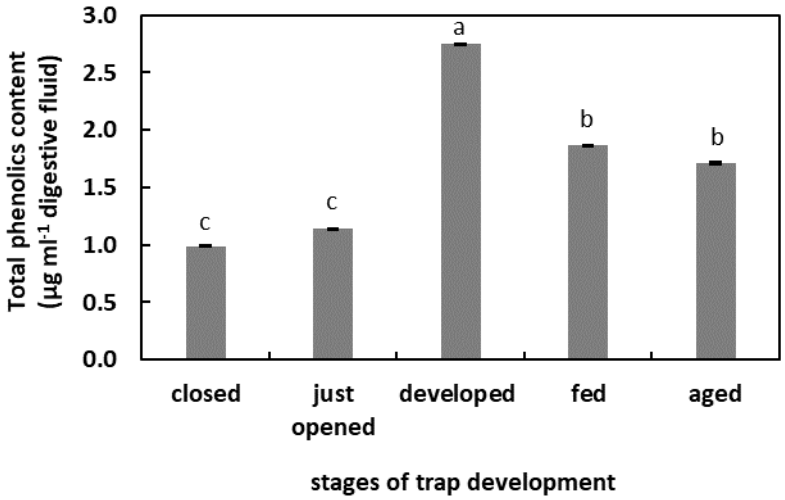

Figure 3. Phenolic compounds (PCs) content in the digestive fluid in the traps at different developmental stages: closed, just opened, developed, fed (one day after feeding), and aged. Values are average ± SD of 3 repetitions. Letters (a–c) indicate homogenous groups determined after ANOVA and post hoc Fisher's LSD test at $p \leq 0.05$.

2.4. The Generation of NO in Digestive Fluid

One day after feeding, the fluorescence of the DAF-FM was the highest in the studied model (more than 20 U) (Figure 4) The lowest fluorescence signal, without significant differences, was in the digestive fluid of the closed traps, freshly opened traps, and aged traps (around 5 U). In the developed traps that were not artificially fed, the fluorescence of the DAF-FM was 12.4 U.

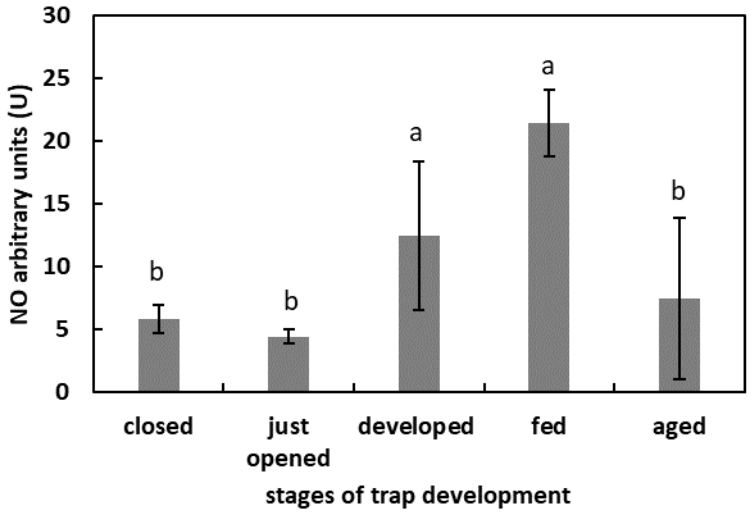

Figure 4. NO generation in digestive fluid in the traps at different developmental stages: closed, just opened, developed, fed (one day after feeding), and aged. Values are average ± SD of 3 repetitions. Letters (a,b) indicate homogenous groups determined after ANOVA and post hoc Fisher's LSD test at $p \leq 0.05$.

2.5. The Generation of $ONOO^-$ in Digestive Fluid

The highest fluorescence of APF was in the digestive fluid of the developed but unfed traps (50.2 U). In the digestive fluid of the closed, freshly opened, fed, and aged traps, the fluorescence of APF was at a similar level, of more than 5 U but below 10 U (Figure 5).

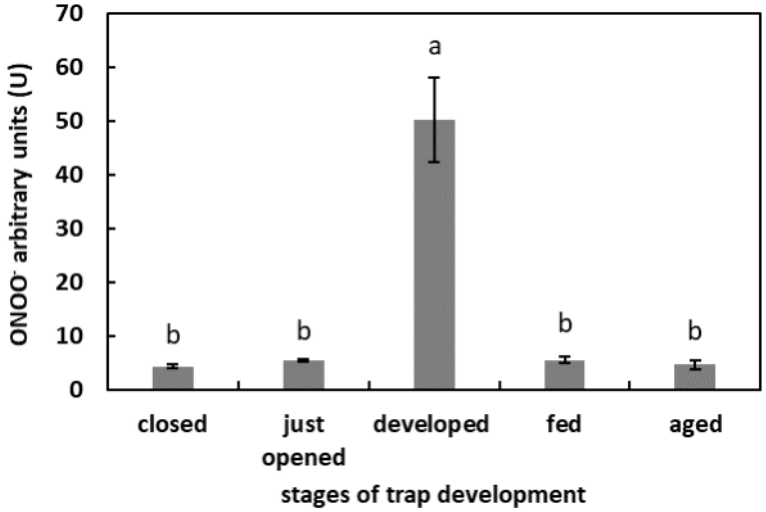

Figure 5. $ONOO^-$ generation in digestive fluid in the traps at different developmental stages: closed, just opened, developed, fed (one day after feeding), and aged. Values are average ± SD of 3 repetitions. Letters (a,b) indicate homogenous groups determined after ANOVA and post hoc Fisher's LSD test at $p \leq 0.05$.

2.6. NO_2^- Content in Digestive Fluid

In the digestive fluid of the developed traps, the NO_2^- concentration was the highest (1.1 nmol mL^{-1} digestive fluid). The NO_2^- concentration in the digestive fluid of closed, just opened, fed, and aged traps was at a similar level, about 18 μg mL^{-1} digestive fluid (Figure 6).

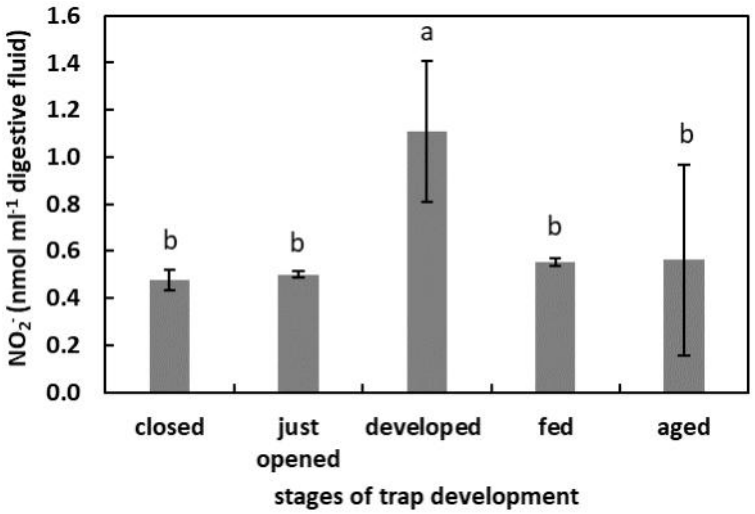

Figure 6. The NO_2^- concentration in digestive fluid in the traps at the different developmental stages: closed, just opened, developed, fed (one day after feeding), and aged. Values are average ± SD of 3 repetitions. Letters (a,b) indicate homogenous groups determined after ANOVA and post hoc Fisher's LSD test at $p \leq 0.05$.

3. Discussion

The natural environment of carnivorous plants is characterized by the poor availability of minerals in the rhizosphere. To survive, they have developed extremely modified "typical" leaves, which, in a way, replaced the roots in the function of absorbing nutrients. Pitcher plants, including *Nepenthes* of different species, developed pitfall traps; the fluid, which fills the lower part of the trap, is a specific mixture that enables the release of nutrients from the bodies of the captured prey [11,23].

ROS are indispensable products of plant cellular metabolism. As signaling molecules, ROS are known for their role in abiotic- and biotic-stress-related reactions. However, they are also involved in numerous processes throughout the plant ontogeny, from embryogenesis and germination through to root, shoot, and flower development.

Apoplastic ROS production is known to have an important regulatory effect [22]. Moreover, ROS are liberated outside the tissues, as was demonstrated for germinated seeds. The germination of radish (*Raphanus sativus* cv Eterna) seeds was accompanied by ROS and POx release [48]. The authors propose that this ROS generation is a developmentally controlled, protective mechanism against pathogen attack. The release of H_2O_2 into the germination medium of apple (*Malus domestica* Borkh.) embryos and from tomato (*Solanum lycopersicum* L.) seedlings was also shown [50,51], pointing to the universality of this phenomenon in the plant kingdom. The presence of ROS in the digestive fluid of carnivorous plants has been confirmed. An et al. [52] demonstrated that among the compounds isolated from the pitcher fluid of *N. alata* Blanco are the digested products of the exogenously oxidized proteins. Subsequently, Chia et al. [26] confirmed the presence of $^{\bullet}OH$ in *N. gracilis* Korth. digestive fluid. The authors proposed that this free radical initiates and facilitates the digestion of proteins or peptides. ROS involvement in proteolysis has been shown as protein degradation is facilitated by irreversible oxidation, mostly carbonylation [28,53]. The results of our research confirmed the presence of ROS in radical form in digestive fluid during the whole trap ontogeny, even in the closed organs. As this phase of trap development is not related to digestion, their function may be associated with

the following: the facilitation of the growth of the trap tissues; the oxidation of the fluid compounds before opening the organ to modify certain compounds, for instance, make them inactive or to alkalinize the pH, in order to prevent the activity of some pH-dependent enzymes. It has been demonstrated for some *Nepenthes* plants that the secreted fluid is bacteria-free and unsuitable for microbial growth [54]. Hence, the ROS could serve as an antibacterial agent. A highly oxidized environment can also be provided by the chlorine ions detected in the fluid of closed *N. alata* pitchers [54]. By contrast, in just-opened traps, the free-radical content strongly decreased. We assume that at this stage of trap development, it is likely that the composition of the microbiome changes [55]. An environment with strong oxidizing properties could negatively affect the growth of beneficial microorganisms. The subsequent phases of the trap's existence are associated with an increase in the ROS content until digestion. This higher ROS level may be due to the higher pH of the fluid, as pH alkalization stimulates the production of some ROS, as was demonstrated for mitochondrial ROS production [56]. The decomposition of prey bodies is linked to a slight decrease in ROS level (as the pH lowers). This physiological phase is also associated with an increase in free-radical-scavenging capacity. To avoid toxic ROS overaccumulation (e.g., plant responses to the mechanical stimuli by the caught prey), antioxidants modulate the free-radical content, and we propose that PCs play an important role in this process. The concentration of the total PCs was lower as digestion proceeded. The activity of PCs in plant responses to different stressors, including biotic stressors, is well known [57]. As substrates, PCs are also used by POxs in peroxidative cycle to reduce H_2O_2 content [22]. We propose that PCs may be used by POx to reduce ROS levels, as demonstrated for Arabidopsis' increased POx activity in response to pathogen attack [58]. The POx activity should be experimentally verified in the pitcher fluid of *N. ventrata*, although a higher level of PCs in the mature but not artificially fed traps may point to the protective role of these compounds in plant–microbe interactions. Some of these compounds may also be of microbial origin [57].

There are no research data that confirm the formation, liberation or generation of NO in pitcher fluid, especially during the digestion process. Our results demonstrated for the first time that RNS are present in *N. ventrata* trap fluid. The presented data strongly suggest that the digestion process is accompanied by increased RNS generation (detected as NO formation using the fluorescence method) in the pitcher fluid. Moreover, during trap development, changes in RNS level were observed. The lowest NO formation was noted in the closed traps and the just-opened traps. Nevertheless, the NO is clearly of plant origin. The question that arises is what the NO function is at the very beginning of the functioning of traps. The mature, opened, but unfed traps was characterized by higher NO levels in the trap fluid than in the fluid of immature or closed traps. The function of NO remains unknown. High NO levels in the fluid during digestion strongly points to the involvement of RNS in this process. We propose two roles of RNS. The first is linked to the ROS modulation by NO; the second is linked to the RNSs' action as protein modifiers. We also do not exclude the involvement of RNS- or NO^--modified molecules in long-distance signaling in the course of prey digestion. By analogy with the action of ROS, the modifications of proteins of a reversible or non-reversible nature fulfill two functions: the modification of the target protein activity and the stimulation of proteolysis. However, the question of whether NO formation after opening the trap is due to the plant's activity or to the activity of microorganisms inside the trap requires further investigation. The release of NO from plants into the surrounding environment as a response to different stimuli (e.g., light) was confirmed [49]. It has been proposed that NO emission, especially in darkness (light-exposure limitation) may be increased by NO_2^- addition, and this is a physiological reaction common to all plants [49]. Our results indicated differences in NO_2^- content in the digestive fluid. Firstly, very low levels of these ions were detected in the closed and just-opened traps. These findings are in agreement with data concerning trace amounts of nitrate (NO_3^-) in the fluid of closed *N. alata* pitchers [54]. Furthermore, the highest level was noted for the mature but unfed traps. A decrease in NO_2^- content was characteristic

for the fed traps, after one day of digestion. These results suggest that the major source of NO in the digestive fluid may be NO_2^-. The protonation of NO_2^- occurs under low pH (similarly to the human stomach), leading to the formation of dinitrogen trioxide (N_2O_3), a strong nitrosating agent (equations below). Further NO and radical nitrogen dioxide ($NO^{\bullet}_2$) are formed in the reaction of disproportionation. This NO formation requires both high NO_2^- concentration and low pH [59].

$$2NO_2^- + 2H^+ \rightleftharpoons N_2O_3 + H_2O$$

$$N_2O_3 \rightleftharpoons NO^{\bullet}_2 + NO$$

The question arises as to the source of NO_2^-. We propose that it is of both plant and prey origin. NO_2^- may be formed from NO (generated in plant tissues or produced by microbes), which reacts with oxygenated hemoproteins in a process called NO deoxygenation, catalyzed by hem-containing proteins, including hemoglobins [59], which are also present in plants [60]. These results are linked to those obtained after the measurement of $ONOO^-$ formation in pitcher fluid. The lowest $ONOO^-$ levels were noted for the closed and just-opened traps, corresponding to the lowest NO emission and NO_2^- content in the analyzed fluid at these developmental stages of the organ. As $ONOO^-$ is a strong nitration agent, it may interfere with the formation of a relationship with microorganisms, or it may modify necessary enzymes and other proteins involved in prey digestion. The highest levels of NO_2^-, the intense generation of $O_2^{\bullet-}$, and $ONOO^-$ formation were characteristic of a fully mature trap "ready" to catch prey. By contrast, even if digestion is accompanied by high NO formation in the digestion fluid, the NO_2^- content and the $ONOO^-$ formation are decreased. This points to the presence of strongly regulated NO biochemistry in digestive fluid during prey decomposition.

The ageing of traps is the last developmental stage along with the decrease in ROS and RNS formation, even as the NO_2^- level increases. It can be assumed that this tissue acts as a nutrient donor before abscission from the plant. High concentrations of NO_2^- at this stage are probably not linked to NO generation but, instead, they may protect against pathogen growth and mold development. Nevertheless, further research should be undertaken to confirm this hypothesis.

4. Materials and Methods

4.1. Plant Culture

Mature pitcher plants *Nepenthes* × *ventrata* Hort. ex Fleming (=(*N. ventricosa* Blanco × *N. alata* Blanco)) were used as plant materials. Plants were grown in a greenhouse under conditions of high humidity (60%), controlled temperature (28 °C), and natural light (spring–summer season). Plants were grown in a mixture of acid peat and perlite with sphagnum moss, and they were watered with rainwater every other day. After opening, pitcher-trap inlets were covered with a single layer of sterile gauze to control prey intake. For experiments, traps at various stages of development were selected: closed traps (Figure 7a), just-opened traps (up to 16 h after opening) (Figure 7b), fully developed, non-artificially-fed traps (Figure 7c), fully developed traps, one day after artificial feeding with two fruit flies (*Drosophilla melanogaster*) (live flies were placed in a fluid of the trap using forceps) or egg-white solution (Figure 7d), and traps with visible symptoms of ageing (browning of the tissues of the lid) (Figure 7e). To measure the generation of $O_2^{\bullet-}$, the NO_2^- content, the free-radical-scavenging capacity and the phenolic-compound content of traps artificially fed with egg-white solution, 40 µg of egg-white solution (1 μg^{-1} µL) was added to the pitchers. To measure NO and $ONOO^-$ generation, traps were fed with fruit files. Each of developmental type of trap was examined on separate plants. The digestive fluid was collected and used for analyses; after short centrifugation (5522× *g*, 5 min at 4 °C), the obtained supernatant was transferred to the sterile tubes for further analyses.

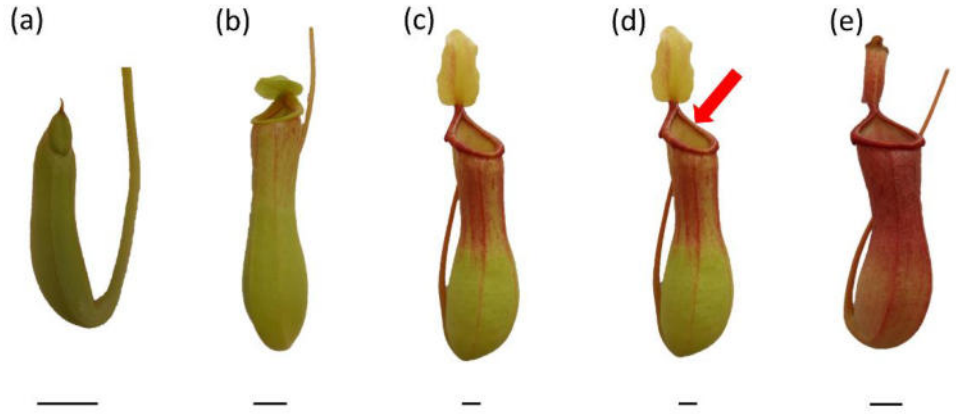

Figure 7. Traps of *Nepenthes* × *ventrata* at various stages of development: closed trap (**a**), just-opened trap (**b**), fully developed, non-artificially fed trap (**c**), fully developed trap artificially fed with two fruit flies (*Drosophilla melanogaster*) or egg-white solution (**d**), and aged trap (**e**). Bar = 1 cm.

4.2. Measurement of $O_2^{\bullet -}$ Generation in Digestive Fluid

Measurement of oxidation of epinephrine by $O_2^{\bullet -}$ was conducted according to Misra and Fridovich [61]. Digestive fluid (2 mL) was mixed with 1 M Tris-HCl pH 7.5 (0.5 mL) to buffer the sample. Next, the sample (585 µL) was mixed with 1 M Tris-HCl pH 7.5 (15 µL), and 300 µL of 60 mM epinephrine dissolved in 50 mM HCl was added. The oxidation of epinephrine to adrenochrome was measured at 480 nm (microplate reader, CLARIO star plus, BMG LABTECH) for 5 min. Autooxidation of epinephrine in the reaction mixture (without digestive fluid) was performed in each assay and obtained values were included in the calculations. The epinephrine-extinction coefficient was $\varepsilon = 4.02\ mM^{-1}\ cm^{-1}$. The results were expressed as the production of nmol $O_2^{\bullet -}\ min^{-1}\ mL^{-1}$ of digestive fluid. The measurements were conducted in three independent experiments, each in at least three biological replicates.

4.3. Measurement of the Free-Radical-Scavenging Capacity (DPPH) in Digestive Fluid

Total capacity of the reduction of the free radicals in the digestive fluid was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) [62]. The digestive fluid (150 µL) was mixed with 60 µM DPPH dissolved in 100% (*w*/*v*) methanol. The reaction mixture was incubated for 15 min in darkness at room temperature. DPPH concentration was measured at 517 nm using a microplate reader (CLARIO star plus, BMG LABTECH). Antioxidant capacity was expressed as reduction of DPPH defined as $((A_0 - A_s)/A_0) \times 100\%$, where A_0 is absorbance of a blank and A_s is absorbance of the sample. Experiments were conducted in four independent replicates with three repetitions in each.

4.4. Measurement of Phenolic Compounds (PCs) Content in Digestive Fluid

Content of total PCs in the digestive fluid was determined using the Folin–Ciocalteu reagent. The digestive fluid (730 µL) was mixed with 70 µL of the F–C reagent (MFCD00132625) and incubated for 5 min in darkness at room temperature. After incubation, 100 µL of 7.5% (*w*/*v*) sodium-carbonate solution was added into the mixture. After incubation of the mixture for 30 min in darkness at room temperature, the content of total PCs was measured at 765 nm using spectrophotometer Hitachi U-2900 (Tokyo, Japan). Blanks were prepared as described above with the replacement of a digestive fluid with distilled water. The standard curve was created using gallic acid (Sigma-Aldrich, St. Louis, MO, USA) as a standard. Total PC content was expressed as µg per pitcher^{-1} (total digestive fluid of the pitcher). Experiments were performed in four independent replicates with three repetitions in each.

4.5. Measurement of NO Generation in Digestive Fluid

NO generation in the digestive fluid was measured as efflux of derivatives of 20 µM 4-amino-5-methylamino-20,70-difluorofluorescein diacetate (DAF-FM DA, Cayman Chemical, Ann Arbor, MI, USA). A stock solution of DAF-FM DA was prepared according to manufacturer's guidelines.

Into the mixture of 1.9 mL of digestive fluid and 100 µL of 100 mM Tris-HCl buffer pH 7.4 was added a 2 nM solution of DAF-FM DA (5 µL). Fluorescence was recorded for 2000 s (excitation 495 nm, emission 515 nm) at constant temperature of 23 °C using Hitachi F-2500 (Tokyo, Japan) spectrofluorometer. As the negative controls, the digestive fluid was replaced with distilled water. The combination of 2.0 mL of 10 mM Tris-HCl buffer, pH 7.4, mixed with the solution of 2 nM DAF-FM DA (5 µL) was used for 1 arbitrary unit calculation. The value of 1U describes the minimum and maximum fluorescence Δ. The results were expressed as arbitrary U per mL^{-1} of digestive fluid. The measurements were performed in 4 repetitions, and their exact reproducibility was confirmed.

4.6. Measurement of $ONOO^-$ Generation in Digestive Fluid

$ONOO^-$ generation in digestive fluid was measured using 30-(p-aminophenyl)fluorescein (APF,), following the manufacturer's instructions. APF sensitivity is associated with the presence of $ONOO^-$ but also $^{\bullet}OH$ and hypochlorite [63].

Fluorescence was recorded for 2000 s (excitation 490 nm, emission 515 nm) using the Hitachi F-2500 (Tokyo, Japan) spectrofluorometer. A final intensity of fluorescence was taken into calculations. The results were calculated per 1 mL^{-1} of digestive fluid and expressed in arbitrary units (U). The 1U was estimated from the result obtained for the maximal intensity of the fluorescence for the probe without APF. Measurements were conducted in 3–4 independent experiments with three repetitions in each.

4.7. Measurement of NO_2^- Content in Digestive Fluid

The NO_2^--concentration measurement was conducted using Griess reagent (modified, Sigma G4410), following the manufacturer's instructions. Digestive fluid (1 mL) was mixed with 50% trichloric acid (2 µL), and vigorously vortexed. Transparent fluid (75 µL) was applied into the 96-well plates containing 75 µL of Griess reagent. After 15 min of incubation in darkness at room temperature, the absorbance was read at 540 nm using microplate reader (CLARIO star plus, BMG LABTECH). Determination was performed in the three independent experiments. NO_2^- concentration was calculated using a standard curve prepared from sodium nitrite ($NaNO_2$) dissolved in 10 mM Tris–HCl pH 7.4. Concentration of NO_2^- was expressed as nmol per mL^{-1} of digestive fluid.

4.8. Statistics

All data were obtained in at least 3 independent experiments. The plants were selected randomly for the experiments from 55 individuals; only "aerial" traps were used. Data were analyzed using Statistica Software. Mean values were calculated and SD was provided. After one-way ANOVA, homogenous groups were evaluated using Fisher's LSD post hoc test.

5. Conclusions

Pitchers of *Nepenthes* may be described as an "external stomach", as these plants digest prey in the lumen of the traps. In our experiment, a comparison of the changes in the ROS and RNS content in the fluid of developed and fed traps point to a contribution of these molecules to the digestion process of pitcher plants. The presented data indicate that digestion is linked to the emission of NO, as the fluid from the fed-trap NO level was higher than that from the developed pitcher fluid. One of the most important sources of NO could be the acidification of NO_2^-, as the concentration of these ions decreased in the fed traps in comparison to the developed traps. Although the nourishment of the plant with an animal or proteins results in the enhanced emission of NO, it is associated with decreased content

of $ONOO^-$, probably due to the lower production of $O_2^{\bullet -}$ at the same time, which may be a consequence of maximal ROS scavenging capacity at this developmental phase of the trap. In addition, because of the relatively low PCs content in the digestive fluid from the fed trap, enzymatic antioxidants could be the most important factors in the regulation of ROS levels during digestion. Thus, there is no doubt that digestion in pitcher plants is linked to ROS and RNS; however, due to the limitation of the experimental data, further investigations of the activity of enzymatic antioxidants and the concentrations of other ROS or ROS/RNS-dependent protein modifications should be carried out, in order to build up a comprehensive view of the regulation of this process.

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Załącznik nr 2 – Wzór oświadczenia o współautorstwie

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Oświadczenie o współautorstwie

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Wal A., Staszek P., Pakula B., Parandowska M., Krasuska U. (2022) ROS and RNS Alterations in the Digestive Fluid of *Nepenthes* × *ventrata* Trap at Different Developmental Stages. Plants 2022, 11(23), 3304; https://doi.org/10.3390/plants11233304.

mój indywidualny udział w jej powstaniu polegał na opracowaniu koncepcji badań, prowadzeniu kultury dzbanecznika brzusznego (*Nepenthes* × *ventrata*), współwykonywaniu pomiarów całkowitej zdolności antyoksydacyjnej cieczy trawiennej, oznaczaniu produkcji anionorodnika ponadtlenkowego, zawartości związków fenolowych i azotynów oraz produkcji tlenku azotu i nadtlenoazotynu w cieczy trawiennej. Brałam również udział w interpretacji wyników, przygotowaniu manuskryptu oraz pełniłam funkcję autora korespondującego.

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mój indywidualny udział w jej powstaniu polegał na opracowaniu koncepcji badań, sprawowaniu opieki merytorycznej w trakcie realizacji badań, udziale w interpretacji wyników, przygotowaniu publikacji oraz redakcji i korekcie całego manuskryptu.

Swój wkład oceniam na 25 %.

Urszula Krasuska

Podpis

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Review

Do Reactive Oxygen and Nitrogen Species Have a Similar Effect on Digestive Processes in Carnivorous *Nepenthes* Plants and Humans?

Urszula Krasuska, Agnieszka Wal *, Paweł Staszek, Katarzyna Ciacka and Agnieszka Gniazdowska

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Simple Summary: Traps of *Nepenthes* (pitcher plants), often referred to as an "external stomach", conduct a unique process of external digestion. In this process in pitcher plants, reactive oxygen species (ROS) primarily act positively, whereas they are considered as "evil characters" in humans. Reactive nitrogen species (RNS) have a dual role, which depends on their concentration and the place of their generation. The digestive process in *Nepenthes* is influenced by reactive oxygen and nitrogen species (RONS), which serve as regulators in both plant and human systems.

Abstract: Carnivorous plants attract animals, trap and kill them, and absorb nutrients from the digested bodies. This unusual (for autotrophs) type of nutrient acquisition evolved through the conversion of photosynthetically active leaves into specialised organs commonly called traps. The genus *Nepenthes* (pitcher plants) consists of approximately 169 species belonging to the group of carnivorous plants. Pitcher plants are characterised by specialised passive traps filled with a digestive fluid. The digestion that occurs inside the traps of carnivorous plants depends on the activities of many enzymes. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) also participate in the digestive process, but their action is poorly recognised. ROS and RNS, named together as RONS, exhibit concentration-dependent bimodal functions (toxic or signalling). They act as antimicrobial agents, participate in protein modification, and are components of signal transduction cascades. In the human stomach, ROS are considered as the cause of different diseases. RNS have multifaceted functions in the gastrointestinal tract, with both positive and negative impacts on digestion. This review describes the documented and potential impacts of RONS on the digestion in pitcher plant traps, which may be considered as an external stomach.

Keywords: carnivorous plants; *Nepenthes*; reactive nitrogen species; reactive oxygen species

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1. Carnivorous Plants

Carnivorous plants (*plantae carnivorae*) are among the most intriguing autotrophic organisms which influence human imagination. The question arises as to how this life form evolved and resembles a kind of mythological "mermaid" that entices animals, kills them, and digests their bodies. The increased interest in these plants is related to the substances synthesised in the digestive process. Some of these compounds are flavonoids or naphthoquinones, which could have potent medical uses as inflammatory therapeutics [1,2]. Pitcher-shaped trap leaves create specific environments that are rich in microorganisms producing enzymes with potential industrial uses, such as lipases resistant to low pH conditions [3].

The phenomenon of animal-eating plants began to be investigated by biologists almost two centuries ago. Darwin described them as "the most wonderful plants in the world" [4,5]. Carnivorous plants do not have one ancestor and do not belong to one systematic group.

Carnivory, as an adaptation, evolved independently in the plant kingdom at least six times in different geographical locations [5]. These plants primarily evolved in moist environments with low nutrient availability. Their typical habitats are swamps or peat bogs with waterlogged soil that is poorly aerated, which are unfavourable conditions for most higher plants [6]. Carnivory involved reprogramming standard leaf physiology from assimilates donor organs to traps. These modified leaves enabled nutrient absorption from organic matter released in the trap. The transformation of assimilatory organs into traps was accompanied by the development of specific features that attract animals to the trap, including altering the leaf colour, generating UV patterns, emitting volatile compounds, producing nectar, and generating specific shapes. These unique heterotrophs are primarily photosynthesising autotrophs that digest animals and absorb nutrients from their bodies [7–9]. The plant carnivory syndrome hypothesis considers the ability of plants to attract prey into a trap, keep it there, kill and digest the prey, and absorb the released nutrients [9,10]. Some plants that had been considered as carnivorous do not achieve all of the properties of the carnivory syndrome. Some do not attract prey, and others are unable to digest trapped organisms by themselves [10]. These plants are often called protocarnivorous or semicarnivorous.

The carnivorous plant group is estimated to contain approximately 860 species [11]. Carnivorous plants are found on almost every continent except for very cold regions [12]. The majority of carnivorous plants have developed different methods of obtaining essential mineral nutrients (e.g., N, P, or sulfur (S)) [13]. Nutrient uptake in carnivorous plants depends on specific glands that secrete digestive enzymes and (or the same) glands that enable nutrient absorption [9]. Traps are commonly grouped into five categories: sticky, adhesive traps (fly-traps), pitcher-shaped containers, moveable snap traps, suction bladders, and eel traps [14].

2. Carnivorous Pitcher Plants

Carnivorous plants that produce a pitfall-formed trap are called "pitcher plants", including Sarraceniaceae, Cephalotaceae, and Nepenthaceae [15]. The *Nepenthes* (Nepenthaceae) create jug-shaped traps that acquire arthropods and other small animals [16]. In this review the term "pitcher plants" is used exclusively for *Nepenthes*. Nepenthaceae are tropical plants that are primarily located in Indonesia, although some species occur in India (*N. khasiana* Hook. f.), Sri Lanka (*N. distillatoria* L.), Seychelles (*N. pervillei* Blume), and Madagascar (*N. madagascarensis* Poir. and *N. masoalensis* Schmid-Hollinger) [17,18] (Figure 1). Pitcher plants grow in habitats of very low nutrient availability, such as heath forests (*kerangas*), peat swamp forests, and montane forests [19].

Figure 1. The worldwide range of the genus *Nepenthes*. POWO (2021) © RBG Kew.

Nepenthes generate dimorphic traps on their leaf tips during ontogeny. The first trap type (terrestrial pitcher) rests on the ground and is commonly ovoid-shaped. The second trap type (funnel-shaped) is located above ground level and is known as an aerial pitcher [19,20]. The terrestrial pitcher trap is characteristic for young plants, which form compact rosettes and straight tendrils tipped with ovary pitchers. Mature plants are characterised by climbing stems with long internodes and curled tendrils, which enable attachment to surrounding plants [19]. Recently, a species of *N. pudica* [21] was also discovered that produces lower pitchers located only underground. Traps are present on wholly or partially achlorophyllous shoots. The pitchers resemble terrestrial pitchers in shape and structure [21].

Nepenthes leaves have similar morphologies, consisting of an extremely enlarged photosynthetically active leaf base called a lamina, and a tendril that carries the trap (Figure 2). Gravity is an important factor in the trapping process as an attracted prey falls into the hollow trap and has no way to climb out due to the trap structure [22]. The *Nepenthes* pitcher trap is divided into three functional parts (basic zones) [23]: a peristome (ribbed upper rim of the pitcher), a waxy zone [24], and a digestive zone [25] (Figure 2A). The trap lid is considered as a separate zone of the pitcher (the fourth functional zone), which primarily protects the trap against rain droplets [22,24] (Figure 2B). The top part of the trap is (usually) a hood-shaped appendix with glands that function to attract a potential prey. These glands are called extrafloral nectaries (EFNs) because they secrete sweet nectar and volatile compounds. EFNs also are located on the tendril [19]. The largest and most tightly packed glands may be present on the peristome [19]. The peristome surface is generally wet and slippery, with inward-pointing hairs that cause the attracted prey to fall into the trap. The outside part is usually rough and hairy and possesses longitudinal rims to facilitate the animal access to the attractive peristome of the trap [22].

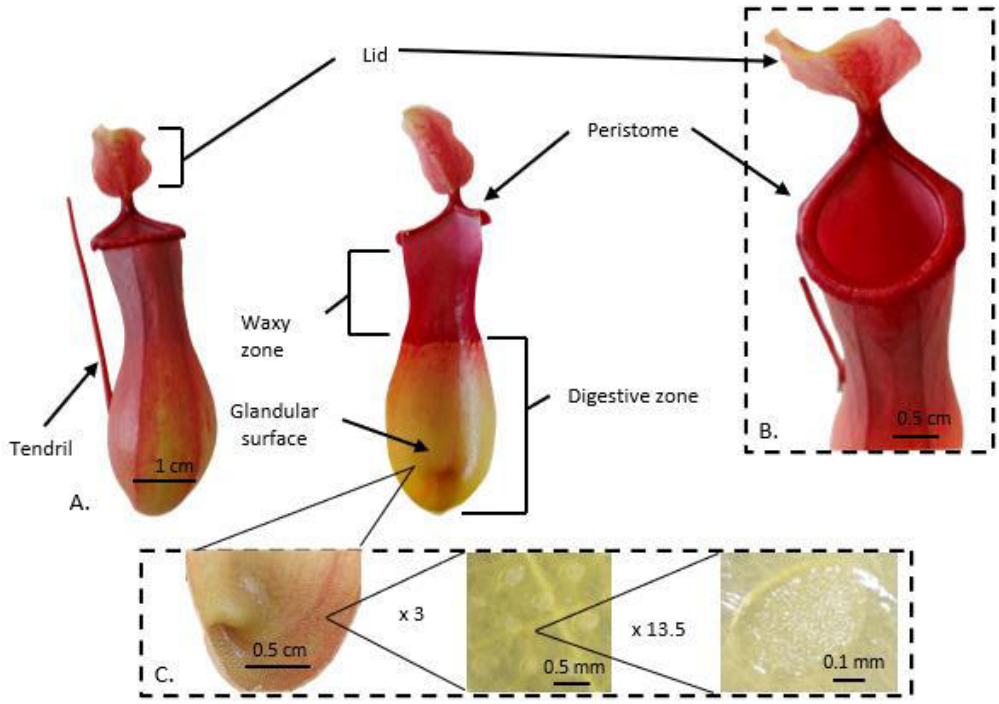

Figure 2. The aerial trap of the pitcher plant. (**A**) External and interior images show characteristics of the mature trap of *Nepenthes ventrata* Hort. ex Fleming (=*N. ventricosa* Blanco x *N. alata* Blanco). A lid is marked in the image of the whole (intact) trap tendril. (**A**) The peristome, glandular surface, and digestive zone are marked in the image of the trap cross-section. (**B**) Close-up image of the upper part of the trap. A peristome and a lid are marked. (**C**) shows images of the digestive glands at magnitudes of approximately 3× and 13.5×.

The upper part of the trap prevents the prey from climbing out because the inner surface is generally covered with downward-pointing hairs or loose wax crystals. The waxy zone contains modified stomata of hypertrophied guard cells (lunate cells) with curved surfaces that block climbing insects [24]. Insect escape is also made more complex by the anisotropic properties of the waxy zone of pitcher plants. The presence of moon cells causes insects to move at high speed and without innards towards the digestive zone, while their movement towards the peristome is difficult or even prevented [26]. The rough and non-cohesive surface of the wax crystals reduces insect adhesion to the waxy zone [25,27]. Even though the waxy zone plays such an essential role in reducing the adhesion of insects and preventing them from moving toward the trap opening, some pitcher plants, for example, *N. ampullaria* Jack and *N. bicalcarata* Hook.f. do not have this zone [28,29]. The released nutrients are adsorbed at the bottom of the trap, which is equipped with a permeable cuticle and glands (Figure 2C) that produce digestive enzymes. Transporters are located across the pitcher wall and actively transport liberated N into plant tissues [30]. The digestive fluid covers the lower part of this zone. The digestive fluid of pitcher plants, due to its unique physicochemical properties, plays not only an important role in digestion but also in the mechanical retention of the prey inside the trap [31]. In some species, e.g., *N. rafflesiana* Jack, the digestive fluid has viscoelastic properties that facilitate the prey drowning [19].

Several digestive enzymes and peptides have been identified in the pitcher fluid of various *Nepenthes* species [2,10,30,32,33]. The presence of thaumatin-like proteins belonging to pathogenesis-related proteins (PRPs) has been reported. PRPs inhibit the growth of microbial competitors (also fungi) in the digestive fluid [2]. Other compounds identified in the digestive fluid are: naphthoquinones, among others plumbagin and droserone, [33,34] and flavonols such as quercetin and kaempferol [35].

Some *Nepenthes* species differ in the morphology and functionality of the pitcher zones and in trapping mechanisms, such as *N. rafflesiana*, which exists in diverse ecological and morphological forms [19]. These alterations enable the examination of the capture strategy of carnivorous plants, by the development of plant–animal cooperation [19] as described below. Some pitcher plants have abandoned the typical "predator" system of nutrient acquisition. *Nepenthes* absorb N from different sources of animal or plant origin [36]. For example, *N. lowii* Hook.f. and other *Nepenthes* produce dimorphic pitchers. Typical terrestrial trap structures that capture arthropods are formed only by immature carnivorous plants. When the mature plant gains the ability to develop aerial pitchers, it changes the method of N uptake. Aerial traps are highly lignified with a modified lid and secrete buttery white exudates that attract the mountain treeshrew (*Tupaia montana*). This small mammal consumes nectar from the pitcher lid and excretes faeces into the pitcher. Nitrogen delivered by treeshew faeces accounts for 57–100% of nutrients absorbed by the plant, and is the primary source of this one macronutrient for leaves [36]. The elongated traps of *N. baramensis* utilise N captured from the faeces of Hardwicke's woolly bats (*Kerivoula hardwickii*) [16,37]. These bats provide approximately 34% of N absorbed by the plant leaves [16,37].

3. *Nepenthes* Trap as a Human Digestive System

Despite the lack of similarity in the structure of the pitcher plant trap and the digestive system of animals, they perform comparable functions. A thorough description of the various digestive systems of carnivorous plants has recently been reviewed by Freund et al. [38]. The processes that occur in the pitcher plant's regimen can be compared with the action of the human gastrointestinal (GI) tract. However, this is an oversimplification of the complex mammalian digestion. Nevertheless, the liberation of nutrients from an animal body in the pitcher bowl is similar to the softening of food in the GI tract (Figure 3). Food digestion and nutrient absorption in humans are mediated by defined components of the GI tract and digestive glands. The GI tract comprises the oral cavity, oesophagus, stomach, small intestine, large intestine, rectum, and anus. Nutrient release from food is mediated by the stomach and other GI compartments and juices secreted from salivary glands, pancreas,

exocrine liver, and mucosal glands [39]. An undisturbed digestive process depends on other components of the GI tract, including smooth muscle activity, epithelial cells, and endocrine cells [39]. Carbohydrates, fats, and proteins are degraded by digestive enzymes such as amylases, lipases, endopeptidases, and proteases [40].

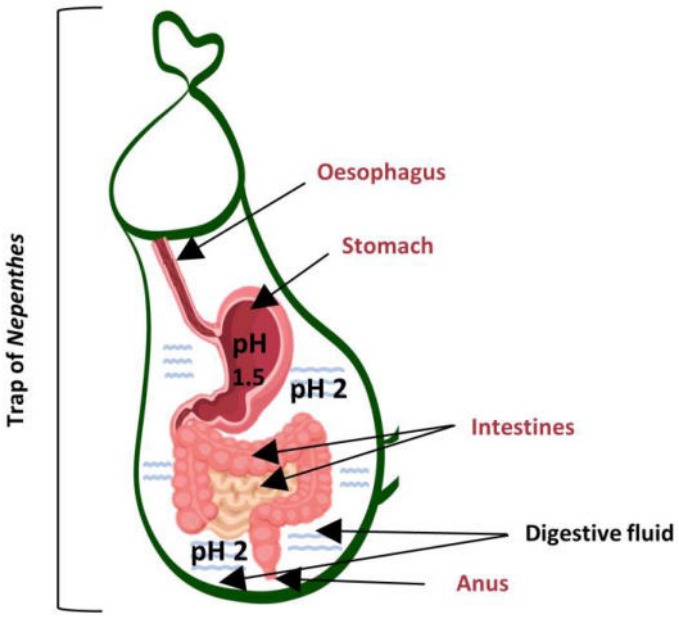

Figure 3. A diagram showing a *Nepenthes* trap as an organ corresponding to the human GI tract consisting of oesophagus, stomach, intestines, and anus. The value of the pH in the stomach is 1.5. A similar pH value, around 2.0, is in the digestive fluid of pitcher plants where the digestion of the prey is carried out. The trap has no human rectal-like area. In pitcher plants, undigested food debris is removed along with the ageing trap.

Human gastric fluid contains a high concentration of hydrochloric acid, which lowers its pH. High acidity maintains sterile conditions in the stomach and promotes the digestion of food proteins. The secretion of gastric acid is modulated by endocrine, paracrine, and nerve pathways, which mediate gastrin secretion, histamine and somatostatin release, and acetylcholine release, respectively. The inhibition of gastric acid secretion is under the control of the proton level in the gastric cavity [41]. Acid secretion is stimulated by histamine via the activation of the histamine H2-type receptors of parietal cells [42].

Stomach pH has been decreasing during three million years of human evolution. High stomach acidity was favoured as it disrupted pathogens, and stomach acidity is considered to function as an ecological filter [43]. The pH value of the stomach lumen is approximately 1.5 for a healthy adult (Figure 3), but higher for a premature infant (pH > 4) or an elderly human, which increases the risk of bacterial (pathogen) infection. Carnivores have a higher stomach acidity than herbivores [43]. This adaptive phenomenon is reflected in carnivorous plants. Young traps with closed lids and visually aged traps have a higher pH in the digestive fluid. Low pH in the digestive fluid is probably linked to the elimination of excess microorganisms and potentially harmful pathogens originated from the prey [44]. The digestion in the pitcher trap is probably highly dependent on the pH of the digestive fluid and the trap age. The digestive fluid of *N. rafflesiana* was monitored, and the pH value decreased rapidly during the first few hours after the trap opening (to a pH of around 4). This value continued to decrease over one week to around pH 2, although it was not induced by the prey capture [45]. Other studies reported that the application of nitrogenous solution (mimicking the prey input) to the digestive fluid or prey capture decreased the pH of the fluid [22]. The acidity of the digestive fluid is related to the species and the food from which the plant derives nutrients [46,47].

The digestive fluid pH may become more acidic as a result of the stimulation of the hydrolytic activity by the prey. The acidification of the digestive fluid in *N. ampullaria* is due to proton secretion by epidermal cells of the pitcher [48]. The plasmalemma H^+-ATPase mediates the acidification of the pitcher fluid in *N. alata* Blanco [48]. The pitcher plant (*N. ampullaria*) also absorbs protons from the digestive fluid to prevent hyperacidity [48]. This regulation is an adaptive strategy that enables symbiotic bacteria and invertebrates to liberate N from the prey [30]. The authors demonstrated that after the pH is reduced to 4 in the pitcher trap, plants actively uptake protons in most zones of the pitcher to increase the pH of the digestive fluid. The lowest proton efflux rate was observed if the digestive fluid pH reached 6 [30].

Closed traps are sterile and contain only the plant-derived compounds [49], which facilitate the identification of specific proteolytic activities in pitcher traps. Protease activity during pitcher plant digestion can be detected and measured by conducting peptide-based fluorescence resonance energy transfer (FRET) [49]. Many hydrolytic enzymes which soften the prey and liberate nutrients have been identified in the trap fluid [50]. The protein identification of the secretome of closed and opened traps collected from *N. mirabilis*, *N. alata*, *N. sanguinea*, *N. bicalcarata*, and *N. albomarginata* confirmed the presence of many hydrolytic enzymes: aspartic proteases, glucanases, a cationic peroxidase, and class I, class III, and class IV chitinases, as well as a PR-1 protein and a thaumatin-like protein [51]. In addition to these proteins, the authors identified, and compared with the *Nepenthes* nucleotide database, peptides corresponding to new, putative digestive enzymes: another aspartic protease, serine carboxypeptidases of types 3, 20 and 47, α- and β-galactosidases, protein phosphatases, nucleotide pyrophosphatases/phosphodiesterases, new peroxidases of type 27 and type N, and lipid transfer proteins. Furthermore, the identification of the partial sequences pointed to additional putative enzymes: a serine carboxypeptidase, a β-xylosidase, an α-glucosidase, β-galactosidases, a β-D-1,3-glucosidase 7-like protein, and a lipase [51]. The best known nepenthesins I and II have been characterised in several *Nepenthes* species [52–54]. The acidic pH of the digestive fluid autoactivates nepenthesins [49]. These acid proteinases have a specificity to aspartic acid residues but the relationship with other aspartic proteinases is rather unclear [55,56]. In plants, under phosphate starvation or mechanical injury, self-incompatibility ribonucleases (S-like RNases) are induced. Moreover, in carnivorous plants, ribonucleases are highly expressed in the trapping organs [50,57]. The transcriptomic data of the pitcher different zones of *N. khasiana* indicated the presence of chitinases' transcripts. The expression of *NkChit2b* was detected prior to the chitin induction, suggesting the participation of these transcripts as a constitutively expressed housekeeping protein [56]. In the digestive zone of the *N. khasiana* trap, high expression of a relatively large number of putative peroxidases was observed [56]. The authors claimed that these enzymes are the key components in the prey digestive machinery and protect against pathogen attack.

4. The Role of Reactive Oxygen Species in External Digestion by Carnivorous Plants

Reactive oxygen species (ROS) are products of the incomplete reduction or excitation of oxygen [58]. High ROS concentrations are primarily linked to inefficient antioxidant systems and lead to the induction of oxidative stress, which may result in cell death. ROS are necessary to perceive "normal" or "typical" functions of an organism because they act as crucial components of signalling transduction cascades [59]. At the physiological level, ROS participate in the removal of a toxic microbiome in animals and plants [60].

ROS include the superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), and peroxyl, alkoxyl, and hydroperoxyl radicals [58]. In plant tissues, ROS are primarily produced in chloroplasts, mitochondria, peroxisomes, and the apoplastic space [61]. ROS are mainly formed in the Fenton and Haber-Weiss reactions [58,62]. The reactivity of $^{\bullet}OH$ is limited to nearby molecules, whereas H_2O_2 has a longer half-life and can be translocated to another cellular compartment [63,64]. The increased activity of the plasma membrane respiratory burst oxidase homolog (Rboh), a nicotinamide adenine din-

ucleotide phosphate (NADPH) oxidase, is linked to higher ROS generation [64]. ROS react with nucleic acids, proteins, lipids, and sugars [59], and are key factors of carcinogenesis in animals [65]. ROS attack on DNA structure results in single- or double-strand DNA breaks and DNA–protein cross-links [66]. Guanine oxidation at the C8 position leads to the formation of 8-hydroxy-2′-deoxyguanosine (8-OHdG) [67], which is mutagenic and related to mutations commonly observed in human cancers [65].

One of the most important modes of ROS action is their reactivity with amino acid residues in peptides/proteins, which leads to protein posttranslational modifications (PTMs). Carbonylation is an irreversible PTM occurring under physiological conditions [68]. Proteins with carbonyl groups formed on arginine, proline, threonine, or lysine residues usually lack normal function and are more prone to degradation [68].

The balance between ROS generation and decomposition is achieved by the antioxidant system, consisting of enzymatic and non-enzymatic components. The best known enzymatic ROS modulators are isoforms of superoxide dismutases, catalases, ascorbate peroxidases, glutathione peroxidases, and glutathione reductases. Thioredoxins, glutaredoxins, and peroxiredoxins are other proteins acting as ROS modulators [69]. Among non-enzymatic antioxidants are reduced ascorbic acid (ASA), reduced glutathione, carotenoids, and α-tocopherols [58,69,70].

The adverse effects of ROS in human digestion have been examined. ROS participate in tissue lesions during inflammatory processes [71]. Important sources of ROS include gastric epithelial cells and activated inflammatory cells such as neutrophils in infected tissues [72]. Carcinogenesis in the digestive system is strongly affected by ROS. Oxidative stress and ROS overproduction is coupled to *Helicobacter pylori* infection, one of the factors in cancer development [65]. The link between ROS generation in mitochondria and several environmental risks for the incidence of development gastric cancer has been proposed. ROS are implicated in gastric cancer invasion and metastasis [65]; their levels increase in chronic gastritis, inflammatory bowel diseases, and chronic liver diseases [73]. The results of animal studies indicate that gastroesophageal reflux stimulates ROS formation and leads to lesions of the oesophageal mucosa [74,75].

The consumption/digestion of meat (consisting of fat, proteins, and free and bound iron) by a human is strongly coupled to oxidative processes. Unstable ROS products of the Fenton reaction generate cytotoxic and genotoxic lipid oxidation products like malondialdehyde or 4-hydroxynonenal [76]. These compounds participate in protein carbonylation. Ageing tissues accumulate carbonylated protein aggregates [68]. ROS also contribute directly to protein degradation, especially of oxidatively modified proteins [77]. Protease activity in vitro is higher when the protein reacts with free radicals [78]. In *N. alata* Blanco pitcher fluid, the enzymatic digestion of products of the exogenous oxidised B chain of bovine insulin was observed [52]. Free radicals are assumed to accelerate protein breakdown, probably by their special modification (e.g., carbonylation) followed by degradation [68,77]. In plants, ROS-dependent changes in the activities of acidic proteases, chymotrypsin-like, peptidyl-glutamyl-peptidehydrolase, caseinolytic-specific, and trypsin-like proteases were reported in sugar-deprived maize (*Zea mays* L.) root tips [79], and germinating apple (*Malus domestica* Borkh.) embryos [80].

ROS are involved in the prey trapping and digestion in the carnivorous plant *N. gracilis* Korth. [81] (Figure 4). Electron paramagnetic resonance (EPR) spin trapping analysis demonstrated that the first step of the prey digestion is accompanied by the generation of free radicals (primarily $^{\bullet}OH$) in the pitcher fluid. To prove that ROS induce protein degradation in the trap digestive fluid, myosin (an abundant protein in insect bodies) was added to the fluid of a young, closed trap along with a cocktail of protease inhibitors, and gel electrophoresis of the fluid protein fraction was performed. Myosin light chains were degraded, indicating that free radicals are beneficial for prey digestion [81]. In the *N. ventrata* Hort. ex Fleming digestive fluid, the presence of $O_2^{\bullet -}$ was confirmed during the whole ontogeny of the trap, starting from the closed organs [44]. The authors proposed

that radical forms of ROS are involved not only in the digestion per se but also play a role as compounds regulating the trap microbiome, although this needs further investigation.

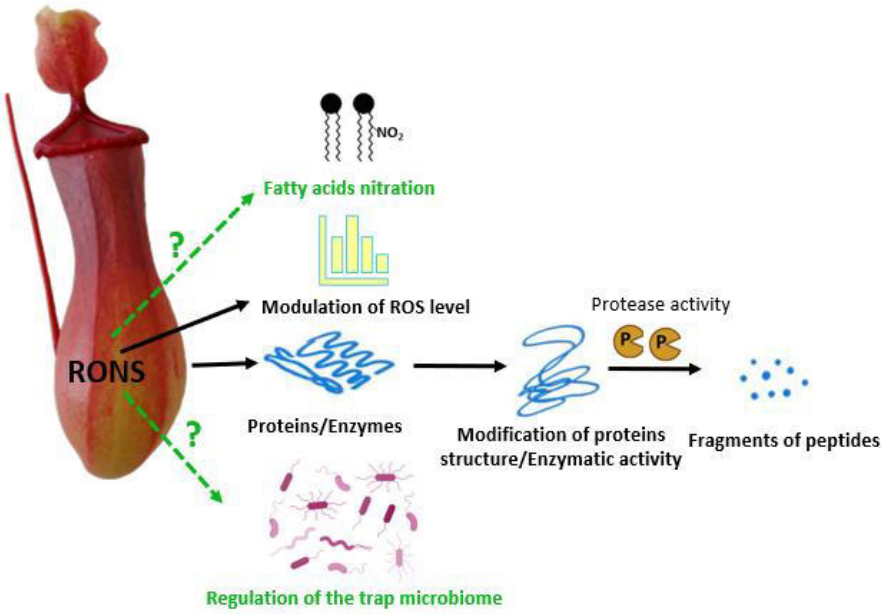

Figure 4. A scheme shows the role that RONS presumably perform (solid black lines) or may perform (dashed green lines with a question mark) in the digestion of prey by pitcher plants. RONS influence the activity of enzymes responsible for the prey decomposition, modify proteins, and facilitate their proteolysis. RNS modify the concentration of ROS in the digestive fluid. RONS alter cellular compounds, which may participate in fatty acid nitration. RNS may also have antimicrobial effects and control the growth of the microbiome in the digestive fluid in the pitcher plant's trap.

5. The Significance of Reactive Nitrogen Species in Carnivorous Plant External Digestion

Reactive nitrogen species (RNS) include nitric oxide (NO) and compounds that are the products of its reaction with oxygen or $O_2^{\bullet -}$. RNS are generated in different cellular compartments of plants and animals [82–84]. The nitrosonium cation (NO^+), nitric oxide radical ($^{\bullet}NO$), nitroxyl anion (NO^-), and peroxynitrite ($ONOO^-$) and its protonated form—peroxinitrous acid (ONOOH)—are known as RNS [82,85]. RNS, particularly NO acting directly or indirectly, have regulatory/signalling functions in various metabolic processes [59,83,85].

In plants, NO synthesis is linked to enzymatic and non-enzymatic pathways or oxidative and reductive pathways. NO is generated by nitrate reductase, mostly the NIA1 isoform, as demonstrated in *Arabidopsis thaliana* ((L.) Heynh.) [86]. Molybdenum cofactor (Moco)-containing enzymes are proposed to be involved in NO generation [87]. In mammalian cells, the main NO-producing enzyme is NO synthase (NOS). This bimodal enzyme is present in three isoforms: constitutive neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) that catalyse the release of NO and citrulline from arginine in the presence of oxygen. This reaction requires many cofactors, such as NADPH or flavin mononucleotide [88]. In higher plants, the existence of a NOS-like enzyme is still not proven [89], but its activity (a reaction requiring all cofactors and co-substrates of mammalian NOS) has been measured [84]. The failure to identify arginine-dependent and oxygen-dependent NO-producing enzymes in higher plants has caused some authors to use the term "nitric oxide generating (NOG)" instead of NOS-like enzyme [90]. One well-described autotrophic organism with a confirmed NOS protein is a green alga, *Ostreococcus tauri* [88].

Nitrite (NO_2^-) is the main non-enzymatic RNS donor. Low pH stimulates the protonation of NO_2^- to nitrous acid (HNO_2). The further formation of different nitrogen

oxides (NO_x) depends on the redox state of the local environment [91,92]. ASA and other reductants increase the rate of NO release from NO_2^- [91].

RNS like ROS modify cellular compounds such as nucleic acids, fatty acids, and proteins. The best-known RNS-initiated PTMs of proteins are *S*-nitrosation and nitration [93]. Similarly to the formation of carbonyl groups in proteins, tyrosine residue nitration is commonly accepted as an irreversible modification [94–96]. Nitro-tyrosine-containing proteins are also believed to be degraded more rapidly as observed for carbonylated proteins.

RNS are generated in various compartments of the human GI tract, including the salivary glands, oesophagus, stomach, small and large intestine, pancreatic glands, and liver. NO has several physiological and pathophysiological roles [39]. The stomach lumen is considered as the main source of NO due to the low pH and high NO_2^- concentration [92,97]. In the oral cavity, nitrate (NO_3^-) is reduced to NO_2^-, which in the gastric compartment is converted into NO. This process is accelerated in the presence of reductants, e.g., dietary polyphenols [98,99]. NO in the stomach promotes PTMs, particularly the nitrosation of gastric mucosal proteins [99]. Stomach pepsinogen and occludin undergo nitration, which modifies their biological function [97,98]. For example, the nitration of pepsinogen resulted in less efficient protease (pepsin) formation, associated with the prevention of acute peptic ulcer disease [98]. It is commonly believed that NO functions as a defence molecule against microbial pathogens. In mammals, NO also may serve as a neurotransmitter in the regulation of blood flow and saliva secretion, and may control GI motility. NO impacts blood flow in most organs of the digestive system, including the stomach, appears to regulate water secretion and exocrine pancreatic secretion [39], and is proposed to protect the stomach against injury caused by stress conditions, HCl, or ethanol [39]. NO may act as a toxic compound in cancer development due to reactions with amines and imides and the formation of N-nitroso compounds (e.g., nitrosamines), which have carcinogenic effects [100,101]. Nitrite salt is used in meat processing to inhibit *Clostridium botulinum* and induces the pink colour of meat [76]. In the acidic stomach environment, HNO_2 forms dinitrogen trioxide (N_2O_3), which is in equilibrium with NO and nitrogen dioxide ($^{\bullet}NO_2$) [101]. Such specific conditions of the stomach favour not only protein nitration but also the nitration of fatty acids. Nitro fatty acids (NO_2-FA) act as signalling molecules in animals and plants [102]. They can alkylate susceptible thiols of regulatory proteins and thus affect gene expression or the metabolic and inflammatory responses in animals. Fazzari et al. [103] demonstrated that olives and olive oil are sources of NO_2-FA. Nitrolinolenic acid at a picomolar concentration was detected in Arabidopsis tissue (seeds, seedlings, and leaves) [102]. Generation of NO_2-FA from the unsaturated fatty acids in the food under acidic gastric digestive conditions opens a new role for these redox-derived metabolites, probably typical for supporters of the Mediterranean diet.

RNS are present in *N. ventrata* Hort. ex Fleming digestive fluid [44]. The authors demonstrated that the highest NO production in the trap fluid (Figure 4) (using 4-amino-5-methylamino-20,70-difluorofluorescein diacetate, DAF-FM DA) was linked to digestion. Moreover, the main source of RNS is probably NO_2^- whose concentration decreased after food application to the trap [44]. The presence of NO_2^- and NO_3^- in the digestive fluid, even in trace amounts, was demonstrated for *N. alata* pitchers [104] and *N. ventrata* [44].

RNS physiological activity may be associated with modulation of the ROS level (Figure 4). To underline the interaction of ROS and RNS, a new nomenclature of RONS for both is proposed [105]. RONS modify protein structure that impacts proteolysis, as was proposed for *N. ventrata* [44]. RNS or NO-modified molecules may act as signalling agents during prey digestion in the traps of *Nepenthes* plants. At present, the totally unknown presence or role of NO_2-FA in the digestive fluid or tissues of the trap may be a new scientific challenge, the explanation of which could change our knowledge on digestion in carnivorous plants.

6. Conclusions

Nepenthes pitchers are sometimes called an "external stomach", as these plants perform external digestion of the prey in the trap lumen. The *Nepenthes* trap functionally corresponds to the human oesophagus, stomach, and intestines (Figure 3). The residual debris of digested animals is removed as the ageing pitchers are lost. Enzymes and other chemicals are secreted into the trap lumen to digest the prey. The digestion is affected by RONS, which function as regulators in both humans and plants. In pitcher plants, ROS primarily act positively, whereas they act negatively in the human GI tract or result from pathophysiological events. RNS have a dual role in the human digestive system, which depends on their concentration and the location of generation. RNS action is probably similar to ROS action in the digestion carried out in pitcher plants (Figure 4). However, due to limited experimental data, it is difficult to draw far-reaching conclusions and further investigations should be conducted.

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Załącznik nr 2 – Wzór oświadczenia o współautorstwie

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Szkoły Głównej Gospodarstwa

Wiejskiego w Warszawie

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy:

Krasuska U., **Wal A.**, Staszek P., Ciacka K., Gniazdowska A. (2023) Do Reactive Oxygen and Nitrogen Species Have a Similar Effect on Digestive Processes in Carnivorous *Nepenthes* Plants and Humans? Biology, 12(10), 1356. doi: 10.3390/biology12101356

mój indywidualny udział w jej powstaniu polegał na opracowaniu koncepcji publikacji, sprawowaniu opieki merytorycznej w trakcie przygotowywania publikacji, przygotowaniu publikacji oraz redakcji i korekcie całego manuskryptu.

Swój wkład oceniam na 40 %.

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Warszawa, 27.05.2025

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Agnieszka Wal

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mój indywidualny udział w jej powstaniu polegał na sprawowaniu opieki merytorycznej w trakcie przygotowywania publikacji, przygotowaniu publikacji oraz redakcji i korekcie całego manuskryptu.

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Podpis

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mój wkład obejmował redakcję i korektę tekstu manuskryptu oraz współtworzenie i opracowanie rycin i schematów.

Swój wkład oceniam na 5 %.

Katarzyna Ciąćka

Podpis

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Wiejskiego w Warszawie

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mój wkład obejmował redakcję i korektę tekstu manuskryptu oraz współtworzenie i opracowanie rycin i schematów oraz sprawowaniu opieki podczas przygotowywania publikacji.

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A. Cwiewolowska

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Nitric oxide action in the digestive fluid of *Nepenthes* × *ventrata* is linked to the modulation of ROS level

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ABSTRACT

Nepenthes are carnivorous plants with photoactive leaves converted into jug-shaped containers filled with the digestive fluid. The digestion requires various enzymes and reactive oxygen species (ROS) that facilitate proteolysis. Reactive nitrogen species are present in the digestive fluid of *Nepenthes* × *ventrata*, and the increased nitric oxide (NO) formation is associated with protein degradation. The aim of the work was to verify the beneficial effect of NO application into the trap on the dynamics of protein digestion and ROS homeostasis. Measurements were done using the digestive fluid or the tissue collected from the mature pitcher plants (fed) grown in a greenhouse. Two independent methods confirmed NO formation in the digestive fluid of fed and non-fed traps. NO supplementation with food into the trap accelerated protein degradation in the digestive fluid by increasing the proteolytic activity. NO modulated free radical formation (as the result of direct impact on NADPH oxidase), stimulated ROS scavenging capacity, increased -SH groups and flavonoids content, particularly at the beginning of the digestion. In non-fed traps, the relatively high level of protein nitration in the digestive fluid may prevent self-protein proteolysis. Whereas, after initiation of the digestion decreasing level of nitrated proteins in the fluid may indicate their accelerated degradation.

Therefore, it can be assumed that NO exhibits a protective effect on the fluid and the trap tissue before digestion, while during digestion, NO is an accelerator of protein decomposition and the ROS balance keeper.

1. Introduction

Pitcher plants (*Nepenthes*) are carnivorous plants that developed passive jug-shaped traps at the end of the main vein of the leaf blade (Mithöfer, 2011). They are native species mainly found in Southeast Asia. The increased interest in carnivorous plants, among them *Nepenthes*, is associated not only with their attractive appearance, but also with substances that have pharmacological potential. Some of them have been used in traditional medicine (Kováčik et al., 2012). The pitcher trap is commonly divided into specific zones due to the functions they exhibit: attracting a prey, facilitating its fall into the digestive fluid, and the prey's digestion/nutrient absorption (Adlassnig et al., 2011; Krasuska et al., 2023). The digestive fluid contains hydrolytic enzymes (e.g. nepenthesins I and II), thaumatin-like proteins belonging to the pathogenesis-related proteins (PR), naphthoquinones, flavonols and other chemicals (Aung et al., 2002; Mithöfer, 2011). Identification of metabolites in the digestive fluid, including phenolic compounds, is a huge challenge. The quantity and quality of phenols depend, besides others, on the plant "diet" and the phase of the digestion (Rosli et al., 2021). In many plant species, apoplastic phenolic compounds play various physiological roles, e.g. by reactive oxygen species (ROS) scavenging resulting in the mitigation of the oxidative stress (Kováčik et al., 2012). The presence of ROS in the digestive fluid, as well as their involvement in prey digestion performed by the *Nepenthes gracilis* Korth. has been demonstrated (Chia et al., 2004). The authors indicated the most crucial role of free radicals (primarily •OH) during the first phase of prey digestion. Recently, the generation of reactive nitrogen species (RNS) in the digestive fluid was confirmed for the *Nepenthes* × *ventrata* Hort. ex Fleming (Wal et al., 2022).

Nitric oxide (NO) and products of its reaction with oxygen or ROS, e.g. superoxide anion ($O_2^{\bullet-}$), belong to RNS. These compounds are generated in different plant cellular compartments, also in the apoplastic space (Del Castello et al., 2019; Kolbert et al., 2019). Depending on the

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Abbreviations:

3-NT - 3-nitroTyr
CTRL - the trap fed with egg white solution
daf - days after feeding
EWS - egg white solution
NF - non-fed trap
NO - nitric oxide
NO sample - the trap fed simulteneusly with food together with NO donor
POx - peroxidases of class III
PR - pathogenesis-related proteins
PTMs - post-translational protein modifications
RNS - reactive nitrogen species
ROS - reactive oxygen species

concentration RNS have a signalling role at low concentrations or are cytotoxic at high concentrations, and participate in many, basic physiological processes (Ciacka et al., 2020a; Del Castello et al., 2019; Stamler et al., 1992). In higher plants, NO synthesis is linked to the enzymatic and non-enzymatic reactions (pathways related to oxidation or reduction). Depending on oxygen availability or metabolic activity one or another pathway dominates. The enzymatic, oxidative NO formation from L-arginine by the activity of NO synthase(-like) is still questioned (Corpas et al., 2022a). Oxygen deprivation is not rare in plant tissues. Thus, the enzymatic NO synthesis may be linked to the reduction of nitrite by nitrate reductase (Mohn et al., 2019). Under such conditions, mitochondria reduce nitrite to NO via electron transport chain, and alternative oxidase may play a special role through this pathway (Gupta and Igamberdiev, 2011; Jayawardhane et al., 2020). Perception of RNS and activation of specific cascades signalling pathways occur via reaction with amino acid residues in proteins, leading to post-translational protein modifications (PTMs), mostly nitration and *S*-nitrosation (Ciacka et al., 2020a). Tyrosine (Tyr) nitration is commonly recognised as an irreversible modification. Proteins with 3-nitroTyr (3-NT) have modified configuration which implicates their structure and turnover (Corpas et al., 2020). NO, as a redox-modulator is involved in ROS level regulation (Ciacka et al., 2020a; Valderrama et al., 2021).

The incomplete reduction or excitation of oxygen lead to the formation of ROS, e.g. $O_2^{\bullet-}$ or hydroxyl radical ($^{\bullet}OH$) (free radicals), and hydrogen peroxide (H_2O_2) (non-radical form) (Mittler, 2017). In plants, ROS are produced in chloroplasts, mitochondria, peroxisomes, and in the apoplastic space (Corpas et al., 2015). The reactivity of free radicals is limited, mainly to the nearby molecules, as these compounds are stable for a very short time. On the contrary, H_2O_2 has a lower reactivity, and a longer half-life but is more mobile, thus engaged in the regulation of various physiological processes as signalling molecule (Kumar et al., 2015; Mittler, 2017; Møller et al., 2011). The multilevel system of antioxidants and prooxidants balances ROS production and scavenging, provides cells redox homeostasis, and prevents free radicals toxicity (Mittler, 2017; Mittler et al., 2004).

Thiol (-SH) groups of cysteine (Cys) residues are the majority form of reduced sulfur in plants. They occur as low molecular weight thiols or/and as proteins' thiols (Corpas et al., 2022b; Tausz et al., 2003). Thiol compounds have reducing properties and are crucial in plant responses to stresses. As antioxidants, e.g. reduced form of glutathione (GSH), thiols are cellular protectors from damage caused by ROS (Pivato et al., 2014). The redox state of cellular compartments serves as valuable information for potential reversible or irreversible oxidative PTMs (Corpas et al., 2022b). One of the irreversible PTMs caused by ROS is carbonylation and proteins with carbonyl groups are more prone to degradation (Ciacka et al., 2020b). In apple (*Malus domestica* Borkh.) embryonic axes higher proteolytic activity linked to the lowering of carbonylated proteins level was demonstrated (Krasuska et al., 2014). Proteases hydrolyse peptide bonds between two amino acids in the substrate peptide, thus participating in protein maturation or degradation (Fernández-Fernández et al., 2023). These enzymes also undergo regulation by PTMs, pH, and redox potential of the catalytic environment (Fernández-Fernández et al., 2023).

Extracellular generation of ROS is linked to the activity of a plasma membrane respiratory burst oxidase homolog (Rboh), a NADPH oxidase. Rboh reduces O_2 to $O_2^{\bullet-}$ using NADPH as the electron donor (Kumar et al., 2015). Elevated ROS production by Rboh occurs in plants mostly under stress conditions (Souri et al., 2021). ROS production due to the overexpression of Rboh coding genes improves plant resistance to pathogen attack (Simontacchi et al., 2015). Enzymes that are involved in the detoxification of H_2O_2 are plant-specific heme oxidoreductases - peroxidases of class III (POx, EC 1.11.1.7). Their activity is related to the oxidation of substrates, scavenging of ROS, and the formation of radicals (Shigeto and Tsutsumi, 2016). POx have a diverse range of functions and play an important role in many physiological processes, e.g. lignification, plant defence, and seed germination (Pandey et al., 2017; Shigeto and Tsutsumi, 2016). It is suggested that they function as indicators of stress responses (De Gara, 2004).

The contribution of ROS in the specific nutrition performed by *Nepenthes* seems to be complex. This is demonstrated by the presence of $O_2^{\bullet-}$ in the traps' digestive fluid of *Nepenthes* × *ventrata* during the ontogeny of the organ (Wal et al., 2022).

ROS action and RNS action during digestion performed by carnivorous plants are closely linked (Krasuska et al., 2023). Since the highest rate of RNS formation was recorded in the *Nepenthes* × *ventrata* fluid after the application of food into the trap, these compounds are most probably involved in digestion (Wal et al., 2022). Thus, the aim of this work was to demonstrate that the rate of protein degradation depends on the current amount of NO formed in the fluid. We have focused our interest on the modulatory role of NO on the dynamics of digestion and the modulation of ROS homeostasis in the *Nepenthes* × *ventrata* trap (digestive fluid and traps' tissues).

2. Materials and methods

2.1. Experimental model and plant culture

Pitcher plants *Nepenthes* × *ventrata* Hort. ex Fleming (=(*Nepenthes ventricosa* Blanco × *Nepenthes alata* Blanco)) were used as the experimental material. Plants were cultivated under controlled conditions in a greenhouse with high humidity (60%) and a temperature of 28 °C. During the spring and summer, they were exposed to natural light, while in autumn and winter, sodium lamps were used to maintain a 16/8 h (day/night) photoperiod. The plants were grown in pots containing a mixture of acidic peat, perlite, and *Sphagnum* moss. They were watered with rainwater every other day. The traps of pitcher plants, immediately after opening, were covered with sterile gauze to avoid contamination by accidental insects (Fig. 1A).

2.2. Application of food and NO_x donor to the digestive fluid

For experiments, digestive fluid and plant tissue (glandular zone), were collected from the mature pitcher plants (3 days after opening the trap), non-fed (NF) (Fig. 1B) traps and fed traps, as described by Wal et al. (2022). The control (CTRL) was fluid collected from the traps fed by adding 40 μl egg white solution (EWS, 1 μg μl^{-1}). For experiments determining NO levels, two fruit flies (*Drosophilla melanogaster*) were added to the digestive fluid directly into a pitcher (live flies were placed into a fluid of the trap using forceps) instead of egg white solution, as this solution showed strong autofluorescence and disrupted the measurements. The NO samples (NO) (Fig. 1B) were fluid with NO_x donor: 50 μl mixture of 0.1 M $NaNO_2$ with 1 mM HCl (Yamasaki, 2000) added together with egg white solution or two fruit flies (food).

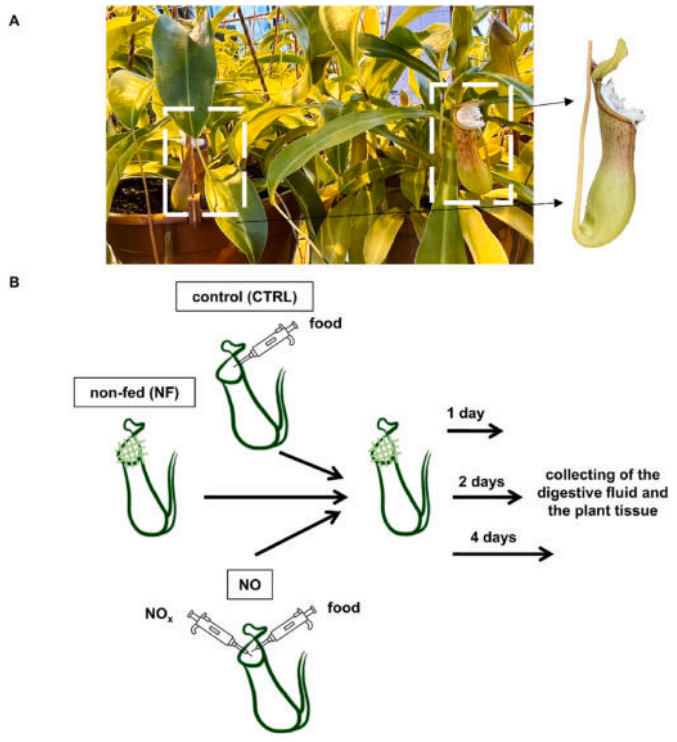

Fig. 1. The culture of *Nepenthes* × *ventrata* plants in the greenhouse. The photo shows traps after opening covered with sterile gauze (A). The scheme of the experimental model related to the samples collection (the digestive fluid and/or the plant tissue). NF (non-fed) - the material collected before the application of food. The control (CTRL) – the material collected after food application, and NO - the material collected after the simultaneous application of food and NO donor (B).

Plant tissue and digestive fluid were collected from the NF trap, from the CTRL, and NO samples after 1, 2 and 4 days after feeding (daf) into sterile test tubes (Fig. 1B). The digestive fluids from NF, CTRL, and NO samples were shortly centrifuged (5522×*g*, 5 min at 4 °C), and the supernatants were used for analyses. Fresh plant tissue was used for histochemical staining. All spectrophotometric measurements were done using the microplate reader, CLARIO star plus, BMG LABTECH.

2.3. Electrophoretic separation of proteins from the digestive fluid

Separation of proteins from the pitcher plant's digestive fluid was done using the SDS-PAGE method (Laemmli, 1970). Protein precipitation: cold acetone 100% was added to the digestive fluid (4:1) and incubated for 24 h at −20 °C (Biteau et al., 2013). Next, the samples were centrifuged 20 min at 12000×*g* at 4 °C. The obtained pellets were briefly dried and then resuspended in 45 μl Laemmli buffer: 63 mM Tris-HCl, pH 6.8, 1% (w/v) SDS, 10% (v/v) glycerol, 0.01% (w/v) bromophenol blue and 20 mM DTT by sonication 15 min at 4 °C. Samples were denatured for 10 min at 70 °C. The whole volume of each sample was loaded on 10% polyacrylamide gels with SDS and separated. After separation, the protein bands were visualised using Coomassie brilliant blue staining. The excess dye was rinsed off using a mixture of methanol (99,5%), acetic acid 80%, and water (6:1:13). Images of representative gels were shown after processing photos in Image Lab Software. The original images of the gels are shown at Fig. S1.

2.4. Measurement of proteolytic activity in the digestive fluid

Prior to conducting analyses, the digestive fluid was buffered by mixing with 1 M buffer in a 9:1 ratio. The buffers were selected to stabilise the pH of the digestive fluid at the level specific to the respective day of the culture. K-phosphate buffer, pH 6.5, was added to the fluid from the non-fed traps, glycine buffer pH 4 was added to the fluid from the 1 daf traps, glycine buffer pH 3 was added to the fluid from the 2 daf traps and K-phosphate buffer, pH 6 was added to the fluid from the 4 daf traps. The substrate for the proteases was prepared by dissolving 40 mg of azocasein in 1 ml of 0.1 M KOH. To 1.4 ml of the buffered digestive fluid, 50 μl of the 4% azocasein solution was added and incubated for 1 h at 37 °C. After incubation and the addition of 75 μl trichloroacetic acid (TCA) (100%), proteins were precipitated for 10 min at room temperature (RT) in the dark. The mixture was then centrifuged at 15000×*g* for 15 min. To 500 μl of supernatant, 500 μl of NaOH was added. Absorbance was measured at 440 nm. The results were expressed as U min^{-1} ml^{-1} digestive fluid, 1 U is $A_{440} = 0.1$.

2.5. Measurement of NO production in the digestive fluid

NO production in the digestive fluid was measured as an efflux of derivatives of 4-amino-5-methylamino-20,70-difluorofluorescein diacetate (DAF-FM DA, Cayman Chemical, Ann Arbor, MI, USA) described by Wal et al. (2022). DAF-FM DA was prepared as a stock solution dissolved in dimethyl sulfoxide (DMSO). A mixture of the digestive fluid (1.9 ml) with 100 mM Tris-HCl buffer pH 7.4 (100 μl) and 2 nM DAF solution (5 μl) was prepared. Control samples were prepared by replacing the digestive fluid with distilled water. Fluorescence was recorded for 2000 s (excitation 495 nm, emission 515 nm, spectrofluorimeter, Hitachi F-2500). The value of 1 U corresponds to the difference between the maximum and minimum fluorescence. To calculate 1 U, a mixture of 2 ml of 10 mM Tris-HCl buffer (pH 7.4), distilled water, and 5 μl of 2 nM DAF-FM DA solution was used.

2.6. Measurement of NO concentration in the digestive fluid

The measurement was carried out using a Nitric Oxide Measuring System (amiNO-700, Innovative Instruments), according to Foresi et al. (2016), with modifications. Before the measurements, the device was calibrated, and the tests were carried out at a constant temperature of 22° C. The standard curve was prepared from the 100 μM $NaNO_2$. $NaNO_2$ was added to the calibration buffer: 3 mM potassium iodide (KI) and 0.1 M sulphuric acid to receive concentrations of 50 nM, 100 nM and 200 nM, and is shown at Fig. S2. The blank was distilled water instead of the digestive fluid. The NO concentration was expressed as nmol l^{-1} digestive fluid.

2.7. Measurement of the production of $O_2^{\bullet-}$ in the digestive fluid

Generation of $O_2^{\bullet-}$ was measured by oxidation of epinephrine according to Misra and Fridovich (1972) with some modifications by Wal et al. (2022).To 2 ml of digestive fluid, 0.5 ml of 1 M Tris-HCl buffer (pH 7.5) was added. The buffered digestive fluid (585 μl) was then mixed with 15 μl of 1 M Tris-HCl buffer (pH 7.5) and 300 μl of 60 mM epinephrine, which had been prepared in 50 mM HCl. The oxidation of epinephrine to adrenochrome was measured at 480 nm for 5 min. Control samples (the autooxidation of epinephrine was measured) were prepared in the same manner, replacing the digestive fluid with distilled water. The epinephrine extinction coefficient was $\varepsilon = 4.02\ mM^{-1}\ cm^{-1}$. The results were expressed as the generation of nmol $O_2^{\bullet-}$ min^{-1} ml^{-1} digestive fluid.

2.8. Measurement of H_2O_2 content in the digestive fluid

Measurement of H_2O_2 concentration was determined using KI (Junglee et al., 2014). The 700 μl of digestive fluid was mixed with 1% TCA, 250 μl of 2 M KI in 10 mM potassium phosphate buffer (pH 7.0, 50 μl). The samples were incubated in the dark at RT for 15 min and absorbance was measured at 390 nm. The blank contained distilled water instead of the digestive fluid. The standard curve was prepared

using H_2O_2 (Fig. S3). The concentration of H_2O_2 was expressed as nmol l^{-1} digestive fluid.

2.9. Localisation of $O_2^{\bullet-}$ in the pitcher plant tissue

The localisation of $O_2^{\bullet-}$ in the ventral pitcher plant's tissue was done using nitrotetrazolium blue chloride (NBT, 0329, VWR) (Bournonville and Díaz-Ricci, 2011). Sections (1 × 2.5 cm) of the glandular zone of the trap were placed in a solution of 5 mM NBT in 10 mM phosphate buffer (pH 7.2). The solution was vacuum infiltrated for 3 min. After 20 min of incubation under light at RT, due to the removal of pigments, sections were placed in the mixture of 96% ethanol, chloroform and 100% TCA (140:35:0.25) for 20 h in the dark at RT. Negative controls were the sections placed only in phosphate buffer, and the other steps were performed as described above.

2.10. Localisation of $O_2^{\bullet-}$ in the pitcher plant tissue in the presence of the inhibitor of Rboh activity

Inhibition of Rboh activity was performed using of diphenyleneiodonium chloride (DPI, 2926, Sigma-Aldrich) (Jiménez-Quesada et al., 2022). The sections of the glandular zone of the trap were placed in a solution of 150 μM diphenyleneiodonium chloride (DPI) dissolved in dimethylsulfoxide (DMSO, D2650, Sigma-Aldrich) and vacuum infiltrated for 3 min. The sections were incubated for 16 h in the dark at RT. The next step was the localisation of $O_2^{\bullet-}$ with NBT, as described above.

2.11. Measurement of the total antioxidant capacity of the digestive fluid

The total free radical scavenging capacity (FRSC) of the digestive fluid was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Molyneux, 2004). To the digestive fluid (480 μl) was added 20 μl of 5 mM DPPH solution dissolved in 100% (w/v) methanol. The samples were then incubated for 30 min at 28 °C in the dark. DPPH concentration was measured at 517 nm. In a blank, distilled water was added instead of the digestive fluid. The FRSC of the digestive fluid was expressed as the reduction of DPPH calculated as: $((A_0 - A_s)/A_0) \times 100\%$, where A_0 was the absorbance of the blank and A_s was the absorbance of the sample.

The free radical-reducing capacity of low molecular weight antioxidants (LMWA) was determined with DPPH (Molyneux, 2004). Firstly, digestive fluid (1.98 ml) was mixed with 20 μl 100% TCA solution. The mixture was centrifuged for 10 min at 15000×*g* at 4 °C. The supernatant was collected (480 μl), and 5 mM DPPH (20 μl) dissolved in 100% (w/v) methanol was added. The reaction mixture was incubated in the dark for 30 min at 28 °C. DPPH content was measured at 517 nm. In the blank, distilled water was added instead of the digestive fluid. LMWA in the digestive fluid was calculated for the FRSC as described above.

2.12. Measurement of the total content of compounds with free non-protein sulfhydryl groups (-SH) in the digestive fluid

The total content of compounds with -SH groups in the digestive fluid was estimated with 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB, D8130, Sigma-Aldrich) (Chan and Wasserman, 1993). The digestive fluid (2 ml) was buffered with 1 M phosphate buffer (pH 7.5). Then, 200 mM phenylmethylsulfonyl fluoride (PMSF) (1.25 μl) and 1 mM EDTA (1.25 μl) were added. The digestive fluid (250 μl) with 6 mM DTNB (50 μl) was buffered using 0.1 M phosphate buffer (pH 7.5). The mixture was incubated in the dark for 10 min at RT. The absorbance of 2-nitro-5-thiobenzoate (TNB) was measured at 420 nm. TNB concentration was calculated using the extinction coefficient $\varepsilon = 14.15\ mM^{-1}\ cm^{-1}$. The content of -SH groups was expressed as μmol TNB ml^{-1} digestive fluid.

2.13. Measurement of POx activity in the digestive fluid

POx activity was measured by oxidation of pyrogallol to purpurogallin (Saunders et al., 1964). The digestive fluid (0.9 ml) was buffered by adding 0.1 ml of the mixture: 1 M K-phosphate buffer (pH 7.0), 30 mM EDTA, 10 mM DTT and 10 mM PMSF. The buffered digestive fluid (300 μl) was mixed with a solution of 7 mM pyrogallol (2.5 ml) (254002, Sigma-Aldrich) in 50 mM K-phosphate buffer (pH 7.0). To the reaction mixture (280 μl) 20 μl H_2O_2 or distilled water (blank) were added to start the reaction. The formation of purpurogallin was monitored at 430 nm. POx activity was expressed as nmol of purpurogallin produced in the digestive fluid (nmol purpurogallin ml^{-1} digestive fluid).

2.14. Measurement of the total phenolic content in the digestive fluid

The total phenolic content was measured with Folin-Ciocioltеu reagent (Staszek et al., 2020). The digestive fluid (730 μl) was mixed with 70 μl 100% Folin-Ciocalteu reagent. The reaction mixture was incubated in the dark for 5 min at 22 °C. Then 100 μl of 7.5% (w/v) sodium carbonate solution was added and incubated for 30 min at the same conditions. The content of phenolic compounds was measured at 765 nm. In the blank, distilled water was added instead of the digestive fluid. The standard curve was prepared using gallic acid (Fig. S4). The total phenolic content was expressed as μg gallic acid ml^{-1} digestive fluid.

2.15. Measurement of the flavonoids level in the digestive fluid

The flavonoids content was measured by the method of Silva-Beltrán et al. (2015) with some modifications. The digestive fluid (1.5 ml) was mixed with 5% $NaNO_2$ solution (75 μl). The mixture was vortexed and incubated for 5 min at 22 °C. Then 150 μl of 10% aluminium chloride was added to the reaction mixture, mixed and incubated for 6 min at 22 °C. Then 1 M NaOH (500 μl) was added and mixed. The flavonoids content was measured at 496 nm. In the blank, distilled water was added instead of the digestive fluid. The standard curve was prepared using quercetin (Fig. S5). The total flavonoids content was expressed as μg quercetin ml^{-1} digestive fluid.

2.16. Immunodetection of 3-nitrotyrosine in the digestive fluid

Immunoblotting technique was done for detection of 3-NT proteins. Isolated proteins (as described above 2.3.) were mixed with 45 μl Laemmli buffer: 63 mM Tris-HCl, pH 6.8, 1% (w/v) SDS, 10% (v/v) glycerol, 0.01% (w/v) bromophenol blue and 20 mM DTT and denatured for 10 min at 70 °C. The whole volume of each sample was loaded into the well on 3% stacking polyacrylamide gel and then 10% resolving gel with 2,2,2-trichloroethanol was used. SDS-PAGE was carried at a constant voltage of 150 V using the Mini-PROTEAN system (BIO-RAD). Then, proteins were electrotransferred to nitrocellulose membranes (Amersham™ Protran® Western blotting membranes, nitrocellulose, pore 0.2 μm) according to Towbin et al. (1979) using a Bio-Rad wet blotting apparatus. Subsequently, the proteins were visualised on the membranes and the gel via the photoreaction of tryptophan with TCE. The membranes were blocked with 5 % non-fat milk in TBST overnight at RT for 1 h. The membranes were incubated overnight at 4 °C with primary antibodies at a dilution of 1:1000 (Monoclonal Anti-3-NT antibodies, Sigma–Aldrich). After being washed in TBST three times, the nitrocellulose membranes were incubated with secondary antibodies (anti-mouse IgG conjugated with Horseradish Peroxidase Sigma-Aldrich) diluted 1:30 000 for 1 h at RT in the dark. Proteins containing 3-NT were visualised using chemiluminescence with the Agrisera kit (AgriseraECL Bright) in Bio-Rad Chemi-Doc XRS system, with the signal collected for 20 s. Images of representative membranes were shown after photos were processed in Image Lab Software. The original images of the membranes were shown at Fig. S6.

2.17. Quantitative measurement of 3-nitrotyrosine in the digestive fluid

3-NT modified proteins were analysed by ELISA method (Krasuska et al., 2016) with modifications. Proteins from the digestive fluid were precipitated as described above (2.3.), with the addition of cold 100% acetone. The obtained pellets were briefly dried and then resuspended in 100 µl of modified Laemmli buffer: 63 mM Tris-HCl, pH 6.8, 1% (w/v) SDS, 10% (v/v) glycerol, and 20 mM DTT by sonication 15 min at 4 °C. The proteins were then desalted from the above solution using 500 µl of 0.1 M ammonium acetate in ethanol (96%). Samples were incubated overnight at - 20 °C and centrifuged 15000×*g* for 15 min at 4 °C. Then 1 ml of 0.1 M ammonium acetate was added to the precipitate, vortexed, and placed into an ultrasonic bath for 10 min at 4 °C. After incubation, the samples were centrifuged 15000×*g* for 15 min at 4 °C and the supernatant was removed and the precipitate dissolved in K-phosphate buffer with 1 mM EDTA, 2% (w/v) PVPP, 2 mM DTT, 1% (v/v) protease inhibitor cocktail (Sigma–Aldrich) and 10% (v/v) glycerol. To the samples 1 M KOH was added to reach a pH level of 9. The samples (200 µl per well) were coated on 96-well plate, and incubated at 4 °C overnight. In addition, a nitrated-BSA was applied to the plate, as a positive control. Nitration was carried out by reducing BSA with 6 mM sodium dithionite and incubated 15 min at RT in the dark. The samples were then desalted by centrifugation in Pierce PES 3K concentrators (Thermo Scientific) 20000×*g* for 20 min at 4 °C. The next step was to add 24 mM sodium nitrite, 0.2 M HCl and 15% sodium chlorite and the mixture was incubated for 60 min in the dark in RT. Proteins were precipitated by adding 50% TCA incubated 20 min at RT and centrifuged 20000×*g* for 10 min at RT. The precipitate was dissolved in the same buffer as the proteins obtained from the digestive fluid and proceeded as described above. After coating, the plate was washed three times with phosphate-buffered saline (PBS) pH 7.4. The next step was blocking with 0.1% (w/v) gelatin in (tris-buffered saline) TBS (250 µl per well) (1 h at 37 °C in dark) and washing three times with TBST (TBS with 0.1% (w/v) Tween 20). Then primary antibodies (Monoclonal Anti-3-NT antibodies, Sigma–Aldrich) were added at a dilution of 1:1000 (200 µl per well), and incubated for 1 h at 37 °C in dark and the plate was washed three times with TBST. Plate was covered with secondary antibodies (anti-mouse IgG conjugated with Alkaline Phosphatase Sigma–Aldrich) at a dilution of 1:30 000 for 1 h at 37 °C in dark. Nitrated groups were visualised using alkaline phosphatase substrate – pNPP (P4744 Sigma-Aldrich) in 0.1 M glycine buffer, pH 10.4, with the addition of 1 mM $MgCl_2$ and 1 mM $ZnCl_2$. The reaction was terminated with 5 µl of 5 M KOH and absorbance measurement at 405 nm. The concentration of 3-NT was calculated based on the content in the nitrated standard. The results were presented as nmol 3-NT protein ml^{-1} digestive fluid.

2.18. Statistics

The plants were selected randomly from 50 individuals; only "aerial" and mature traps were used. One trap was collected from one plant, and it was one biological repetition. Measurements were done in 3–5 biological replicates, with four technical replicates for each. Data were analysed using Statistica Software. Mean values were calculated, and SD was provided. After one-way ANOVA, homogenous groups were evaluated using Fisher's LSD post hoc test.

3. Results

3.1. The profile of proteins isolated from the digestive fluid

The separation of proteins of egg white solution dissolved in deionised water (W + EWS) showed mostly proteins of 70 kDa and less (Fig. 2A). Tight bands of low intensity were present in protein extracts from the digestive fluid from the NF trap. Well-visualised bands were observed after the separation of proteins isolated from the CTRL 1 daf. The most intense and thick bands were between 55 and 35 kDa. After the separation of proteins isolated 1 daf from the NO sample, more bands were visible than in the CTRL. However, they were less intense and thinner. Moreover, 2–3 bands appeared between 55 and 25 kDa. In the CTRL and the NO sample 2 daf, the protein bands were visibly thinner and weaker in comparison to the CTRL 1 daf. The proteins' band pattern for the CTRL 2 daf was similar to the pattern for the CTRL 4 daf. The bands become thinner. Significantly fewer protein bands and their decreasing intensity were characteristic for the NO samples 2 daf (Fig. 2A). Only two bright bands were visible in the protein pattern obtained 4 daf from the NO sample.

3.2. The proteolytic activity in the digestive fluid

The highest proteolytic activity was detected 1 daf in the NO sample and was more than twice as high as in the digestive fluid from NF trap and almost twice as high as in the CTRL (Fig. 2B). Generally in the NO sample the proteolytic activity decreased as the period after feeding was getting longer. In the digestive fluid from the CTRL and the NO sample 2 or 4 daf proteolytic activity was at a similar level. The lowest proteolytic activity was noticed in the fluid from NF trap (Fig. 2B).

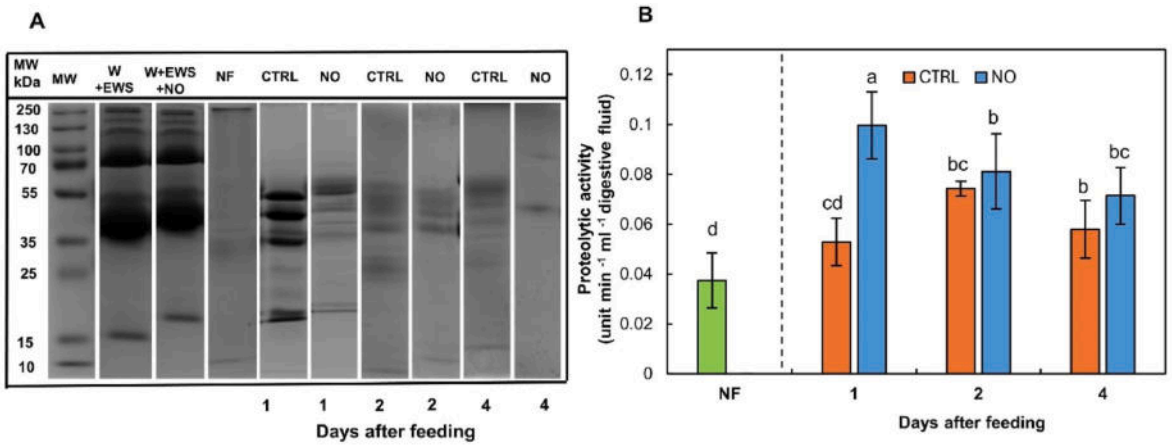

Fig. 2. Proteins bands after SDS-PAGE separation (10% gel) and Coomassie brilliant blue staining (A). Line MW: molecular markers, line W + EWS proteins separated from deionised water mixed with egg white solution, line W + EWS + NO proteins separated from deionised water mixed with egg white solution and NO_x donor. The proteolytic activity (B) of the digestive fluid collected from the non-fed trap (NF), trap fed with egg white solution (CTRL) and trap fed with egg white solution + NO_x donor (NO) determined 1, 2, and 4 days after feeding (daf). Values are average ± SD of 4 repetitions. Letters (a–d) indicate homogenous groups determined after ANOVA and post hoc Fisher's LSD test at $p \leq 0.05$.

3.3. Generation of NO in the digestive fluid

The lowest fluorescence signal (15 U) was obtained for the digestive fluid from NF trap (Fig. 3A). The level of the DAF-FM fluorescence of NF traps' fluid was similar to the fluorescence measured for the CTRL 2 daf (17.75 U). The highest fluorescence signal (35.5 U) was observed for the digestive fluid of the NO sample 1 daf (Fig. 3A). The fluorescence signal of the NO sample 2 daf decreased 1.5 times as compared to 1 daf. The DAF-FM fluorescence signal detected 4 daf, irrespective of the sample, was at a similar level (around 25.25 U) (Fig. 3A).

3.4. The concentration of NO in the digestive fluid

The lowest NO content was observed in the digestive fluid from the NF trap and the CTRL 2 daf (0.77 nmol l^{-1} digestive fluid) (Fig. 3B). The NO concentration in the CTRL 1 daf was 0.92 nmol l^{-1} digestive fluid. The highest NO level was in the NO sample 1 daf (1.33 nmol l^{-1} digestive fluid). 4 daf the NO content in the CTRL was higher than in NO sample (Fig. 3B). In CTRL fluctuations in NO level in the digestive fluid were detected, with the minimum 2 daf. In the digestive fluid of NO sample, NO content decreased gradually as the period after feeding was prolonged, dropping down to around 0.8 nmol l^{-1} digestive fluid, an equivalent to the minimum for the CTR.

3.5. $O_2^{\bullet-}$ production in the digestive fluid

The lowest generation of $O_2^{\bullet-}$ was detected in the digestive fluid from the NF trap (10.5 nmol min^{-1} ml^{-1}digestive fluid) (Fig. 3C). The production of $O_2^{\bullet-}$ in the CTRL and the NO samples 1 and 2 daf was similar. The highest $O_2^{\bullet-}$ generation was noticed for the CTRL 4 daf and was almost three-fold higher than in the fluid from the NF trap. In the NO sample 4 daf the $O_2^{\bullet-}$ production decreased as compared to the earlier period after feeding and was almost twice lower than in the CTRL (Fig. 3C).

3.6. H_2O_2 concentration in the digestive fluid

The concentration of H_2O_2 in the digestive fluid of CTRL fluctuated during the observation. The lowest concentration of H_2O_2 (2.1 nmol l^{-1}digestive fluid) was detected for the CTRL 2 daf (Fig. 3D). In the CTRL and NO samples 1 daf the H_2O_2 content was the same. The highest level of H_2O_2 was noticed for the NO sample 4 daf and was higher than in the CTRL (Fig. 3D).

3.7. Visualisation of $O_2^{\bullet-}$ in the pitcher plant tissue and the effect of the Rboh inhibition by DPI on $O_2^{\bullet-}$ formation

The lowest intensity of formazan generation was observed 1 daf in the glands of the CTRL and the NF trap's tissue (Fig. 4A). The most intense formazan spots were noted in the glandular zone from the trap of NO samples 4 daf. After treatment with the DPI, formazan formation was not detected regardless of feeding-type and digestion duration (Fig. 4B).

3.8. Total antioxidant capacity of the digestive fluid

Total free radical scavenging capacity (FRSC) was the lowest in the digestive fluid from the NF trap (Fig. 5A). Feeding stimulated FRSC in both the CTRL and NO samples, but DPPH reduction was higher for NO

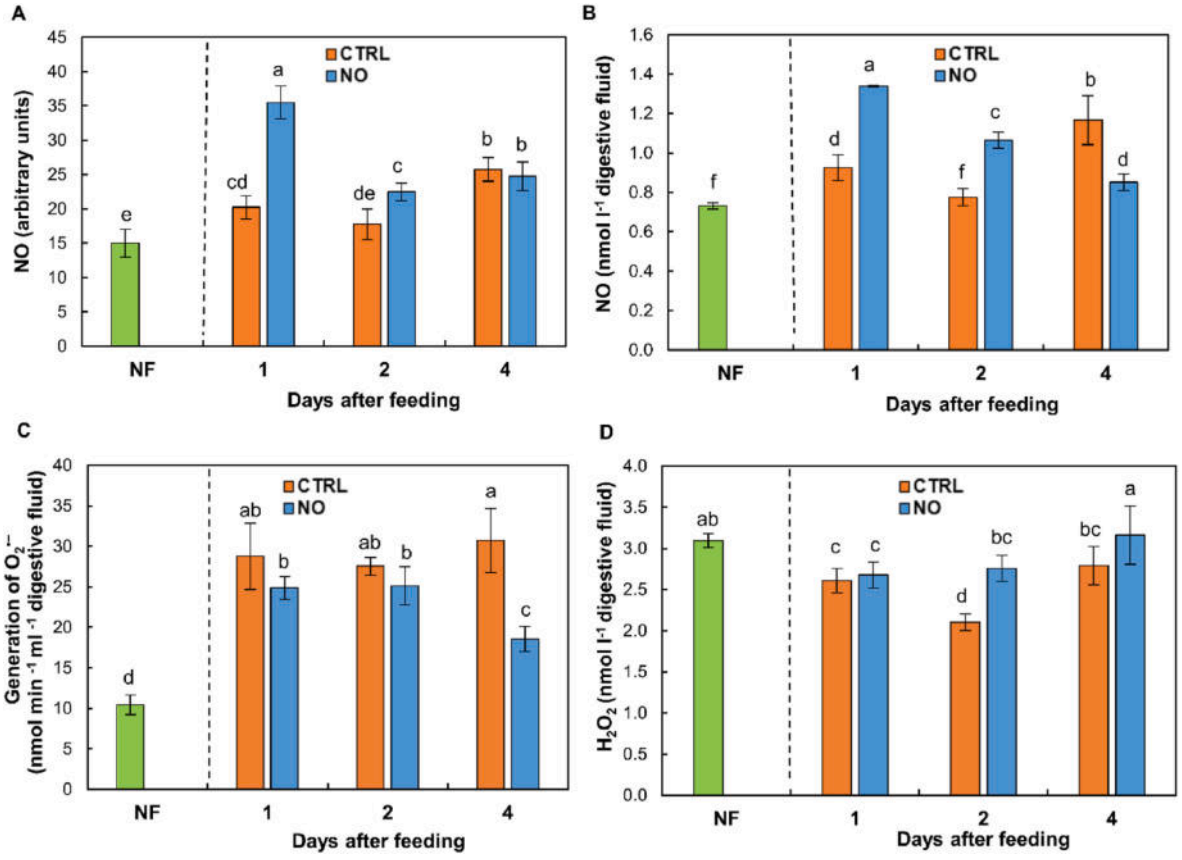

Fig. 3. The fluorescence of DAF-FM (A), NO concentration (B), superoxide anion ($O_2^{\bullet-}$) production (C) and the content of hydrogen peroxide (H_2O_2) (D) in the digestive fluid in the non-fed traps (NF), traps fed with two fruit flies (CTRL), and traps fed with two fruit flies together with NO (NO) (A and B) or traps fed with egg white solution (CTRL) and with egg white solution and NO donor (NO) (C and D). Measurements were done 1, 2, and 4 days after feeding (daf). Values are average ± SD of 4 repetitions. Letters (a–f) indicate homogenous groups determined after ANOVA and post hoc Fisher's LSD test at $p \leq 0.05$.

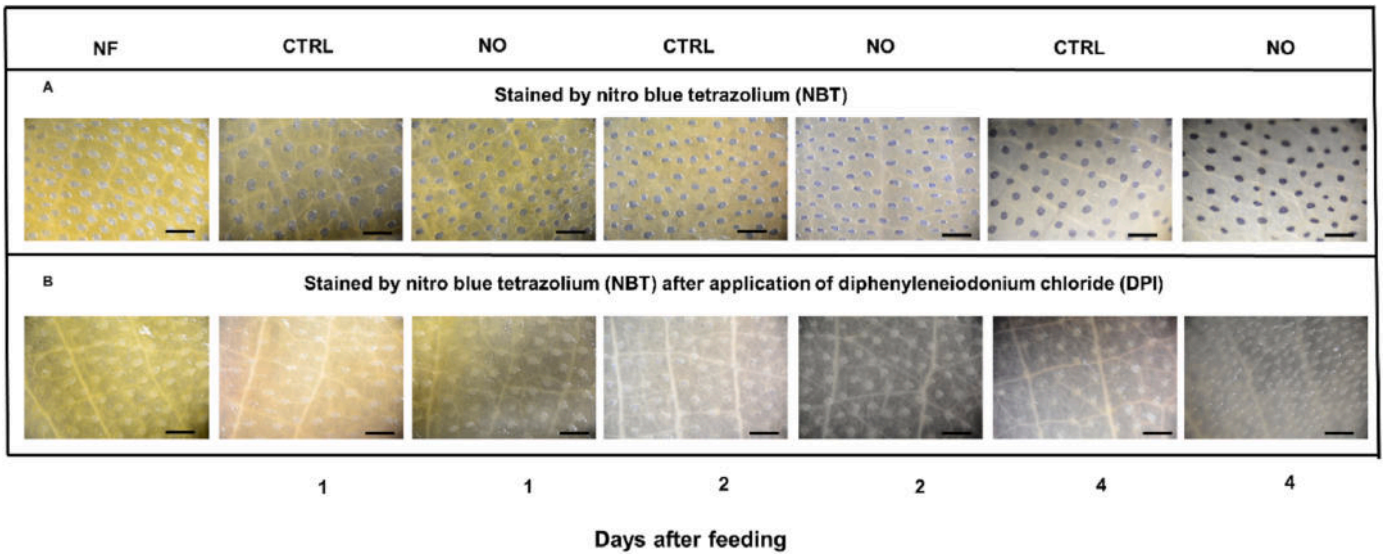

Fig. 4. The traps' sections with secretory glands from the glandular zone of the non-fed (NF) trap, CTRL and NO samples 1, 2, and 4 days after feeding (daf) (zoom 3 x). The slices were stained with NBT (A) or Rboh inhibitor (DPI) followed by NBT staining (B). Representative photographs were shown. Photographs were taken with a stereoscopic microscope with an integrated Nikon H550S camera (ISO, 2000; aperture f/3, exposure time 1/10 s). Bar = 1 mm.

samples than for CTRL (Fig. 5A).

The highest radical scavenging capacity of low molecular weight antioxidants (LMWA) was in the digestive fluid of the NF trap (Fig. 5B), while the lowest was observed in the digestive fluid of the CTRL 1 daf and NO samples 4 daf. It decreased in NO samples, whereas increased in CTRL as duration of digestion was prolonged. LMWA for CTRL and NO samples 2 daf was at a similar level (Fig. 5B).

3.9. The concentration of the compounds with -SH groups in the digestive fluid

The lowest level of the compounds with the -SH group was in the digestive fluid from NF trap (Fig. 5C). The concentration of TNB 1 daf was 0.061 μmol ml^{-1} digestive fluid for the CTRL, and it was slightly lower compared to the NO sample. The highest content of the compounds with -SH was noted for the NO sample 4 daf (0.095 μmol TNB ml^{-1}digestive fluid) (Fig. 5C).

3.10. POx activity in the digestive fluid

POx activity decreased in the digestive fluid of CTRL as the duration of digestion was prolonged (Fig. 5D), whereas POx activity in the digestive fluid of NO sample fluctuated slightly, dropping down 4 daf. The highest POx activity was measured for the CTRL sample 1 daf, and it was almost three times higher than in the digestive fluid from NF trap which value was the lowest (Fig. 5D). In the digestive fluid from the CTRL and the NO samples POx activity 4 daf was similar (Fig. 5D).

3.11. The total phenolic content in the digestive fluid

The total phenolic content was high in the digestive fluid of NF traps (2 μg ml^{-1} digestive fluid) and decreased drastically in CTRL and NO samples as the digestion period was extended for 2 and more days (Fig. 5E). The highest level of phenols was observed 1 daf for both the CTRL and NO sample (Fig. 5E). There were no statistical differences in the phenolic content in the CTRL and NO sample 2 daf, but 4 daf the total phenolic content in the NO sample was almost twice higher than in the CTRL (Fig. 5E).

3.12. Flavonoids concentration in the digestive fluid

Flavonoids concentration in the digestive fluid of the NF trap was 15 μg ml^{-1} digestive fluid (Fig. 5 F). The same concentration of flavonoids was observed in the digestive fluid in CTRL 2 daf, and it was the maximum of flavonoids content in CTRL detected during the digestion period. The opposite pattern of changes in flavonoids content in the digestive fluid from NO sample was observed as an experiment was prolonged. Flavonoids concentration 2 daf in the NO sample was lower (10 μg ml^{-1} digestive fluid) than in the CTRL (Fig. 5F), while at the beginning and at the end of the digestion process it was almost doubled.

3.13. The pattern of nitrated proteins in the digestive fluid

Two bands showing 3-NT proteins were observed in proteins isolated from egg white solution dissolved in deionised water (W + EWS) and from egg white solution with the NO_x donor dissolved in deionised water (W + EWS + NO). The most intense bands were observed from the sample of digestive fluid from NF trap, two visualised bands corresponded to proteins of approximately 55 kDa and 25 kDa (Fig. 6A). Tight bands of low intensity were present in protein extracts from the digestive fluid from the CTRL and NO sample 1 daf. In the digestive fluid from CTRL 4 daf, the protein bands were visibly thinner and weaker in comparison to the NO sample 4 daf. Four thin and bright bands were observed after the separation of proteins isolated from the digestive fluid of CTRL 2 daf, while in the NO sample 2 daf only two bands were visible, corresponding to proteins of approximately 55 kDa and 30 kDa (Fig. 6A).

3.14. The concentration of total 3-NT in the digestive fluid

The concentration of the 3-NT protein in the digestive fluid from NF, CTRL, NO samples 1 and 4 daf was similar (Fig. 6B). The lowest level of 3-NT protein was observed in the protein isolated from the CTRL 2 daf and was around 40% lower than in the protein isolated from the NO sample 2 daf.

4. Discussion

In Nepenthes's trap we have distinguished a functional, specific apoplastic zone, consisting of glands and digestive fluid into which

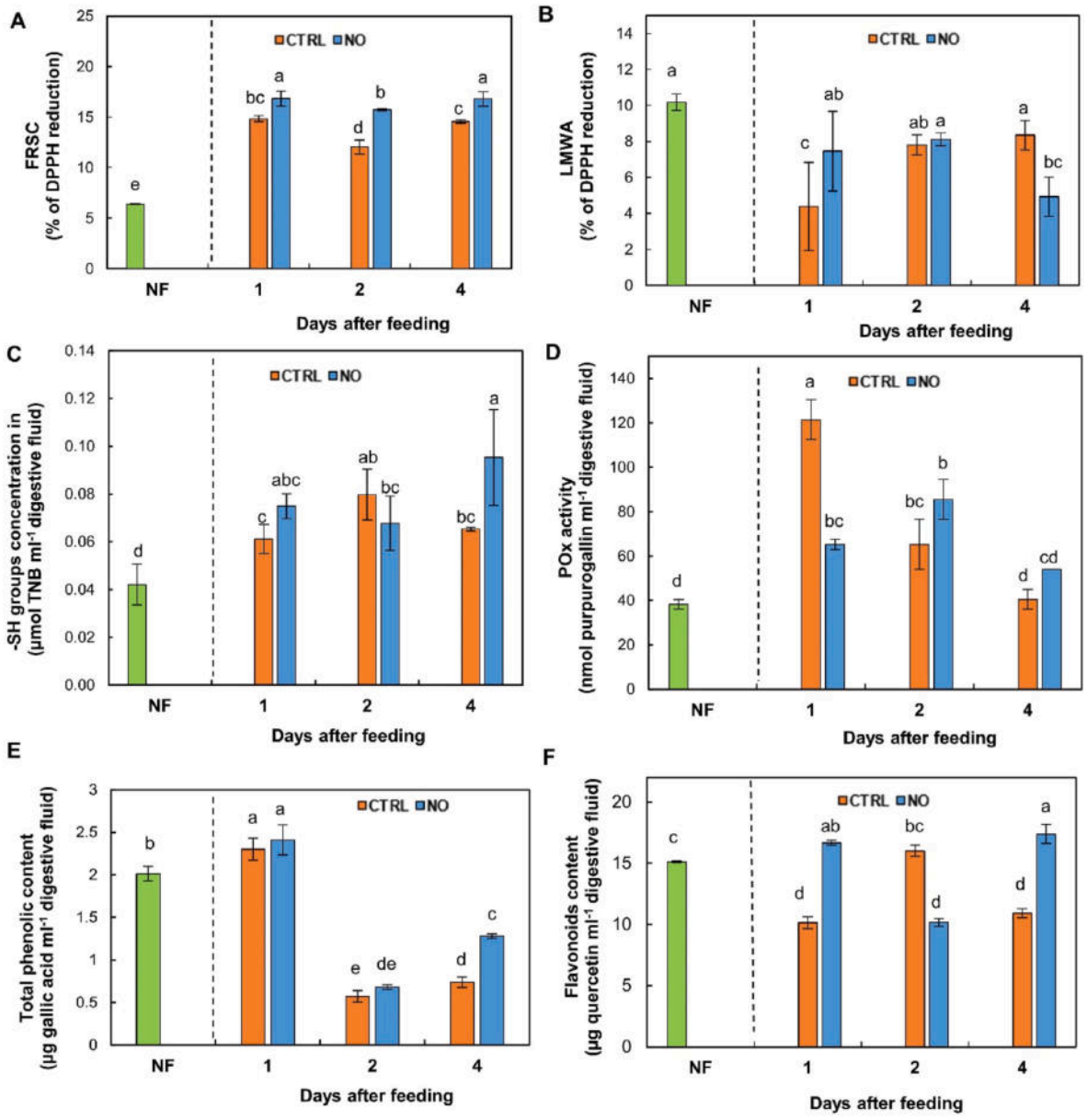

Fig. 5. The total free radical scavenging capacity (FRSC) of the digestive fluid (A) and free radical scavenging capacity of low molecular weight antioxidants (LMWA) (B) the concentration of compounds with a sulfhydryl group (C), POx activity (D), the total phenolics level (E), the flavonoids concentration (F) in the digestive fluid in the non-fed traps (NF), traps fed with egg white solution (CTRL), and traps fed with egg white solution with the NO_x donor (NO). Measurements were done in the digestive fluid from traps 1, 2, and 4 days after feeding (daf). Values are average ± SD of 4 repetitions. Letters (a–e) indicate homogenous groups determined after ANOVA and post hoc Fisher's LSD test at $p \leq 0.05$.

various proteins and other metabolites are secreted. ROS and RNS have been described among the well-characterised components of the digestive fluid (Chia et al., 2004; Wal et al., 2022). The increase of NO formation in the traps' fluid after food application indicates that this compound participates in the release of nutrients from the prey body (Wal et al., 2022). According to the presented data, we propose that NO accelerates digestion, as the alterations of the proteins' band profiles (decomposition of proteins of egg white solution) were observed after electrophoretic separation. The addition of NO together with food resulted in an increased number of thin bands in the protein profile from the digestive fluid 1 daf. Moreover, in the protein profile from the digestive fluid of NO sample collected 4 daf fewer bands were observed compared to the CTRL (Fig. 2A). Furthermore, NO increased the proteolytic activity during the digestion performed in the fluid of pitcher plants, especially 1 daf (Fig. 2B). This was linked to the high concentration of NO in the digestion fluid (Fig. 3A and B). The exact mechanism of the acceleration of the digestion in pitcher plants following NO application remains unknown, but it may be due to NO-dependent PTMs of food proteins. In the digestive fluid, NO may participate in PTMs of proteins liberated from food or secreted by glands to perform digestion. Nitration is RNS-linked PTMs, known as irreversible at physiological conditions. It modifies protein structure and function (Gupta et al., 2020). The presence of 3-NT containing proteins in plant material has been verified, and the putative function of this modification has been described (León, 2022). Our data are the first demonstration of the presence of nitrated proteins in the digestive fluid of carnivorous plants. NO may have a direct impact on proteolytic enzymes, particularly on aspartic proteases - nepenthesines. Nitration of Tyr^{168} in the conservative domain of aspartic protease Cathepsin D did not lead to its degradation but enhanced its proteolytic activity, as was

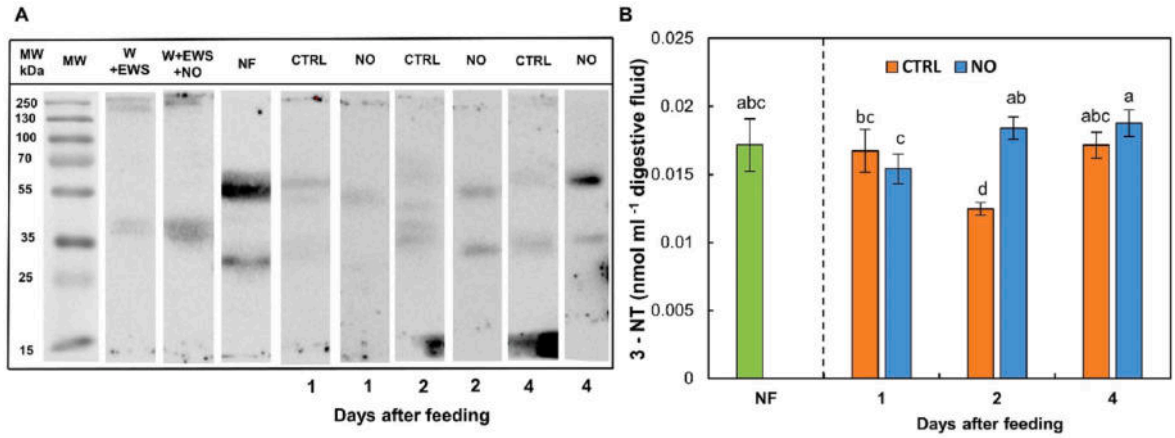

Fig. 6. The pattern of 3-NT modified proteins isolated from the digestive fluid (A) photos of the membrane were taken at an exposure of 20 s. Line MW: molecular markers, line W + EWS proteins separated from deionised water mixed with egg white solution, line W + EWS + NO proteins separated from deionised water mixed with egg white solution and NO_x donor. The content of 3-NT in proteins (B) isolated from the digestive fluid from the non-fed traps (NF), traps fed with egg white solution (CTRL), and traps fed with egg white solution with addition of NOx donor (NO). Measurements were done in the digestive fluid 1, 2, and 4 days after feeding (daf). Values are average ± SD of 4 repetitions. Letters (a–d) indicate homogenous groups determined after ANOVA and post hoc Fisher's LSD test at $p \leq 0.05$.

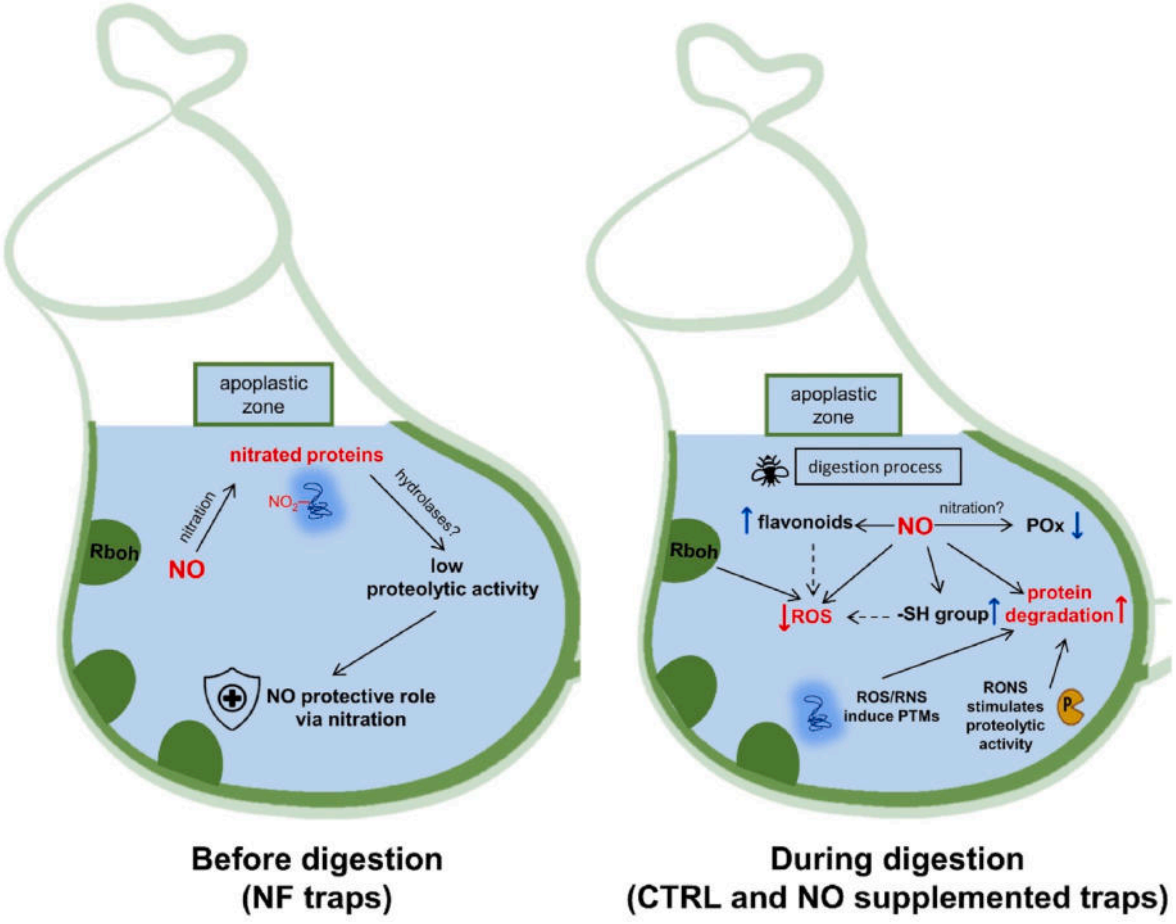

Fig. 7. A schema showing the role (solid black arrows) and putative mode of action (dashed black arrows) of NO in the apoplastic zone of *Nepenthes* × *ventrata* before and during the digestion process. NO nitrates proteins, presumably from the hydrolase class, lowering proteolytic activity in the trap fluid before digestion. The low activity of proteolytic enzyme in non-digesting (NF) traps may be a protective role of NO for the trap. NO decreases the activity of peroxidases of class III (POx), possibly through protein nitration. NO accelerates protein degradation due to the higher proteolytic activity stimulated by nitration. NO action in the stimulation of the digestion process in the fluid may also be due to post-translational protein modifications (PTMs) of the prey proteins. NO in the fluid lowers ROS level. Indirect effects of NO in the digestive fluid can occur through higher levels of flavonoids and compounds containing -SH groups at the initial period of digestion. Plasma membrane respiratory burst oxidase homologs (Rboh) are probably the source of superoxide anion ($O_2^{\bullet-}$) in the apoplastic zone of the trap.

shown for mammary (rat) glands after lactation (Zaragozá et al., 2009). As peroxynitrite and NO were detected in *Nepenthes* × *ventrata* digestive fluid (Wal et al., 2022), nitration of proteases in the fluid could be possible thus, increased proteases activity may occur similarly to mammalian tissue. On the other hand, nitration of specific proteins (e.g. hydroxylases) before digestion may have a protective function. In vitro studies have shown that nitrated pepsinogen (at acidic pH corresponding to the pH in the rat stomach) was a less active form of pepsin than non-modified one. This points to nitration as a gastroprotective factor (Rocha et al., 2013, 2016). Moreover, in vivo studies have shown that RNS were involved in the nitration of pepsinogen in the stomach, as the addition of uric acid decreased nitrogen dioxide radical levels and inhibited pepsin nitration (Rocha et al., 2016). The digestive fluid from NF pitcher plants (before digestion, Fig. 7) had the highest content of nitrated proteins. It can be assumed that nitration also has a protective effect in the pitcher fluid, altering the activity of proteolytic enzymes in the absence of food in the trap.

Currently, it is recommended that at least two different techniques should be used to determine the NO content in the biological samples (Gupta and Igamberdiev, 2013). The method based on a fluorescent dye in the digestive fluid requires various positive and negative controls (due to the autofluorescence of the digestive fluid) (Wal et al., 2022). In this work, we additionally applied amperometric NO detection using an electrochemical NO sensor. We confirmed the presence of RNS in the fluid and the fluctuations in the level of these compounds during food digestion (both for the CTRL and the NO samples). The highest NO content was recorded 1 daf in the digestive fluid from the trap supplemented with NO. Evidently, such an "extra" introduction of NO at the beginning of the digestion accelerated food decomposition, which was clearly visible as the decline of the protein bands in the gel profile after the SDS-PAGE separation (Fig. 2A).

We do not know whether the NO in the traps' fluid may be of plant or microorganism origin. However, the presence of nitrite ions in the fluid of *Nepenthes* × *ventrata*, which may constitute an essential source of RNS at acidic pH, has been described (Wal et al., 2022). NO was detectable in the *Nephenthes x ventrata* trap at all stages of its development, and RNS impact on ROS homeostasis was also shown (Wal et al., 2022). ROS participated in the digestion process performed by *Nepenthes gracilis* Korth. (Chia et al., 2004). The authors indicated that •OH was responsible for the first step of protein (myosin) decomposition in the digestive fluid even after the addition of the protease inhibitors.

In the digestive fluid of *Nepenthes* × *ventrata* $O_2^{\bullet-}$ was recorded during the trap ontogeny (Wal et al., 2022). Food application into the digestive fluid of *Nepenthes* × *ventrata* increased the formation of $O_2^{\bullet-}$ while parallel to food NO application only slightly inhibited this process (Fig. 3C). Histochemical localisation of $O_2^{\bullet-}$ revealed that the traps' glands are responsible for the generation of this compound. The secretory activity of the glands is extracellular thus, their products directly alter the fluid, collectively forming a functional apoplastic zone (Fig. 7). The use of DPI (Rboh inhibitor) indicated that $O_2^{\bullet-}$ generated in the trap glands resulted from the activity of Rboh. The highest intensity of visible spots (formazan precipitates) (Fig. 4) was observed 4 daf in glands of the NO samples. Our data indicate that NO has no significant effect on $O_2^{\bullet-}$ formation *per se* until the 2 daf. The modulatory role of NO, in relation to free radicals is, therefore, more related to the detoxification of ROS. The incompatibility of $O_2^{\bullet-}$ generation in the tissue and the digestive fluid could be the result of Rboh activation (Fig. 4 vs 3C) and may also be explained by the data for the total free-radical scavenging capacity. We observed a slightly higher antioxidant capacity in the digestive fluid of the NO samples 1 daf. NO may regulate oxygen free radicals levels. An excess of ROS not only has a toxic effect but may also contribute to the oxidation of metabolites to harmful forms, e.g. *meta*-tyrosine, which may be incorporated into protein structures (Ciacka et al., 2022). We propose that specialised sensors of ROS or redox status in the apoplastic zone (most probably in the digestive fluid) may include specific proteins that undergo oxidative PTMs and/or various oxidized metabolites (Fig. 7).

The cellular ROS homeostasis and signal transduction are regulated by proteins and peptides containing Cys residues (Waszczak et al., 2018). The increased content of compounds with -SH groups in the NO samples 4 daf was associated with the lower (compared to the CTRL) generation of $O_2^{\bullet-}$. The GSH and its oxidised form, together with phenolic compounds (naphthoquinones) participate in cell redox homeostasis (Eilenberg et al., 2010). The presence of -SH groups in the digestive fluid allows us to speculate that ROS also have a signalling role in the apoplastic zone of the trap (Fig. 6). It is not known whether it is only a local signal or it also involves long-distance transduction. Currently, there is no information regarding GSH detection in *Nepenthes'* digestive fluid. Scherzer et al. (2017) confirmed the presence of GSH in the tissue and the secreted fluid of Venus flytrap (*Dionaea muscipula* J. Ellis). The authors hypothesised that antioxidants, due to their stability in the acidic pH of the fluid, maintained ROS homeostasis for the effective action of hydrolytic enzymes during digestion (Scherzer et al., 2017) A very low concentration of H_2O_2 detected in the digestive fluid precludes induction of oxidative stress and may suggest H_2O_2 signaling function. Similar low concentration (nmoles l^{-1}) of H_2O_2 were recorded in exudates from the roots of non-stressed tomato (*Solanum lycopersicum* L.) seedlings (Krasuska et al., 2016). At the same time, the concentration of H_2O_2 in the tomato root extracts achieved 75–80 pmol mg^{-1} FW (equivalent to 78–83 μM). Such a large difference (25-fold) in the ROS concentration between the tissue and the "outside zone" was also demonstrated for apple embryos (H_2O_2 concentration in the embryos in comparison to the medium in which their culture was carried out) (Gniazdowska et al., 2010). In the digestive fluid, H_2O_2 may be formed from spontaneous dismutation of $O_2^{\bullet-}$ or by the microbiome of the digestive fluid, as some bacteria can produce extracellular H_2O_2 (Erttmann and Gekara, 2019; Juven and Pierson, 1996). The level of H_2O_2 in the digestive fluid was higher in the NO samples 2 daf. We believe that ROS production in the *Nepenthes'* trap apoplastic zone results from POx and Rbohs activities. Hatano and Hamada (2012) detected the presence of POx in the digestive fluid of *Nephenthes alata*. POx genes expression were induced by pathogens, temperature, anoxia and other stressors (Shigeto and Tsutsumi, 2016). In plant-pathogen relations, POx are known to enhance ROS level (Hiraga et al., 2001). Acting as a peroxidases, POx reduce H_2O_2 to water, oxidising substrates of the reaction, e.g. phenolic compounds (Takahama and Oniki, 2000). When POx uses thiol or salicylic acid as substrates, the derivative radicals may react with O_2 to form free radicals (Shigeto and Tsutsumi, 2016). Thus, POx are involved in both ROS scavenging and ROS generation. NO is known as an inhibitor of the activity of heme-containing enzymes, e.g. POx (Pintus et al., 2012). The activity of POx in the digestive fluid increased 1 daf, particularly in CTRL traps. However, when NO was added, the activity of this enzyme in the digestive fluid was significantly lower compared to the CTRL. RNS donors negatively impacted POx activity in various plants. Peroxynitrite donor and *S*-nitrosoglutathione inhibited the activity of POx IV in bell pepper (*Capsicum annum* L.) fruits by 50% (González-Gordo et al., 2023). The authors suggested that this inhibition resulted from *S*-nitrosation and nitration of the enzyme. Moreover, in bell pepper, the activity of POx IV was entirely inhibited by GSH and Cys, suggesting that *S*-nitrosation, despite limiting POx activity, had a positive effect by protecting the enzyme from other PTMs (González-Gordo et al., 2023). In the digestive fluid of *Nepenthes* × *ventrata*, protein nitration may serve a similar function, as evidenced by the constant POx activity till 4 daf in the NO samples. A stable POx activity in the fluid protects against excessive generation of ROS and accumulation of oxidised metabolites and, therefore, may prevent disruption of the digestive process.

In carnivorous plants, mostly in the traps' tissues, phenolic compounds containing flavonoids and their derivatives of various physiological functions are synthesised (Wójciak et al., 2023). In the trap fluid of *Nepenthes khasiana*, droserone and 5-O-methyl-droserone and in the fluid of *Nepenthes ventricosa* plumbagin and 7-methyl-juglone were identified (Buch et al., 2013; Eilenberg et al., 2010) showing

antimicrobial activity. The biological, antibacterial and antifungal properties of phenolic compounds are associated with generating free radicals (Staszek et al., 2020). The presence of phenolic compounds in the digestive fluid was confirmed during the development and growth of the *Nepenthes* × *ventrata* trap (Wal et al., 2022). The highest concentration of total phenolics was noticed in the fluid of the mature trap. Nevertheless, the digestion of food was also accompanied by a high level of phenolic compounds (Wal et al., 2022). The application of food into the digestive fluid of *Nepenthes* × *ventrata* (1 daf) resulted in the accumulation of phenolic compounds. Whereas a rapid decline of total phenolics content (to the level below that one observed in the fluid from the NF trap) occurred during the prolongation of the digestion. This reaction was similar to those observed in plant-microbe interaction (Mandal et al., 2010). Among the phenolic compounds, flavonoids are the largest group, and they are characterised by the highest antioxidant activity (Robards and Antolovich, 1997). In the genus *Nepenthes*, the presence of numerous flavonoids such as kaempferol, quercetins, epicatechin 3-gallate, and quercetin gallate esters were demonstrated in the tissues (Wójciak et al., 2023). The dynamics of flavonoid accumulation in the digestive fluid of *Nepenthes* × *ventrata* plants differed in the CTRL and the NO samples. In the CTRL 1 daf the concentration of flavonoids decreased, potentially being consumed by POx, whose activity increased after food application. When the digestive fluid was supplemented with NO, the pool of flavonoids remained unchanged at 1 daf, decreased the next day, and returned to the level observed in the CTRL after next 2 days. Our data on the content and generation of ROS demonstrated that in the digestive fluid from the NO samples, the rates of the examined parameters returned more rapidly to the level characteristic for the fluid from the NF trap than for the CTRL. Thus, we propose that NO, as the modulator of the flavonoids' level, implicates the dynamics of the digestion process and ROS homeostasis in the digestive fluid of *Nepnthes x ventrata* (Fig. 7).

5. Conclusions

ROS, together with RNS play a supporting and regulatory role in the trap fluid before and during digestion. We suggest that NO in the fluid (before digestion) may have a protective effect through the nitration of proteolytic enzymes (which may be evidenced by the low activity of proteases in the NF traps) (Fig. 7). During digestion performed in the apoplastic zone inside the *Nepenthes* trap NO exhibit dual role as an accelerator of protein decomposition and the ROS balance keeper (Fig. 7). We propose that the food digestion in the trap depends on the actual level of NO.

Author contributions

Conceptualisation, U.K., P.S. and A.W.; methodology, P.S., U.K. and A.W.; investigation, A.W., M.P., K.C., J.B. data curation, A.W. and U.K.; writing—original draft preparation, A.W.; writing—review and editing, P.S. A.G. and U.K.; supervision, U.K. A.G. and P.S. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2024.109088.

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Nitric oxide action in the digestive fluid of *Nepenthes x ventrata* is linked to the modulation of ROS level

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Supplementary information:

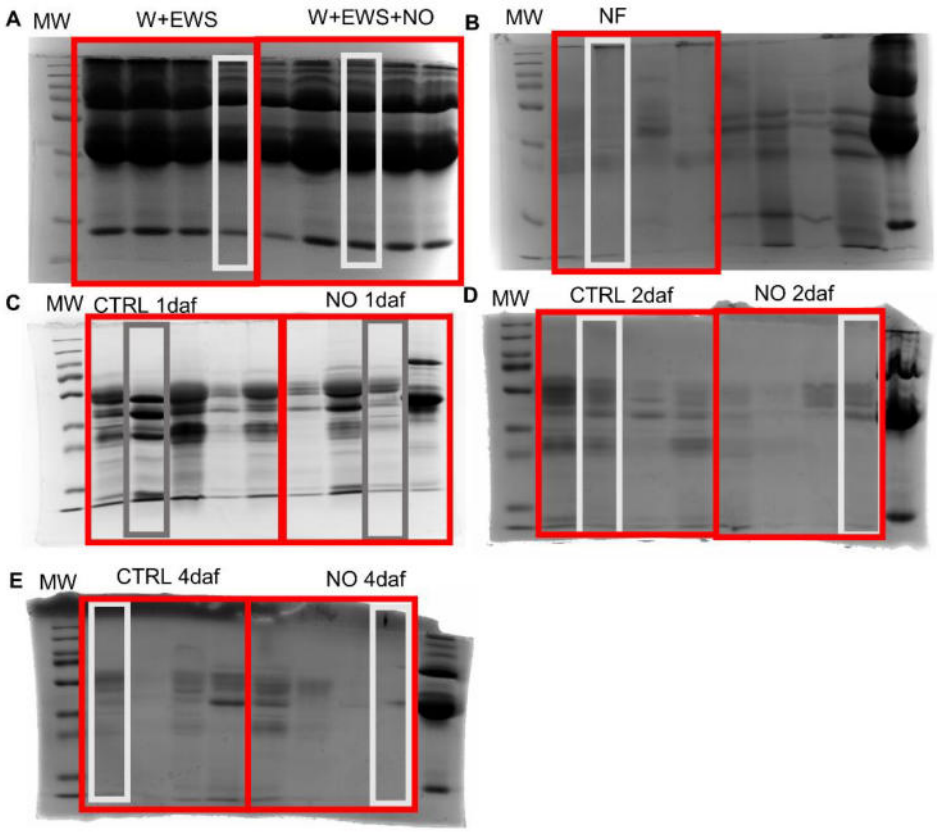

Fig. S1. Proteins bands after SDS-PAGE separation (10% gel) and coomassie brilliant blue staining of proteins from water mixed: with (i) egg white solution and with (ii) egg white solution + NO_x donor (A), the digestive fluid from the non-fed (NF) traps (B), and after 1 (C), 2 (D), and 4 (E) days after feeding: traps fed with egg white solution (CTRL) and traps fed with egg white solution + NO_x donor (NO). Representative lines, which were used for the preparation of Fig. 2, were indicated.

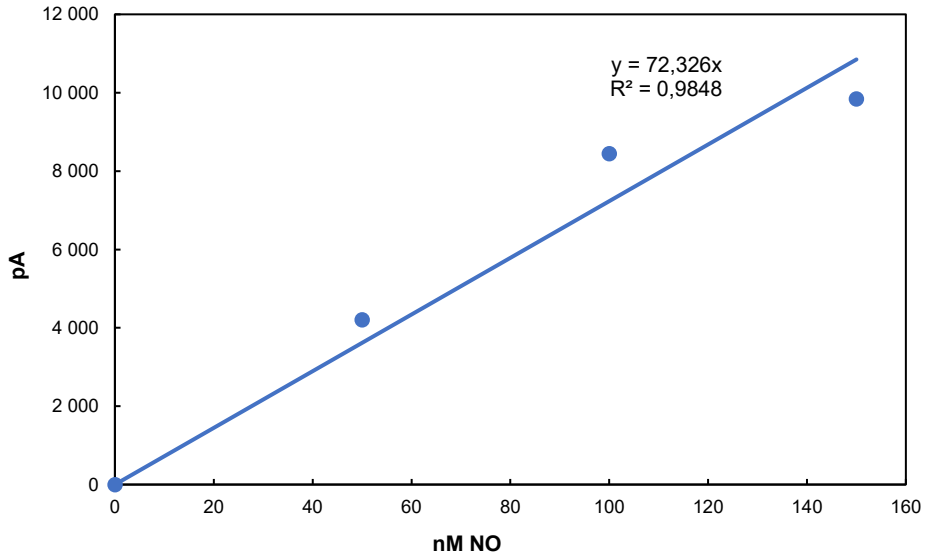

Fig. S2. The standard curve for NO level determination prepared using $NaNO_2$.

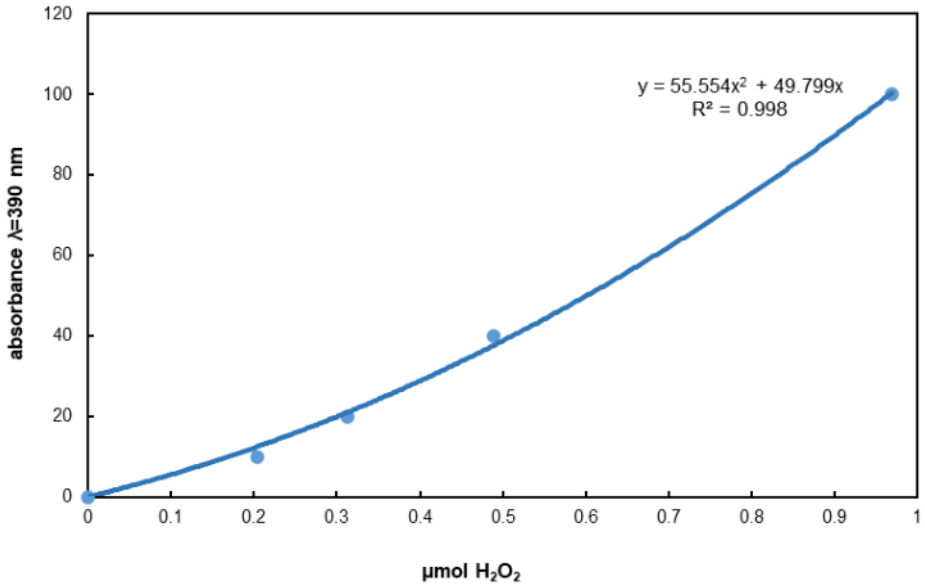

Fig. S3. The standard curve for H_2O_2 concentration determination prepared using H_2O_2.

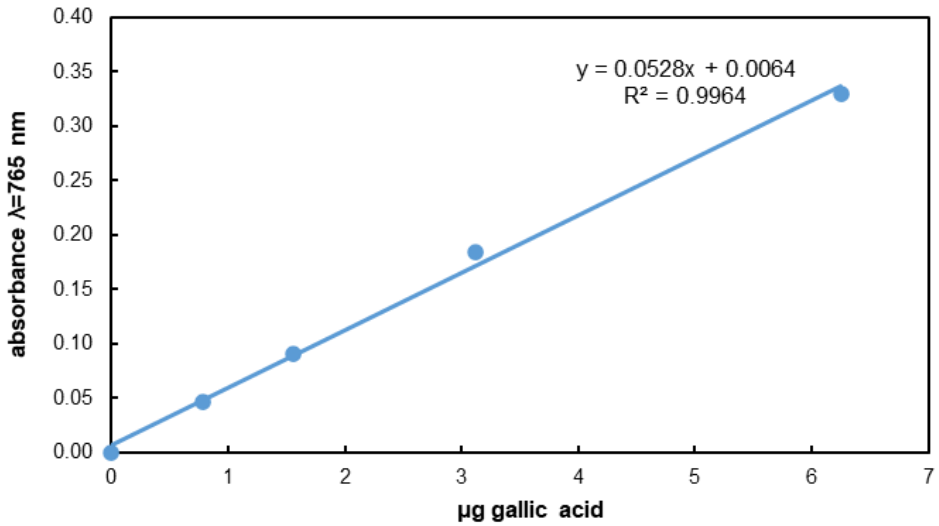

Fig. S4. The standard curve for phenolic content determination prepared using gallic acid.

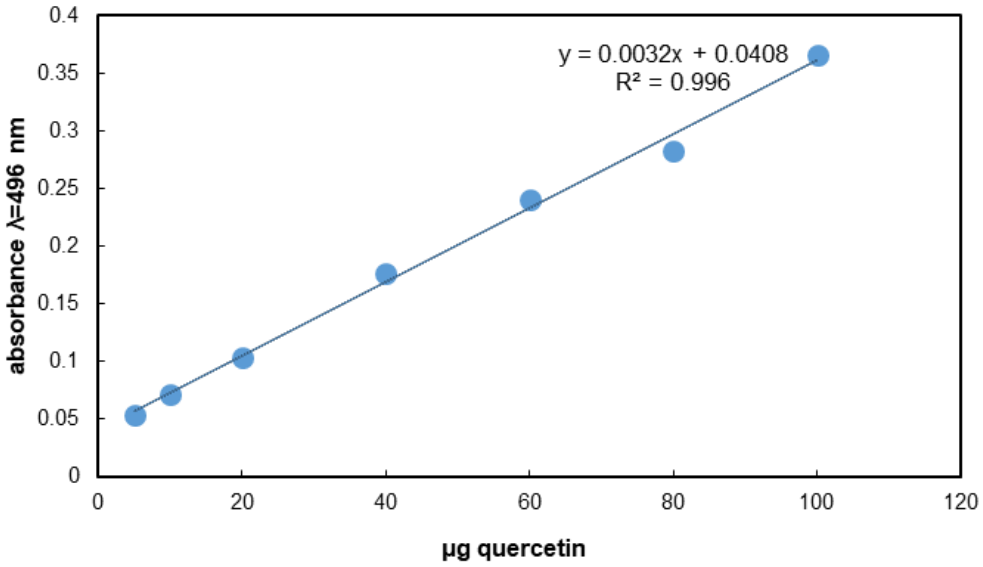

Fig.S5. The standard curve for flavonoids level determination prepared using quercetin.

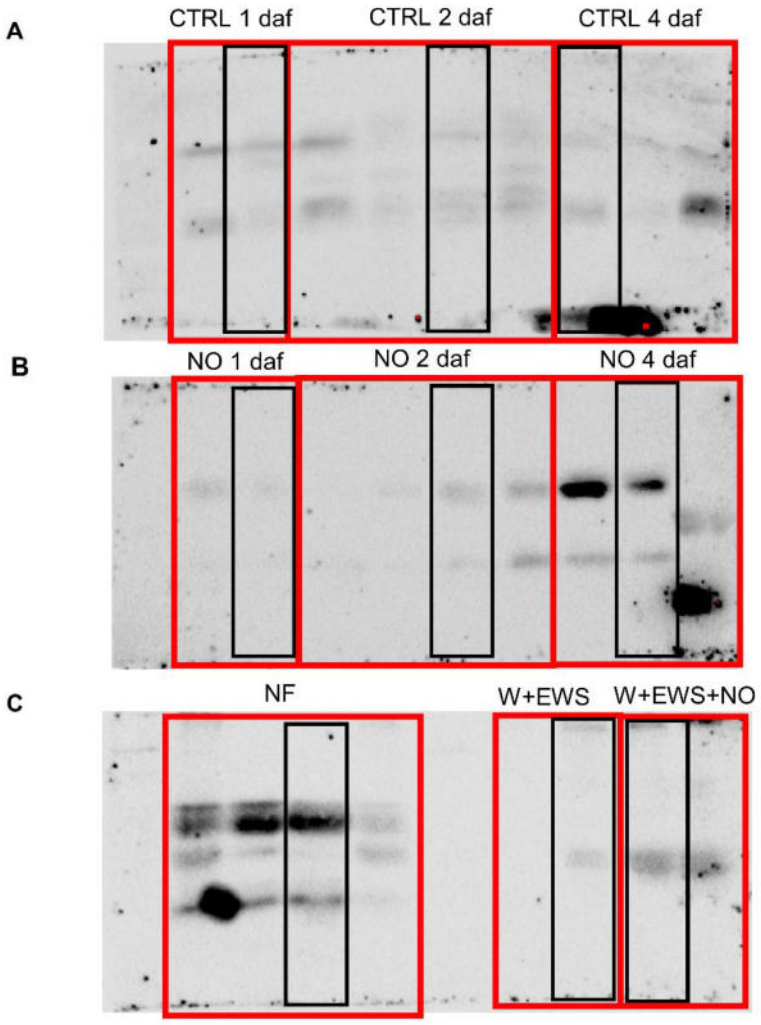

Fig. S6. The pattern of 3-NT modified proteins isolated from the digestive fluid, photos of the membrane were taken at an exposure of 20 s. Lines CTRL from the digestive fluid from the trap 1, 2, and 4 days after feeding (daf) (A). Lines NO from the digestive fluid from the trap 1, 2, and 4 days after feeding (daf) (B). Lines NF from the digestive fluid from the non-fed traps, W+EWS proteins from deionised water mixed with egg white solution, line W+EWS+NO proteins from deionised water mixed with egg white solution and NO_x donor (C). Representative lines, which were used for the preparation of Fig. 6. were indicated.

Załącznik nr 2 – Wzór oświadczenia o współautorstwie

Warszawa, 27.05.2025

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Wiejskiego w Warszawie

Oświadczenie o współautorstwie

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Wal A., Piekarniak M., Staszek P., Chodór K., Bieniek J., Gniazdowska A., Krasuska U. (2024) Nitric oxide action in the digestive fluid of *Nepenthes* × *ventrata* is linked to the modulation of ROS level. Plant Physiology and Biochemistry 216:109088. doi: doi.org/10.1016/j.plaphy.2024.109088

mój indywidualny udział w jej powstaniu polegał na współuczestniczeniu w opracowywaniu koncepcji badań, prowadzeniu kultury dzbanecznika brzusznego (*Nepenthes* × *ventrata*), współwykonywaniu pomiarów całkowitej zdolności antyoksydacyjnej, aktywności peroksydaz klasy III, związków z grupą tiolową, aktywności proteolitycznej cieczy trawiennej, wykonaniu rozdziału elektroforetycznego SDS-PAGE białek z cieczy trawiennej, oznaczenie produkcji oraz stężenia tlenku azotu, oznaczaniu i wizualizacji produkcji anionorodnika ponadtlenkowego, oznaczeniu stężenia nadtlenku wodoru, zawartości fenoli i flawonoidów, a także wykonaniu immunolokalizacji oraz oznaczeniu zawartości białek z grupą nitrową w cieczy trawiennej. Brałam również udział w interpretacji wyników, przygotowaniu manuskryptu oraz pełniłam funkcję autora korespondującego.

Swój wkład oceniam na 50 %.

Podpis

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mój indywidualny udział w jej powstaniu polegał na współuczestnictwie w wykonywaniu pomiarów całkowitej zdolności antyoksydacyjnej, aktywności peroksydaz klasy III oraz związków z grupą tiolową.

Swój wkład oceniam na 5 %.

Maciej Piekarniak

Podpis

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Wiejskiego w Warszawie

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mój indywidualny udział w jej powstaniu polegał na współuczestnictwie w opracowaniu koncepcji pracy, sprawowaniu opieki merytorycznej podczas przeprowadzania eksperymentów oraz przygotowaniu publikacji oraz redakcji i korekcie całego manuskryptu.

Swój wkład oceniam na 10 %.

Podpis

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Oświadczenie o współautorstwie

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mój indywidualny udział w jej powstaniu polegał na współwykonywaniu pomiaru aktywności proteolitycznej cieczy trawiennej, swój wkład oceniam na 5 %.

Podpis

Chodór Kamila

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Warszawa, 10.09.2024

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Oświadczenie o współautorstwie

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mój indywidualny udział w jej powstaniu polegał na współwykonywaniu pomiaru aktywności proteolitycznej cieczy trawiennej, swój wkład oceniam na 5 %.

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Jakub Bieniek

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mój indywidualny udział w jej powstaniu polegał na: udziale w redakcji i korekcie całego manuskryptu oraz sprawowaniu opieki podczas przygotowywania publikacji.

Swój wkład oceniam na 5 %.

A. Gniazdowska

Podpis

Załącznik nr 2 – Wzór oświadczenia o współautorstwie

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mój indywidualny udział w jej powstaniu polegał na opracowaniu koncepcji badań, sprawowaniu opieki merytorycznej w trakcie realizacji badań, udziale w interpretacji wyników, przygotowaniu publikacji oraz redakcji i korekcie całego manuskryptu.

Swój wkład oceniam na 20 %.

Urszula Krasuska

Podpis

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Nitric oxide stimulates digestion modifying the nutrient composition of the traps' fluid of *Nepenthes x ventrata*

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ABSTRACT

External digestion performed by autotrophs is a characteristic feature of carnivorous plants, such as those of the *Nepenthes spp*. These plants developed jug-shaped traps filled with digestive fluid that consists of water, various proteins (mostly enzymes), and nutrients. Moreover, the presence of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the traps' fluid of *N. ventrata* has been demonstrated. RNS, among them nitric oxide (NO), accelerates digestion e.g. by the alteration of ROS levels. The aim of the study was to demonstrate the stimulation of external digestion by NO_x supplementation linked to the modulation of the nutrient composition of the trap fluid, digestive enzyme activity and gene transcription. Using the digestion fluid of *N. ventrata* mature traps we indicated that NO_x temporarily increases K, Fe, Cu and ammonia that may be involved in the modulation of free radicals content. The stimulatory effect of NO_x on the activities of enzymes responsible for digestion, and on the transcripts' levels of *Nepenthesin I* and *II*, *Purple Acid Phosphatase*, and *S-like Ribonuclease* was shown. The decrease in the level of carbonylated proteins (from food source) in the trap' fluid during digestion suggests their absorption by *Nepenthes* trap tissues. We also demonstrated the presence of carbonylated proteins in the trap fluid before feeding.

1. Introduction

Autotrophs with the ability to attract, trap, kill, digest, and absorb the released nutrients from the prey are known as carnivorous plants. They naturally occur in areas of nutrient-poor availability. Among them, *Nepenthes spp.* (commonly known as pitcher plants) underwent evolutionary changes in which photosynthetically active leaves gradually developed into passive jug-shaped containers located at the end of the main vein of the leaf blade. The natural habitats of these plants are located mainly in Southeast Asia (Krasuska et al., 2023; Mithöfer, 2011 and references herein). The growing interest in various pitcher plants is mainly due to their ability to synthesize and release different antimicrobial compounds (Buch et al., 2013; Pilarska et al., 2022). Digestion takes place within the mature, opened trap, which creates an environment specifically adapted for extracting nutrients from the prey. The trap consists of the characteristic zones reflecting the physiological functions: (i) attracting the prey and facilitating its fall inside the organ (peristome), (ii) preventing the escape of the prey (waxy zone), and (iii) digesting the prey and absorbing nutrients (digestive/glandular zone) (Krasuska et al., 2023; Moran and Clarke, 2010). The specific composition of the trap fluid is the adaptation to the plant's nutritional strategies. The presence of enzymes responsible for food decomposition in the trap of *N. alata* has been confirmed (Zulkapli et al., 2021). Most enzymes responsible for proteolysis in carnivorous plants belong to two

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classes: the aspartic proteases and serine carboxypeptidases. Among them the best characterised are Nepenthesin I (Nep I) and Nepenthesin II (Nep II), the members of a subfamily of aspartic proteases, with a high content of cysteine (Cys) residues (Athauda et al., 2004; Zulkapli et al., 2021). In the traps' fluid of *N. ventricosa* the activities of Cys proteinase and S-like Ribonuclease (RNase) have been noticed (Stephenson and Hogan, 2006), as well as various esterases, and chitinases (Krasuska et al., 2023; Rottloff et al., 2016 and references herein). Phosphorus (P) mobilization from the prey body depends on the activity of phosphatases that catalyse the hydrolysis of phosphate esters (Płachno et al., 2006; Rottloff et al., 2016). Other components of the digestive fluid are naphthoquinones, flavonols, and small sulphur-containing compounds (Aung et al., 2002; Mithöfer, 2011; Wal et al., 2024). Beyond, various ions are found in the trap fluid, even within the immature closed pitchers (Buch et al., 2013). Macro and micro-elements play a key role in plant metabolism, they also participate in signal transduction. The limitation of soil nitrogen availability for roots is compensated by the specific transporters (ammonium transporters, amino acid transporters or/and peptide transporters) in the carnivorous plants' traps (Schulze et al., 1999). In addition to nitrogen (preferentially ammonium ions - NH_4^+) and P, the absorption of other nutrients: iron (Fe), manganese (Mn) and magnesium (Mg) was shown in pitcher plants (*Cephalotus follicularis* La Billadiere, *Sarracenia purpurea* L., *Heliamphora nutans* Bentham) (Adlassnig et al., 2009; Schulze et al., 1999).

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are crucial regulators of various physiological processes. Their presence in the digestive fluid of pitcher plants has been confirmed (Chia et al., 2004; Wal et al., 2024, 2022). Chia et al. (2004) pointed at the involvement of the oxygen free radicals in the early phase of digestion in *N. gracilis* Korth. The formation of ROS, and alterations in their content in the digestive fluid of the *N. ventrata* during trap ontogeny as well during digestion stimulated by nitric oxide (NO) were demonstrated (Wal et al., 2024, 2022). The oxygen incomplete reduction or excitation generates various ROS. Superoxide anion ($O_2^{\bullet-}$) and hydroxyl radical ($^{\bullet}OH$) are included intro ROS free radicals; contrary hydrogen peroxide (H_2O_2) is a non-radical compound (Mittler, 2017). ROS molecules differ in half-life. The half-life of $^{\bullet}OH$ is very short, thus this molecule reacts mostly at its formation site. In contrast H_2O_2 has longer half-life, and can be relocated, thus facilitates the signal transduction (Hübner and Haase, 2021; Mittler, 2017). Besides its signalling role, H_2O_2 in the presence of Fe(II) is involved in $^{\bullet}OH$ or ferryl intermediate $[Fe^{4+}=O]^{2+}$ generation by the Fenton reaction (Prousek, 2007). In addition to Fe^{2+}, copper ion (Cu^+) may promote free radicals formation due to the Fenton–like or Fenton–type reactions (Bosio et al., 2023). Plants generate ROS in chloroplasts, peroxisomes, mitochondria, and in the apoplastic space releasing them also into the surroundings (culture media, digestive fluid) (Corpas et al., 2015; Liszkay et al., 2004; Wal et al., 2024). Reactive compounds and antioxidants modulate ROS level, form the redox potential hubs, and are responsible for the organelle-to-organelle and cell-to-cell signal transduction (Fichman et al., 2022; Mittler, 2017). Plant responses to the environmental stimuli is mediated by ROS and calcium ions (Ca^{2+}) via the central ROS receptor that is a leucine-rich-repeat receptor-like kinase H_2O_2-INDUCED CA^{2+} INCREASES 1 (HPCA1) (*Arabidopsis thaliana*) (Fichman et al., 2022). ROS participate in the post-translational modifications of proteins, including reversible and non-reversible alterations (Ciacka et al., 2020). Protein carbonylation is a form of irreversible (in vivo) protein oxidation. Direct ROS attack or metal-catalysed oxidation (MCO) of proline, arginine (Arg), lysine or threonine generate carbonyl groups in target proteins. Thus, protein carbonylation is stimulated in the presence of ROS, reduced metal ions and also reducing sugars, leading to adduction of advanced glycation end products (AGEs). Carbonylated proteins lose their function and are prone to degradation (Ciacka et al., 2020b). Over accumulation of ROS is mitigated by antioxidants which presence was proven in the digestive fluid of carnivorous plants (Wal et al., 2024).

Based on the reduction potential of the environment, NO forms various RNS, which similarly to ROS, are known as common cellular regulators. In plants RNS are generated in almost all compartments, including the apoplastic space (Del Castello et al., 2019; Kolbert et al., 2019; Wal et al., 2024). At low concentrations, NO and other RNS act as signals modifying proteins via nitration or *S*-nitrosation (Ciacka et al., 2020a; Corpas et al., 2023). Depending on the oxygen availability or the metabolic activity NO in plants is formed enzymatically or non-enzymatically (by oxidative or reductive pathways). The oxidative Arg-dependent pathway is still an open question, nevertheless possible (Corpas et al., 2023). RNS together with ROS influence redox potential of the biological systems (Ciacka et al., 2020a; Samanta et al., 2024). Previously we have demonstrated RNS impact on ROS metabolism during digestion performed by *N. ventrata* (Wal et al., 2024, 2022). RNS accelerated protein degradation in the trap fluid. We suggested that NO_x regulated digestion due to, e.g. nitration of food proteins (Wal et al., 2024).

In this work we continue the studies of the mechanisms of NO_x action on external digestion performed in the trap of *N. ventrata* plants. The aim of this research was to verify the beneficial role of NO_x supplementation on the digestion process in the context of the modulation of the oxidative environment in the trap fluid. We have focused on the impact of NO_x on the basic nutrients (macro and microelements) and NH_4^+ content in the fluid during digestion. Our goal was to connect the changes observed in the level of microelement (Cu, zinc (Zn), Fe), effecting redox potential, to the reducing capacity of the tissue and the alterations in carbonylated protein content in the digestive fluid. The phosphatase activity in the fluid and its enzymatic visualisation in the trap tissue after plants feeding supplemented with NO_x were analyzed. Gene expression of *Nep I* and *II*, *Purple Acid Phosphatase* (*Pap*), and *S-like RNase,* in glandular zone of trap, was investigated to link a positive role of NO_x to prey's digestion via signal from fluid to trap tissue.

2. Materials and methods

2.1. Experimental model and plant culture

Nepenthes × *ventrata* Hort. ex Fleming (a hybrid of *N. ventricosa* Blanco and *N. alata* Blanco) plants were used as the experimental material. The cultivation was conducted in a greenhouse that provided controlled environmental conditions: a constant 60 % humidity, 28°C temperature, and additional sodium lighting to maintain a 16-hour day and 8-hour night photoperiod during autumn and winter. In spring and summer, plants were exposed only to natural light. They were grown in pots with acidic peat, perlite, and Sphagnum moss. Plants were watered every other day with distilled water. During the period between the experiments (a minimum of three weeks in between tests), the plants were fertilised with mineral fertiliser. The mineral fertilizer (Biotom) used contained the following composition: total nitrogen 0.4 %—including ammonium nitrogen 0.1 %, nitrate nitrogen 0.1 %, and amide nitrogen 0.2 %; water-soluble phosphorus pentoxide 0.6 %; and water-soluble potassium oxide 0.6 %. Additionally, it contained micronutrients: boron 0.001 %, copper 0.0002 %, iron 0.002 %, manganese 0.001 %, molybdenum 0.0001 %, and zinc 0.0002 %. This dilution was experimentally determined during the optimization of plant culture conditions. To prevent unintended contamination, gauze was placed over the traps immediately upon opening to block accidental insect entry (Fig. 1). Digestive fluid and trap tissue were collected from mature pitchers (aerial type, around 9 cm in length) fed or non-fed (NF). Feeding consisted of the addition of 40 μL of egg white solution (1 μg μL^1) (CTRL) or egg white solution with nitric oxide donor (NO_x): 50 μL mixture of 0.1 M $NaNO_2$ with 1 mM HCl (NO) (Wal et al., 2024) directly into the pitcher. Samples were collected 1, 2 and 4 days after feeding (daf). The digestive fluids were shortly centrifuged (5522 × *g*, 5 min at 4°C), and the supernatants were used for analyses. The tissue from the glandular zone of the trap was used immediately after collection for the experiments or frozen in liquid nitrogen and stored in a

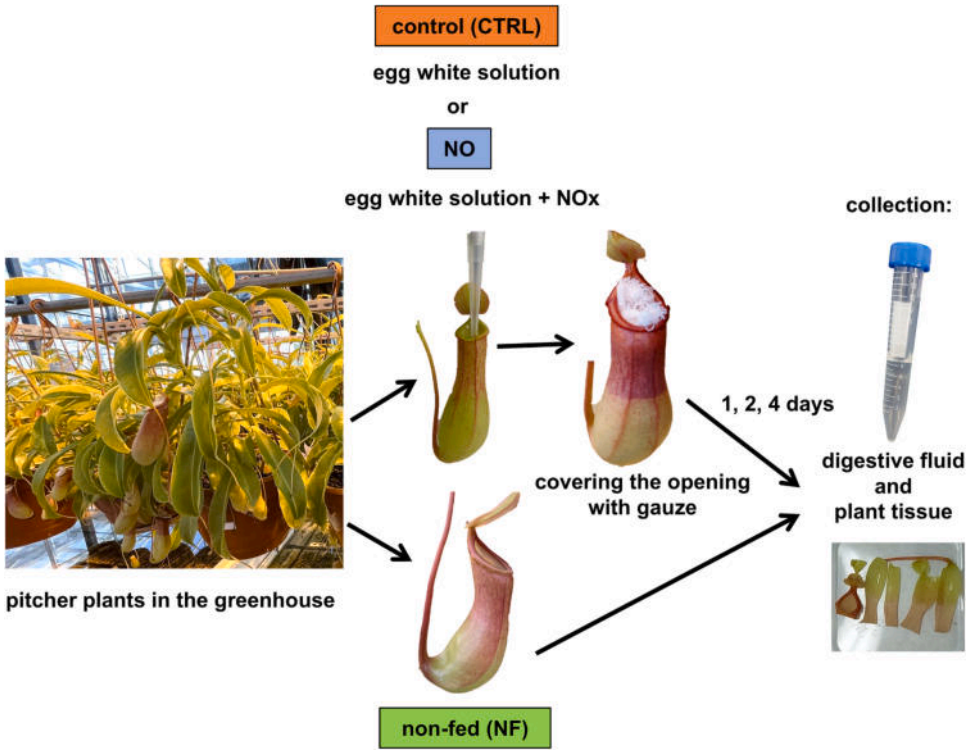

Fig. 1. The photography illustrates the cultivation of *Nepenthes* × *ventrata* plants in the greenhouse. The diagram outlines the steps for collecting experimental materials: digestive fluids and tissues from the trap. We randomly selected plants and traps for the experiment that were not treated - non-fed (NF), control (CTRL) to which egg white solution was added and samples to which egg white solution and nitric oxide (NO) were added. The openings of the traps were covered with sterile gauze.

−70°C freezer.

2.2. Measurement of oxygen concentration in the digestive fluid

Oxygen content in the digestive fluid was measured using liquid-phase oxygen electrode systems (Hansatech Instruments Ltd Oxygen Electrode Measurement System). The electrode was calibrated on de-aerated water (addition of a 50 mM sodium dithionite solution to the measuring chamber). Measurements were taken in a volume of 1.5 mL of digestive fluid, at constant temperature and pressure. The experiments were done in six biological replicates. The results were expressed as mmol oxygen L^{-1}.

2.3. Measurement of reducing capacity in the digestive fluid

The digestion fluid was buffered with 2 M sodium phosphate buffer pH 6.6 at a ratio of 1:9. An equal volume of 1 % potassium ferricyanide solution was added to the buffered fluid (Ferreira et al., 2007). The mixture was incubated at 50°C for 20 min in the dark. Then 250 μL 100 % TCA was added and incubated for 5 min at RT at dark. The mixture was then centrifuged at 15000 ×*g* for 15 min. To the supernatant (2.5 mL), 1 mL of 0.1 % of ferric chloride was added and incubated for 10 min at RT. Absorbance was measured at 700 nm by a microplate reader CLARIO star plus, BMG LABTECH. The experiments were done in three biological replicates and in three technical repetitions. The results were presented as % reducing capacity relative to the 0.125 mM ascorbic acid solution, which accounted for 100 %.

2.4. Immunodetection of protein carbonyl groups in the digestive fluid

Immunoblotting technique was done for detection of protein carbonyl groups. Separation of proteins from the digestive fluid was done using the SDS-PAGE method (Laemmli, 1970). Protein precipitation was made with cold acetone 100 %, it was added to the digestive fluid (4:1) and incubated for 24 h at −20°C (Biteau et al., 2013). Next, the samples were centrifuged 20 min at 12000 × *g* at 4°C. The supernatant was removed and the precipitate dried, then 1 mL of 0.1 M ammonium acetate was added to the precipitate, vortexed, and placed into an ultrasonic bath for 10 min at 4 °C. After incubation, the samples were centrifuged 15000 × *g* for 15 min at 4°C and the supernatant was removed and the precipitate dissolved in K-phosphate buffer with 1 mM EDTA, 2 % (w/v) PVPP, 2 mM DTT, and 10 % (v/v) glycerol. Isolated proteins with CO groups were labelled with 20 mM 2,4-dinitrophenylhydrazine (DNPH Sigma-Aldrich) dissolved in 2 M HCl for 30 min at 35 °C in darkness. At the same time, samples prepared without DNPH were incubated in 2 M HCl. Proteins were precipitated 10 min with 10 % (w/v) TCA, and the pellets obtained after centrifugation 10000 × *g*; 10 min at 4°C were washed four times with 1:1 (v/v) ethanol: ethyl acetate. After that, the samples were centrifuged for 5 min at 10000 × *g* at 4°C. The obtained pellets were briefly dried and then resuspended in 45 μL Laemmli buffer: 63 mM Tris-HCl, pH 6.8, 1 % (w/v) SDS, 10 % (v/v) glycerol, 0.01 % (w/v) bromophenol blue and 20 mM DTT by sonication 15 min at 4°C. Samples were denatured for 10 min at 70°C. The same steps as described above were done for the mixture of 5 mL water with the addition of egg white solution with or without NO_x (concentration and volume the same as was added to the trap. The whole volume of each sample was loaded on 10 % polyacrylamide gels with SDS and separated. SDS-PAGE was carried at a constant voltage of 150 V using the Mini-PROTEAN system (BIO-RAD). Then, proteins were electrotransferred to nitrocellulose membranes (Amersham™ Protran® Western blotting membranes, nitrocellulose, pore 0.2 μm) according to Towbin et al. (1979) using a Bio-Rad wet blotting apparatus. Subsequently, the proteins were visualised on the membranes and the gel via the photoreaction of tryptophan with Trichloroethylene (TCE). The membranes were blocked with 5 % non-fat milk in TBST at RT for 1 h. The membranes were incubated with

antibodies monoclonal anti-DNP, and antibodies were diluted in TBST of 1:60000 (Monoclonal Anti-dinitrophenyl (DNP) antibodies, A2831 Sigma-Aldrich) at RT for 1 h and overnight at 4°C. After being washed in TBST three times, the nitrocellulose membranes were incubated in solution: 0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$, 0.2 mM nitroblue tetrazolium (NBT), and 0.21 mM BCIP. Assays were performed in four or five independent biological replications. Images of representative fragments of membranse were shown after photos were processed in Image Lab Software. The original images of the membranes were shown at Fig. S1.

2.5. Determination of elements: Ca, Cu, Fe K, Mg, Mn, Na, P, S and Zn in the digestive fluid

The digestive fluid was filtered with syringe filters (0.45 μm) and diluted 1:9 with 2 % HNO_3. Samples that contained only water with egg white solution or egg white solution and NO_x were prepared the same way as the digestive fluid samples. The content of selected elements was measured using inductively coupled plasma optical emission spectrometry (ICP OES iCAP 7000 Thermo Fisher Scientific). The experiments were done in three biological replicates. All samples were preserved at 4°C before analysis. The results are shown in mg L^{-1}.

2.6. Measurement of ammonium ions content in the digestive fluid

The digestive fluid was buffered by mixing with 1 M K-phosphate buffer, pH 7.5 in a 9:1 ratio. To the buffered fluid, 100 μL phenol reagent (7 g phenol and 34 mg disodium pentacyanonitrosylferrate were dissolved in 100 mL deionised water) and 200 μL hypochlorite reagent (2.96 g NaOH and 29.74 g Na_2HPO_4 12 H_2O were dissolved in 140 mL deionised water and 16.6 mL of 12 % NaOCl solution was added (Witte and Medina-Escobar, 2001). The samples were gently shaken and incubated for 1 h at 50 °C. After the samples had cooled down to RT, absorbance was measured at 636 nm by microplate reader CLARIO star plus, BMG LABTECH. In the blank, distilled water was added instead of the digestive fluid. The experiments were done in three biological replicates and in three technical repetitions. The standard curve was prepared using ammonium sulphate (Fig. S2). The ammonium ions content was expressed as μmol NH_4^+ $trap^{-1}$.

2.7. Analysis of gene expression in the trap tissue from the glandular zone

Total RNA was isolated from 1 g glandular tissue of the trap. Samples were ground in liquid nitrogen and transferred to a cooled falcon tube and 5 mL RNA extraction buffer: 100 mM Tris-HCl pH 8.0, 25 mM EDTA, 2 M NaCl, 2 % cetyltrimethylammonium bromide (CTAB), 2,5 % Polyvinylpyrrolidone (PVP-40) and 2 % β-Mercaptoethanol was added (Abdul-Rahman et al., 2017). The buffer was heated to 60 °C before use. Samples were shaken on Vortex. The samples were then incubated for 1 h at 65 °C, mixing every 10 min. After incubation, an equal volume of chloroform-isoamyl alcohol mixture (ratio of 24:1) was added. The samples were centrifuged for 15 min, 13000 × *g*, 4 °C (MPW-350R). After centrifugation, the supernatant was transferred to a new faclon tube and an equal volume of the chloroform-isoamyl alcohol mixture was added again. The samples were centrifuged again for 15 min, 13 000 × *g*, 4 °C, the upper phase was transferred to tubes. Next, 12 M lithium chloride (LiCl) was added in a volume representing 1/3 of the sample volume, gently mixed and incubated overnight in a freezer at −20°C. The samples were centrifuged for 90 min, 13 000 × *g*, 4 °C. After centrifugation, the liquid phase was carefully removed and the precipitate was suspended in 1 mL of 0.1 % DEPC water. Samples were transferred to tubes and an equal volume of the chloroform-isoamyl alcohol mixture (24:1) was added. Samples were centrifuged at 13000 × *g*, at 4 °C, for 15 min. The supernatant was transferred to tubes. Then 12 M LiCl was added to the samples in a volume representing 1/3 of the sample volume, gently stirred and incubated overnight in a freezer at −20°C. The samples were centrifuged for 30 min, 13000 × *g*, 4 °C. The liquid phase was removed, and the precipitate was washed with 500 μL of 75 % ethanol and centrifuged for 15 min, 13000 × *g*, 4 °C. After centrifugation, the ethanol was removed. Samples were again washed with ethanol as described above. The precipitates were dried and dissolved in 15 μL of 0.1 % DEPC water and stored in the freezer, at −70 °C.

Samples were treated with DNAase I (Thermo Scientific EN0523). The reaction was carried out for 30 min, 37 °C. After incubation to inhibit the reaction, 1 μL of 50 mM EDTA was added to the samples, and they were incubated for 10 min at 65 °C. Total RNA (200 ng) was used for cDNA synthesis. The cDNA was obtained using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, K1622) in a total volume of 10 μL in a thermocycler (T100 Thermal Cycler, Bio-Rad). The finished product was diluted 8 times. A qPCR was carried out in a Bio-Rad CFX Connect™ Real-Time PCR Detection System. The iTaq™ universal SYBR® Green supermix (Bio-Rad, 172–5124) was used for the reaction in a total volume of 12 μL (6 μL PCR supermix, 2 μL cDNA, 1 μL primer, and 3 μL sterile H_2O). The primers used were designed based on literature data (Dkhar et al., 2020; Yilamujiang et al., 2016) (Table S1).

The expression levels were normalised using two reference genes: GAPDH enzyme (*GAPC*) and 18 s rRNA (*18 s*) (Table S1) and calculated using the method described by Hellemans et al. (2007). The experiments were done in four biological replicates in three technical repetitions.

2.8. Measurement of acid phosphatases activity in the digestive fluid

The digestive fluid 75 μL, was buffered by mixing with 475 μL 50 mM acetate buffer pH 3. The substrate for the acid phosphatase activity 4-nitrophenyl phosphate (p-NPP) (Sigma-Aldrich, P4744) was dissolved in 50 mM acetate buffer to 5 mM concentration (Saganová et al., 2018). To the buffered fluid, 400 μL of substrate was added. The mixture was incubated for 20 min at 25 °C. The blank was prepared in the same way as the test sample, but 75 μL of acetate buffer was added instead of the digestive fluid. The reaction was stopped by adding 160 μL of 1 M NaOH. Absorbance was measured at 405 nm by microplate reader CLARIO star plus, BMG LABTECH. The experiments were done in three biological replicates and in three technical repetitions. Results were calculated using the molar absorption coefficient of p-nitrophenol (pNP) $\varepsilon = 1.725\ M^{-1}cm^{-1}$ and presented as nmol pNP min^{-1} $trap^{-1}$.

2.9. Localisation of acid phosphatases activity in trap glandular tissue

Section (1 × 2.5 cm) of the glandular zone of the trap were placed in a solution of 0.1 % Triton X-100 for 10 min. After that, sections were washed four times with distilled water and incubated in a 0.01 % 5-bromo-4-chloro-3-indoyl phosphate (BCIP) solution for 24 h, then washed with distilled water (Wang and Liu, 2017). The stains were done in four biological replicates and in three technical repetitions. Images of stained sections were taken with a stereoscopic microscope with an integrated Nikon H550S camera with zoom in x13 (ISO, 2000; aperture f/3, exposure time 1/10 s).

2.10. Measurement of esterases activity in the digestive fluid

The digestive fluid was buffered by mixing with 1 M K-phosphate buffer, pH 7, in a 9:1 ratio. The esterase substrate was prepared by suspending 100 mM 4-nitrophenyl butyrate (Sigma-Aldrich, 335339) in 50 mM K-phosphate buffer, pH 7 (Morohoshi et al., 2011). To 630 μl of buffered digestive fluid, 70 μL of substrate was added and the mixture was incubated for 10 min at 37 °C. In the blank, instead of fluid, distilled water was used. After incubation, absorbance was measured at 410 nm by a microplate reader with CLARIO star plus, BMG LABTECH. The experiments were done in four biological replicates and in three technical repetitions. The results were expressed as U min^{-1} $trap^{-1}$, 1 U was $A_{410} = 0.1$.

2.11. Statistical analysis

The plants were selected randomly from 50 individuals; only "aerial" and mature traps were used. One trap was collected from one plant, and it was one biological repetition. Measurements were done in 3–6 biological replicates, with three technical replicates for each. Data were analysed using Statistica Software. Mean values were calculated, and SD was provided. After two-way ANOVA, homogenous groups were evaluated using Fisher's LSD post hoc test.

3. Results

Feeding the trap with white egg solution (CTRL) or with white egg solution + NO_x (NO) did not influence the appearance and growth rate of plants and traps in relation to non-fed (NF) plants. No differences in plant's morphology were observed 1, 2 and 4 daf (Fig. 2).

3.1. Oxygen content in the digestive fluid

The highest oxygen concentration (around 780 mmol O_2 L^{-1}) was noticed in the digestive fluid from the NF trap and in the fluid from the CTRL trap 2 daf (Fig. 3). In general, trap feeding (with NO_x and without) resulted in a fluctuation in the oxygen level. A decline of oxygen content 1 daf, followed by the increased oxygen concentration 2 daf, and finally the drop down to the value of 344–317 mmol O_2 L^{-1} 4 daf were observed (Fig. 3).

3.2. Reducing capacity in the digestive fluid

The reducing capacity in the fluid from the non-fed (NF) trap was around 22 %. In the digestive fluid from CTRL traps it droped down for about 50 % 1 daf, but increased twice 2 and 4 daf (Fig. 4). The reducing capacity in the fluid from the trap fed with white egg solution and NO_x (NO) increased slightly just 1 daf as compared to fluid from the NF trap. During the experiment it was at the level similar to that one observed in the fluid from the NF trap, but lower than in the fluid from the CTRL trap (Fig. 4).

3.3. The carbonylated proteins pattern in the digestive fluid

Egg white solution proteins were rich in proteins with carbonyl groups. The mass of the two thickest bands were between 100 and 70kDa and between 30 and 55 kDa. In proteins isolated from digestive fluid from the NF trap, two thin bands representing carbonylated proteins of approximately 70 kDa were detected (Fig. 5A). A similar pattern was observed after the separation of proteins isolated from digestive fluid from the trap without NOx (CTRL) and with the addition of NOx (NO) 2 daf. Although, the protein bands corresponding to carbonylated proteins were weaker in the digestive fluid from the trap fed with NOx 2 daf than in the CTRL trap. Two bands of high intensity, approximately 70 kDa in size, were present in protein extracts from the digestive fluid from the trap 4 daf, irrespectivly of the feeding (CTRL and NO). In contrast, four bands indicating carbonylated proteins, also around 70 kDa and between 55 and 35 kDa were observed after separation of proteins isolated from digestive fluid from CTRL and NO traps 2 daf (Fig. 5). In contrast, four bands indicating carbonylated proteins were observed after separation of proteins isolated from digestive fluid from NO traps 2 daf (Fig. 5).

3.4. The concentration of elements Ca, Cu, Fe, K, Mg, Mn, Na, P, S, and Zn in the digestive fluid

A very low amount of Ca (around 3 mg L^{-1}) was added to the fluid during feeding. In the fluid from the NF trap, the Ca level was 102 mg L^{-1}. The highest concentration of Ca was in the digestive fluid from the trap, with the addition of egg white solution (CTRL) 1 daf and was almost 150 mg L^{-1} (Table 1). Generally, in the digestive fluid from the trap with the addition of NO_x (NO), the Ca content was lower than in the fluid from the CTRL trap.

In the digestive fluid from the NF trap and the trap, to which fluid NO_x was added 1 daf the highest Cu concentration was observed and it was over 0.010 mg L^{-1} (Table 1). Apart from this, in the digestive fluid from the trap with various feeding combinations, the Cu content was similar and remained constant regardless of the duration of the experiment.

The lowest K content was in the fluid from the trap, with the addition of egg white solution (CTRL) and was more than twice as low as that observed in the digestive fluid from the NF trap (Table 1). In the digestive fluid from the trap, to which fluid NO_x was added (NO) 1 and 2 daf the K content was higher than in the fluid without NO_x (CTRL). However, 4 daf in the digestive fluid from the trap, with the addition of egg white solution (CTRL) the level of K was the highest (around 150 mg L^{-1}).

The highest Mg content was in the digestive fluid from the trap 1 daf (both NO and CTRL) and 4 daf in the CTRL trap; it was around 22.5 mg $L^{-1}$and it was higher than in the fluid of the NF trap. Two and four daf in the digestive fluid from traps with the addition of NO_x (NO) Mg content was lower than in the fluid from the CTRL trap (Table 1).

Fig. 2. The photography presents plants with the non-fed (NF) trap, traps fed with white egg solution (CTRL) or with white egg solution + NO_x (NO) 4 days after feeding (daf).

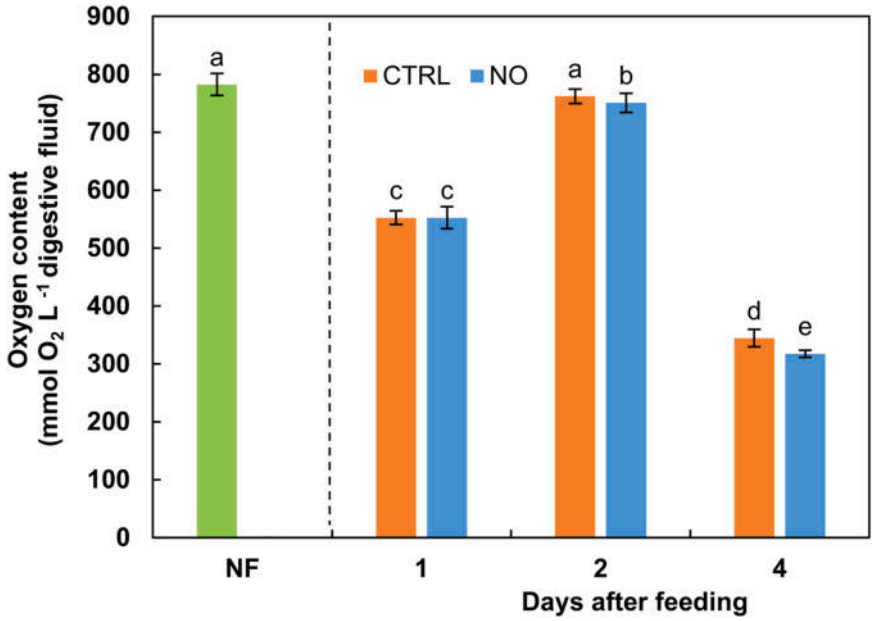

Fig. 3. Oxygen content in the digestive fluid from the non-fed (NF) trap and traps fed with white egg solution (CTRL) or with white egg solution + NO_x donors (NO) determined 1, 2, and 4 days after feeding (daf). Values are average ± SD of 6 repetitions. Letters (a–e) indicate homogenous groups determined after ANOVA and post hoc Fisher's LSD test at $p \leq 0.05$.

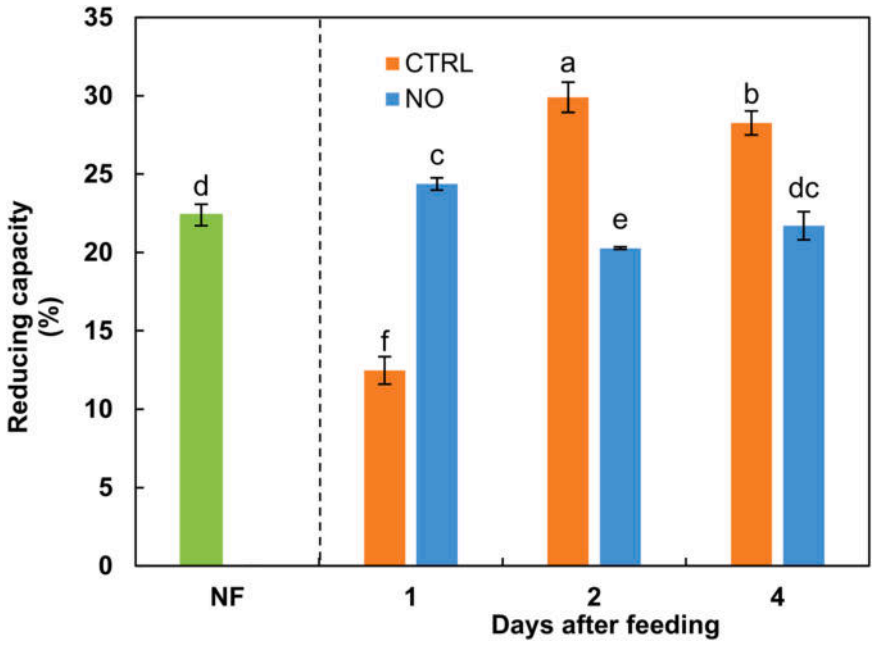

Fig. 4. Reducing capacity in the digestive fluid from the non-fed (NF) trap and traps fed with white egg solution (CTRL) or with white egg solution + NO_x donors (NO) determined 1, 2, and 4 days after feeding (daf). Values are average ± SD of 4 repetitions. Letters (a–f) indicate homogenous groups determined after ANOVA and post hoc Fisher's LSD test at $p \leq 0.05$.

The highest Mn concentation was in the digestive fluids from the CTRL trap 1 daf, and at the same period Mn concentration in the fluid in the NO trap was slightly lower, but still high. Prolongation of the experiment resulted in lowering Mn content in the digestive fluid of both CTR and NO traps (Table 1).

One daf the concentration of Na in the digestive fluid of fed (CTRL and NO) traps was twice or more higher than in the fluid from the NF trap (Table 1). In the fluid of NO traps it decreased as the duration of the culture was prolonged but was similar 2 and 4 daf. In the fluid from the CTRL trap Na concentration decreased almost 4 times 2 daf and later on increased to the value of 29.5 mg L^{-1} after additinal two days.

In the digestive fluid from the NF trap, the level of P was undetectable (Table 1). With the addition of egg white solution, around 0.06 mg L^{-1} of P was added to the digestive fluid, and this content was noted in the fluid from the trap with the addition only of egg white solution (CTRL) 1 daf. However, in the fluid from the trap to which the NO_x was added, the concentration of P increased rapidly 1 daf (was around 2 mg L^{-1}). In the fluid from both CTRL and NO traps P level decreased to the same value and was constant 2 and 4 daf.

No significant differences in Fe concentration were found between

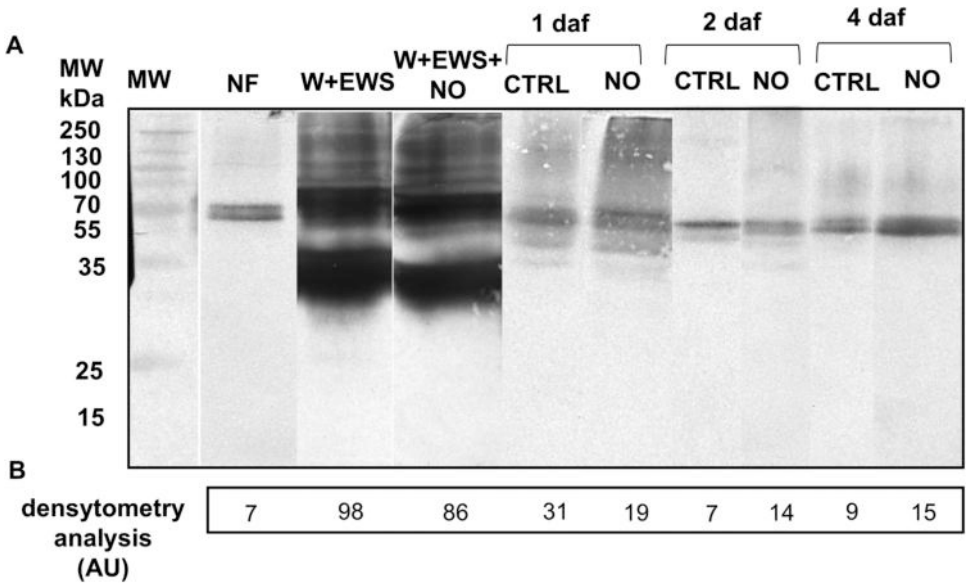

Fig. 5. The pattern of protein with carbonyl groups isolated from the digestive fluid (A), photos of the membrane were taken at an exposure of 0.2 s. Line MW: molecular markers, line W+EWS: proteins separated from deionised water mixed with egg white solution, line W+EWS+NO: proteins separated from deionised water mixed with egg white solution and NOx donor, line NF: proteins separated from the digestive fluid from the non-fed trap, line CTRL proteins isolated from the digestive fluid from the trap fed with white egg solution, line NO: proteins separated from the digestive fluid from the trap fed with white egg solution + NOx donors 1, 2, and 4 days after feeding (daf). The densytometry analysis arbitrary unit (AU) of the intensity of the line from the membrane with protein with carbonyl groups (B).

Table 1
Content of elements: Ca, Cu, Fe, K, Mg, Mn, Na, P, S and Zn in the digestive fluid from non-fed (NF) trap and the digestive fluids in the traps fed with white egg solution (CTRL) or with white egg solution + NO_x (NO) 1, 2, and 4 days after feeding (daf). Values are average ± SD of 3 repetitions. Letters (a–h) indicate homogenous groups determined after ANOVA and post hoc Fisher's LSD test at $p \leq 0.05$. Statistical analysis was performed separately for each element.

combination of feeding	Ca mg L^{-1}	Cu mg L^{-1}	Fe mg L^{-1}	K mg L^{-1}	Mg mg L^{-1}	Mn mg L^{-1}	Na mg L^{-1}	P mg L^{-1}	S mg L^{-1}	Zn mg L^{-1}
water + egg white solution	3.336 ± 0.325 h	0.005 ± 0.004 b	0.033 ± 0.003 b	6.574 ± 0.429 e	0.627 ± 0.099 e	0.002 ± 0.002 g	3.497 ± 0.645 f	0.060 ± 0.020 cd	1.123 ± 0.302 a	0.033 ± 0.041 b
water + egg white solution + NO_x	3.670 ± 0.100 h	0.001 ± 0.002 e	0.044 ± 0.027 ab	8.063 ± 1.116 e	0.764 ± 0.006 e	0	3.865 ± 0.0738 f	0.057 ± 0.015 d	1.271 ± 0.0430 a	0.087 ± 0.027 ab
NF	102.429 ± 1.381 e	0.014 ± 0.001 a	0.035 ± 0.004 b	210.619 ± 15.804 b	18.863 ± 1.441 b	0.130 ± 0.008 d	24.813 ± 2.227 d	0	0.418 ± 0.016 b	0.132 ± 0.039 ab
CTRL 1 daf	146.817 ± 1.087 a	0.001 ± 0.001 cde	0.027 ± 0.001 b	93.198 ± 8.128 d	21.961 ± 1.583 a	0.299 ± 0.020 a	78.787 ± 3.921 a	0.067 ± 0.015 cd	0.717 ± 0.088 b	0.119 ± 0.062 ab
NO 1daf	128.114 ± 1.690 b	0.011 ± 0.004 a	0.078 ± 0.042 a	157.203 ± 12.617 c	22.133 ± 1.700 a	0.213 ± 0.003 b	45.998 ± 3.482 b	2.027 ± 0.158 a	0.597 ± 0.066 b	0.131 ± 0.097 ab
CTRL 2daf	123.704 ± 2.630 c	0.003 ± 0.002 bcd	0.042 ± 0.038 ab	164.413 ± 14.828 c	18.615 ± 1.551 b	0.165 ± 0.003 c	20.543 ± 1.926 e	0.202 ± 0.033 b	0.519 ± 0.171 b	0.081 ± 0.071 ab
NO 2daf	87.421 ± 2.818 g	0.001 ± 0.002 e	0.030 ± 0.026 b	205.275 ± 18.161 b	15.693 ± 1.427 c	0.093 ± 0.010 e	21.041 ± 2.182 de	0.216 ± 0.041 b	0.665 ± 0.137 b	0.050 ± 0.071 ab
CTRL 4 daf	112.867 ± 3.834 d	0.003 ± 0.004 bc	0.063 ± 0.026 ab	240.842 ± 4.300 a	23.252 ± 1.234 a	0.076 ± 0.007 f	29.526 ± 1.883 c	0.188 ± 0.009 b	0.601 ± 0.294 b	0.169 ± 0.137 a
NO 4 daf	95.654 ± 3.431 f	0.001 ± 0.001 de	0.049 ± 0.014 ab	201.565 ±1.413 b	13.513 ± 0.503 d	0.124 ± 0.014 d	20.217 ± 0.859 e	0.155 ± 0.016 bc	0.453± 0.181 b	0.067 ± 0.058 ab

all tested digestive fluids. Zn content did not change in the digestive fluids regardless of feeding type and duration of the experiement (Table 1). In all tested digestive fluids, S concentrations were similar and 30–50 % lower than those in white egg solution added during feeding (Table 1).

3.5. The content of ammonium ions in the digestive fluid

The lowest concentration of NH_4^+ was in the fluid from traps 4 daf (both CTRL and NO) (Fig. 6) and was similar to the result for the fluid from the NF trap. The highest NH_4^+ content was 1 daf in the digestive fluid from the trap to which the NO_x was added, and in the fluid from the

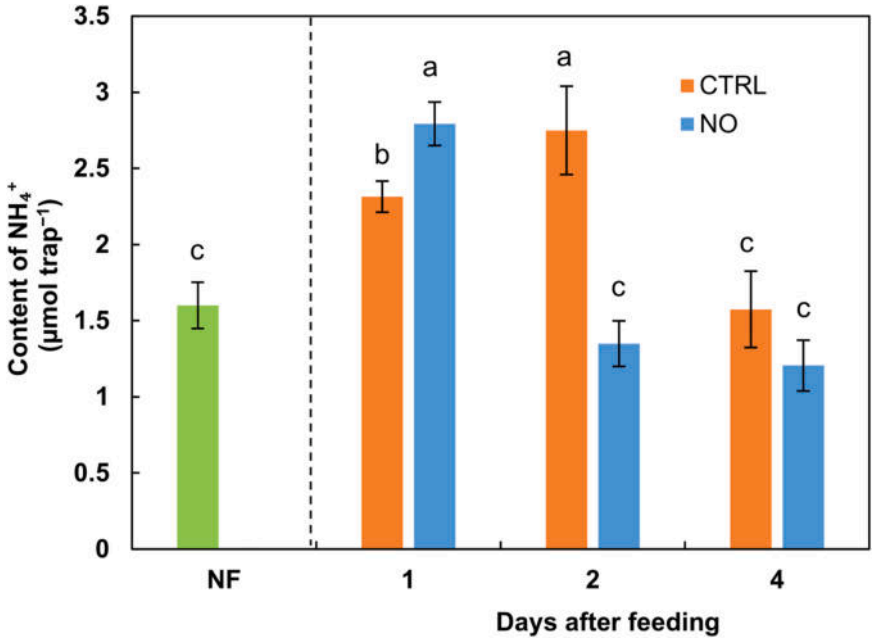

Fig. 6. The content of ammonium ions in the digestive fluid from the non-fed (NF) trap and in the digestive fluids in the traps fed with white egg solution (CTRL) or with white egg solution + NO_x (NO) 1, 2, and 4 days after feeding (daf) (A). Values are average ± SD of 3 repetitions. Letters (a–c) indicate homogenous groups determined after ANOVA and post hoc Fisher's LSD test at $p \leq 0.05$.

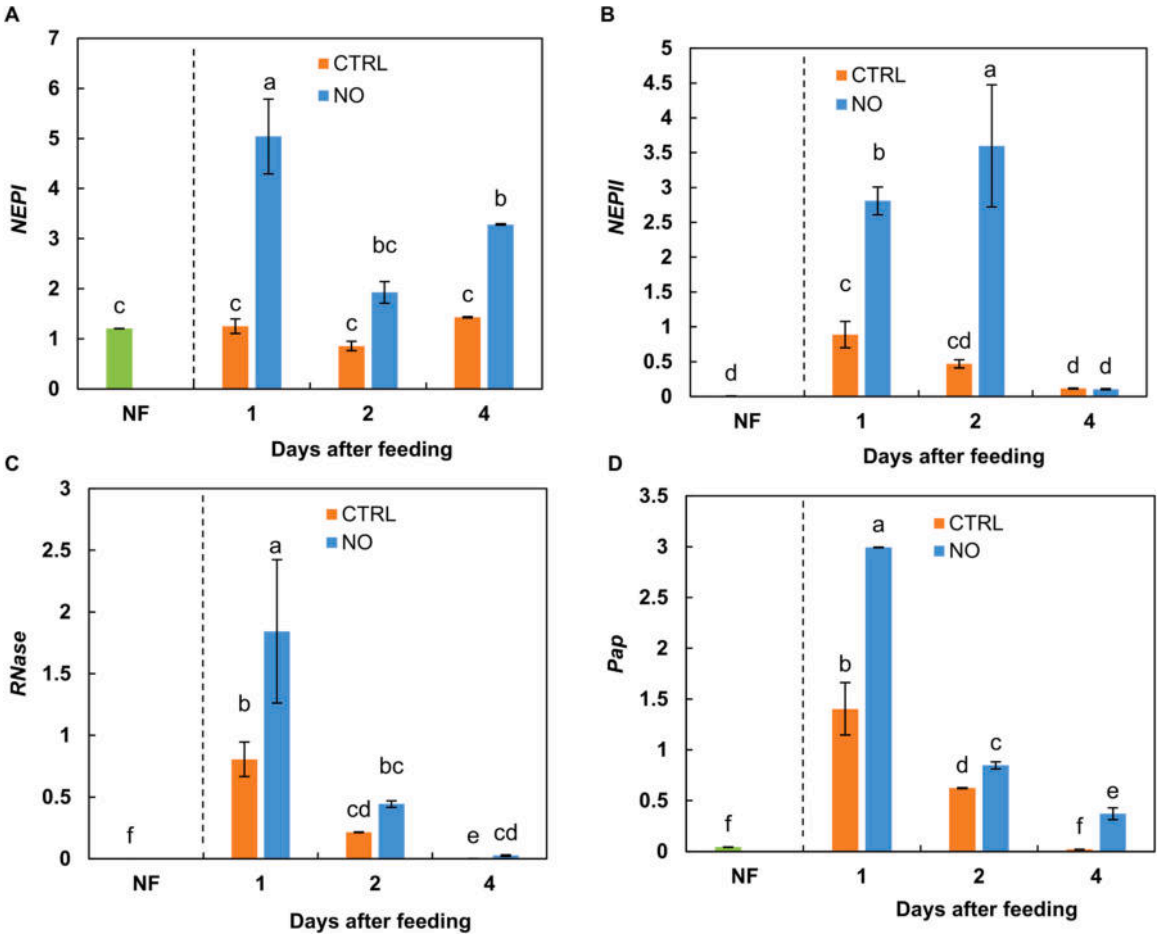

Fig. 7. Transcripts level (relative gene expression) of *Nepenthesin I* (*NEPI*) (A), *Nepenthesin II* (*NEPII*) (B), *S-like Ribonuclease* (*RNase*) (C), *Purple Acid Phosphatase* (*Pap*) (D) in the glandular tissue of the traps: non-fed (NF), fed with white egg solution (CTRL) or fed with white egg solution + NO_x donors (NO) determined 1, 2 and 4 daf. Values are average ± SD of 4 repetitions. Letters (a–f) indicate homogenous groups determined after ANOVA and post hoc Fisher's LSD test at $p \leq 0.05$.

trap with the addition of egg white solution, only 2 daf.

3.6. Transcripts levels in the trap glandular tissue

NEPI transcripts level in the glandular tissue from the trap, to which NO_x was added (NO), was higher than in the tissue without the NO_x (CTRL) regardless of the period after feeding (Fig. 7A). The highest level of *NEPI* transcripts was in the glandular tissue from the trap fed with NO_x (NO) 1 daf. In the tissue from the trap, to which egg white solution was added (CTRL) *NEPI* transcripts level did not differ 1, 2 and 4 daf and was similar to *NEPI* transcripts level in the tissue from the NF trap (Fig. 7A).

In the tissue from the trap, fed with NO_x (NO), the level of *NEPII* transcripts was higher than in the glandular tissue from the CTRL trap 1 and 2 daf (Fig. 7B). The gene transcripts level in the glandular tissue from the NF and NO as well CTRL traps 4 daf was minimal.

The highest transcripts level of *RNAse* was in the glandular tissue from the trap fed with NO_x donor 1 daf (Fig. 7C). The gene transcripts level in the tissue from the CTRL traps was lower than in glandular tissue from the trap, to which NO_x was added (NO), although 2 daf the difference was not statistically significant.

Generally, in the tissue from the trap with the addition of NOx donor, the *Pap* transcripts level was higher compared to the tissue from the traps without NO_x donor or non-fed (NF) (Fig. 7D). One daf *Pap* transcripts level in the tissue from the trap fed with NO_x donor (NO) was more than twice as high as in the tissue from the trap to which egg white solution was applied (CTRL) (Fig. 7D). Tanscript level in the tissue from the NF trap and tissue from the CTRL trap 4 daf was similar and very low (Fig. 7D).

3.7. Acid phosphatase activity in the digestive fluid

The highest phosphatase activity was in the fluid from the NO traps 1 and 2 daf (around 4 nmol pNP trap $^{-1}$). Generally, in the digestive fluid from the trap with the addition of NO_x donor, acid phosphatase activity was higher than in the digestive fluid from the trap without the addition of NO_x (Fig. 8A). In the digestive fluid from CTRL traps the phosphatase activity was stable during the experiement and similar to the activity in the fluid in NF traps (Fig. 8A).

3.8. Localisation of acid phosphatase activity in tissue

Phosphatase activity was observed exclusively within the glands. In the gland from NF and CTRL traps, regardless of the time after feeding, the most intense colour of chromogenic substrate for acid phosphatase was observed (Fig. 8B). In all tissue from the trap, to which NO_x was added, the intensity of staining demonstrating the presence of active acid phosphatases was lower than that of glands from the trap without the addition of NO_x (CTRL). Almost no staining was observed in the glands from the trap fed with NO_x 1 daf.

3.9. Esterases activity in the digestive fluid

The highest esterases activity was observed in the fluid from the traps 2 daf (CTRL 0.98 and NO 1.19 U min^{-1} trap^{-1}). In the digestive fluid 1 daf, regardless of the addition of NO_x or not, the activity of esterases was similar (Fig. 9), and it was almost twice as low as in the fluid from the NF trap. In the digestive fluid from CTRL and NO traps 4 daf esterases activity was the lowest.

4. Discussion

The trap of *N. ventrata* forms phytotelma (an aquatic environment created by a plant), and at the same time, it constitutes an external, specific apoplastic zone (Adlassnig et al., 2011; Gilbert et al., 2020; Wal et al., 2024). The quality of these small water environment is linked to

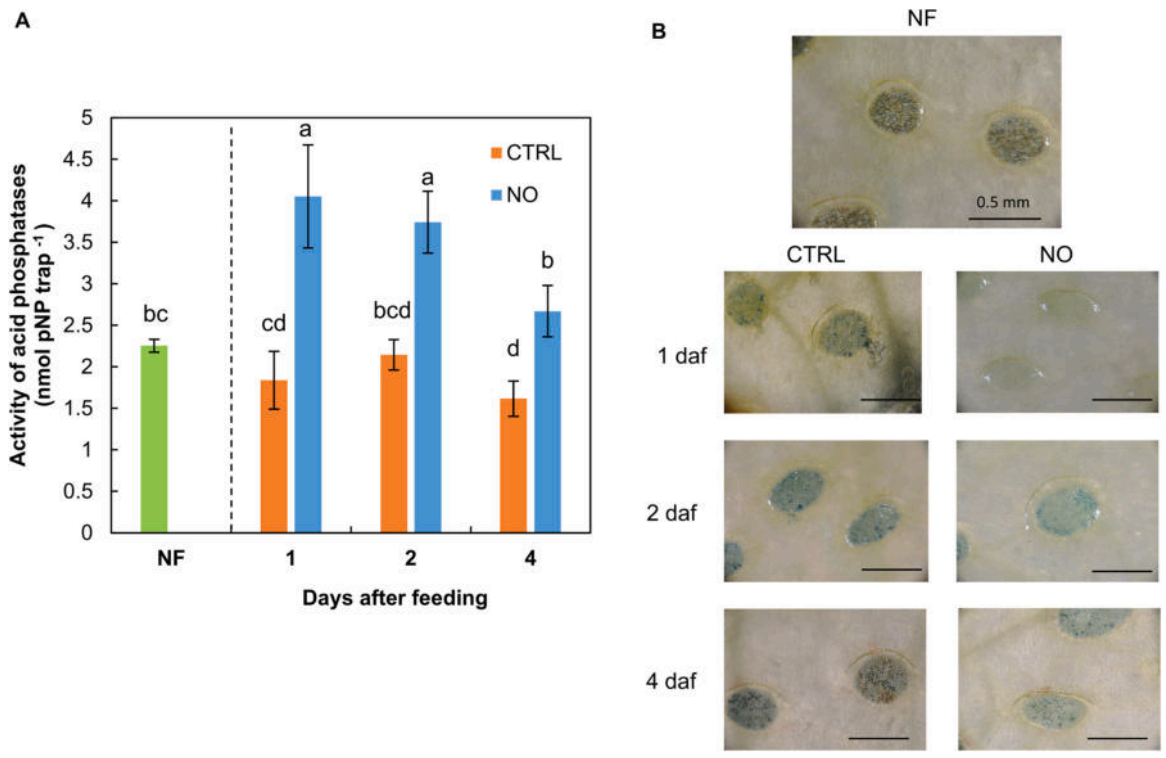

Fig. 8. Acid phosphatase activity in the digestive fluid from the non-fed (NF) trap and in the digestive fluids in the traps fed with white egg solution (CTRL) or with white egg solution + NO_x (NO) 1, 2, and 4 days after feeding (daf) (A). Values are average ± SD of 4 repetitions. Letters (a–d) indicate homogenous groups determined after ANOVA and post hoc Fisher's LSD test at $p \leq 0.05$. Localisation of acid phosphatase activity in tissue from non-fed (NF) trap and in the tissue from the traps fed with white egg solution (CTRL) or with white egg solution + NO_x (NO) 1, 2, and 4 days after feeding (daf) (B). Bar= 0.5 mm.

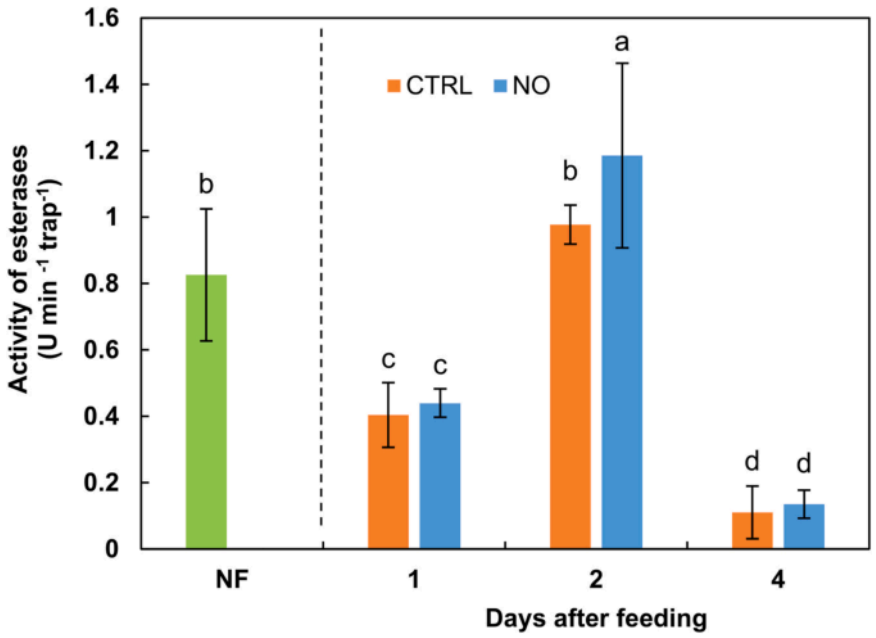

Fig. 9. Activity of esterases in the digestive fluid from the non-fed (NF) trap and in the digestive fluid in the traps fed with white egg solution (CTRL) or with white egg solution + NO_x (NO) 1, 2, and 4 days after feeding (daf). Values are average ± SD of 4 repetitions. Letters (a–d) indicate homogenous groups determined after ANOVA and post hoc Fisher's LSD test at $p \leq 0.05$.

oxygen concentration. Dissolved oxygen content in the trap fluid of various species of *Nepenthes* is actively modified, depending on the developmental stage. The lowest oxygen content (relatively hypoxic conditions) is observed in newly opened pitchers and it increases in the mature ones (Gilbert et al., 2020). In our experiment high oxygen content was recorded for the *N. ventrata* fluid of mature traps to which food had not yet been introduced (NF). At this phase increased oxygen level can be linked to the higher free radical scavenging capacity of low molecular weight antioxidants, which prevents the excessive formation of $O_2^{\bullet-}$ as we previously observed (Wal et al., 2024), the alterations in the oxygen concentration are related to the plant ability to modulate the level of microorganisms inhabiting the fluid of the trap (Gilbert et al., 2020). Low oxygen conditions decreases the number of aerobic microbes and are deadly to living insects to prevent their existence as the potent competitors (Gilbert et al., 2020). In *N. ventrata* trap fluid, we observed a decrease in oxygen content 1daf. Lower oxygen level, in the trap fluid, 1 daf correlated to ROS formation (Wal et al., 2024). In the presence of reductants (e.g. thiol compounds) and metal transition ions quinone derivatives are known to form ROS via non-enzymatic reactions (Hasegawa et al., 2004). Naphthoquinones were detected in the pitcher fluid (Mithöfer, 2011; Staszek et al., 2023), as well as small molecular thiol-containing compounds (Wal et al., 2024). The lowest oxygen concentration in the digestive fluid of *N. ventrata* was observed at the final stage of digestion, especially after NO_x application. Thus, we demonstrated that in the fluid of the mature trap the oxygen level was depended on the digestion progression, and plant strategy related to the carnivory. Using various amounts of *Dionea muscipula* extract and in the presence of 20 U of *Clostridium kluyveri* diaphorase the alteration in the oxygen uptake was observed (Galek et al., 1990). An increase in oxygen uptake was linked to the presence of juglone or plumbagin, flavoproteins that undergo autooxidation. In our model, the increase in the content of flavonoids in the fluid of *N. ventrata* trap 4 daf, and after NO_x application were also noted (Wal et al., 2024). Thus, the alterations related to the oxygen level may depend on the content of antioxidants and pro-oxidants. NO_x application, together with egg white solution, maintained the redox environment of the *N. ventrata* fluid close to this one in the NF traps. Higher reduction capacity of the fluid of NF traps and the fluid with NO_x 1 daf points to the protective role of NO_x against uncontrolled oxidation. An altered redox environment is involved in the redox signaling as digestion progressed. The systems of redox regulation, together with thiol-containing proteins and small molecular compounds are part of the plant sensing/responses mechanism to various signals (Peláez-Vico et al., 2022).

In addition to proteolysis, a non-enzymatic mechanism for nutrient release from food, involving the presence of ROS occurring in the trap fluid, has been proposed (Chia et al., 2004; Galek et al., 1990; Wal et al., 2024, 2022). Oxidation facilitates protein disruption (Wolff and Dean, 1986). Under aerobic conditions, in extracts from *D. muscipula* leaves, in the presence of quinones, the oxygen reduction to H_2O_2 i $O_2^{\bullet-}$ was described. This was accompanied by an increase in trypsin degradation of bovine serum albumin (BSA) by a combined oxidative-proteolytic reaction sequence (Galek et al., 1990). Going further, The authors suggested that ROS play a role in the digestion processes in carnivorous plants, through the modification of proteins (Galek et al., 1990). The oxi-modifications of proteins are proposed to impact food digestibility. This is achieved by altering the specific recognition sites of digestive enzymes or by forming cross-linking and/or aggregation, which may mask the recognition sites (Duque-Estrada et al., 2019; Sante-Lhoutellier et al., 2007). Contrary, protein unfolding caused by the oxi-dependent proteins' modifications increases the accessibility of specific sites to digestive enzymes (Soladoye et al., 2015). One of the most persistent ROS derivatives are oxi-modifed proteins, especially those with carbonyl groups (Ciacka et al., 2020b). The enzymatic degradation of products of the oxidized B chain of bovine insulin in *N. alata* pitcher fluid was noted (An et al., 2002).The first step of the digestion performed by *N. gracilis* was accompanied by the generation of free radicals in the pitcher fluid (Chia et al., 2004). Thus, protein oxidation stimulates or even initiates enzymatic degradation (Galek et al.1990). Feeding of *N. ventrata* is linked to the application of carbonylated proteins from egg white solution. Nevertheless, starting from the first daf, the decrease in the content of carbonylated proteins in the digestive fluid was observed, Previously the highest rate of proteolytic activity in *N. ventrata* trap was observed 1 daf with egg white solution supplemented with NO_x (Wal et al., 2024). We assumed that carbonylated peptides stimulated proteolytic activity. Carbonylated proteins cleavage may be performed by an ATP- and ubiquitin independent pathway by the 20S 'core' proteasome (Davies,

2001). As there were no proteins marked with ubiquitin in the digestive fluid (Fig. S3 i S4), it is possible that the carbonyl groups in the proteins would stimulate 20S proteasome, which requires verification. In addition, we propose that carbonylated peptides or amino acids with a carbonyl group may be absorbed by the *Nepenthes* traps' tissues. Many carbonylated proteins are of the food source, thus we are unable to determine whether carbonylation of digestive proteins takes place in the fluid and whether this modification could affect enzymatic proteins and finally impact their activity during digestion. Undoubtedly, there were also carbonylated proteins in the fluid prior food application which, indicates the regulatory role of this modification to some extent. This is also confirmed by the presence of such modified proteins of a higher molecular weight in the digestive fluid 4 daf. Therefore, not all carbonylated proteins seem to be degraded or are formed *de novo*. Similarly, nitrated proteins were present in the fluid from the NF trap and in the fluid from the fed traps 4 daf, which we explained by the regulatory function of this irreversible modification (Wal et al., 2024). The previously noted increased in $O_2^{\bullet-}$ concentration (Wal et al., 2024), may be related to the supplementation of proteins with carbonyl groups in a food source.

Our experimental model assumes plants' feeding with egg white solution, and the trap itself is isolated from uncontrolled access to insects. The chitinolytic activity was low (Fig. S5), noted only 2 daf in the digestive fluid from both the CTRL and NO traps. This indicates "economical" use of digestive enzymes, the compostion of which depends on the food type. The application of ammonium chloride (NH_4Cl) had no effect on chitinolytic activity in the absence of chitin measured in various *Nepenthes* species (Saganová et al., 2018). The stimulation of endochitinase activity was observed after the addition of the worm meal, BSA solution or chitin (Saganová et al., 2018). Plants in our experiment were fed with almost ten thousand less protein concentration. Thus, it may be assumed that the enzymatic composition of the digestive fluid is modified according to food quality and quantity.

It is supposed that NH_4^+ is the major donor of N for pitcher plants, that in the fluid reach the content of approximately 250 mmol L^{-1} (Schulze et al., 1997). The incubation of casein with the secreted fluid of *D. muscipula* resulted in the increase in NH_4^+ coming from the deamination of liberated amino acids (Scherzer et al., 2013). The increase in NH_4^+ content is linked to the digestion process or the activity of nitrogen-fixing bacteria located inside the trap (Prankevicius and Cameron, 1991). Feeding of *N. alata* increased the content of NH_4^+ (An et al., 2001). The transient rise, and then fast decrease in NH_4^+ concentration in the trap' fluid of *N. ventrata* after NO_x application may be due to the acceleration of the digestion rate and then, an increase in the absorption of these ions.

As the application of food together with NO_x to *N. ventrata* fluid stimulated proteolytic activity (Wal et al., 2024), thus we analysed transcript levels of *Nep I* and *Nep II* in the glandular tissue of the trap. The transcripts levels of *Nep II* was almost undetectable 4 daf and before feeding. Takahashi et al. (2005) observed that relatively high activity (85 %) of Nep II was only at pH 3, and pH above 5 led to a loss of its activity, in contrast to Nep I whose activity was high (79 %) even at pH 10. NO_x enhanced transcripts level coding both aspartic proteases. *Nep I* reached the highest level 1 daf, and *Nep II*, 2 daf. Saganová et al. (2018) found the highest levels of *Nep I* and *NepII* transcripts in the tissue from the digestive zone of *Nepenthes* after the application of NH_4Cl, compared to living prey or BSA. As NO_x increased the concentration of NH_4^+ in the trap fluid, it points that NO_x play a regulatory role in the stimulation of digestion. The more, NH_4^+ application resulted in rapid depolarization of the membrane in *Nepenthes* (Saganová et al., 2018). This electrochemical signalling pathway may also engage secondary messengers, including H_2O_2 (noted in the fluid of *N. ventrata* by Wal et al., 2024) or Ca^{2+}.

The digestive fluid of *Nepenthes* closed traps contains very low (undetectable) amount of N and P (Buch et al., 2013). In the pitcher fluid of the mature traps of *N. sumatrana*, *N. spectabilis*, and *N. tobaica* occurring in their natural environment, the concentration of P was between 8.30 – 6.30 mg L^{-1} (Mansur et al., 2022), much higher than in the fluid of *N. ventrata* in our study, regarding the greenhouse culture of the plants. In the fluid of NF traps total P content was also very low (below detection range). NO_x application enhanced total P content in the fluid 1 daf. This reflects the higher rate of digestion and the beneficial role of NO in this process. A gradual decrease in the amount of P in the fluid as digestion is continued indicates the utilization of this element. Together with the higher P level, the increase in acid phosphatases activity in the digestive fluid of *N. ventrata*, after NO_x application has been noted. Moreover, this was accompanied by phosphatase activity in the glands of the lower (digestive) part of the trap. Acid phosphatases participate in the P recycling and transport. It is proposed that plant true carnivory relay on the activity of this enzyme (Płachno et al., 2006). Application of prey to the trap strongly stimulated phosphatase activity in the digestive fluid of various *Nepenthes* (Saganová et al., 2018). We observed the stimulatory effect of NO_x implementation on the expression of "digestive" genes (e.g. *Pap*), especially 1 daf.

Another enzyme involved in the plant carnivory is S-like RNase (Arai et al., 2015). In the typical autotrophs it is responsible for the responses to leaf and flower senescence, P starvation, or wounding. As was shown for *Drosera adelae*, the digestive fluid contains RNase, which activity was also proved. In some carnivorous plants (*D. adelae* and *Cephalotus follicularis*) *S-like RNases* are constitutively expressed in glandular cells (Nishimura et al., 2013; Okabe et al., 2005). In traps of *Dionea muscipula* prey (capture) stimulated the expression of *S-like RNase*, then protein was secreted into the digestive fluid (Nishimura et al., 2013). The increased level of transcripts of *S-like RNase* from *N. ventrata* in the trap tissue was noted 1 daf with NO_x application. Then, starting 2 daf, a significant decrease almost to undetectable level has been observed. This suggests that NO_x is strongly involved in the carnivory response stimulating digestion by regulation of enzymatic activity and transcripts level.

Digestion is linked to the lowering of pH and to activation of various enzymes in the fluid, which impact the inner surface of the trap. To create a barrier between harsh environment and tissues, plants use esterases/lipases, which catalyse the formation of the hydrophobic coatings (Duong et al., 2018). It is expected these enzymes are used by carnivorous plants to protect the trap tissues from digestive enzymes and acidic environment. The highest activity of total esterases/lipases in the fluid of *N. ventrata* was noted 2 daf, which points to their protective role. It might be related to the fact that pH in the fluid started to rise from about pH 3 to pH 5. Morohoshi et al. (2011) showed that the highest, activity of esterases isolated from the digestive fluid of the pitcher plant *N. hybrida* was at pH between 4 and 6. Surprisingly, also quite high activity of esterases/lipases was measured in the trap fluid before food application (NF) (constant protective effect).

Sulphur (S) is not only found in essential amino acids, including Cys, but also as a hydrogen sulphide (H_2S), which plays the signalling role of a gasotransmitter (Corpas et al., 2020; de Bang et al., 2021). Increasing the content of carbonylated proteins in meat fed to mice impacted the gut microbiome. The authors observed a decrease in the amount of *Desulfovibrio* bacteria, which produce H_2S (Ge et al., 2021). The effect of carbonylated proteins as H_2S modulators in the digestive fluid of *N. ventrata* requires further research. Thiol groups are involved in the regulation of redox potential, in peptides and proteins may serve as redox sensors (Corpas et al., 2022). The presence of SO_4^{2-} in the pitcher fluids from the genus *Nepenthes* was confirmed, as well as in the closed *N. alata* pitchers (Buch et al., 2013). Moreover, the total S content in the closed *N. alata* pitchers was high 2.41 mg L^{-1}. The lowest content of the total S in the fluid from NF *N. ventrata* plants corresponds well with the lowest -SH groups content noted previously (Wal et al., 2024). It should be underlined, that the food source (egg white solution) introduced high amounts of total S into the trap fluid. The continuous decline of the concentration of this element in the fluid was characteristic as digestion was progressed.

The presence of Ca^{2+} and Mg^{2+} in carnivorous plants was confirmed long time ago (Smith, 1893). The role of Ca^{2+} includes its function as a signaling compound, also linked to ROS action. Just recently, we demonstrated altered peroxidase (of class III) activity in the trap fluid of *N. ventrata* (Wal et al., 2024). Peroxidases of class III under specific conditions take part in oxygen radicals formation (Shigeto and Tsutsumi, 2016). POx identified from the secretion of *N. mirabilis* have two conserved Ca-binding sites in distal and proximal positions (Rottloff et al., 2016). This suggests the involvement of Ca^{2+} in the regulation of POx activity, thus in the ROS level in the fluid of pitcher plants. Generally, carnivorous plants are considered calcifuges. However, it is proposed that Ca liberated from food serves other functions beyond nutritional purposes (Meir et al., 1991). High amounts of total Ca were noted for fluids from *N. sumatrana, N. spectabilis, and N. tobaica* (Mansur et al., 2022). The egg white solution provided very low amount of total Ca into the fluid of *N. vetrata*. In the fluid from the traps 1 daf total Ca content was the highest, and higher for fluid from the CTRL than the NO supplemented trap. NO_x application stimulated decline of total Ca content, starting 2 daf. As was demonstrated for *D. spatulata*, the prey capture modified the dynamic of Ca^{2+} responses both local and systemic (Procko et al., 2022). The authors also wonder how plant distinguish Ca^{2+} signal to respond appropriately. We are curious if NO_x helps differentiate these signals.

The osmotic regulation, anion-cation balancing, and proper binding of biomolecules are regulated by potassium ions (K^+) (de Bang et al., 2021). Passive traps of pitcher plants usually contain high concentrations of this element, even in closed ones e.g. in *N. alata* (1019 mg L^{-1}) (Buch et al., 2013). In the fluids of mature, opened traps of *N. sumatrana, N. spectabilis*, and *N. tobaica* total K was from about 1700 mg L^{-1} to about 1600 mg L^{-1} (Mansur et al., 2022). In our study, the egg white solution did not provide high amounts of total K (6.5 – 8.0 mg L^{-1}). The decrease of total K both in the fluid from the CTRL and NO_x supplemented trap was noted. However, NO_x prevented the rapid decline of K concentration, and at the same time decreased the total sodium (Na) level. Both, K and Na are responsible for osmotic regulation and electrolyte balance, but Na is not essential for plant growth and development (Maathuis et al., 2014). Only 1 daf the increase in the total Na content in the trap fluid was noted (as compared to the NF trap). We assume that this transient increase in the total Na, together with relatively high amounts of K stabilize the osmotic potential of the fluid. Also, lower amounts of Na in the fluid of *N. ventrata* plants our experimental model may be related to the diet. In the fluids of mature, opened traps of naturally grown *N. sumatrana*, *N. spectabilis*, and *N. tobaica* plants total Na was much higher c.a. 1000–900 mg L^{-1} (Mansur et al., 2022). These plants digested the food of animal origin, rich in Na.

Pitcher plants absorb Fe, Mn, and Mg (Adlassnig et al., 2009), using them for nutritional purposes. We did not observe any spectacular changes in the total Mg content in the fluid during food digestion. In the fluids of *N. sumatrana*, *N. spectabilis*, and *N. tobaica* plants the total level of Mg was lower in comparison to K and Na (Mansur et al., 2022). We believe that Mg plays a buffering role in the aqueous environment of the trap fluid. In addition to the nutritional role of Fe, Cu, Mn, and Zn, these elements implicate redox chemistry. Fe and Cu, are cofactors in electron transport chains, and are present in the antioxidant proteins (Palmer and Guerinot, 2009; Pérez-Almeida et al., 2019). The bivalent cation Zn is not redox active in the biological systems (Hübner and Haase, 2021; Palmer and Guerinot, 2009). Nevertheless, Zn bounded to the protein may affect its redox regulation, thus participates in the oxidation of targets compounds (Palmer and Guerinot, 2009, Hübner and Haase, 2021). The regulatory role of Mn^{2+} in the maintenance of ROS homeostasis may be related to their ability to be oxidised from Mn^{2+} to Mn^{3+} or by the enhancement of MnSOD activity (Coassin et al., 1992). In *N. ventrata* trap fluid, all redox active elements could be involved in the regulation of ROS metabolism. The transient differences in the total content of Fe, Cu, Mn and Zn in the fluid from the NO supplemented traps in comparison to the CTR were observed only 1 daf. Thus, we suppose that NO also indirectly (via elements) influences the regulation of the redox potential of the fluid by modifying the ROS content and accelerates the non-enzymatic degradation of proteins.

5. Conclusions

Plant carnivory depends on the ability to digest the food and to absorb the nutrients. This is a complex process under sophisticated regulation. RNS participate in the development of this scarce lifestyle, as a regulatory compound. NO accelerates digestion, preventing the too long presence of the prey (food) in the trap. This may protect against the uncontrolled growth of microorganisms. Based on the presented data, we propose that NO implicates the levels of compounds that function as signals, like NH_4^+or/and Ca^{2+} (Fig. 10). Moreover, NO strongly influences ROS level, which, in turn, participates in the regulation of digestive enzymes activity and transcription of encoding genes. This regulation is related to the oxygen content in the trap environment as well as the pH and the presence of certain elements (Fe, Cu, Zn, and Mn) (Fig. 10). Future research could explore the role of ion channels in the absorption of prey-derived nutrients, particularly in the context of RNS-mediated signaling. Particular focus should be put on channels involved in NH_4^+, amino acids, or peptides and Ca^{2+} transport, as they may be crucial regulators linking NO activity with nutrient uptake efficiency. Additionally, investigating how NO modulates ion fluxes under varying trap conditions, such as oxygen level, pH, and presence of metal ions, may reveal novel mechanisms of adaptive regulation in carnivorous plants.

CRediT authorship contribution statement

Urszula Krasuska: Writing – review & editing, Writing – original draft, Supervision, Methodology, Conceptualization. **Jakub Bieniek:**

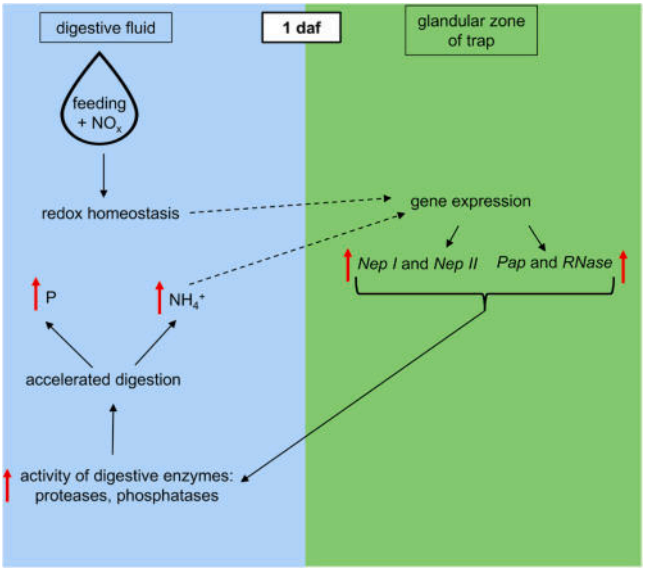

Fig. 10. A schema illustrating the role (solid thin lines) and proposed (dashed thin line) mechanism of action (solid red lines) of NO introduced together with egg white solution in the trap fluid of *N. ventrata* 1 day after feeding (daf). The addition of NO leads to a change in the concentration of ROS and RNS in the digestive fluid (Wal et al., 2024). This increases the expression of genes (in the tissue – in the glandular zone marked in green color) encoding enzymes involved in digestion: proteases Nepenthensin I and Nepenthensin II (Nep I and Nep II) and Purple Acid Phosphatase (Pap). Increased gene expression results in the activation of proteolytic enzymes and phosphatases in the digestive fluid (marked in blue color), leading to accelerated digestion. As a result of the enzymes activation, NH_4^+ and P are released into the fluid. Wide arrows indicate increased level, activity or expression.

Investigation. **Adéla Šípková:** Investigation. **Vladislav Chrastný:** Supervision. **Agnieszka Gniazdowska:** Writing – review & editing, Supervision. **Pawel Staszek:** Supervision, Conceptualization. **Agnieszka Wal:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plantsci.2025.112558.

Data availability

Data will be made available on request.

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Nitric oxide stimulates digestion modifying the nutrient composition of the traps' fluid of *Nepenthes ventrata*

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Supplementary information:

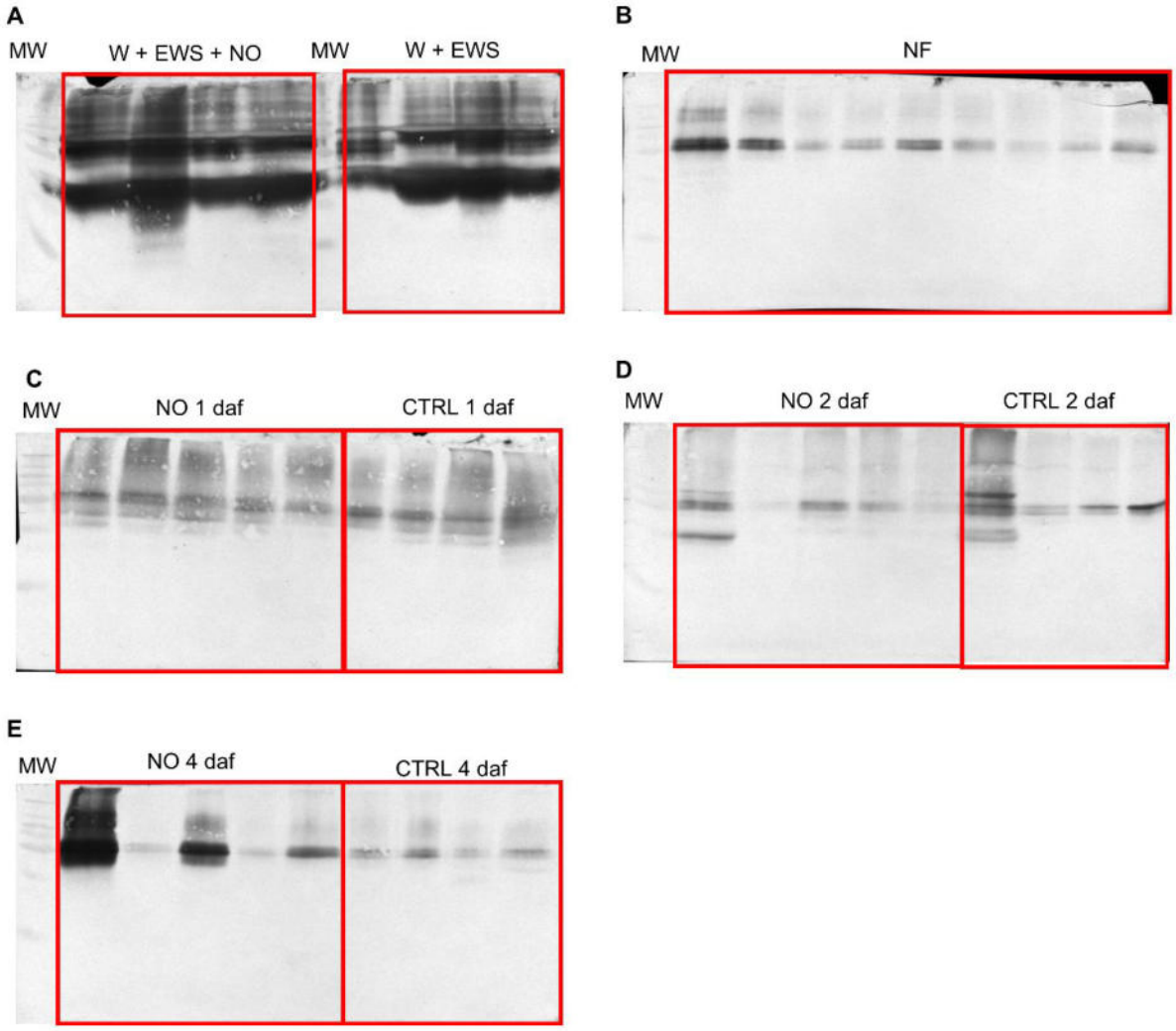

Fig. S1. The pattern of protein with carbonyl groups isolated from the digestive fluid from the non-fed traps (NF), traps fed with egg white solution (CTRL), and traps fed with egg white solution with the addition of NO_x donor (NO). Measurements were done in the digestive fluid 1, 2, and 4 days after feeding (daf). Line MW: molecular markers, line W+EWS proteins

separated from deionised water mixed with egg white solution, line W+EWS+NO proteins separated from deionised water mixed with egg white solution and NO_x donor. Line MW: molecular markers, line W+EWS proteins separated from deionised water mixed with egg white solution, line W+EWS+NO proteins separated from deionised water mixed with egg white solution and NO_x donor. Photos of the membrane were taken at an exposure of 0.2 s.

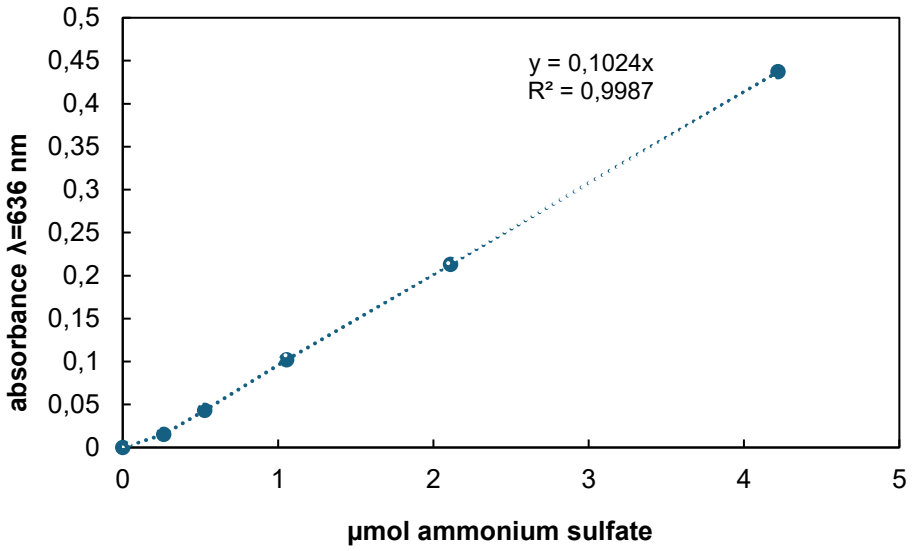

Fig. S2. The standard curve for ammonium sulfate concentration determination prepared using ammonium sulfate.

Immunodetection of ubiquitin–containing protein in the digestive fluid

Immunoblotting technique was done for detection of ubiquitin–containing protein. Protein precipitation from the pitcher plant's digestive fluid was made with cold acetone 100%, it was added to the digestive fluid (4:1) and incubated for 24 h at -20ºC (Biteau et al., 2013). Next, the samples were centrifuged 20 min at 12000× *g* at 4°C. The obtained pellets were briefly dried and then resuspended in 45 µL Laemmli buffer: 63 mM Tris-HCl, pH 6.8, 1% (w/v) SDS, 10% (v/v) glycerol, 0.01% (w/v) bromophenol blue and 20 mM DTT by sonication 15 min at 4°C. Samples were denatured for 10 min at 70°C. The whole volume of each sample was loaded on 10% polyacrylamide gels with SDS and separated using SDS-PAGE method (Laemmli, 1970). In addition, the ubiquitin protein (Ubiqutin AS08 307S) was loaded on gels as positive control, 2 µL per well (concentration 0.8µg/µL). SDS-PAGE was carried at a constant voltage of 150 V using the Mini-PROTEAN system (BIO-RAD). Then, proteins were electrotransferred to nitrocellulose membranes (Amersham™ Protran® Western blotting membranes, nitrocellulose, pore 0.2 µm) according to Towbin et al. (1979) using a Bio-Rad wet blotting apparatus.

Subsequently, the proteins were visualised on the membranes via the photoreaction of tryptophan with Trichloroethylene (TCE) (Fig. S3). The membranes were blocked with 5 % non-fat milk in TBST at RT for 1 h. The membranes were incubated with antibodies Ubiqutin (AS08 307) were diluted in TBST of 1:10000 at RT for 1 h and overnight at 4°C. After being washed in TBST three times, the nitrocellulose membranes were incubated with secondary antibodies 1:30000 (anti-rabbit IgG conjugated with horseradish peroxidase Sigma–Aldrich) for 1 h at RT in the dark. Ubiquitin–protein were visualised using chemiluminescence with the Agrisera kit (AgriseraECL Bright) in Bio-Rad Chemi-Doc XRS system, with the signal collected for 100 s. Images of representative membranes were shown after photos were processed in Image Lab Software. Assays were performed in four -five independent biological replication.

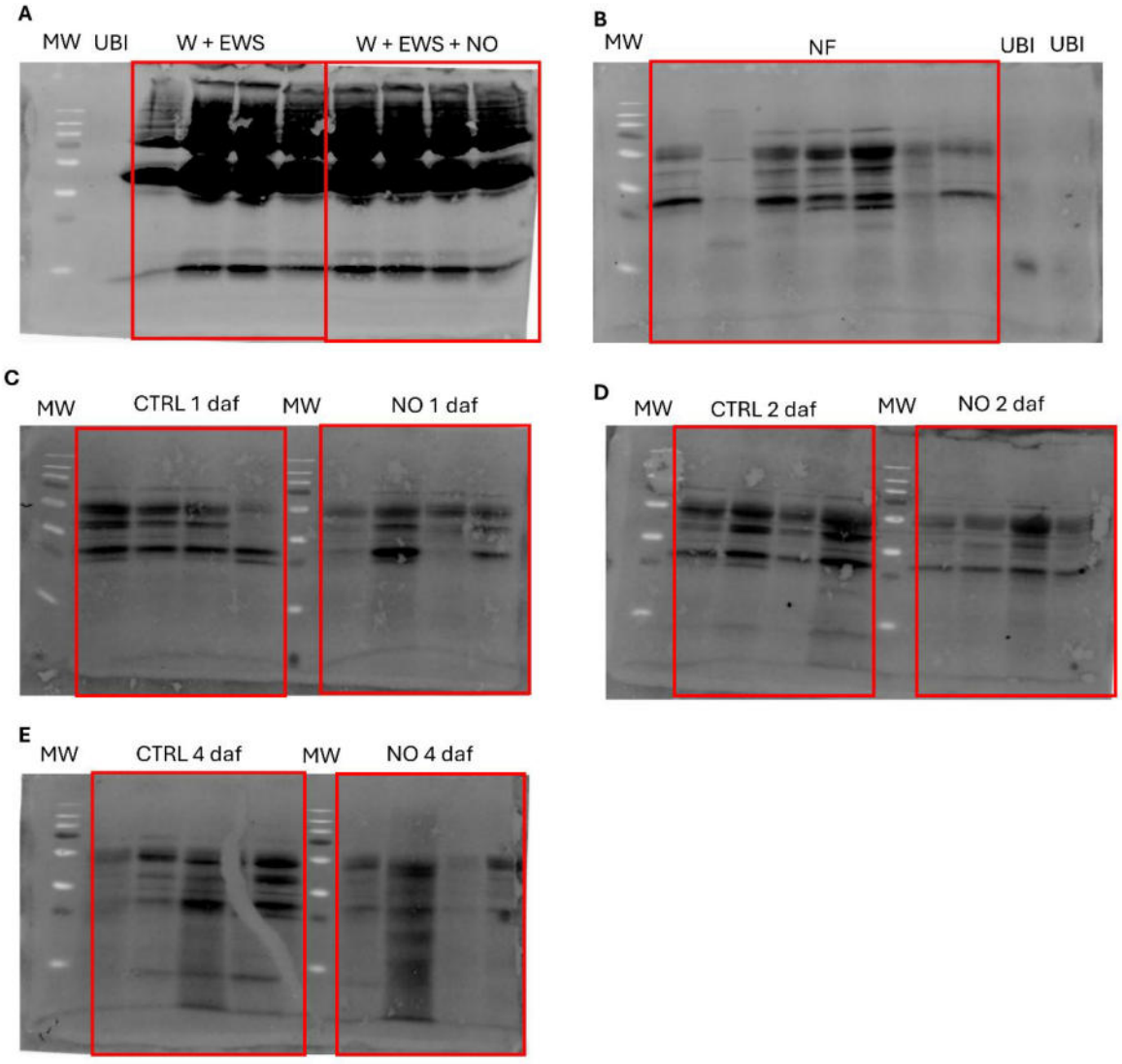

Fig. S3. The proteins were after electrotransferred visualised on the membranes via the photoreaction of tryptophan TCE. Lines MW: molecular markers. lines UBI lines ubiquitin protein - positive control. W+EWS proteins from deionised water mixed with egg white solution, line W+EWS+NO proteins from deionised water mixed with egg white solution and NO_x donor (A). Lines NF from the digestive fluid from the non-fed traps, (B). Lines CTRL and NO from the digestive fluid from the trap 1, 2, and 4 days after feeding (daf) (C, D, E).

Ubiquitin–containing proteins in the digestive fluid

The bands showing positive control were very clearly visible (Fig. S4A, B). In protein isolated from deionised water mixed with egg white solution (W+EWS) and from deionised water mixed with egg white solution and NO_x (W+EWS+NO) there were no bands (Fig. S4A). In the protein isolated from digestive fluid from CTRL and NO trap 1, 2 and 4 daf were only a few bands in random places.

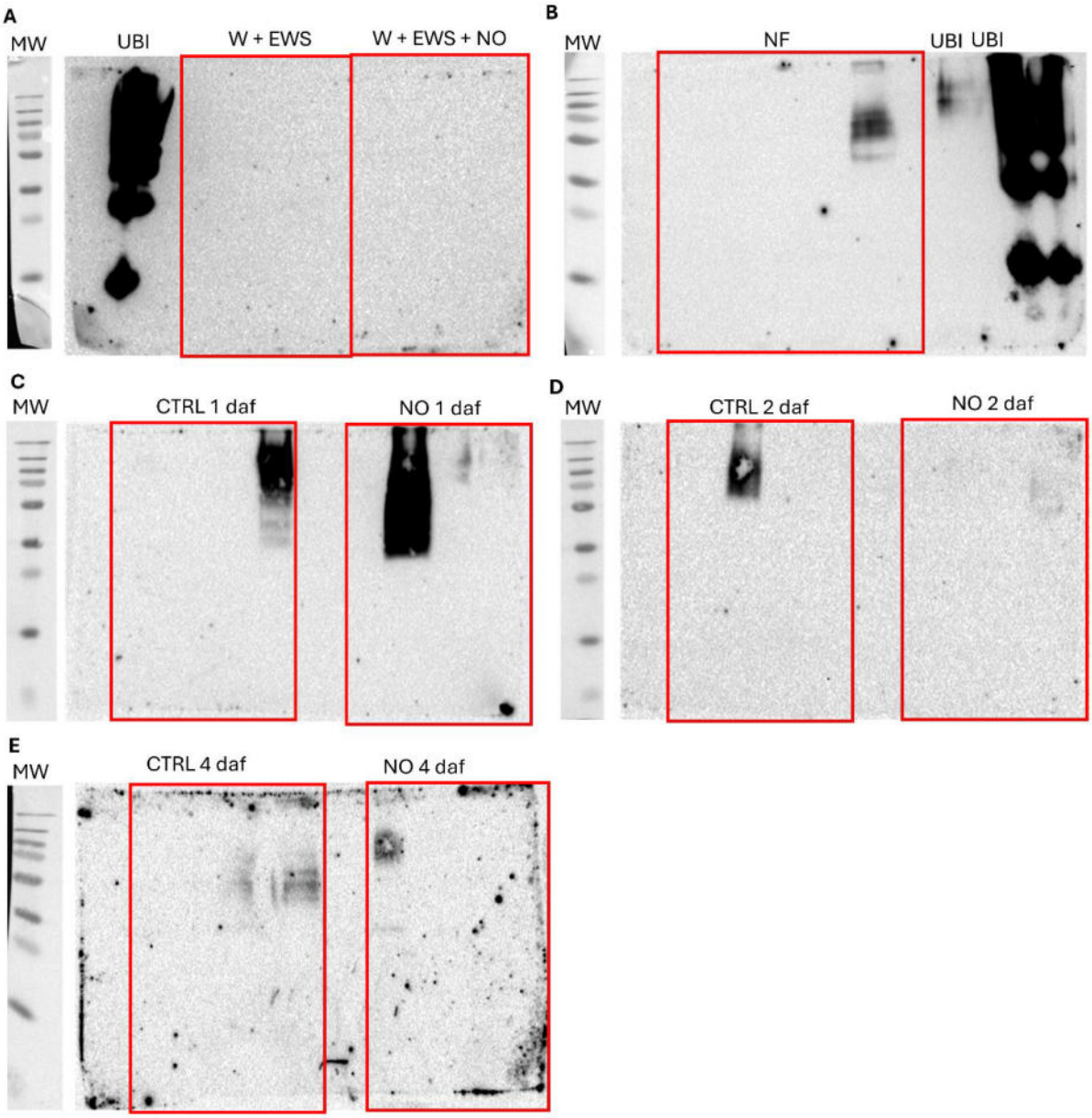

Fig. S4. Ubiquitin–containing proteins isolated from the digestive fluid, photos of the membrane were taken at an exposure of 20 s. Line MW: molecular markers, line W+EWS: proteins separated from deionised water mixed with egg white solution, line W+EWS+NO: proteins separated from deionised water mixed with egg white solution and NO_x donor, line NF: proteins separated from the digestive fluid from non-fed trap, line CTRL proteins isolated from the digestive fluid from the trap fed with white egg solution, line NO: proteins separated from the digestive fluid from the trap fed with white egg solution + NO_x donors 1, 2, and 4 days after feeding (daf).

Measurement of chitinases activity in the digestive fluid

Chitinase activity was measured by a spectrophotometric method using colloidal chitin azure (Shen et al., 2010). The substrate was prepared by weighing 15 mg of chitin azur (Sigma-Aldrich, C3020) and adding 150 µL of 37% HCl. The solution was shaken on a Vortex and then incubated in the dark at room temperature (RT) for 50 min. In the next step 3.75 mL of deionised water was added and shaken on Vortex. The solution was centrifuged for 20 min at 8000× *g* at RT. The supernatant was decanted into a tube, and 3.75 mL of deionised water was added to the remaining precipitate. Both tubes were centrifuged for 20 min at 8000× *g* at RT. The contents of the tubes were shaken on Vortex and transferred to an evaporator, which was placed in a water bath for 30 min at 100ºC. To the resulting precipitate, 0.2 M glycine buffer pH 3 (1 mL buffer per 3 mg precipitate) was added. The precipitate was dissolved by shaking on a Vortex and incubating for 10 min in an ultrasonic bath. The digestive fluid was buffered by mixing with 1 M K-phosphate buffer, pH 7, buffer in a 9:1 ratio. To a buffered fluid of 600 µL was added 100 µl of colloidal chitin azur and incubated with shaking at 25ºC for 15 min in the dark. Distilled water was used instead of fluid in the blank samples. Samples were then incubated for 5 min at 100ºC and centrifuged for 5 min at 12000 × *g* at RT. Absorbance was measured at 560 nm by microplate reader CLARIO star plus, BMG LABTECH. The experiments were done in four biological replicates and in three technical repetitions. The results were expressed as U min^{-1} trap $^{-1}$, 1 U is A_{560} = 0.1.

Chitinases activity in the digestive fluid

In all tasted digestive fluid except the digestive fluids collected 2 daf, chitinases activity was low and at the same level (Fig. S5). The activity of the enzyme in the fluid from the trap to which NO_x was added (NO) 2 daf was about 30% higher that in the fluid of CTRL trap and around ten times higher than in fluid from NF trap.

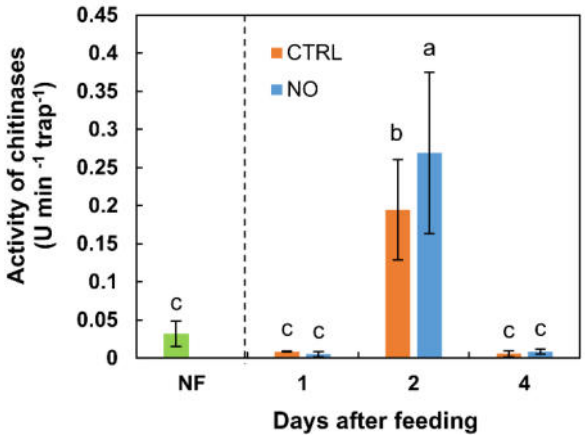

Fig. S5. The activity of chitinases in the digestive fluid from non-fed (NF) trap and traps fed with egg white solution (CTRL), and traps fed with egg white solution with the addition of NO_x donor (NO) 1, 2, and 4 days after feeding (daf). Values are average ± SD of 4 repetitions. Letters (a–d) indicate homogenous groups determined after ANOVA and post hoc Fisher's LSD test at $p \leq 0.05$.

Table S1. List of primers.

Gene symbol	Primer sequence 5'-3'	Encoded protein	Species	Literature source
GAPC	F: GTGGTGCTAAGAAGGTCGTCATC R: TCTCATTAACACCAACAACGAACA	GAPDH enzyme	*Nepenthes khasiana*	Dkhar et.al., 2020
18s	F: CTTGATTCTATGGGTGGTGGTG R: GTTAGCAGGCTGAGGTCTC	18s rRNA	*Nepenthes ventricosa*	Yilamujiang et. al., 2016
NEPI	F: GATTTGGAGTTGCCCAGTGTG R: CGGTGTCGTAAACGACTAG	Nepenthesin I	*Nepenthes khasiana*	Dkhar et.al., 2020
NEPII	F: ATATCTGTCCGGTGAGCAGCTG R: CTATCAATAGTCTCCATCACTTCAG	Nepenthesin II	*Nepenthes khasiana*	Dkhar et.al., 2020
PAP	F: GGCCTCGCCACAACGTATAT R: GAGTGAAACCCAGTAGTTGG	Purple Acid Phosphatase	*Nepenthes khasiana*	Dkhar et.al., 2020
RNase	F: CGTGCAACACCGACGAATAC R: AGAATGGCCGACACAAATGTAA	S-like Ribonuclease	*Nepenthes khasiana*	Dkhar et.al., 2020

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Załącznik nr 2 – Wzór oświadczenia o współautorstwie

Warszawa, 27.05.2025

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Szkoły Głównej Gospodarstwa

Wiejskiego w Warszawie

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy:

Wal A., Staszek P., Gniazdowska A., Chrastný V., Šípková A., Bieniek J., Krasuska U. Nitric oxide stimulates digestion modifying the nutrient composition of the traps' fluid of *Nepenthes x ventrata*. Plant Science 2025, 112558.
https://doi.org/10.1016/j.plantsci.2025.112558

mój indywidualny udział w jej powstaniu polegał na wykonaniu immunolokalizacji białek z grupą karbonylową, oznaczeniu zawartości tlenu i jonów amonowych w cieczy trawiennej, oznaczeniu zdolności redukcyjnej cieczy trawiennej, oznaczeniu ekspresji genów kodujących nepentezyny (I i II), fosfatazy kwaśne oraz rybonukleazy. Współwykonywałam również pomiary aktywności chitynaz w cieczy trawiennej oraz oznaczenia zawartość pierwiastków (Ca, Cu, Fe, K, Mg, Mn, Na, P, S i Zn). Wykonywałam także oznaczenia i lokalizację aktywności fosfataz kwaśnych oraz w oznaczałam aktywność esteraz. Brałam udział w interpretacji wyników, przygotowaniu manuskryptu oraz pełniłam funkcję autora korespondującego.

Swój wkład oceniam na 50 %.

Podpis

Agnieszka Wal

Załącznik nr 2 – Wzór oświadczenia o współautorstwie

Warszawa, 26.05.2025

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Wiejskiego w Warszawie

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Niniejszym oświadczam, że w pracy:

Wal A., Staszek P., Gniazdowska A., Chrastný V., Šípková A., Bieniek J., Krasuska U. Nitric oxide stimulates digestion modifying the nutrient composition of the traps' fluid of *Nepenthes x ventrata*. Plant Science 2025, 112558.
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mój indywidualny udział w jej powstaniu polegał na współuczestnictwie w opracowaniu koncepcji pracy oraz sprawowaniu opieki merytorycznej podczas przeprowadzania eksperymentów.

Swój wkład oceniam na 10 %.

Podpis

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Wal A., Staszek P., Gniazdowska A., Chrastný V., Šípková A., Bieniek J., Krasuska U. Nitric oxide stimulates digestion modifying the nutrient composition of the traps' fluid of *Nepenthes x ventrata*. Plant Science 2025, 112558.
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mój indywidualny udział w jej powstaniu polegał na: udziale w redakcji i korekcie całego manuskryptu oraz sprawowaniu opieki podczas przygotowywania publikacji.

Swój wkład oceniam na 5 %.

A. Gniazdowska

Podpis

Annexe 2 - Declaration of co-authorship

Prof. Chrastný Vladislav

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Statement of Co-Authorship

I acknowledge my involvement in the work:
Wal A., Staszek P., Gniazdowska A., Chrastný V., Šípková A., Bieniek J., Krasuska U. Nitric oxide stimulates digestion modifying the nutrient composition of the traps' fluid of *Nepenthes x ventrata*. Plant Science 2025, 112558. https://doi.org/10.1016/j.plantsci.2025.112558
my individual contribution consisted of providing supervision and technical support in the determination of element content (Ca, Cu, Fe, K, Mg, Mn, Na, P, S, and Zn).

I estimate my contribution at 5%.

Signature

Annexe 2 - Declaration of co-authorship

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Statement of Co-Authorship

I acknowledge my involvement in the work:
Wal A., Staszek P., Gniazdowska A., Chrastný V., Šípková A., Bieniek J., Krasuska Nitric oxide stimulates digestion modifying the nutrient composition of the traps' fluid of Nepenthes x ventrata. U. Plant Science 2025. 112558. https://doi.org/10.1016/j.plantsci.2025.112558
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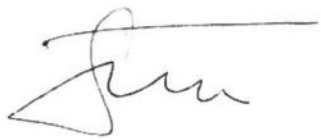
I estimate my contribution at 5%.



Signature

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Niniejszym oświadczam, że w pracy:

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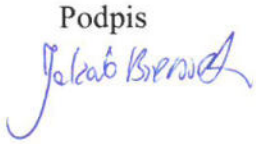
mój indywidualny udział w jej powstaniu polegał na współwykonywaniu pomiaru aktywności chitynaz w cieczy trawiennej, swój wkład oceniam na 5 %.

Podpis



Załącznik nr 2 – Wzór oświadczenia o współautorstwie

Warszawa, 27.05.2025

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mój indywidualny udział w jej powstaniu polegał na opracowaniu koncepcji badań, sprawowaniu opieki merytorycznej w trakcie realizacji badań, udziale w interpretacji wyników, przygotowaniu publikacji oraz redakcji i korekcie całego manuskryptu.

Swój wkład oceniam na 20 %.

Podpis