Szkoła Główna Gospodarstwa Wiejskiego

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Instytut Biologii

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Tlenek azotu – cząsteczka modyfikująca ilość, jakość i funkcjonalność białek osi zarodkowych nasion jabłoni (*Malus domestica* Borkh.) poddanych starzeniu

Nitric oxide – a molecule modifying the quantity, quality, and functionality of proteins in the embryonic axes of apple seeds (*Malus domestica* Borkh.) subjected to ageing

Rozprawa doktorska

Doctoral thesis

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Spis treści

Streszczenie w języku polskim

Starzenie dotyczy każdego organu roślin, w tym również nasion, których biologiczną funkcją jest zachowanie i dyspersja gatunków w środowisku, sprzyjające utrzymaniu bioróżnorodności. Nasiona są kluczowe dla wielu gałęzi przemysłu, w szczególności przemysłu rolnego. Stąd, konieczne jest prowadzenie badań dotyczących mechanizmów ich starzenia, mających na celu zrozumienie molekularnych podstaw zachowania żywotności nasion. Nasiona typu *orthodox*, na przykładzie nasion jabłoni (*Malus domestica* Borkh.), charakteryzują się wysokim stopniem odwodnienia, który osiągają podczas dojrzewania. Cytoplazma w komórkach nasion tego typu osiąga tzw. stan szklisty, co sprzyja ograniczeniu przemian metabolicznych oraz zachowaniu struktury i funkcji białek. Niska aktywność metaboliczna nasion jabłoni jest wynikiem głębokiego spoczynku, tymczasowej, fizjologicznej bariery zakłócającej kiełkowanie izolowanych zarodków. Stąd też, w warunkach laboratoryjnych, w celu indukcji starzenia nasion tego typu (w tym nasion jabłoni) wykorzystuje się wystandaryzowane protokoły. Przeważnie dotyczą one poddawania nasion działaniu podwyższonej wilgotności i temperatury, aby osiągnąć efekt przyspieszenia procesów metabolicznych, których skutkiem są zmiany degeneracyjne.

Starzenie jest procesem prowadzącym do stopniowej utraty wigoru, a następnie żywotności nasion. Zmniejsza się ich zdolność kiełkowania. Głównym czynnikiem odpowiadającym za uszkodzenia wewnątrzkomórkowe podczas starzenia są reaktywne formy tlenu (ROS) i produkty ich reakcji, np. *meta*-tyrozyna (*m*-Tyr), będąca znacznikiem stresu oksydacyjnego. Postępujące starzenie nasion jest w dużej mierze konsekwencją zaburzenia homeostazy redoks, która wynika m.in. z zachwiania równowagi pomiędzy generowaniem a detoksykacją reaktywnych form tlenu i azotu (RONS). Kluczową cząsteczką sygnałową w biologii nasion jest tlenek azotu (NO), którego pochodne tworzą reaktywne formy azotu (RNS). Jednocześnie przełamanie spoczynku nasion wielu gatunków roślin wymaga osiągnięcia optymalnego (dla danego gatunku) stężenia RONS, mieszczącego się w zakresie tzw. „okna oksydacyjnego" i „drzwi nitrozacyjnych". Zatem zbyt niskie stężenie RONS wiąże się z utrzymaniem spoczynku nasion, natomiast zbyt wysokie tworzy stres nitrooksydacyjny, przyczyniając się do nagromadzania uszkodzeń i ostatecznie śmierci komórek. Jednakże na podstawie danych literaturowych proponuje się, że starzenie wynika m.in. ze zbyt niskiego stężenia NO w komórkach. Ponadto potwierdzono, że krótkotrwałe traktowanie nasion

(lub zarodków) różnych gatunków roślin donorami NO ma korzystne działanie stymulujące metabolizm oraz mechanizmy naprawcze. W rezultacie postarzane nasiona charakteryzuje wyższa zdolność kiełkowania, zatem kluczowe dla zachowania żywotności nasion jest utrzymanie proteostazy, w tym regulacja aktywności białek związanych z homeostazą RONS.

Celem pracy było wykazanie roli egzogennego NO jako cząsteczki poprawiającej żywotność zarodków izolowanych z postarzanych nasion jabłoni w odniesieniu do ilości, jakości i funkcjonalności białek związanych z zaburzeniem homeostazy RONS.

Aby podnieść poziom wiedzy dotyczącej korzystnego efektu działania NO jako cząsteczki poprawiającej jakość postarzanych nasion typu *orthodox* weryfikacji poddano pięć **hipotez badawczych:**

1. Starzenie nasion jabłoni wiąże się ze wzrostem zawartości *m*-Tyr w osiach zarodkowych. *(Hipoteza zweryfikowana pozytywnie)*
2. Krótkotrwałe traktowanie zarodków jabłoni izolowanych z postarzanych nasion NO ogranicza wzrost zawartości *m*-Tyr w osiach zarodkowych. *(Hipoteza zweryfikowana negatywnie)*
3. Krótkotrwałe traktowanie zarodków jabłoni izolowanych z postarzanych nasion NO reguluje przemiany glutationu i wpływa na utrzymanie równowagi GSH/GSSG w osiach zarodkowych. *(Hipoteza zweryfikowana pozytywnie)*
4. Krótkotrwałe traktowanie zarodków jabłoni izolowanych z postarzanych nasion NO zmienia jakościowo białka karbonylowane i nitrowane izolowane z osi zarodkowych. *(Hipoteza zweryfikowana pozytywnie)*
5. Krótkotrwałe traktowanie zarodków jabłoni izolowanych z postarzanych nasion NO reguluje degradację białek w osiach zarodkowych. *(Hipoteza zweryfikowana pozytywnie)*

Do badań wykorzystano osie zarodkowe, które izolowano z zarodków nasion jabłoni poddanych zabiegowi przyspieszonego starzenia w warunkach podwyższonej wilgotności (50 %) i temperatury (33-35°C) przez 7, 14 i 21 dni. Pozbawione okryw nasiennych zarodki krótkotrwale (3 h) traktowano NO (uwalnianym z zakwaszonego azotynu sodu). Następnie, zarodki izolowane z postarzonych nasion nietraktowane (kontrola) i zarodki traktowane NO wykładano na szalki Petriego i prowadzono ich

kulturę przez 48 h. Po tym czasie z zarodków izolowano osie zarodkowe, które stanowiły materiał doświadczalny.

W celu zweryfikowania hipotez zastosowano metody badawcze, takie jak: techniki separacyjne z wykorzystaniem wysokosprawnej chromatografii cieczowej (HPLC), metody spektrofotometryczne, analiza RT-qPCR, metody spektrofluorescencyjne oraz elektroforetyczna separacja białek jednokierunkowa, w żelu poliakrylamidowym w warunkach denaturujących (SDS-PAGE) wraz z elektrotransferem (Western Blot) oraz dwukierunkowa (2D SDS-PAGE), poprzedzająca technikę spektometrii mas (MS) MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight).

Wyniki uzyskane w ramach niniejszej pracy potwierdziły, że każdy etap starzenia nasion charakteryzują odrębne zmiany na poziomie biochemiczno-molekularnym zachodzące w osiach izolowanych zarodków. Potwierdzono, że wraz z wydłużaniem starzenia do 21 dni obserwuje się postępującą utratę żywotności wyizolowanych zarodków oraz wzrost liczby siewek obarczonych anomaliami rozwojowymi. Jednocześnie traktowanie izolowanych zarodków NO poprawia ich zdolność kiełkowania, ale efekty jego działania zależą od czasu trwania zabiegu starzenia nasion.

Początkowy etap starzenia (**7 dni zabiegu**), przebiegający w warunkach podwyższonej temperatury i związany z uwodnieniem tkanek (imbibicja), może inicjować zmiany metaboliczne, podobne do tych, które zachodzą podczas ustępowania spoczynku. Ten etap nie wiąże się jeszcze z nagromadzaniem trwałych uszkodzeń wewnątrzkomórkowych, stąd tylko u nielicznych izolowanych zarodków kontrolnych obserwuje się efekty starzenia. Stymulacja prawidłowego kiełkowania, obserwowana po traktowaniu zarodków poddanych starzeniu przez **7 dni** NO, wiąże się ze wzrostem aktywności reduktazy glutationowej (GR) i roślinnej peroksydazy glutationowej (GPx-like) oraz zmianami zawartości zredukowanego (GSH) i utlenionego (GSSG) glutationu, należących do systemu antyoksydacyjnego. Stabilnym markerem stresu oksydacyjnego w komórkach jest *m*-Tyr, powstająca w wyniku utleniania fenyloalaniny (Phe). Toksyczność *m*-Tyr polega na jej wbudowaniu do łańcucha polipeptydowego w miejsce Phe, co prowadzi do nieprawidłowego fałdowania białka. Obserwowany wzrost zawartości *m*-Tyr w osiach zarodków traktowanych NO, na tym etapie starzenia był niwelowany podwyższeniem zawartości białkogennej tyrozyny (Tyr), co podtrzymywało korzystny stosunek *m*-Tyr/Tyr.

Wyniki przeprowadzonych badań wykazały, że obniżonej żywotności zarodków izolowanych z nasion poddanych starzeniu przez **14 i 21 dni** towarzyszy niska aktywność

GR i GPx-like. Traktowanie zarodków NO na tym etapie starzenia moduluje aktywność tych enzymów w osiach zarodkowych. Wykazano, że aktywność GPx-like była podwyższona, niezależnie od czasu trwania starzenia, natomiast aktywność GR była stymulowana jedynie po **14 dniach** starzenia. Może to prowadzić do ograniczenia odtwarzania puli GSH, cząsteczki biorącej udział w utrzymaniu homeostazy redoks.

Starzenie nasion jabłoni przez **21 dni** prowadzi do wzrostu stężenia *m*-Tyr w osiach zarodkowych. Niwelowanie skutków starzenia związanych ze wzrostem zawartości *m*-Tyr przez NO przejawiało się obniżeniem stosunku *m*-Tyr/Tyr oraz zwiększeniem stężenia Phe.

Stres nitrooksydacyjny, który towarzyszy starzeniu nasion, sprzyja RONS-zależnym modyfikacjom białek, w tym karbonylacji i nitracji. Karbonylacja jest nieodwracalną modyfikacją potranslacyjną (PTM) świadczącą o nadmiarze ROS, polegającą na wbudowywaniu grup karbonylowych (=CO) w łańcuch reszt niektórych aminokwasów. Podobnie jak karbonylacja, nitracja białek jest nieodwracalna w warunkach fizjologicznych i może funkcjonować jako znacznik przyspieszający degradację białek. Głównym czynnikiem nitrującym jest nadtlenoazotyn ($ONOO^-$) powstający w reakcji NO z anionorodnikiem ponadtlenkowym ($O_2^{\bullet-}$) należącym do ROS.

Zazwyczaj starzenie nasion wiąże się ze wzrostem stężenia białek karbonylowanych, chociaż takiej zależności nie wykazano w przypadku nasion jabłoni. Karbonylacji w nasionach podlegają białka zapasowe, co z uwagi na ich wysoką zawartość, może stanowić dodatkowy mechanizm chroniący kluczowe biomolekuły przed interakcją z ROS. Analiza jakościowa wykazała, że rola NO w łagodzeniu skutków starzenia nasion jabłoni może dotyczyć karbonylowanej pruniny 1 (białka zapasowego). Zaobserwowano względny wzrost zawartości tego białka izolowanego z osi zarodków po traktowaniu NO w porównaniu do kontroli. Ponadto, wykazano, że białka szoku cieplnego (klasy II – HSP) zawierają również grupy =CO, co nie zostało zaobserwowane w osiach zarodków kontrolnych. Karbonylacja białek może stanowić mechanizm znakujący je do degradacji przez proteasom 20S (proces niezależny od ATP). Łagodzenie efektów starzenia nasion jabłoni przez **14 i 21 dni** w wyniku traktowania zarodków NO przejawiało się wzmożoną aktywnością proteasomu 20S, regulując zawartość białek karbonylowanych oraz prawdopodobnie nitrowanych (utrzymanie proteostazy). W osiach zarodków nasion poddanych starzeniu zidentyfikowano m.in. nitrowaną pruninę 1. Trudno znaleźć jedno wytłumaczenie, dlaczego dane białko jest nieodwracalnie znakowane przez obie tego typu modyfikacje, które prowadzą do przyspieszonej jego hydrolizy. Może

być to związane z uwalnianiem peptydów zawierających grupy =CO i grupy nitrowe, których udział w sygnalizacji komórkowej jest prawdopodobny, ale niezweryfikowany. Dodatkowo wykazano, że starzenie nasion jabłoni wiąże się z obecnością nitrowanych białek szlaków wytwarzania energii m.in. dehydrogenazy mleczanowej, enolazy czy podjednostki ß syntazy ATP. Traktowanie zarodków NO (po **14 dniach** starzenia) prowadziło do zwiększenia względnej zawartości białka zidentyfikowanego jako podjednostka ß syntazy ATP, podlegającej nitracji. Zatem, prawdopodobne jest ograniczenie degradacji białek zależne od ATP, promując ich hydrolizę przez proteasom 20S.

Biorąc pod uwagę powyższe, niwelowanie efektów starzenia przez NO w osiach zarodków jabłoni jest związane z łagodzeniem toksycznego działania *m*-Tyr, modulacją zawartości glutationu i aktywności enzymów z nim związanych. Ponadto, regulacyjne działanie NO może wiązać się ze zmianami ilości, jakości i funkcjonalności białek, poprzez ich specyficzne PTM np. znakowanie ubikwityną czy nitrację. Może wiązać się to z kierowaniem do degradacji białek zapasowych (prunina 1), białek których działanie nie jest już niezbędne w osiach kiełkujących zarodków, lub takich, które utraciły swoją funkcję (utrzymanie proteostazy).

Słowa kluczowe: degradacja białek, potranslacyjne modyfikacje białek, przyspieszone starzenie nasion, RONS, system antyoksydacyjny, zmiany degeneracyjne,

Streszczenie w języku angielskim

Ageing affects every plant organ, including seeds, whose biological function is to ensure the preservation and dispersal of species in the environment, thereby contributing to the maintenance of biodiversity. Seeds are also crucial to numerous industries, particularly the agricultural sector. It is therefore essential to conduct research into the mechanisms of seed ageing to understand the molecular basis of seed viability preservation. *Orthodox*-type seeds, such as apple seeds (*Malus domestica* Borkh.), are highly dehydrated, as they acquire this during maturation. In the cells of this seed type, the cytoplasm enters the so-called glassy state, which limits metabolic activity and promotes the preservation of protein structure and function. The low metabolic activity of apple seeds results from deep dormancy, a temporary physiological barrier that prevents the germination of isolated embryos. Therefore, under laboratory conditions, standardised protocols are used to induce ageing in seeds of this type, including apple seeds. These protocols typically involve exposing seeds to elevated humidity and temperature in order to accelerate metabolic processes that ultimately lead to degenerative changes.

Ageing is a process that leads to a gradual loss of seed vigour, followed by a decline in seed viability. Consequently, seed germination capacity decreases. The main factors responsible for intracellular damage during ageing are reactive oxygen species (ROS) and ROS-reacting compounds, such as *meta*-tyrosine (*m*-Tyr), which is considered a marker of oxidative stress. Progressive seed ageing is largely a consequence of disturbed redox homeostasis, resulting, among other factors, from an imbalance between the generation and detoxification of reactive oxygen and nitrogen species (RONS). Nitric oxide (NO), whose derivatives constitute reactive nitrogen species (RNS), is a particularly important signalling molecule in seed biology. At the same time, the dormancy alleviation in seeds requires the presence of an optimal, species-specific RONS concentration within the range of the so-called “oxidative window” and “nitrosative door”. Thus, too low RONS levels are associated with the maintenance of seed dormancy, whereas excessively high levels induce nitro-oxidative stress, thereby contributing to the accumulation of cellular damage and, ultimately, cell death. However, based on the available literature, it has been proposed that seed ageing may result, at least in part, from low intracellular NO levels. Moreover, short-term treatment of seeds or embryos of various plant species with NO donors has been shown to have a beneficial effect, stimulating metabolism and repair

mechanisms. As a result, aged, NO-treated seeds exhibit higher germination capacity. Therefore, maintaining proteostasis, including regulating proteins involved in RONS homeostasis, is crucial for preserving seed viability.

The aim of this study was to demonstrate the role of exogenous NO as a molecule improving the viability of embryos isolated from aged apple seeds, focusing on the quantity, quality, and functionality of proteins associated with disrupted RONS homeostasis.

To expand the current understanding of the beneficial effect of NO as a molecule improving the quality of aged *orthodox*-type seeds, five **research hypotheses** were verified:

1. Ageing of apple seeds is associated with an increase in *m*-Tyr content in embryonic axes. *(Hypothesis positively verified)*
2. Short-term NO treatment of apple embryos isolated from aged seeds limits the increase in *m*-Tyr content in embryonic axes. *(Hypothesis negatively verified)*
3. Short-term NO treatment of apple embryos isolated from aged seeds regulates glutathione alterations and contributes to the maintenance of the GSH/GSSG balance in embryonic axes. *(Hypothesis positively verified)*
4. Short-term NO treatment of apple embryos isolated from aged seeds induces qualitative changes in carbonylated and nitrated proteins isolated from embryonic axes. *(Hypothesis positively verified)*
5. Short-term NO treatment of apple embryos isolated from aged seeds regulates protein degradation in embryonic axes. *(Hypothesis positively verified)*

As an experimental material, embryonic axes isolated from apple seed embryos were subjected to accelerated ageing at elevated humidity and temperature for 7, 14, and 21 days. After removal of the seed coats, aged embryos were subjected to short-term NO treatment for 3 h. Nitric oxide was released from sodium nitrite during a reaction carried out under acidic conditions. Subsequently, embryos isolated from aged seeds (control) and NO-treated embryos were placed on Petri dishes and cultured for 48 h. After this period, embryonic axes were isolated from the embryos and used for the experiments.

To verify the hypotheses, a range of research methods was applied, including separation techniques based on high-performance liquid chromatography (HPLC), spectrophotometric methods, RT-qPCR analysis, spectrofluorometric methods, one-dimensional electrophoretic separation of proteins in polyacrylamide gel under

denaturing conditions (SDS-PAGE), followed by electrotransfer (Western blot), and two-dimensional electrophoresis (2D SDS-PAGE) preceding MALDI-TOF (Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight. mass spectrometry (MS) analysis.

The results obtained during this work confirmed that each stage of seed ageing is characterised by distinct biochemical and molecular changes occurring in the axes of isolated embryos. It was demonstrated that prolonging ageing to 21 days was accompanied by a progressive loss of viability in isolated embryos and an increase in the number of seedlings exhibiting abnormalities. At the same time, NO treatment of isolated embryos improved their germination capacity; however, the effects of NO depended on the duration of the seed ageing treatment.

The first stage of ageing, corresponding to **7 days** of treatment under elevated temperature and associated with tissue hydration (imbibition), may initiate metabolic changes similar to those occurring during dormancy alleviation. This state is not yet associated with permanent intracellular damage; therefore, ageing-related effects are observed only in a limited number of isolated control embryos.

The stimulation of proper germination observed after NO treatment of embryos subjected to **7 days** of ageing was associated with increased activities of glutathione reductase (GR) and plant glutathione peroxidase-like enzyme (GPx-like), as well as with changes in the contents of reduced (GSH) and oxidised (GSSG) glutathione, which are components of the antioxidant system.

A stable marker of oxidative stress in cells is *m*-Tyr, which is produced during the oxidation of phenylalanine (Phe). The toxicity of *m*-Tyr is related to its incorporation into the polypeptide chain in place of Phe, leading to improper protein folding. The observed increase in *m*-Tyr content in the axes of NO-treated embryos was counterbalanced by an elevated content of the proteinogenic amino acid tyrosine (Tyr), which maintained a favourable *m*-Tyr/Tyr ratio.

The conducted analyses showed that reduced viability of embryos isolated from seeds aged for 14 and 21 days was accompanied by low GR and GPx-like activities. NO treatment of embryos at this stage of ageing modulated the activities of these enzymes in embryonic axes. GPx-like activity was increased irrespective of the duration of ageing, whereas GR activity was stimulated only after 14 days of ageing. This may limit the restoration of the GSH pool, a molecule involved in maintaining redox homeostasis.

Ageing of apple seeds for 21 days led to an increase in *m*-Tyr concentration in embryonic axes. The NO-mediated mitigation of ageing effects associated with elevated *m*-Tyr content was reflected by a decreased *m*-Tyr/Tyr ratio and an increased Phe concentration.

Nitro-oxidative stress accompanying seed ageing promotes RONS-dependent protein modifications, including carbonylation and nitration. Carbonylation is an irreversible post-translational modification (PTM) that reveals ROS excess and involves the incorporation of carbonyl groups (=CO) into the side chains of selected amino acid residues.

Similar to carbonylation, protein nitration is an irreversible modification that may function as a tag accelerating protein degradation. The major nitrating agent is peroxynitrite ($ONOO^-$), which is formed in the reaction of NO with the superoxide anion radical ($O_2^{\bullet-}$), a member of ROS.

Seed ageing is usually associated with an increase in the concentration of carbonylated proteins, although such a case was not demonstrated in apple seeds. In seeds, storage proteins undergo carbonylation, which, given their high abundance, may constitute an additional mechanism protecting key biomolecules from ROS-mediated interactions. Qualitative analysis showed that the role of NO in mitigating the effects of apple seeds ageing may involve carbonylated prunin 1, a well-known storage protein. A relative increase in the abundance of this protein was observed in embryonic axes after NO treatment in comparison with the control. Moreover, it was shown that class II heat shock proteins (HSP) also contained =CO groups, which was not observed in the axes of control, aged embryos.

Protein carbonylation may serve as a mechanism for tagging proteins for degradation by the 20S proteasome, an ATP-independent process. The mitigation of the effects of apple seed ageing after 14 and 21 days by NO treatment of embryos was reflected in enhanced 20S proteasome activity, thereby regulating the abundance of carbonylated and, probably, nitrated proteins, and contributing to the maintenance of proteostasis. In the axes of embryos isolated from aged seeds, nitrated prunin 1 was identified, among other proteins. It is difficult to provide a single explanation for why a given protein is irreversibly tagged by both types of modifications, each of which may promote its accelerated hydrolysis. This may be associated with the release of peptides containing =CO and nitro groups, whose involvement in cellular signalling is suggested but has not yet been verified.

Moreover, apple seed ageing was shown to be associated with the presence of nitrated proteins involved in energy-generating pathways, including lactate dehydrogenase, enolase, and the beta subunit of ATP synthase. NO treatment of embryos after 14 days of ageing led to an increase in the relative abundance of a nitrated protein identified as the beta subunit of ATP synthase. Thus, ATP-dependent protein degradation is likely to be limited, thereby promoting protein hydrolysis via the 20S proteasome.

The mitigation of ageing effects by NO in apple embryonic axes is associated with the diminishment of the toxic potential of *m*-Tyr, as well as with the modulation of glutathione content and the activities of glutathione-related enzymes. Moreover, the regulatory action of NO may be linked to changes in protein quantity, quality, and function via specific PTM, such as ubiquitin tagging or nitration. This may involve targeting storage proteins, such as prunin 1, for degradation, as well as proteins whose function is no longer required in the axes of germinating embryos or that have lost their function, thereby contributing to the maintenance of proteostasis.

Keywords: antioxidant system, accelerated seed ageing, degenerative changes, post-translational protein modifications, protein degradation, RONS

Spis rysunków

Spis tabel

Spis skrótów stosowanych w pracy

-SH – grupy tiolowe
$^{\bullet}NO$ – rodnik tlenku azotu
$^{\bullet}NO_2$ – rodnik ditlenku azotu
$^{\bullet}OH$ – rodnik hydroksylowy
=CO – grupy karbonylowe
$^1O_2$ – tlen singletowy
3-NT – 3-nitrotyrozyna
8-oxoG – 8-oksy-7,8-dihydroguanina
AA – *Accelerated Ageing,* procedura przyspieszonego starzenia nasion
ABA – kwas abscysynowy
AOX – oksydaza alternatywna
AQC – 6-aminochinolilo-N-hydroksysukcynimidylokarbaminian
Arg – arginina
ASA – kwas askorbinowy
ATP – adenozynotrifosforan
BSA – albumina surowicy bydlęcej
CAT – katalaza
CDT – *Controlled Deterioration Treatment,* procedura kontrolowanego pogarszania jakości nasion
CHCA – kwas α-cyjano-4-hydroksycynamonowy
Cys – cysteina
DNPH – 2,4-dinitrofenylohydrazyna
$E_{GSSG/2GSH}$ – potencjał redoks glutationu
GA – gibereliny
GPx-like – roślinna peroksydaza glutationowa
GR – reduktaza glutationowa
GSH – glutation w formie zredukowanej
GSNO – *S*-nitrozoglutation
GSNOR – reduktaza GSNO
GSSG – glutation w formie utlenionej
H_2O_2 – nadtlenek wodoru
HNE – 4-hydroksynonenal
HNO – nitroksyl
HNO_2 – kwas azotowy
HPLC – wysokosprawna chromatografia cieczowa
HSP – białka szoku cieplnego
IEF – ogniskowanie izoelektryczne
ISTA – *International Seed Testing Associacion*, Międzynarodowy Związek Oceny Nasion
LEA – białka późnej embriogenezy
Lys – lizyna
m-Tyr – *meta*-tyrozyna

MALDI-TOF – *Matrix-Assisted Laser Desorption/Ionization Time-of-Flight,* desorpcja/ jonizacja laserowa wspomagana matrycą
MBBr – monobromobiman
MDA – dialdehyd malonowy
N_2O_3 – tritlenek diazotu
NADPH – fosforan dinukleotydu nikotynoamidoadeninowego - forma zredukowana
NO – tlenek azotu
NO^- – anion nitrozylowy
NO^+ – kation nitrozoniowy
NO_2^- – jony azotynowe
NOS – syntaza NO
NR – reduktaza azotanowa
o-Tyr – *orto*-tyrozyna
O_2 – tlen
$O_2^{\bullet-}$ – anionorodnik ponadtlenkowy
O_3 – ozon
$ONOO^-$ – nadtlenoazotyn
p-Tyr – *para*-tyrozyna
Phe – fenyloalanina
POx – peroksydazy klasy III
Pro – prolina
PMF – *Peptide Mass Fingerprint*, metoda odcisku palca
PTM – potranslacyjne modyfikacje białek
RNS – reaktywne formy azotu
RONS – reaktywne formy tlenu i azotu
ROS – reaktywne formy tlenu
SBP – białka zawierające biotynę
SDS-PAGE – rozdział elektroforetyczny w żelu poliakrylamidowym w warunkach denaturujących w obecności dodecylosiarczanu sodu
SNP – nitroprusydek sodu
SOD – dysmutaza ponadtlenkowa
Thr – treonina
Trp – tryptofan
Tyr – tyrozyna

Spis publikacji wchodzących w skład rozprawy doktorskiej

Publikacja 1

Tyminski Marcin, Ciacka Katarzyna, Staszek Pawel, Gniazdowska Agnieszka, Krasuska Urszula. 2021. Toxicity of *meta-Tyrosine*. Plants 10: 2800. https://doi.org/10.3390/plants10122800 (IF_{2021}: **4.658**, pkt MNiSW: **70**)

Publikacja 2 - *Praca wchodzi w skład rozprawy w zakresie obejmującym analizę zawartości aminokwasów (meta-tyrozyny, tyrozyny, fenyloalaniny)*

Ciacka Katarzyna, **Tyminski Marcin**, Wal Agnieszka, Gniazdowska Agnieszka, Krasuska Urszula. 2022. Nitric oxide-an antidote to seed aging modifies *meta*-tyrosine content and expression of aging-linked genes in apple embryos. Frontiers in Plant Science 13: 929245. https://doi.org/10.3389/fpls.2022.929245 (IF_{2022}: **5.6**, pkt MNiSW: **100**)

Publikacja 3

Tyminski Marcin, Ciacka Katarzyna, Krasuska Urszula. 2024. Reactive nitrogen species act as the enhancers of glutathione pool in embryonic axes of apple seeds subjected to accelerated ageing. Planta 260: 51. https://doi.org/10.1007/s00425-024-04472-5 (IF_{2024}: **3.8**, pkt MNiSW: **100**)

Publikacja 4

Tyminski Marcin, Staszek Pawel, Ciacka Katarzyna, Gniazdowska Agnieszka, Krasuska Urszula. 2026. NO treatment of embryos isolated from artificially aged apple (*Malus domestica* Borkh.) seeds modifies the pattern of nitrated and carbonylated proteins. Plant Science 362: 112828. https://doi.org/10.1016/j.plantsci.2025.112828 (IF_{2024}: **4.1**, pkt MNiSW: **100**)

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1. Wstęp teoretyczny

1.1. Znaczenie badań nad starzeniem nasion

Podstawową funkcją nasion jako specyficznych organów roślin jest zachowanie i dyspersja gatunków w środowisku (El-Maarouf-Bouteau 2022). Poprzez szereg mechanizmów związanych z budową i regulacją aktywności metabolicznej nasion, w określonym zakresie warunków zewnętrznych, rośliny zwiększają swoje szanse na przetrwanie oraz zdobywanie nowych nisz ekologicznych (Finch-Savage i Leubner-Metzger 2006; Thomas 2013; El-Maarouf-Bouteau 2022). Nasiona, które dla roślin nasiennych stanowią kardynalny element cyklu życiowego, dla ludzi mają zastosowanie w wielu gałęziach przemysłu, np. rolnego. Wykorzystywane jako źródło oleju, skrobi, mąki, dodatek do pasz czy też jako produkt końcowy spożywany przez człowieka, powinny być przechowywane w optymalnych dla danego gatunku warunkach, aby zachować ich wysoką jakość (w tym żywotność) (FAO 2014; De Vitis i inni 2020; El-Maarouf-Bouteau 2022; Corbineau 2024). Równie istotne jest wykorzystanie tych organów, będących wynikiem rozmnażania generatywnego, w utrzymaniu bioróżnorodności. Powstające w tym celu banki nasion muszą zostać tak zaprojektowane, aby podczas przechowywania materiału roślinnego w indywidualnie dobranych dla każdego gatunku warunkach, jak najdłużej zachować jego zdolność kiełkowania. Zachowanie żywotności nasion w określonym czasie jest determinowane m.in. zawartością wody w tkankach, co przekłada się na regulację procesów biochemicznych w komórkach (El-Maarouf-Bouteau 2022). Niezależnie od stopnia desykacji, nawet jeśli reakcje metaboliczne zachodzą w minimalnym stopniu, aktywność ta wiąże się z postępującym procesem starzenia nasion i utratą przez nie żywotności. Stąd niezwykle aktualna jest potrzeba badań dotyczących problematyki starzenia nasion i czasowych możliwości ich przechowywania, poprzez które próbuje się odpowiedzieć na pytanie: „*Jak długo żyją nasiona?*” (Nadarajan i inni 2023).

Szczególnym przykładem są doniesienia o nasionach, których żywotność została zachowana przez wiele wieków. Wykorzystując technikę datowania radioizotopowego, wykazano, że nasiono lotosu orzechodajnego (*Nelumbo nucifera* Gaertn.) pochodzące z Azji (Chiny), które wykiełkowało i z którego rozwinęła się siewka pozbawiona anomalii rozwojowych, miało ponad 1000 lat (Shen-Miller i inni 1995). Podobnie, nasiona daktylowca właściwego (*Phoenix dactylifera* L.), które odnaleziono podczas

badań archeologicznych na Bliskim Wschodzie, wykiełkowały, a ich wiek został oszacowany na około 2000 lat (Sallon i inni 2008).

Badanie mechanizmów starzenia nasion jest skomplikowane, głównie ze względu na wyzwania organizacyjne oraz długi czas trwania takich eksperymentów. Z tego powodu opracowano wystandaryzowane procedury laboratoryjne, których efektem jest osiągnięcie zmian zachodzących podczas naturalnego starzenia (El-Maarouf-Bouteau i inni 2011; Corbineau 2012). Zazwyczaj polegają one na poddaniu nasion podwyższonej temperaturze i wilgotności otoczenia, aby przyspieszyć zachodzące w nich procesy metaboliczne (Bailly i inni 2008; Corbineau 2012; Groot i inni 2012). Zgodnie z zaleceniami Międzynarodowego Związku Oceny Nasion (ISTA – *International Seed Testing Associacion*) procedura przyspieszania starzenia nasion (AA, *Accelerated Ageing*) powinna obejmować przechowywanie tych organów w podwyższonej temperaturze i w 100% wilgotności przez okres właściwy dla danego gatunku (ISTA 2025). Podobnie przeprowadzana jest procedura związana z kontrolowanym pogarszaniem jakości nasion – (CDT, *Controlled Deterioration Treatment*), podczas której w badanym materiale, przed rozpoczęciem traktowania podwyższoną wilgotnością i temperaturą, należy wyrównać zawartość wody (Groot i inni 2012; ISTA 2025). W zależności od potrzeb stosuje się także inne techniki starzenia nasion, na przykład przechowywanie ich w warunkach podwyższonego ciśnienia parcjalnego tlenu (O_2) (Groot i inni 2012).

1.2. Podstawowe pojęcia związane z biologią nasion

Zmienność warunków środowiskowych występujących w obrębie danej szerokości geograficznej sprawiła, że u wielu gatunków roślin moment kiełkowania został oddzielony w czasie od zakończenia dojrzewania nasion, co przekłada się na zmiany fizjologiczno-biochemiczne zachodzące na poziomie komórek zarodka. Okres minimalizacji aktywności metabolicznej nasion w zależności od czynników zewnętrznych, regulowany na poziomie molekularnym, jest definiowany jako spoczynek. Zatem **spoczynkiem nasion** określa się stan, w którym żywotne nasiona nie kiełkują, pomimo sprzyjających warunków środowiska (Finch-Savage i Leubner-Metzger 2006). Regulacja głębokości spoczynku pozwala na rozpoczęcie kiełkowania w czasie najbardziej sprzyjającym wzrostowi roślin, zwiększając tym samym szanse przeżycia siewek oraz sukces reprodukcyjny gatunku (Penfield 2017). Zjawisko to uwarunkowane jest między innymi czynnikami wewnętrznymi, obejmującymi genotyp oraz związane

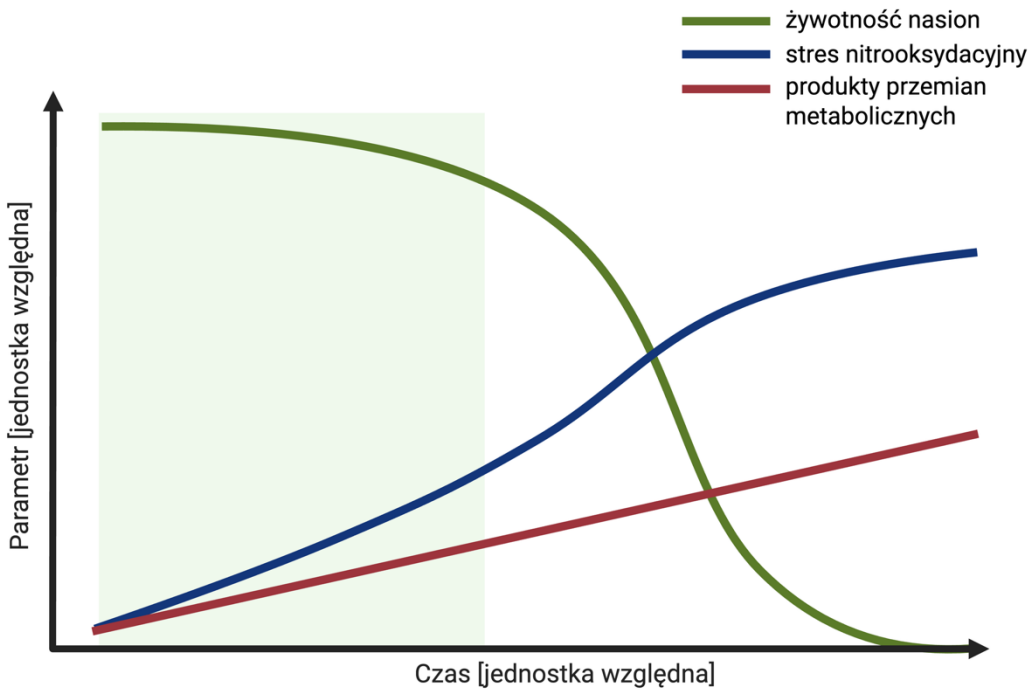
z nim właściwości strukturalne nasion (Finch-Savage i Leubner-Metzger 2006; El-Maarouf-Bouteau 2022). Kluczowym czynnikiem jest równowaga hormonalna, gdyż spoczynek nasion wynika z przewagi stężenia kwasu abscysynowego (ABA) nad giberelinami (GA). Stopień spoczynku reguluje również białko DELAY OF GERMINATION 1 (DOG1) (Footitt i inni 2011; Soppe i Bentsink 2020), co zostało potwierdzone m.in. badaniami transkryptomicznymi (Bentsink i inni 2006; Finch-Savage i Footitt 2017 i cytowania tam zawarte; Sacharowski i inni 2025). Wykazano, że ustępowanie spoczynku wiąże się z obniżeniem zawartości ABA i wzrostem stężenia GA (Kermode 2005; Tan-Wilson i Wilson 2012; Yan i Chen 2017). Na ustąpienie spoczynku wpływa także obecność cząsteczek sygnałowych, takich jak reaktywne formy tlenu (ROS, *Reactive Oxygen Species*) oraz reaktywne formy azotu (RNS, *Reactive Nitrogen Species*) (El-Maarouf-Bouteau i Bailly 2008; Dębska i inni 2013; Krasuska i inni 2015; Bailly 2023).

Ustąpienie spoczynku najczęściej prowadzi do inicjacji kiełkowania, rozumianego jako współdziałanie wielu procesów fizjologicznych zachodzących w określonej kolejności. Zatem **kiełkowanie nasion** *sensu stricto* to ściśle regulowana aktywacja zarodka, zakończona przebiciem okrywy nasiennej przez korzeń zarodkowy (Lewak 2011; Bewley i inni 2013). Pierwszym etapem kiełkowania jest pęcznienie (imbibicja), stanowiące proces fizyczny. Następnie w uwodnionych tkankach nasion następuje intensyfikacja procesów katabolicznych i związana z tym hydroliza substancji zapasowych. Kiełkowanie kończy tzw. faza anaboliczna (wzrost syntezy metabolitów) i przebicie łupiny nasiennej przez wydłużający się korzeń zarodkowy (Bewley i inni 2013; El-Maarouf-Bouteau 2022).

Kolejne pojęcia dotyczące biologii nasion wiążą się z ich jakością i obejmują: wigor nasion, ich żywotność oraz długowieczność.

Wigor nasion określa zdolność nasion do kiełkowania i wytworzenia prawidłowo rozwijających się siewek w zróżnicowanych warunkach środowiskowych (Finch-Savage i inni 2010; Finch-Savage i Bassel 2016; Basu i Groot 2023). Cecha ta jest ściśle związana z **żywotnością nasion** – zdolnością kiełkowania i formowania organizmu, który następnie wytwarza potomstwo (Dumont i inni 2015). Istotnym parametrem jakości nasion jest również ich **długowieczność**, określająca czas, przez który nasiona zachowują żywotność podczas przechowywania lub przebywania w środowisku naturalnym (Finch-Savage i Bassel 2016).

W trakcie przechowywania nasiona stopniowo ulegają **starzeniu**, co prowadzi do obniżania wigoru, a następnie utraty żywotności. Po osiągnięciu punktu krytycznego starzenia następuje gwałtowna utrata żywotności kończąca się śmiercią komórek (Walters i inni 2010; Yin i inni 2016). Tempo utraty żywotności można zobrazować kształtem odwróconej litery „S" (Rysunek 1.1) (Yin i inni 2016, 2017). Postępujące starzenie nasion jest w dużej mierze konsekwencją zaburzenia homeostazy redoks, która wynika m.in. z nasilenia stresu nitrooksydacyjnego (Rysunek 1.1) oraz zachwiania równowagi między generowaniem reaktywnych form tlenu i azotu (RONS), a wydajnością mechanizmów antyoksydacyjnych/detoksykacyjnych (Bailly 2004; El-Maarouf-Bouteau i Bailly 2008; Rajjou i Debeaujon 2008; Sano i inni 2016). Skutkiem tego jest nagromadzanie produktów przemian metabolicznych oraz powstawanie trwałych uszkodzeń oksydacyjnych, obejmujących m.in. utlenione lipidy, zmodyfikowane białka i uszkodzone kwasy nukleinowe, co stopniowo przyczynia się do obniżania żywotności i wigoru nasion (Rysunek 1.1) (Kurek i inni 2019; Ciacka i inni 2020a; Waterworth i inni 2024).



Rysunek 1.1 Schemat przedstawiający typowy przebieg zmian żywotności dojrzałych nasion typu *orthodox* podczas ich przechowywania. Początkowo zmiany związane ze wzrostem stresu nitrooksydacyjnego i nagromadzaniem produktów przemiany materii są niewielkie (zielone pole) i nie mają istotnego wpływu na żywotność nasion. Z czasem dochodzi do przyspieszenia starzenia, zakończonego całkowitą utratą żywotności nasion (Walters i inni 2010 - zmodyfikowano). Rysunek przygotowany przy użyciu aplikacji Biorender.

Na żywotność nasion i starzenie wpływa szereg interakcji pomiędzy czynnikami wewnętrznymi zarodka a parametrami otoczenia (Walters i inni 2010; Sano i inni 2016). Kluczowe są warunki, w jakich nasiona są przechowywane, w tym wilgotność, temperatura oraz dostępność tlenu (O_2) (Basu i Groot 2023; Pirredda i inni 2023). Tempo starzenia nasion i ich długowieczność są również determinowane przez budowę anatomiczną, skład zgromadzonych substancji zapasowych oraz uwarunkowania genetyczne (Sano i inni 2016). Duży wpływ mają także warunki, w jakich znajdowała się roślina mateczna podczas dojrzewania nasion (Sano i inni 2016) oraz głębokość spoczynku. Jednym z kluczowych parametrów decydujących o podatności nasion na utratę żywotności jest zawartość wody w tkankach. Stopień uwodnienia tkanek wpływa na tempo przemian metabolicznych, a niekontrolowany wzrost stężenia produktów przemiany materii może przyspieszać utratę żywotności nasion (El-Maarouf-Bouteau 2022; Nadarajan i inni 2023; Corbineau 2024).

1.3. Klasyfikacja fizjologiczna nasion

Odwadnianie jest ostatnim etapem dojrzewania nasion. W zależności od ostatecznej zawartości wody w tkankach nasiona klasyfikuje się na typ *orthodox* – takie, które wykazują znaczną tolerancję i niską wrażliwość na utratę wody – oraz na typ *recalcitrant* – wrażliwe na desykację. Ponadto wyróżnia się nasiona typu pośredniego, które znoszą odwodnienie, ale ulegają uszkodzeniu podczas przechowywania w niskich temperaturach (Rajjou i Debeaujon 2008; Sano i inni 2016; Corbineau 2024). Przykłady nasion o trzech typach wrażliwości na desykację przedstawiono w Tabeli 1.1.

Tabela 1.1 Wybrane przykłady gatunków roślin wytwarzających nasiona *orthodox*, *recalcitrant* i nasiona typu pośredniego

Nasiona typu *recalcitrant*	Nasiona typu *orthodox*	Nasiona typu pośredniego
Quercus robur L. dąb szypułkowy	***Malus domestica* Borkh.** **jabłoń domowa**	*Fagus sylvatica* L. buk zwyczajny
Theobroma cacao L. kakaowiec właściwy	*Arabidopsis thaliana* L. rzodkiewnik pospolity	*Coffea arabica* L. kawa arabska
Castanea sativa Mill. kasztan jadalny	*Zea mays* L. kukurydza zwyczajna	
Persea americana Mill. smaczliwka wdzięczna	*Acer platanoides* L. klon zwyczajny	
	Triticum aestivum L. pszenica zwyczajna	

W dojrzałych nasionach typu *recalcitrant* zawartość wody nie powinna zostać obniżona poniżej wartości 30-65%. W warunkach naturalnych nasiona te szybko tracą żywotność, jeśli kiełkowanie nie jest inicjowane bezpośrednio po przedostaniu się do gleby. Z tego względu ich przechowywanie z wykorzystaniem standardowych metod jest znacznie utrudnione lub czasami wręcz niemożliwe. Ogranicza to zarówno możliwości długoterminowego zabezpieczania zasobów genowych, jak i krótkoterminowego magazynowania materiału siewnego na potrzeby obrotu handlowego oraz wykorzystania w kolejnych sezonach (Berjak i Pammenter 2013; Corbineau 2024). Dodatkowo wiele nasion typu *recalcitrant* jest wrażliwych na chłód. W zależności od gatunku i zmian adaptacyjnych związanych z występowaniem w danej szerokości geograficznej, nasiona te nie tolerują temperatur niższych niż 5-15°C. Ponieważ nie wykształciły mechanizmów chroniących je przed tworzeniem kryształów lodu we wnętrzu komórki, w przypadku przemarzania dochodzi do powstawania krytycznych uszkodzeń i śmierci organizmu (Berjak i Pammenter 2013).

Około 80% wszystkich roślin nasiennych na świecie wytwarza nasiona typu *orthodox*. Wykazano, że zawartość wody w takich nasionach osiąga jedynie 4-10% (El-Maarouf-Bouteau 2022; Matilla 2022). Tak wysoki stopień odwodnienia pozwala na ich przechowywanie w temperaturze poniżej zera. Mechanizmy tolerancji na desykację umożliwiają długotrwałe utrzymanie żywotności nasion bez utraty wigoru (Matilla 2022). Ta adaptacja obejmuje obecność białek ochronnych, m.in. dehydryn, białek szoku cieplnego (HSP), czy białek późnej embriogenezy (LEA). Komórki dojrzałych nasion typu *orthodox* charakteryzują się ekstremalnie wysoką lepkością cytoplazmy, określaną jako stan szklisty (*glassy state*) (Pirredda i inni 2023). W tym stanie aktywność komórkowa i intensywność przemian metabolicznych ulegają znacznemu ograniczeniu, co sprzyja zachowaniu struktury i funkcji białek (Buitink i Leprince 2008; Ballesteros i Walters 2011). Oprócz wspomnianych wcześniej białek ochronnych, ważną rolę w utrzymaniu stanu szklistego cytoplazmy odgrywają cukry, takie jak rafinoza, stachioza i werbaskoza, które m.in. stabilizują lipidy obecne w błonach komórkowych (Corbineau 2024 i cytowania tam zawarte).

Nasiona jabłoni (*Malus domestica* Borkh.) wykazują głęboki spoczynek fizjologiczny, który dodatkowo jest pogłębiony obecnością okrywy nasiennej, istotnej bariery dla wody i gazów. Należą do nasion typu *orthodox* (Tabela 1.1), a ich główny materiał zapasowy stanowią tłuszcze magazynowane w liścieniach zarodków (Lewak 2011). Z tego względu są też przypisane do kategorii nasion oleistych. Głęboki spoczynek

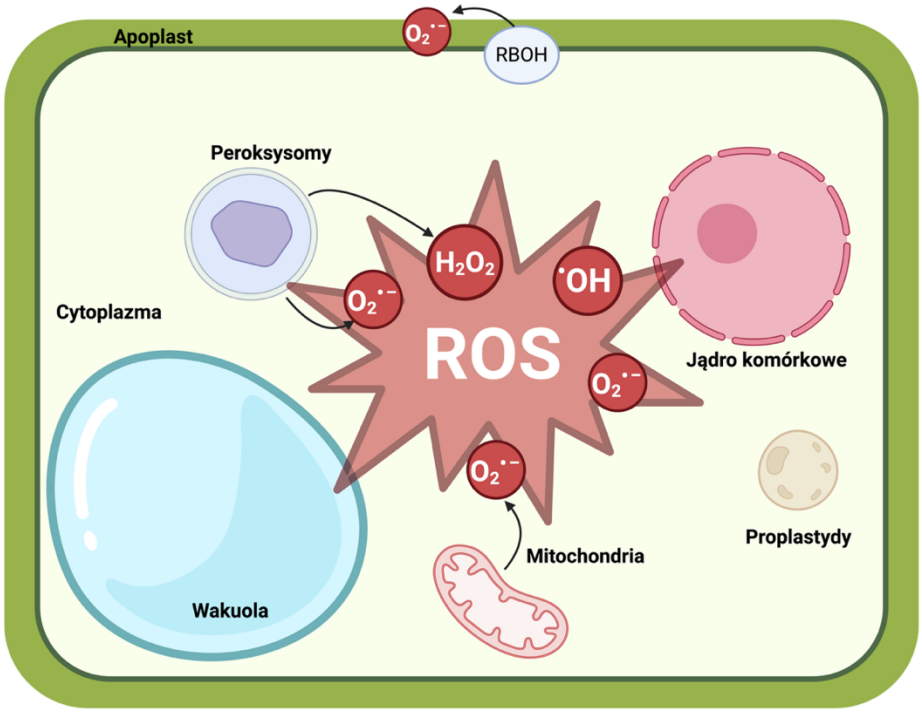
nasion jabłoni ustępuje podczas zabiegu stratyfikacji w niskiej, ale dodatniej temperaturze (stratyfikacja w chłodzie) (Lewak 2011). Poddanie nasion temu zabiegowi przez 90 dni w warunkach 60% wilgotności i w temperaturze 5°C prowadzi do kiełkowania wszystkich izolowanych z nich zarodków już po dwóch dniach prowadzenia kultury, a rozwijające się siewki cechują się brakiem zaburzeń rozwojowych (Dębska i inni 2013). Wykazano, że kiełkowanie zarodków wyizolowanych z nasion, których spoczynek nie został przełamany, było spowolnione, a rozwijające się z nich siewki charakteryzowała obecność anomalii takich jak nierównomierne zazielenienie i asymetryczny wzrost liścieni czy brak wydłużonego korzenia zarodkowego. Wskazuje to na zaburzoną aktywację zarodka (Gniazdowska i inni 2010b; Lewak 2011). Z kolei zabieg przechowywania nasion jabłoni w podwyższonej temperaturze (powyżej 25°C) i zwiększonej wilgotności podłoża prowadził do silnego obniżania ich żywotności. Jednocześnie część izolowanych z takich nasion zarodków nadal pozostawała w stanie spoczynku, o czym świadczyła obecność anomalii rozwojowych (Dębska i inni 2013; Ciacka i inni 2022b).

1.4. Reaktywne formy tlenu (ROS)

Pomimo potencjalnie destrukcyjnego charakteru, ROS pełnią ważną rolę w komórkach roślin i zwierząt. Powstają głównie podczas niepełnej redukcji O_2 (Noctor i inni 2018). W tej grupie związków znajdują się wolne rodniki, a więc cząsteczki posiadające niesparowane elektrony na orbitalu walencyjnym, czego przykładem jest anionorodnik ponadtlenkowy ($O_2^{\bullet-}$) lub rodnik hydroksylowy ($^{\bullet}OH$). Ponadto do ROS zalicza się związki niebędące rodnikami, o niższej reaktywności i dłuższym czasie półtrwania – np. ozon (O_3), nadtlenek wodoru (H_2O_2) i tlen singletowy ($^1O_2$) (Corpas i inni 2015; Mittler 2017; Considine i Foyer 2021).

W komórkach roślinnych ROS powstają w wielu przedziałach, spośród których istotne znaczenie dla nasion mają mitochondria, peroksysomy (w tym glikoksysomy), proplastydy oraz apoplast (Rysunek 1.2) (Corpas i inni 2015; Noctor i inni 2018; Peng i inni 2024). Wytwarzanie $O_2^{\bullet-}$ jest związane z zaburzeniem przepływu elektronów w mitochondrialnym łańcuchu oddechowym, dotyczy to w szczególności kompleksów błonowych I i III (Navrot i inni 2007; Noctor i inni 2018; Bailly 2019, 2023). Peroksysomy, które w nasionach mogą przybierać wyspecjalizowaną formę glikosysomów, to organelle, w których zachodzą intensywne przemiany metaboliczne, w tym β-oksydacja kwasów tłuszczowych. Są to miejsca dynamicznego powstawania

ROS, zwłaszcza podczas drugiej fazy kiełkowania nasion (Ma i inni 2016). W czasie tego procesu, w apoplaście wzrasta również stężenie ROS, co wiąże się z aktywnością oksydaz fosforanu dinukleotydu nikotynoamidoadeninowego (NADPH) RBOH (*Respiratory Burst Oxidase Homolog*). Powstające wolne rodniki uczestniczą w rozluźnianiu struktury ścian komórkowych, umożliwiając wzrost wydłużeniowy komórek (Muller i inni 2009). W cytoplazmie $^{\bullet}OH$ może powstawać z H_2O_2 podczas reakcji Habera-Weissa lub w wyniku reakcji Fentona, w obecności jonów metali przejściowych (głównie Fe^{2+}, Cu^{+}) (Mittler 2017).



Rysunek 1.2 Uproszczony schemat uwodnionej komórki osi zarodkowej nasiona przedstawiający najważniejsze przedziały komórkowe, w których powstają ROS. Najczęściej do powstawania wolnych rodników dochodzi w apoplaście, peroksysomach, mitochondriach i cytoplazmie. Rysunek przygotowany przy użyciu aplikacji Biorender.

W zależności od budowy chemicznej ROS, ich reaktywności i czasu półtrwania oddziaływanie na inne cząsteczki jest zróżnicowane. Wolne rodniki są najbardziej reaktywne, ale jednocześnie jako nietrwałe, reagują jedynie z cząsteczkami w ich bezpośrednim sąsiedztwie, głównie w miejscu ich powstawania (Møller i inni 2007). Stosunkowo długi czas półtrwania (około 1 ms) i brak ładunku umożliwiają przenikanie H_2O_2 przez błony komórkowe, dzięki czemu cząsteczka ta pełni istotną rolę w przekazywaniu sygnałów komórkowych i międzykomórkowych (Møller i inni 2007; Corpas i inni 2015). Stąd ROS są powszechnie nazywane cząsteczkami o „dwoistej

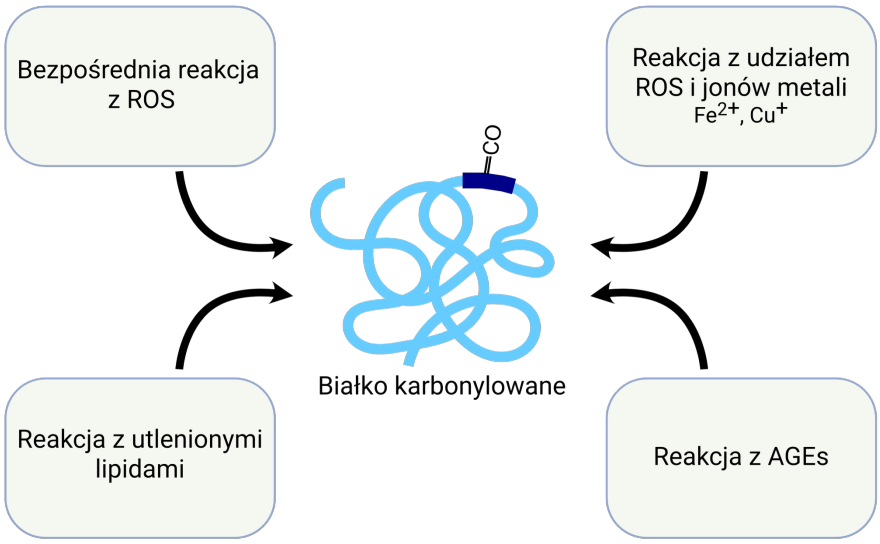
naturze". W niskich, fizjologicznych stężeniach pełnią funkcje sygnałowe, natomiast nadmierny i niekontrolowany wzrost ich zawartości powoduje uszkodzenia, które przy braku mechanizmów naprawczych prowadzą do śmierci komórek (Bailly i inni 2008; Mittler 2017).

Działanie ROS ściśle wiąże się z ich zdolnością do reagowania z innymi, otaczającymi je cząsteczkami, w tym lipidami, kwasami nukleinowymi, cukrami oraz białkami (Møller i inni 2007; Møller i Sweetlove 2010; Mittler 2017). Jednocześnie w przypadku nieprawidłowego działania mechanizmów kontrolujących ich stężenie, jest to mechanizm toksyczności. Powstałe w reakcjach z ROS pochodne makrocząsteczek (np. utlenione białka) najczęściej tracą pierwotną strukturę i funkcję (Ciacka i inni 2020b). Poza toksycznym działaniem, ROS modyfikują biocząsteczki, które w następstwie tych zmian mogą pełnić funkcję sygnałową. Do takich związków zalicza się oksylipiny, peptydy powstające z utlenionych białek oraz utlenione mRNA (Møller i Sweetlove 2010; Chmielowska-Bąk i inni 2015).

ROS inicjują peroksydację lipidów, przede wszystkim wielonienasyconych kwasów tłuszczowych obecnych w fosfolipidach błon komórkowych. W wyniku tego procesu powstają niestabilne nadtlenki lipidowe, które ulegają przekształceniom do aldehydów (Pizzimenti i inni 2013). Należą do nich: dialdehyd malonowy (MDA), akroleina czy 4-hydroksynonenal (HNE), które ze względu na swoje właściwości chemiczne biorą udział w modyfikacjach białek oraz innych makrocząsteczek komórkowych. MDA, ze względu na stabilność, jest powszechnie uznawany za wskaźnik stopnia uszkodzeń komórkowych spowodowanych stresem oksydacyjnym (Noctor i inni 2016).

Wykazano, że w warunkach stresu oksydacyjnego oraz podczas procesów starzenia dochodzi do częstszego powstawania modyfikacji potranslacyjnych białek (PTM), zmieniających ich konformację, aktywność, oddziaływania międzycząsteczkowe oraz podatność na degradację (Ciacka i inni 2020b i cytowania tam zawarte). Modyfikacje ROS-zależne dotyczą reszty cysteiny (Cys), argininy (Arg), lizyny (Lys), treoniny (Thr) i proliny (Pro). Odwracalne PTM biorą udział m.in. w utrzymaniu homeostazy redoks, co najczęściej dotyczy grupy tiolowej (-SH) Cys. Do tego typu modyfikacji zalicza się tworzenie mostków disiarczkowych, sulfenylację Cys czy *S*-glutationylację (Liu i Liao 2025). Karbonylacja białek jest przykładem PTM, która polega na wbudowywaniu grup karbonylowych (=CO) w łańcuch reszt niektórych aminokwasów (Møller i inni 2007; Ciacka i inni 2020b). Ta modyfikacja w warunkach fizjologicznych jest nieodwracalna i prowadzi do zmian w strukturze przestrzennej białek, ponieważ

zależności pomiędzy hydrofilowymi i hydrofobowymi resztami łańcucha aminokwasowego zostają zaburzone w porównaniu z prawidłowo sfałdowanym białkiem. Sprzyja to wzajemnym oddziaływaniom między tak zmodyfikowanymi białkami i tworzeniu nierozpuszczalnych, toksycznych agregatów (Grune i inni 2004; Nyström 2005). Karbonylacja białek zachodzi w wyniku bezpośredniej reakcji z ROS ($^{\bullet}OH$, $O_2^{\bullet-}$, H_2O_2); podczas reakcji z udziałem ROS i jonów metali (Fe^{2+}, Cu^{+}, H_2O_2); w reakcji z utlenionymi lipidami, czy z produktami zaawansowanej glikacji białek (AGEs) (Rysunek 1.3) (Ciacka i inni 2020b i cytowania tam zawarte).

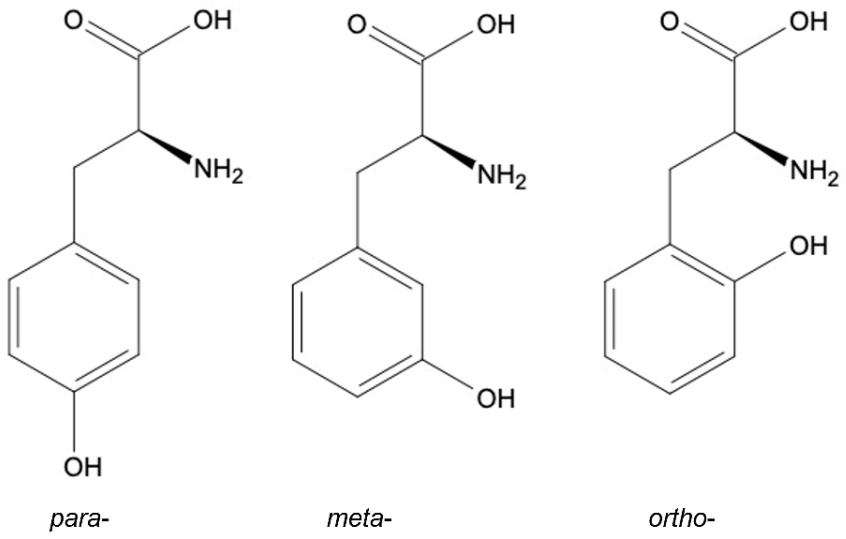


Rysunek 1.3 Schemat przedstawiający główne drogi powstawania białek karbonylowanych (Ciacka i inni 2020b - zmodyfikowano).

Oprócz białek również kwasy nukleinowe ulegają uszkodzeniom zależnym od ROS, co potwierdzono w nasionach (Kurek i inni 2019). ROS reagują z zasadami azotowymi, czego konsekwencją może być pęknięcie nici kwasu nukleinowego (Guo i inni 2017). Markerem takich zmian jest produkt utleniania guaniny, 8-oksy-7,8-dihydroguanina (8-oxoG). Wysoka podatność na tego typu uszkodzenia dotyczy RNA, który ze względu na swoją strukturę jest bardziej wrażliwy na działanie ROS niż dwuniciowy DNA (El-Maarouf-Bouteau i inni 2013). Utlenianie RNA może też regulować proces translacji i pośrednio wpływać na proteom w komórkach (Chmielowska-Bąk i inni 2015).

Tyrozyna (Tyr) i fenyloalanina (Phe) to aromatyczne aminokwasy, których synteza zachodzi jedynie u roślin i mikroorganizmów (**Publikacja 1)**. Utlenianie Phe prowadzi do powstawania strukturalnych izomerów białkogennej *para*-Tyr: *meta*-Tyr (*m*-Tyr)

lub *orto*-Tyr (*o*-Tyr) (Rysunek 1.4), związków zaliczanych do antymetabolitów oraz markerów stresu oksydacyjnego (Ipson i Fisher 2016). Toksyczność *m*-Tyr polega na jej wbudowaniu do łańcucha polipeptydowego w miejsce Phe, co prowadzi do nieprawidłowego fałdowania białka i zaburzenia jego biologicznej funkcji **(Publikacja 1 i cytowania tam zawarte).** Mechanizm włączania antymetabolitów w procesie translacji wciąż nie jest w pełni wyjaśniony. Badania z wykorzystaniem *Escherichia coli* wykazały, że *m*-Tyr może zostać wprowadzona do łańcucha białkowego poprzez swoje powinowactwo do $tRNA^{Phe}$ (Bullwinkle i inni 2014).



Rysunek 1.4 Strukturalne izomery Tyr: *para*-Tyr, *meta*-Tyr, *orto*-Tyr **(Publikacja 1, Fig. 2).**

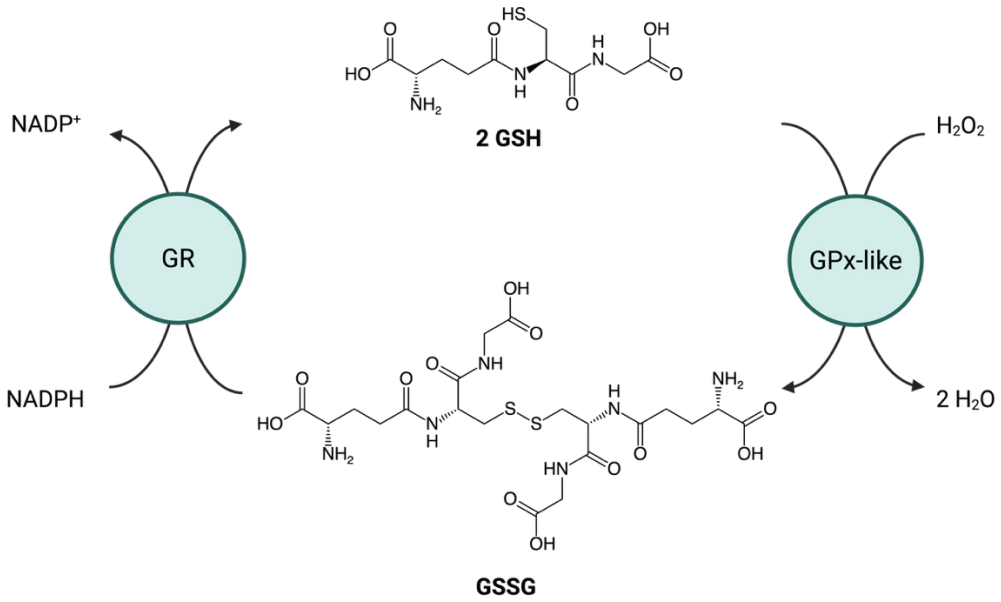
1.5. System antyoksydacyjny

System antyoksydacyjny odpowiada za utrzymanie homeostazy ROS w komórkach. Do najważniejszych enzymów związanych z detoksykacją ROS zalicza się: dysmutazę ponadtlenkową (SOD), katalazę (CAT) oraz różne peroksydazy, w tym peroksydazy klasy III (POx). Jednocześnie komórki zawierają związki o niskiej masie cząsteczkowej, zdolne do bezpośredniego reagowania z ROS i pełniące rolę nieenzymatycznego systemu antyoksydacyjnego. Do tej grupy należą między innymi karotenoidy, tokoferole, kwas askorbinowy (ASA) oraz glutation w formie zredukowanej (GSH) (Rudenko i inni 2023).

Nasiona typu *orthodox*, w stanie spoczynku, ze względu na wysoki stopień odwodnienia, cechuje niska aktywność enzymatyczna. Z tego względu nieenzymatyczny system antyoksydacyjny stanowi filar zapewniający ochronę przed nadmiarem ROS (Foyer i Noctor 2011). W tej grupie modulatorów ROS znajdują się związki

z grupami -SH (np. Cys), gdyż biorą udział w reakcjach utleniania i redukcji i są wrażliwe na zmiany potencjału redoks (sensory potencjału redoks) (Considine i Foyer 2021). Ich znaczenie dla nasion typu *orthodox* jest kluczowe, gdyż podczas ostatniego etapu dojrzewania, w suchych nasionach, znacznemu obniżaniu ulega stężenie ASA do wartości niemalże niewykrywalnych standardowymi technikami (Tommasi i inni 1999).

Glutation w formie zredukowanej (GSH) jest tripeptydem, zbudowanym z kwasu glutaminowego, Cys i glicyny. Jego utleniona forma (GSSG) powstaje podczas odwracalnej reakcji dimeryzacji dwóch cząsteczek GSH z utworzeniem wiązania disiarczkowego (Kranner i inni 2006). GSH jest związkiem powszechnie występującym w komórkach organizmów tlenowych, gdzie pełni również funkcję sygnałową (Li i inni 2025). Reakcja dimeryzacji cząsteczki GSH może zachodzić bezpośrednio z ROS bądź jako wynik aktywności enzymów, które w komórkach roślinnych należą do grupy peroksydaz typu ssaczej peroksydazy glutationowej (GPx-like) (Rysunek 1.5). Biologicznie istotne jest stałe odtwarzanie puli GSH, tak aby ta cząsteczka mogła pełnić funkcję ochronną (antyoksydacyjną) i regulatorową w komórkach. Ta reakcja, do której niezbędny jest NADPH, jest katalizowana przez reduktazę glutationową (GR) (Rysunek 1.5).



Rysunek 1.5 Uproszczony cykl przemian GSH i GSSG w obecności ROS oraz enzymów GPx-like i GR. GPx-like – roślinna peroksydaza glutationowa, GR – reduktaza glutationowa, NADPH – zredukowana forma fosforanu dinukleotydu nikotynoamidoadeninowego. Rysunek przygotowany przy użyciu aplikacji Biorender.

Wskaźnikiem stopnia żywotności nasion jest potencjał redoks glutationu ($E_{GSSG/2GSH}$). Ten parametr pozwala ocenić stopień zaawansowania stresu oksydacyjnego w komórkach. W żywych nasionach wartość ta wynosi około -250 mV (Kranner i inni 2006; Noctor i inni 2012). Wzrost wartości potencjału redoks $E_{GSSG/2GSH}$ do zakresu od -180 do -140 mV wiąże się z występowaniem nieodwracalnych uszkodzeń komórkowych (Kranner i inni 2006) .

1.6. Reaktywne formy azotu (RNS)

Drugą grupą reaktywnych cząsteczek są RNS, związki również istotne z punktu widzenia biologii nasion. Podstawowym reprezentantem tej grupy jest tlenek azotu (NO) – gazowy związek zdolny do dyfuzji przez błony komórkowe. Występuje jako wolny rodnik ($^{\bullet}NO$), anion nitrozylowy (NO^-) bądź kation nitrozoniowy (NO^+) (Durzan i Pedroso 2002; Procházková i inni 2015). Do RNS należą także produkty reakcji NO z O_2 bądź ROS, między innymi nadtlenoazotyn ($ONOO^-$) oraz nitroksyl (HNO) (Stamler i inni 1992; Kolbert i inni 2019; Arasimowicz-Jelonek i inni 2023).

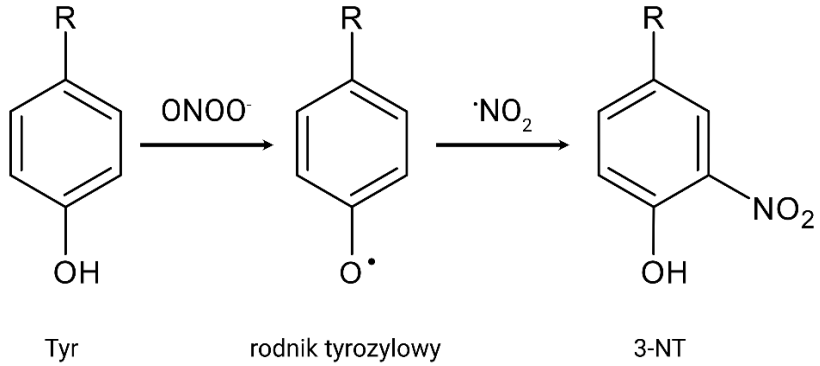
Ze względu na swoje właściwości fizykochemiczne – lipofilność, wysoki stopień dyfuzji, reaktywność – NO pełni rolę cząsteczki sygnałowej, uczestnicząc w regulacji wielu procesów rozwojowych i fizjologicznych (Stamler i inni 1992). Związek ten bierze udział m.in. w ustępowaniu spoczynku nasion wielu gatunków roślin, inicjacji kiełkowania, kontroli wydłużania komórek korzenia, regulacji kwitnienia czy dojrzewania owoców (Hancock i Neill 2019; Kolbert i inni 2019; Hancock 2025). Ponadto wykazano, że NO jest istotnym związkiem regulującym starzenie nasion (Ciacka i inni 2022a, b).

W komórkach roślin wyższych do tej pory nie udało się zidentyfikować enzymów należących do klasy syntaz NO (NOS), charakterystycznych dla komórek zwierzęcych (Hancock i inni 2025). Enzym ten, w warunkach tlenowych, katalizuje dwuetapową reakcję przekształcenia Arg do cytruliny, podczas której uwalniana jest cząsteczka NO (Rivero 2006; Santolini i inni 2017; Dusak i inni 2026). Wyniki wielu badań, z zachowaniem warunków reakcji i kofaktorów typowych dla ssaczych NOS oraz przy użyciu specyficznych inhibitorów tego enzymu, wskazują na prawdopodobieństwo istnienia tej drogi syntezy NO u roślin, w tym w kiełkujących zarodkach jabłoni (Krasuska i inni 2016; Hancock i Neill 2019; Corpas i inni 2022 i cytowania tam zawarte). Stąd proponuje się, że u wyższych autotrofów występują enzymy charakteryzujące się funkcjonalnym podobieństwem do NOS, ale prawdopodobnie różniące się budową

(Corpas i Barroso 2017; Hancock i Neill 2019, Hancock i inni 2025). Ponadto, uważa się, że w syntezę NO w warunkach normoksji są zaangażowane inne enzymy, np. peroksydazy klasy III (Dusak i inni 2026). Wzrost wytwarzania NO w nasionach zachodzi już na samym początku kiełkowania, nawet w warunkach ograniczonej dostępności O_2 (Bykova i inni 2015). Hipoksja sprzyja syntezie NO w mitochondriach w wyniku przekształcania jonów azotynowych (NO_2^-) przez kompleksy III i IV oraz oksydazę alternatywną (AOX). Ten mechanizm pozwala utrzymać elektrochemiczny gradient protonów pomiędzy błonami mitochondriów, który jest wykorzystywany przez syntazę adenozynotrifosforanu (ATP) do generowania tego nukleotydu (Stoimenova i inni 2007; Gupta i Igamberdiev 2016; Gupta i inni 2022). W kwaśnym środowisku dochodzi do uwalniania RNS z NO_2^-, zwłaszcza w obecności reduktantów (Yamasaki 2000). Wykazano, że w warunkach wysokiego stężenia NO_2^- i niedoboru O_2, znajdująca się w cytoplazmie reduktaza azotanowa (NR) katalizuje reakcję przekształcania NO_2^- do NO (Chamizo-Ampudia i inni 2017; Mohn i inni 2019).

RNS wykazują podobny efekt działania na procesy fizjologiczne roślin jak ROS; powstają również w tych samych przedziałach komórkowych. W optymalnym stężeniu pełnią funkcję przekaźników sygnałów i regulatorów procesów komórkowych, natomiast niekontrolowany wzrost ich zawartości może prowadzić do głębokich uszkodzeń komórkowych, zakończonych śmiercią. Podobnie jak ROS, RNS modyfikują białka, tłuszcze i kwasy nukleinowe (Gupta i inni 2020; Hancock 2025). RNS reagują z białkami, a najczęściej opisywanymi w literaturze PTM są nitracja i *S*-nitrozacja (Corpas i Barroso 2017). Wykazano, że nagromadzenie białek nitrowanych może wskazywać na występowanie stresu nitrooksydacyjnego, co potwierdzono zarówno w tkankach roślinnych, jak i zwierzęcych (Bartesaghi i Radi 2018; Arasimowicz-Jelonek i Floryszak-Wieczorek 2019; Corpas i inni 2021). Najbardziej podatne na nitrowanie są aromatyczne aminokwasy: Tyr i tryptofan (Trp). Reakcja nitracji przebiega wieloetapowo, najczęściej zachodzi przy udziale $ONOO^-$ i rodnika dwutlenku azotu ($^{\bullet}NO_2$) (Winterbourn i Kettle 2003; Radi 2018; Corpas i inni 2021) (Rysunek 1.6), prowadząc do przyłączenia grupy nitrowej do pierścienia aromatycznego. W wyniku nitracji powstaje stabilny w warunkach fizjologicznych metabolit – 3-nitrotyrozyna (3-NT). Nitracja zmienia aktywność białek (Corpas i inni 2021 i cytowania tam zawarte), ale może również funkcjonować jako znacznik ich przyspieszonej degradacji (Castillo i inni 2015; Kolbert i inni 2017). Na dzień dzisiejszy nie ma danych dotyczących możliwości tworzenia nitrowanych peptydów sygnałowych, uwolnionych z hydrolizowanych, nitrowanych

białek, tak jak sugeruje się w przypadku peptydów powstałych z białek karbonylowanych (Møller i Sweetlove 2010).



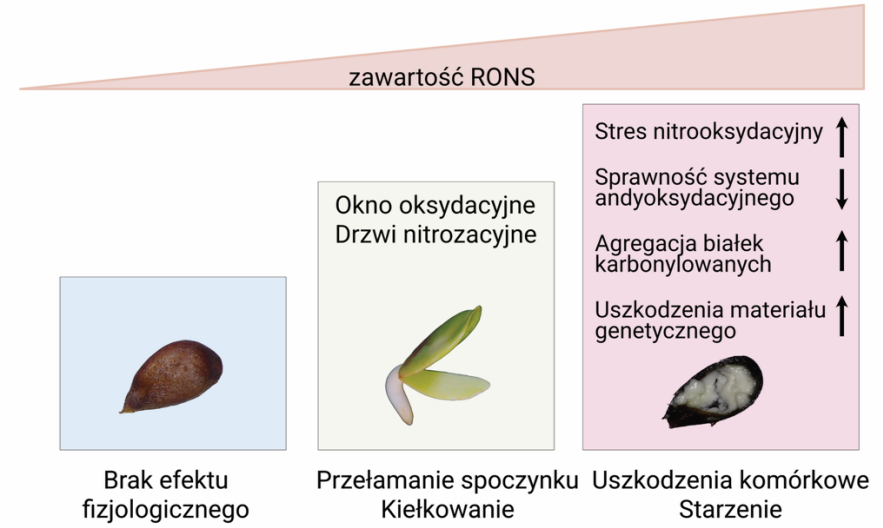
Rysunek 1.6 Uproszczony schemat reakcji nitracji reszty Tyr będącej częścią łańcucha białkowego (R) i powstawania 3-NT.

S-nitrozacja białek lub peptydów polega na przyłączeniu NO do grupy -SH reszty Cys. W przeciwieństwie do nitracji, jest to modyfikacja odwracalna, w zależności od warunków środowiska reakcji (Leterrier i inni 2011; Corpas 2016), dzięki czemu tak zmodyfikowane związki mogą stanowić swego rodzaju „magazyn" NO. Przykładem jest *S*-nitrozoglutation (GSNO), cząsteczka należąca do grupy *S*-nitrozotioli. GSNO dyfunduje przez błony komórkowe, przez co jest jednocześnie uważany za formę transportową NO. Przemiany GSNO mogą zachodzić na drodze reakcji katalizowanych przez enzymy (na przykład reduktaza GSNO – GSNOR) lub podczas bezpośrednich reakcji GSNO z resztami Cys innych białek – transnitrozacja białek. W reakcji z udziałem GSNOR pula całkowitego NO ulega obniżeniu, ponieważ GSNO jest przekształcony do GSSG i hydroksyloaminy/jonów amonowych, w zależności od dostępności GSH i NADH (Lindermayr 2018; Jahnová i inni 2019).

1.7. Wybrane aspekty działania RONS w starzeniu nasion

Wyniki licznych badań jednoznacznie wskazują, że RONS odgrywają kluczową rolę w biologii nasion, włączając w to starzenie fizjologiczne i starzenie wywołane zabiegami przyspieszającymi procesy metaboliczne (starzenie patofizjologiczne) (El-Maarouf-Bouteau i Bailly 2008; Bailly i inni 2008; Krasuska i inni 2015; Bailly 2023). Zależna od stężenia aktywność biologiczna tych reaktywnych cząsteczek została dobrze udokumentowana w literaturze. Przełamanie spoczynku nasion wielu gatunków roślin wymaga osiągnięcia optymalnego (dla danego gatunku) stężenia RONS, mieszczącego

się w zakresie tzw. „okna oksydacyjnego” i „drzwi nitrozacyjnych” (Rysunek 1.7) (Bailly i inni 2008; Krasuska i inni 2015; Puglia 2024 i cytowania tam zawarte). Zbyt niskie stężenie RONS odpowiada za utrzymanie spoczynku nasion, natomiast zbyt wysokie wiąże się ze wzrostem stresu nitrooksydacyjnego, postępującymi uszkodzeniami i ostatecznie śmiercią komórek (Bailly i inni 2008; Krasuska i inni 2015).



Rysunek 1.7 Uproszczony schemat działania RONS w zależności od ich komórkowego stężenia. Zbyt niskie stężenie nie wywołuje efektu fizjologicznego, a zbyt wysokie prowadzi do stresu nitrooksydacyjnego i uszkodzeń wewnątrzkomórkowych. Optymalne dla danego gatunku rośliny stężenie, które mieści się w granicach „okna oksydacyjnego” i „drzwi nitrozacyjnych”, pełni funkcje o charakterze regulatorowym, prowadząc m. in. do przełamania spoczynku i w konsekwencji inicjacji kiełkowania nasion.

Stres nitrozacyjny, jako czynnik odpowiedzialny za przyspieszone starzenie nasion, prowadzi do potencjalnie odmiennych efektów fizjologicznych. W przypadku osiągnięcia przez RNS stężenia toksycznego dla komórek, mechanizm działania byłby podobny do tego, który inicjują ROS. Jednakże to zbyt niskie stężenie NO jest zazwyczaj odnotowywane w nasionach postarzanych (Dębska i inni 2013; Ciacka i inni 2020a). Wykazano, że starzeniu nasion wiązu syberyjskiego (*Ulmus pumila* L.) towarzyszyło znaczne obniżanie zawartości białek *S*-nitrozowanych (He i inni 2018). Autorzy wykazali, że *S*-nitrozacja dotyczyła białek o istotnym znaczeniu metabolicznym, potencjalnie również zaangażowanych w proces starzenia nasion.

Wielokrotnie potwierdzono, że krótkotrwałe traktowanie nasion (lub zarodków) różnych gatunków roślin donorami NO ma korzystne działanie stymulujące metabolizm i w rezultacie przyspieszające kiełkowanie (Gniazdowska i inni 2010a; Ciacka i inni 2020a i cytowania tam zawarte). Podobnie, egzogenny NO działa korzystnie w nasionach

poddanych przyspieszonemu starzeniu (Ciacka i inni 2020a). Zastosowanie nitroprusydku sodu (SNP), będącego donorem NO, przed zabiegiem sztucznego postarzania nasion wiązu syberyjskiego ograniczało obniżanie ich żywotności w stosunku do nasion starzonych, ale nietraktowanych (He i inni 2018). Ponadto, pozytywne działanie egzogennego NO wydaje się być niezależne od momentu jego zastosowania względem zabiegu starzenia, czego dowiedziono na przykładzie ziarniaków owsa zwyczajnego (*Avena sativa* L.) (Mao i inni 2018). Autorzy wykazali, że wykorzystanie SNP do traktowania ziarniaków poddanych wcześniej starzeniu poprawiało aktywność mitochondrialnego systemu antyoksydacyjnego. Łagodzenie skutków stresu oksydacyjnego (jeden z mechanizmów działania RNS) prowadziło do poprawy żywotności ziarniaków owsa zwyczajnego (Mao i inni 2018). Efekt stosowania NO w kontekście utrzymania żywotności zarodków izolowanych z postarzonych nasion opisano również dla jabłoni (Ciacka i inni 2022b). Fumigacja NO zarodków jabłoni izolowanych z postarzanych nasion przez 14 lub 21 dni sprzyjała wzrostowi wydajności systemu antyoksydacyjnego (Ciacka i inni 2022b). W związku z wynikami dotyczącymi egzogennej roli NO, sugeruje się, że starzenie nasion jest związane z obniżeniem zawartości tej cząsteczki w komórkach zarodków i w konsekwencji z zaburzeniem aktywacji mechanizmów naprawczych (Ciacka i inni 2020a). Wykazano, że podczas przyspieszonego starzenia nasion jabłoni dochodzi do wzrostu zawartości utlenionego RNA (Ciacka i inni 2022b). Fumigacja NO zarodków izolowanych z nasion po zabiegu ich starzenia sprzyjała obniżaniu zawartości 8-oxoG, co wskazuje na rolę tej cząsteczki w regulacji mechanizmów naprawczych lub detoksykujących (Ciacka i inni 2022b). Zatem, regulacyjna rola RNS w łagodzeniu skutków starzenia nasion wiąże się m.in. z modulacją zawartości ROS i utlenionych pochodnych podstawowych biocząsteczek (Ciacka i inni 2022a, b). Dane literaturowe dotyczące działania NO w postarzanych nasionach są nadal ograniczone i nie pozwalają jednoznacznie wyjaśnić mechanizmu tego zjawiska. Dla nasion kluczowe jest osiągnięcie stężenia NO odpowiadającego „drzwiom nitrozacyjnym" – w tym ujęciu NO można traktować jako cząsteczkę poprawiającą żywotność nasion poddanych starzeniu.

Starzenie nasion jest związane z zaburzeniem działania systemu antyoksydacyjnego, co skutkuje wzrostem stężenia reaktywnych cząsteczek. Szczególnie narażone na uszkodzenia oksydacyjne są mitochondria (Ratajczak i inni 2019; Kurek i inni 2019), stanowiące istotne miejsce powstawania ROS w trakcie życia nasion (Dan Dunn i inni 2015; Ratajczak i inni 2019; Małecka i inni 2021). Badania prowadzone

na mitochondriach izolowanych z nasion buka zwyczajnego (*Fagus sylvatica* L.) przechowywanych przez 17 lat wykazały wzrost zawartości H_2O_2 i MDA (Małecka i inni 2021). Autorki zaobserwowały także zmiany strukturalne mitochondriów.

W prawidłowo funkcjonującej komórce roślinnej, białka karbonylowane są degradowane przez proteasom 20S na drodze niezależnej od ATP (Smakowska i inni 2014; Oracz i Stawska 2016). Zaburzenia w usuwaniu tak zmodyfikowanych białek prowadzą do ich agregacji, co jest podstawą cytotoksyczności (Smakowska i inni 2014). Starzenie nasion powiązano ze wzrostem zawartości białek karbonylowanych, co wykazano dla buka zwyczajnego (Kalemba i Pukacka 2014), rzodkiewnika pospolitego (*Arabidopsis thaliana* (L.) Heynh.) (Rajjou i inni 2008; Morscher i inni 2015) i wiązu syberyjskiego (Li i inni 2017). Takiej zależności nie odnotowano w przypadku postarzanych nasion jabłoni, niezależnie od czasu trwania zabiegu (Ciacka i inni 2022b). Całkowita zawartość białek karbonylowanych i nitrowanych w osiach zarodków izolowanych z postarzanych nasion jabłoni przez 14 i 21 dni, sugeruje bardziej złożony mechanizm powstawania uszkodzeń podczas starzenia (Ciacka i inni 2022b). Zatem to szczegółowa analiza jakościowa białek karbonylowanych i nitrowanych stanowiła kolejny etap w poznaniu mechanizmów starzenia nasion jabłoni.

2. Cel pracy

Starzenie nasion (również typu *orthodox*) jest procesem postępującym w czasie, nawet jeśli są przechowywane w optymalnych warunkach. Stanowi to problem natury ekonomiczno-ekologicznej, dlatego badanie mechanizmów tego procesu i/lub rozwijanie technik pozwalających na precyzyjną ocenę żywotności nasion ma duże znaczenie. Uszkodzenia komórek związane ze starzeniem nasion są spowodowane m.in. niekontrolowanym wzrostem stężenia ROS (Bailly i inni 2008; Ciacka i inni 2020b). W nasionach typu *orthodox*, które podczas spoczynku pozostają w stanie głębokiej desykacji tkanek, procesy antyoksydacyjne opierają się głównie o związki drobnocząsteczkowe, takie jak GSH. Dostępność wody w środowisku i aktywacja metabolizmu sprawiają, że stosunek GSH/GSSG może być regulowany w wyniku aktywności GPx-like i GR, białek o modulatorowej roli w przemianach ROS.

Molekularne znaczniki postępującego starzenia nasion powinny być stabilne, zwłaszcza podczas ich izolacji z badanego materiału. Białka karbonylowane i nitrowane są bardziej trwałe niż związki rodnikowe, co w dużej mierze sprzyja ich prawidłowej, powtarzalnej i odtwarzalnej identyfikacji. Podobną stabilnością cechuje się *m*-Tyr, która może być wbudowywana w białka. W efekcie, ocena żywotności przechowywanych nasion, bazująca na tych znacznikach, jest bardziej precyzyjna i wskazuje na stopień uszkodzeń komórkowych.

Początkowo uważano, że za starzenie nasion odpowiada nadmiar NO w tkankach, jednakże na podstawie danych literaturowych sugeruje się, że to jego niewystarczające stężenie przyspiesza ten proces. Traktowanie zarodków izolowanych z postarzanych nasion jabłoni NO prowadziło do łagodzenia skutków starzenia, co zależało od czasu trwania tego zabiegu (Ciacka i inni 2022b). Niestety, dokładny mechanizm korzystnego działania NO w tym kontekście wciąż nie został w pełni poznany.

Celem pracy było wykazanie roli egzogennego NO jako cząsteczki poprawiającej żywotność zarodków izolowanych z postarzanych nasion jabłoni w odniesieniu do ilości, jakości i funkcjonalności białek związanych z zaburzeniem homeostazy RONS.

Cel pracy został osiągnięty poprzez uzyskanie odpowiedzi na poniższe **pytania badawcze**:

1. Jak zmienia się zawartość wybranych aminokwasów uznanych za markery stresu oksydacyjnego w osiach zarodkowych nasion jabłoni poddanych starzeniu, po krótkotrwałym traktowaniu izolowanych zarodków NO?
2. Jak zmienia się sprawność systemu antyoksydacyjnego, związanego z glutationem, w osiach zarodkowych nasion jabłoni poddanych starzeniu, po krótkotrwałym traktowaniu izolowanych zarodków NO?
3. Jakie białka ulegają stabilnym modyfikacjom (karbonylacja i nitracja) w osiach zarodkowych nasion jabłoni poddanych starzeniu, po krótkotrwałym traktowaniu izolowanych zarodków NO?
4. Czy krótkotrwałe traktowanie zarodków nasion jabłoni poddanych starzeniu NO wpływa na procesy związane z degradacją białek?

Oraz pytanie odnoszące się do wszystkich punktów powyżej:

W jaki sposób czas zabiegu przyspieszonego starzenia nasion wpływa na działanie NO, którym traktuje się zarodki bezpośrednio po pozbawieniu ich okryw nasiennych?

Zakres pracy

W pracy dokonano pomiaru zawartości *m*-Tyr (**Publikacja 2)** – metabolitu, którego wyższa zawartość może świadczyć o zaawansowaniu stresu oksydacyjnego (**Publikacja 1**). Kolejnym elementem było zbadanie wpływu NO na aktywność GR i GPx-like oraz poziom transkryptów kodujących te enzymy. Efekt aktywności badanych enzymów oceniono w kontekście analizy zmian stężenia GSH i GSSG. Ponadto, korzystając z równania Nernsta obliczono współczynnik $E_{GSSG/2GSH}$, który może być traktowany jako wyznacznik żywotności nasion (**Publikacja 3)**.

Następną fazę badań stanowiły analizy proteomiczne (**Publikacja 4**), w ramach których określono zmiany zawartości białek, aktywność proteolityczną oraz aktywność proteasomu 20S. Ocenę stopnia degradacji białek oparto także na immunodetekcji białek znakowanych ubikwityną. Ponadto, **przeanalizowano jakościowe (identyfikacja białek)** zmiany białek karbonylowanych i nitrowanych izolowanych z osi zarodków kontrolnych (postarzanych i nietraktowanych NO) i osi zarodków krótkotrwale traktowanych NO, w zależności od czasu trwania zabiegu postarzania nasion.

W celu poznania korzystnego efektu działania NO jako cząsteczki poprawiającej jakość postarzanych nasion typu *orthodox* weryfikacji poddano pięć postawionych hipotez badawczych.

Hipotezy badawcze:

Hipoteza 1.

Starzenie nasion jabłoni wiąże się ze wzrostem zawartości *m*-Tyr w osiach zarodkowych.

Hipoteza 2.

Krótkotrwałe traktowanie zarodków jabłoni izolowanych z postarzanych nasion NO ogranicza wzrost zawartości *m*-Tyr w osiach zarodkowych.

Hipoteza 3.

Krótkotrwałe traktowanie zarodków jabłoni izolowanych z postarzanych nasion NO reguluje przemiany glutationu i wpływa na utrzymanie równowagi GSH/GSSG w osiach zarodkowych.

Hipoteza 4.

Krótkotrwałe traktowanie zarodków jabłoni izolowanych z postarzanych nasion NO zmienia jakościowo białka karbonylowane i nitrowane izolowane z osi zarodkowych.

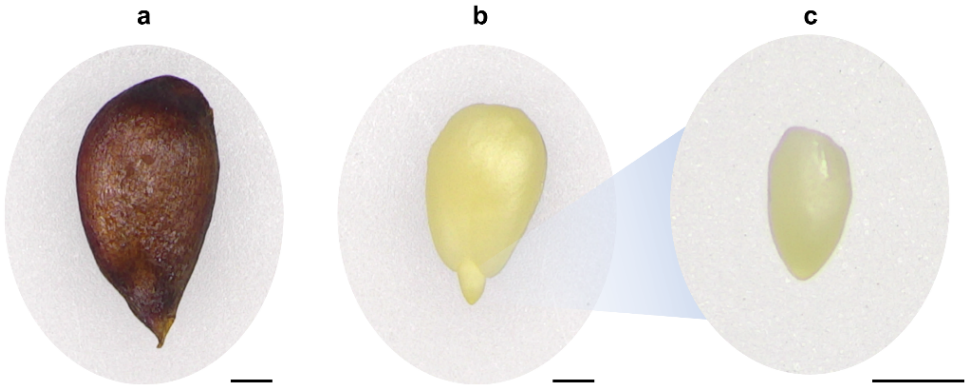
Hipoteza 5.

Krótkotrwałe traktowanie zarodków jabłoni izolowanych z postarzanych nasion NO reguluje degradację białek w osiach zarodkowych.

3. Opis materiału doświadczalnego i wykorzystane metody badawcze

3.1. Materiał doświadczalny

Do badań wykorzystano osie zarodkowe nasion jabłoni (*Malus domestica* Borkh.), odmiany Antonówka, pozyskane z sadów z okolic Grójca (Mazowieckie, Polska) w latach 2019-2023. Nasiona były izolowane z owoców i suszone na powietrzu w temperaturze pokojowej. Następnie nasiona przechowywano w 4°C do czasu przeprowadzania doświadczeń. Materiał doświadczalny przedstawiono na Rysunku 3.1.



Rysunek 3.1 Zdjęcia materiału doświadczalnego. Nasiono jabłoni (a), zarodek wyizolowany z nasiona (b), oś zarodkowa (c). Skala reprezentuje 1 mm (**Publikacja 3 Fig. 1**).

3.2. Procedura przyspieszonego starzenia nasion

Nasiona poddawano przyspieszonemu starzeniu zgodnie z opisem zamieszczonym w pracy Ciacka i inni (2022b). W tym celu, nasiona mieszano w szalce Petriego (ø 15 cm) ze sterylnym piaskiem o wilgotności 50%. Nasiona poddawano starzeniu przez 7, 14, 21 dni w temperaturze 33-35°C, mieszając je co trzy dni. Temperaturę dobierano eksperymentalnie dla każdego nowego materiału nasiennego (pozyskiwanego w danym roku). W miarę potrzeby dodawano wodę destylowaną, tak, by utrzymać stałą wilgotność (metoda wagowa). Po określonym czasie zabiegu nasiona wyjmowano z podłoża i po przepłukaniu w wodzie izolowano zarodki (mechanicznie usuwano okrywy nasienne). Zarodki izolowane z postarzonych nasion (kontrola) i zarodki krótkotrwale traktowane NO (Rozdział 3.3) wykładano na szalki Petriego na bibułę zwilżoną wodą destylowaną.

Kulturę zarodków prowadzono w komorze fitotronowej Sanyo (MLR-35OH) przez 48 h w warunkach: temperatura 25/20°C, wilgotność 60%, fotoperiod 12/12 h, natężenie światła 150 μmol PAR $m^{-1}s^{-1}$. Po 48 h prowadzenia kultury z zarodków izolowano osie

zarodkowe, które ważono i mrożono w ciekłym azocie. Do czasu przeprowadzenia doświadczeń osie przechowywano w -80°C.

3.3. Traktowanie zarodków jabłoni tlenkiem azotu (NO)

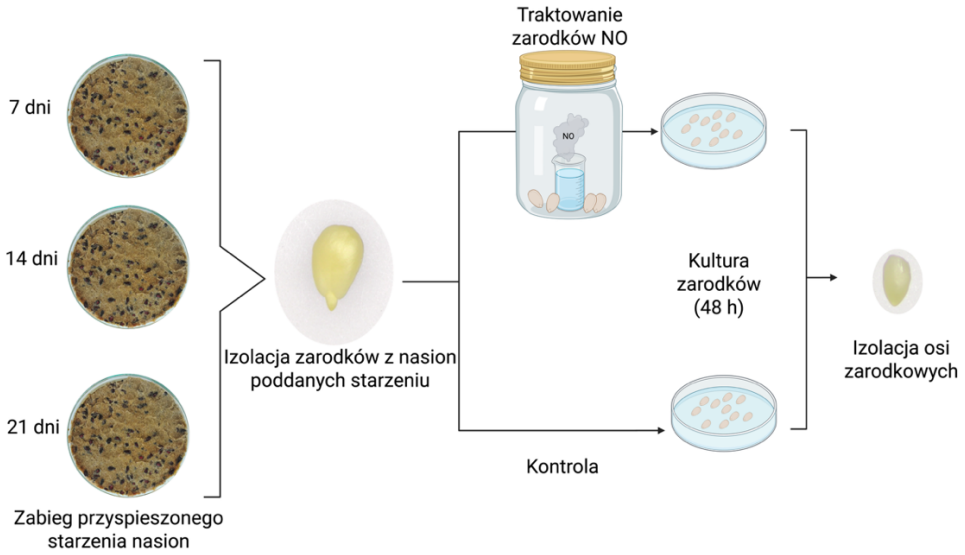
Izolowane z nasion poddanych starzeniu zarodki traktowano NO jak opisano w pracy Ciacka i inni (2022b). Izolowane zarodki umieszczano w szklanym naczyniu (500 ml) (Rysunek 3.2), wyłożonym bibułą filtracyjną, zwilżoną 0,05 M buforem K-fosforanowym pH 7,0. Do naczynia wprowadzano szklaną zlewkę z wodnym roztworem azotynu sodu o stężeniu 14,5 mM, który zakwaszano roztworem 0,2 M kwasu solnego. Uwalnianie NO (fumigacja zarodków) wiąże się z reakcją przedstawioną poniżej (Yamasaki 2000):

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

$$2HNO_2 \leftrightarrow N_2O_3 + H_2O$$

$$N_2O_3 \leftrightarrow \mathbf{NO}\uparrow + NO_2$$

Zarodki umieszczone w szklanym zamkniętym naczyniu, traktowano NO przez 3 h. Po tym czasie wyjmowano je, przepłukiwano wodą destylowaną i przenoszono na szalki Petriego na zwilżoną wodą bibułę filtracyjną i prowadzono kulturę w warunkach opisanych powyżej. Schemat modelu doświadczalnego przedstawiono na Rysunku 3.2.



Rysunek 3.2 Schemat przygotowania materiału doświadczalnego (model badawczy). Nasiona jabłoni poddawano procedurze przyspieszonego starzenia przez 7, 14 i 21 dni. Po tym czasie z nasion izolowano zarodki, które traktowano NO. Kulturę zarodków prowadzono w kontrolowanych warunkach przez 48 h. Zarodki kontrolne (kontrola) nie były traktowane NO. Ostatnim etapem przygotowania materiału doświadczalnego było izolowanie osi zarodkowych. Na podstawie Ciacka i inni (2022b) – zmodyfikowano. Rysunek przygotowany przy użyciu aplikacji Biorender.

3.4. Metody badawcze wykorzystane w publikacjach wchodzących w skład rozprawy doktorskiej

W celu zweryfikowania hipotez badawczych zastosowano metody badawcze, takie jak: techniki separacyjne z wykorzystaniem wysokosprawnej chromatografii cieczowej (HPLC), metody spektrofotometryczne, analiza RT-qPCR, metody spektrofluorescencyjne oraz elektroforetyczna separacja białek jednokierunkowa, w żelu poliakrylamidowym w warunkach denaturujących (SDS-PAGE) wraz z elektrotransferem (Western Blot) oraz dwukierunkowa (2D SDS-PAGE), poprzedzająca technikę spektometrii mas (MS) MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight).

W każdym roku, w którym pozyskiwano nasiona, przeprowadzano ich testy kiełkowania w celu oceny głębokości spoczynku, doboru warunków przyspieszonego starzenia (temperatura) oraz stężenia azotynu sodu (fumigacja NO). Kulturę zarodków pozyskanych z nasion poddawanych przyspieszonemu starzeniu (kontrola) jak i traktowanych NO prowadzono przez 7 dni w warunkach kontrolowanych, opisanych powyżej. Opis przeprowadzonych doświadczeń zawarto w **Publikacji 3 i 4.**

Metody chromatograficzne

Oznaczenie **zawartości aminokwasów (*m*-Tyr, Phe i Tyr)** przeprowadzano techniką HPLC z wykorzystaniem 6-aminochinolilo-N-hydroksysukcynimidylokarbaminianu (AQC) do tworzenia analizowanych pochodnych. Dokładny opis przeprowadzonego doświadczenia znajduje się w **Publikacji 2**.

Stężenie GSH i GSSG oznaczono techniką HPLC z wykorzystaniem monobromobimanu (MBBr). Dokładny opis doświadczenia znajduje się w **Publikacji 3.** Obliczono **potencjał redukcyjny glutationu [$E_{GSSG/2GSH}$].** Do wyznaczenia tego parametru wykorzystano równanie Nernsta. Dokładny opis znajduje się w **Publikacji 3.**

Metody spektrofotometryczne

Zawartość białka zmierzono korzystając z odczynnika Bradford, wykorzystując albuminę surowicy bydlęcej (BSA) do przygotowania krzywej wzorcowej (Bradford 1976). Pomiaru dokonano za pomocą czytnika ClarioStar Plus, a dokładny opis przeprowadzonych doświadczeń zawarto w **Publikacjach 3 i 4.**

Pomiar **aktywności GR i GPx-like** wykonano z wykorzystaniem czytnika ClarioStar Plus. Do pomiaru wykorzystano zależność utleniania NADPH do $NADP^+$. Dokładny opis doświadczeń znajduje się w **Publikacji 3.**

Aktywność proteolityczną zbadano, wykorzystując azokazeinę jako substrat w reakcji. Opis przeprowadzonego doświadczenia znajduje się w **Publikacji 4.**

Reakcja łańcuchowej polimerazy w czasie rzeczywistym (RT-qPCR)

Analizę poziomu transkryptów genów kodujących GR zlokalizowaną w cytoplazmie (*GRc*) i plastydach (*GRp*), a także genów kodujących GPx-like (*GPX2, GPX6, GPX7, GPX8*) przeprowadzono wykorzystując technikę RT-qPCR (Bio-Rad CFX Connect™ Real-Time PCR Detection System). Dokładny opis tej techniki został umieszczony w **Publikacji 3.**

Metody spektrofluorescencyjne

Aktywność proteasomu 20S oznaczono, korzystając z zestawu Proteasome 20S Activity Assay Kit (Sigma-Aldrich, MAK172), zawierającego sondę LLVY-R110, z wykorzystaniem czytnika Clario Star Plus. Szczegółowy opis przeprowadzonego doświadczenia zawarto w **Publikacji 4**.

Separacja białek i metody ich detekcji

Detekcję białek związanych z ubikwityną przeprowadzono techniką rozdziału białek SDS-PAGE (Laemmli 1970) oraz elektrotransferu na membranę nitrocelulozową techniką Western Blot (Mini Protean Bio-Rad) (Towbin i inni 1979). Następnie białka ubikwitynowane oznaczano przy użyciu specyficznych przeciwciał (Agrisera AS08307A). Szczegółowy opis doświadczenia znajduje się w **Publikacji 4.**

Istotnym elementem pracy było zidentyfikowanie białek nitrowanych i karbonylowanych. Białka do analizy izolowano zgodnie z procedurą opisaną w Wu i inni (2014). **Białka nitrowane** izolowano techniką immunoprecypitacji z przeciwciałami specyficznymi względem 3-NT (Agrisera AS10 706), a następnie dokonano rozdziału zgodnie z ich punktem izoelektrycznym (IEF) i masą z wykorzystaniem techniki 2D SDS-PAGE (Bio-Rad PROTEAN i12 IEF Cell). Po rozdziale białka wybarwiano barwnikiem Coomasie i wycinano plamki w celu identyfikacji z wykorzystaniem techniki MS. Szczegółowy opis doświadczenia znajduje się w **Publikacji 4.**

Detekcję białek karbonylowanych przeprowadzono poprzez rozdział ekstraktu białkowego techniką 2D SDS-PAGE. Grupy karbonylowe w białkach zostały wyznakowane za pomocą 2,4-dinitrofenylohydrazyny (DNPH). Następnie przeprowadzono elektrotransfer białek techniką Western Blot. Plamki na żelu po rozdziale 2D SDS-PAGE, odpowiadające wykrytym białkom karbonylowanym, były wycinane i poddawane identyfikacji techniką MS, jak opisano w **Publikacji 4.**

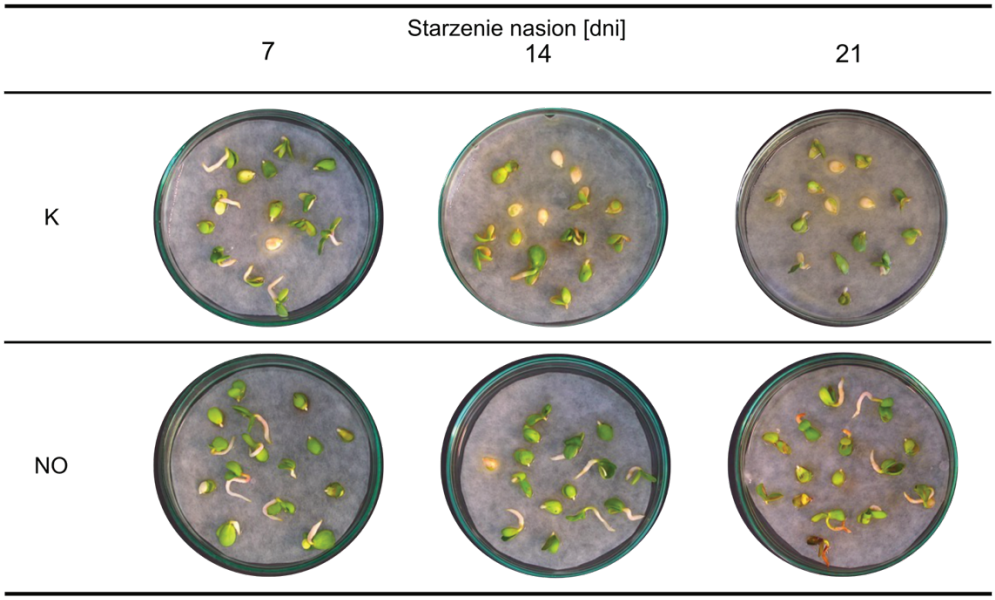
Identyfikacja białek techniką MS

Elementem poprzedzającym **identyfikację białek** było ich trawienie trypsyną. Uzyskane peptydy mieszano z matrycą (kwasem α-cyjano-4-hydroksycynamonowym – CHCA) i dokonywano ich jonizacji w spektrometrze mas MALDI-TOF-TOF (Shimadzu AXIMA PERFORMANCE). Białka identyfikowano metodą odcisku palca (*Peptide Mass Fingerprint* – PMF), wykorzystując oprogramowanie Mascot. Szczegółowy opis przeprowadzonego doświadczenia znajduje się w **Publikacji 4**.

4. Opis najważniejszych wyników z elementami dyskusji

4.1. Łagodzenie efektów starzenia nasion jabłoni przez NO wiąże się z regulacją zawartości *m*-Tyr, Tyr i Phe

Starzenie nasion, również typu *orthodox*, stanowi złożony proces, którego efekty postępują w czasie. Badania opisane w niniejszej pracy zostały przeprowadzone z wykorzystaniem protokołu przyspieszonego starzenia nasion jabłoni w warunkach podwyższonej wilgotności i temperatury (Ciacka i inni 2022b). Autorzy wykazali, że starzenie nasion do 21 dni powoduje przyspieszenie utraty żywotności wyizolowanych zarodków oraz sprzyja występowaniu licznych anomalii rozwojowych u siewek. Jednocześnie opisano, że traktowanie izolowanych zarodków NO poprawia ich zdolność kiełkowania. Jednakże przedłużenie procedury starzenia nasion do 40 dni skutkowało zamieraniem zarodków w ponad 90%. W tym przypadku, traktowanie NO takich zarodków nie tylko nie wywoływało efektu łagodzenia negatywnych skutków starzenia, ale wręcz pogłębiało zmiany degeneracyjne (Ciacka i inni 2022b). Przeprowadzone testy kiełkowania potwierdziły skuteczność i powtarzalność opracowanego protokołu (Rysunek 4.1) (**Publikacja 3, Fig. 2; Publikacja 4, suplement**). Jednocześnie wykazano, że efekty starzenia nasion, przy zachowaniu warunków tej procedury, nie zachodzą liniowo, zwłaszcza na poziomie komórkowym. Pierwsze efekty starzenia nasion jabłoni widać już po 7 dniach procedury (Rysunek 4.1) (**Publikacja 3, Fig. 2)**. Z drugiej strony, testy kiełkowania potwierdziły, że na wczesnych etapach starzenia, część zarodków wykazuje symptomy ustępowania spoczynku. Potwierdziły to obserwacje braku charakterystycznych anomalii rozwojowych u niektórych rozwijających się siewek. To oznacza, że 7-dniowe starzenie nasion nie przełamuje w pełni spoczynku izolowanych zarodków. Indukcja kiełkowania (pobudzenie metabolizmu i uruchamianie mechanizmów naprawczych) została osiągnięta po krótkotrwałym traktowaniu zarodków jabłoni izolowanych z postarzanych nasion NO (Rysunek 4.1) (**Publikacja 3, Fig. 2).**



Rysunek 4.1 Reprezentatywne zdjęcia siewek rozwijających się z zarodków jabłoni poddanych procedurze przyspieszonego starzenia przez 7, 14 i 21 dni (kontrola – K) i traktowanych NO (NO). Test kiełkowania (kulturę zarodków) prowadzono przez 7 dni, w warunkach opisanych powyżej. **Publikacja 3, Fig.2 – zmodyfikowano.**

Nieprawidłowo rozwinięte siewki jabłoni obserwowano już wcześniej, przy porównaniu wpływu zabiegu stratyfikacji nasion w chłodzie i stratyfikacji w cieple (Dębska i inni 2013). W przeciwieństwie do stratyfikacji nasion w 4°C, warunki podwyższonej wilgotności i temperatury nie prowadzą do ustąpienia spoczynku. Potwierdza to znaczenie temperatury w fizjologii nasion jabłoni (Lewak 2011). W niskiej, dodatniej temperaturze aktywowane są enzymy, które hydrolizują koniugaty cyjankowe (glikozydy cyjanogenne obecne w zarodkach jabłoni) i uwalniają cyjanowodór (HCN). Liczne dane literaturowe wskazują na istotny udział HCN w przełamywaniu głębokiego spoczynku nasion, w tym nasion i/lub zarodków jabłoni (Lewak 2011; Krasuska i inni 2023). Po krótkotrwałym traktowaniu spoczynkowych zarodków izolowanych z niestarzonych nasion jabłoni HCN, obserwowano wzrost emisji NO z osi zarodkowych (Krasuska i Gniazdowska 2012).

Wyniki uzyskane przez Dębską i innych (2013) wykazały wzrost stężenia H_2O_2 w zarodkach pochodzących z nasion poddanych stratyfikacji w cieple powyżej 7 dni – warunków zbliżonych do tych opisanych w protokole starzenia nasion jabłoni (Ciacka i inni 2022b). Dane opublikowane przez Dębską i innych (2013) stanowiły początek dalszych badań dotyczących starzenia nasion jabłoni realizowanych w Katedrze

Fizjologii Roślin (obecnie Katedra Botaniki i Fizjologii Roślin) Instytutu Biologii Szkoły Głównej Gospodarstwa Wiejskiego w Warszawie.

Doświadczenia realizowane w ramach niniejszej rozprawy prowadzono na osiach zarodków izolowanych z nasion postarzanych przez 7, 14 i 21 dni, po 48 h kultury. Po tym czasie kultury zaobserwowano pierwsze korzystne efekty działania NO na poziomie komórkowym w osiach zarodków izolowanych z postarzanych nasion. Starzenie nasion przez 21 dni wiązało się ze wzrostem stężenia *m*-Tyr w osiach zarodkowych (**Publikacja 2, Fig. 1**), co odpowiadało zmianom oksydacyjnym obserwowanym na tym etapie doświadczenia (Ciacka i inni 2022b). Wzrost ten był o 50% wyższy niż odnotowany dla osi zarodkowych nasion poddanych starzeniu przez 7 i 14 dni. Jak wspomniano, wprowadzanie tego antymetabolitu do sekwencji białkowej może prowadzić do zaburzenia funkcji biologicznej białek (**Publikacja 1**), co jest znanym mechanizmem toksyczności. Nie tylko zmiany zawartości samej *m*-Tyr wskazują na stymulację procesów patofizjologicznych. Wraz z postępującym stresem oksydacyjnym rośnie też stosunek *m*-Tyr do Tyr w osiach zarodków postarzanych nasion jabłoni (kontrola; **Publikacja 2, Fig. 1).** Ta zmiana, choć nieróżniąca się istotnie, może zaburzać przebieg szlaków biochemicznych opartych o te aminokwasy. Toksyczność *m*-Tyr potwierdzono zarówno dla zwierząt jak i dla roślin (Ipson i Fisher 2016 i cytowania tam zawarte, **Publikacja 1**), chociaż do tej pory nie była łączona ze starzeniem nasion. Potencjalnym źródłem *m*-Tyr może być proteoliza białek zawierających ten aminokwas. Jednak nadal nie został opisany dalszy los tego antymetabolitu, w szczególności, czy podlega detoksykacji, czy może być ponownie włączany do białek syntetyzowanych *de novo* (Samardzic i Rodgers 2017 i cytowania tam zawarte). Jednym z mechanizmów przeciwdziałania cytotoksyczności *m*-Tyr jest zwiększenie puli Phe dostępnej w komórce. Obniża to prawdopodobieństwo wbudowania *m*-Tyr do łańcucha białkowego (**Publikacja 1**). Doświadczenia przeprowadzone na siewkach pomidora (*Solanum lycopersicum* L.) (Andrzejczak i inni 2018) i rzodkiewnika pospolitego (Zer i inni 2020) wykazały, że egzogenna Phe znosiła efekt działania *m*-Tyr obecnej w podłożu. Krótkotrwałe traktowanie NO zarodków jabłoni izolowanych z postarzanych nasion przez 21 dni sprzyjało zwiększaniu stężenia Phe i korzystnym zmianom stosunku *m*-Tyr/Tyr w osiach (**Publikacja 2, Fig. 1).** Zatem modulowanie puli wolnych aminokwasów może stanowić jeden z mechanizmów działania NO, który jako cząsteczka poprawiająca jakość nasion łagodzi negatywne skutki postępującego starzenia.

4.2. NO modyfikuje zawartość glutationu w osiach zarodków jabłoni izolowanych z nasion poddanych starzeniu

Glutation, głównie GSH, jest znanym drobnocząsteczkowym antyoksydantem. Zmiany stosunku GSH/GSSG świadczą o zaawansowaniu stresu oksydacyjnego. Ze względu na swoją budowę chemiczną GSH wchodzi w reakcje z ROS i RNS. Wykazano, że starzenie nasion pomidora wiązało się z obniżeniem zawartości GSH i wzrostem stężenia GSSG (De Vos i inni 1994). Podobną zależność zaobserwowano także dla nasion grochu zwyczajnego (*Pisum sativum* L.) (Kranner i inni 2006), słonecznika (*Helianthus annuus* L.) (Morscher i inni 2015) i rzepaku (*Brassica napus* L.) (Naghisharifi i inni 2024). Starzenie nasion jabłoni nie wpłynęło istotnie na zmiany zawartości GSH ani GSSG w osiach zarodkowych (**Publikacja 3, Fig. 4)**. Taki wynik, choć odmienny od przytoczonych wcześniej, nie jest niespodziewany. Jak wykazano, naturalne starzenie nie zawsze prowadzi do obniżania całkowitej puli glutationu (GSH + GSSG). Zaobserwowano, że GSH + GSSG jest względnie stała m.in. w nasionach marchwi zwyczajnej (*Daucus carota* L.) i ogórka siewnego (*Cucumis sativus* L.), nawet przy ich przechowywaniu powyżej 10 lat (Stegner i inni 2022). Wskazuje to, że zmiany w metabolizmie tego tripeptydu podczas starzenia zależą od gatunku, stopnia zaawansowania starzenia oraz fizjologicznego stanu nasion. Aktywność biologiczna NO jako cząsteczki łagodzącej negatywne skutki starzenia jest związana z modulowaniem zawartości ROS (Mao i inni 2018; He i inni 2018; Ciacka i inni 2022b). Jednocześnie wpływ NO na zawartość glutationu w osiach zarodkowych nasion jabłoni poddanych starzeniu był wielowymiarowy i zależał od czasu trwania tego zabiegu (**Publikacja 3, Fig. 7)**. Istotny statystycznie wzrost stężenia GSH, po traktowaniu NO, stwierdzono w osiach wyizolowanych z zarodków poddanych starzeniu przez 7 i 21 dni. Analogiczny efekt zaobserwowano dla GSSG. Z kolei, traktowanie NO zarodków pozyskanych z nasion poddanych starzeniu przez 14 dni nie prowadziło do podobnych zmian. Jest to potwierdzeniem, że negatywne efekty starzenia zależą od fazy (etapu) tego procesu (czasu trwania zaburzeń/nagromadzania metabolitów i ewentualnego działania mechanizmów naprawczych) i powinny być analizowane oddzielnie **(Publikacja 3)**. Każdy punkt starzenia (dzień 7, dzień 14 i dzień 21) to odrębna faza postępujących zmian (Ciacka i inni 2022b). W zarodkach jabłoni niepoddawanych starzeniu, wzrost zawartości GSH łączono z ustępowaniem spoczynku i inicjacją kiełkowania (Gniazdowska i inni 2010b; Ciacka i inni 2022c). NO-zależny

efekt łagodzenia zaburzeń metabolicznych wywołanych starzeniem może zatem wynikać z aktywacji procesów podobnych do tych, które zachodzą podczas inicjacji kiełkowania.

Wzrost zawartości ROS podczas starzenia nasion oraz wiążące się z tym uszkodzenia komórkowe odzwierciedlają obniżającą się aktywność enzymatycznego systemu antyoksydacyjnego, w tym GR i GPx-like. Po 14 dniach starzenia nasion jabłoni zaobserwowano istotne obniżenie aktywności GR w osiach zarodkowych w porównaniu z aktywnością mierzoną dla osi zarodków nasion starzonych przez 7 dni (**Publikacja 3, Fig. 5)**. Traktowanie zarodków izolowanych z nasion postarzanych przez 7 i 14 dni NO stymulowało aktywność GR w osiach zarodkowych. Podobny wynik zaobserwowano u postarzanych ziarniaków owsa zwyczajnego, które traktowano SNP (Mao i inni 2018). Z kolei, wzrostu aktywności GR w osiach zarodków jabłoni izolowanych z nasion postarzanych przez 21 i traktowanych NO już nie odnotowano, nawet przy jednoczesnym, istotnie wyższym poziomie transkryptów kodujących ten enzym (**Publikacja 3, Fig. 5-6).** Na tym etapie starzenia obserwowane są zaawansowane zmiany degeneracyjne, a traktowanie NO, mimo, że zwiększało zdolność kiełkowania zarodków jabłoni w porównaniu z kontrolą, nie zawsze uruchamiało wszystkie mechanizmy naprawcze (Ciacka i inni 2022b). Podstawową rolą GPx-like jest detoksykacja wodoronadtlenków lipidowych i H_2O_2, przy udziale GSH (Ali i inni 2025). Wzrost aktywności tego enzymu w osiach zarodkowych izolowanych z postarzonych nasion jabłoni po traktowaniu NO, wraz ze zwiększeniem poziomu transkryptów kodujących to białko, może świadczyć o ochronnej roli NO w komórce (**Publikacja 3)**. Jednakże by przeciwdziałać wzrostowi stężenia GSSG, powinno to korelować ze wzrostem aktywności GR.

4.3. NO prowadzi do zmian jakościowych białek karbonylowanych i nitrowanych w osiach zarodkowych nasion poddanych przyspieszonemu starzeniu

Wzrost zawartości ROS w starzejących się nasionach jest zwykle łączony ze zwiększeniem ilości białek karbonylowanych, chociaż takiej zależności nie potwierdzono w przypadku nasion jabłoni poddanych przyspieszonemu starzeniu (Ciacka i inni 2022b). Uważa się, że karbonylacja białek nie zawsze zachodzi w sposób przypadkowy. Wyniki badań prowadzonych na nasionach rzodkiewnika wskazały, że kluczowe dla prawidłowego przebiegu kiełkowania lub starzenia mogą być zmiany w profilu (zmiany jakościowe) tak zmodyfikowanych białek (Job i inni 2005). Autorzy wykazali, że karbonylacji ulegały głównie białka zapasowe należące do globulin.

W przypadku osi zarodkowych jabłoni izolowanych z postarzanych nasion również zasugerowano, że za brakiem widocznych zmian ilościowych białek karbonylowanych może stać przewaga białek zapasowych (Ciacka i inni 2022b). Te przypuszczenia zostały potwierdzone poprzez identyfikację białek karbonylowanych techniką MS (**Publikacja 4)**. Już na wczesnym etapie starzenia nasion (7 dni) w osiach zarodkowych karbonylacji ulega prunina 1 – białko należące do klasy globulin 11S (**Publikacja 4, Table 2)**. Podobny wynik uzyskano dla naturalnie postarzonych nasion buka zwyczajnego (Kalemba i Pukacka 2014). Poza globulinami, wykazano, że traktowanie NO zarodków izolowanych z nasion jabłoni poddanych starzeniu prowadzi do karbonylacji HSP (klasy II-like). Karbonylacji tego białka nie potwierdzono dla osi zarodków kontrolnych (nietraktowanych NO). Te białka były już wcześniej łączone ze starzeniem nasion i opisane w tym kontekście również dla nasion buka (Kalemba i Pukacka 2008) oraz ziarniaków ryżu (*Oryza sativa* L.) (Kaur i inni 2015). Traktowanie zarodków jabłoni, izolowanych z nasion poddanych wcześniej zabiegowi starzenia przez 7 dni NO prowadziło do karbonylacji białek zawierających biotynę SBP65 – należących do rodziny białek LEA. Białka SBP są typowe dla nasion i ulegają degradacji podczas kiełkowania – uwalniając biotynę, która jest kofaktorem niezbędnym w wielu przemianach metabolicznych (Duval i inni 1997; Alban i inni 2000). Karbonylację tych białek potwierdzono również dla osi izolowanych z zarodków nasion starzonych przez 14 i 21 dni, oraz traktowanych NO (**Publikacja 4, Table 2)**. Ponadto zidentyfikowano kolejne białka z rodziny LEA, takie jak białko em H5, białko em-like GEA1 oraz LEA 2-like, które ulegają karbonylacji. Degradacja tych białek może mieć różne konsekwencje w kontekście starzenia nasion (np. zaburzać ich działanie ochronne i naprawcze). Jednocześnie, jak wspomniano, efektem działania NO jest częściowa aktywacja metabolizmu spoczynkowych zarodków (nawet podczas ich starzenia). Zatem przyspieszona degradacja wspomnianych wyżej białek stanowi element mechanizmu działania NO w łagodzeniu negatywnych skutków starzenia. Tego typu rozważania wymagają dalszych analiz, chociaż degradacja białek LEA podczas kiełkowania nasion została dobrze udokumentowana w literaturze (Kalemba i Pukacka 2008, 2014; Rajjou i inni 2008).

Podobnie jak karbonylacja, nitracja jest PTM znakującą białka do degradacji. W związku z tym, zaskakującym było zidentyfikowanie pruniny 1 jako białka, które podlegało nitracji i karbonylacji (**Publikacja 4**). Nie jest jasne, dlaczego dane białko jest nieodwracalnie znakowane przez obie tego typu modyfikacje. Kwestią wymagającą

dalszych badań pozostaje potencjalna rola zmodyfikowanych peptydów powstających w wyniku degradacji takiego białka. Możliwym wydaje się udział zmodyfikowanych produktów hydrolizy białek w przekazywaniu sygnałów, jak sugeruje się w przypadku peptydów karbonylowanych, chociaż nie zostało to jeszcze zweryfikowane eksperymentalnie (**Publikacja 4, Fig. 8)** (Møller i Sweetlove 2010). Wykazano, że starzenie nasion jabłoni wiąże się z obecnością nitrowanego białka serpiny-ZX-like – będącego inhibitorem proteaz serynowych i cysteinowych, oraz białek HSP klasy II i białek szlaków wytwarzania energii m.in. dehydrogenazy mleczanowej, enolazy czy podjednostki ß syntazy ATP (**Publikacja 4, Table 3).** Traktowanie zarodków NO (po 14 dniach starzenia) prowadziło do zwiększania względnej zawartości białka zidentyfikowanego jako podjednostka ß syntazy ATP, podlegającego nitracji, co może świadczyć o zaburzeniach powstawania tej cząsteczki. Zatem prawdopodobne jest ograniczenie degradacji białek zależne od ATP, promując ich hydrolizę przez proteasom 20S.

4.4. NO moduluje degradację białek w osiach zarodkowych nasion jabłoni poddanych starzeniu

Hydroliza białek, jako proces kontroli jakości, zachodzi stale w prawidłowo funkcjonującej komórce. Intensywnym przemianom katabolicznym podlegają białka podczas kiełkowania nasion (Tan-Wilson i Wilson 2012). W nasionach gromadzone są białka, które pełnią rolę zapasową, będąc źródłem azotu dla syntezowanych *de novo* produktów translacji (Jain 2023). Starzenie nasion wiąże się z uszkodzeniami wewnątrzkomórkowymi i degradacją białek, co zostało wykazane między innymi dla zarodków jabłoni (Dawidowicz-Grzegorzewska 1989). Analizując strukturę komórkową zarodków poddanych działaniu podwyższonej temperatury i znajdujących się w środowisku uwodnionym (warunki odpowiadające przyspieszonemu starzeniu), autorka wykazała zmiany morfologiczne mitochondriów oraz częściowy zanik wakuoli magazynujących białka, przy jednoczesnym utrzymaniu stanu spoczynku (Dawidowicz-Grzegorzewska 1989). Degradacja białek w uwodnionych zarodkach (na przykład izolowanych z nasion poddanych starzeniu, bądź nasion kiełkujących) przebiega przy udziale peptydaz, aktywnych w różnym pH środowiska (Tan-Wilson i Wilson 2012). W osiach zarodkowych nasion poddanych przyspieszonemu starzeniu aktywność proteolityczna w kwaśnym pH pozostawała stała (**Publikacja 4, Fig. 1).** Starzenie wiązało się natomiast ze wzrostem aktywności proteolitycznej w pH 8,8. Pomimo,

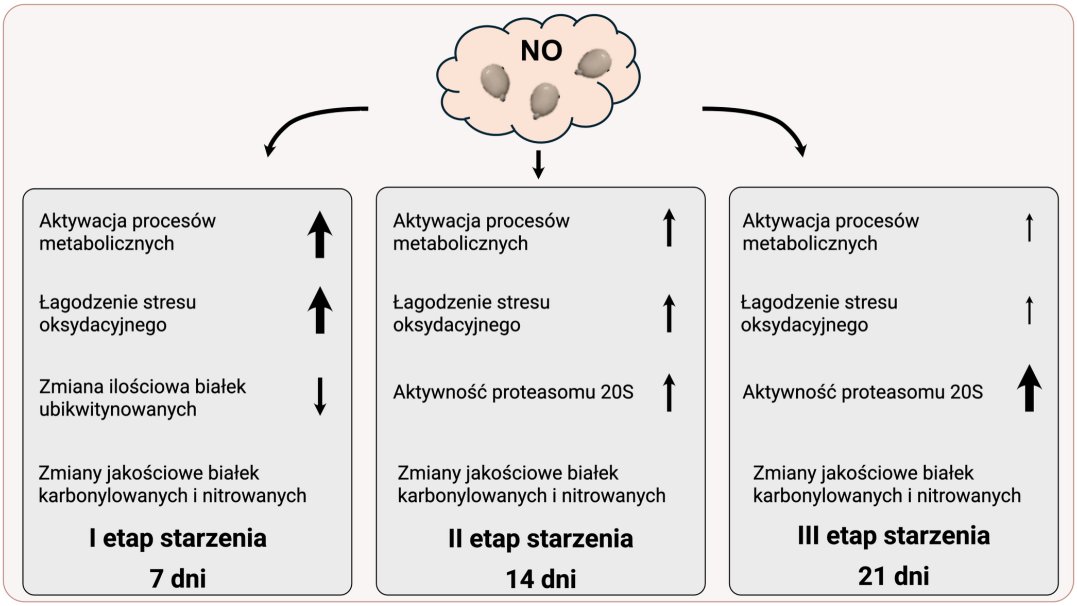
że aktywność proteolityczna w osiach zarodków kontrolnych i traktowanych NO izolowanych z postarzonych nasion była zbliżona, mogła dotyczyć odmiennych procesów fizjologicznych (**Publikacja 4)**. Częściowe przełamanie spoczynku wynikające z działania NO najprawdopodobniej było efektem proteolizy białek zapasowych, a produkty tej reakcji pokrywały zapotrzebowanie na azot kiełkujących zarodków. Jednocześnie w przypadku zarodków kontrolnych, których spoczynek nie został zniesiony w obecności NO, wzrost aktywności proteolitycznej, dotyczącej również białek zapasowych, prawdopodobnie prowadził do utraty cennego materiału zapasowego. Hydroliza materiałów zapasowych ma znaczenie fizjologiczne wtedy, gdy zostają odblokowane procesy rozwojowe, a uwalniane związki mogą zostać wykorzystane na potrzeby wzrostu siewki.

Degradacja błędnie sfałdowanych bądź w inny sposób zmienionych białek zazwyczaj zachodzi przy udziale proteasomu (Raynes i inni 2016). W prawidłowo funkcjonującej komórce roślinnej proces ten zachodzi z udziałem proteasomu 26S, zależnego od ATP. Do hydrolizy tą drogą metaboliczną, kierowane są białka znakowane ubikwityną (Sadanandom i inni 2012; Oracz i Stawska 2016). Ubikwitynacja białek stanowi sposób regulacji głębokości spoczynku nasion. Modyfikacja ta dotyczy m.in. białek zaangażowanych w regulację ścieżki transdukcji ABA i GA (Morris i inni 2011; Chiu i inni 2016). Traktowanie zarodków nasion jabłoni postarzonych przez 7 dni (pierwszy etap starzenia) NO prowadziło do istotnego obniżania zawartości białek związanych z ubikwityną (**Publikacja 4, Fig. 6)**. Takiej zmiany nie wykazano dla osi nasion starzonych przez 14 i 21 dni (postępujące starzenie) i traktowanych NO. Sposób oddziaływania tego gazu po 7 dniach starzenia nasion (pierwszy etap starzenia) na białka ubikwitynowane może dotyczyć m.in. stymulacji degradacji inhibitorów kiełkowania. Brak zmiany profilu białek ubikwitynowanych w osiach zarodków poddanych starzeniu przez 14 i 21 dni oraz traktowanych NO może wiązać się z nagromadzaniem uszkodzonych białek, których kierowanie do hydrolizy stanowiłoby mechanizm naprawczy (utrzymanie proteostazy). Zatem udział NO w łagodzeniu negatywnych skutków starzenia ma charakter wielokierunkowy, a molekularne cele działania tego gazu zależą od stopnia zaawansowania procesu starzenia. Wyniki badań dotyczących degradacji białek karbonylowanych i nitrowanych, wskazują, że może ona zachodzić na drodze niezależnej od ubikwityny i ATP, poprzez proteasom 20S (Oracz i Stawska 2016; Kumar Deshmukh i inni 2019; Pepelnjak i inni 2024). Jednocześnie, wykazano, że warunki stresu oksydacyjnego sprzyjały zwiększeniu aktywności proteasomu 20S

(Wang i inni 2010), chociaż dokładny mechanizm reakcji wciąż nie został w pełni wyjaśniony (Kumar Deshmukh i inni 2019). W starzejących się nasionach jabłoni, szczególnie po 21 dniach, wykazano obniżenie aktywności proteasomu 20S (**Publikacja 4, Fig. 7)**. W wyniku tego mogą powstawać toksyczne agregaty białek karbonylowanych, co sprzyja uszkodzeniom komórkowym. Jednocześnie traktowanie zarodków poddanych starzeniu NO prowadziło do znaczącej poprawy aktywności proteasomu 20S (**Publikacja 4, Fig. 7)**. Zatem NO pełni istotną rolę w łagodzeniu niekorzystnych skutków starzenia nasion, m.in. regulując proces degradacji białek na drodze niezależnej od ATP.

5. Podsumowanie

Starzenie nasion jabłoni w warunkach podwyższonej temperatury i wilgotności przebiega etapowo. Wyniki uzyskane w ramach niniejszej pracy potwierdziły, że każdy etap postarzania nasion charakteryzują odrębne zmiany na poziomie biochemiczno-molekularnym w osiach izolowanych zarodków. Cząsteczką poprawiającą żywotność zarodków izolowanych z nasion poddanych starzeniu jest NO, a efekty jego działania zależą od czasu trwania tego zabiegu (Ciacka i inni 2022b) (Rysunek 5.1). Krótkotrwałe starzenie nasion (I etap starzenia – 7 dni zabiegu) warunkuje uwodnienie tkanek (imbibicję), które przebiegając w warunkach podwyższonej temperatury może inicjować zmiany metaboliczne związane z ustępowaniem spoczynku. Czas trwania tego zabiegu nie prowadzi jednak do powstawania zaawansowanych zmian degeneracyjnych. Traktowanie zarodków jabłoni izolowanych z nasion poddanych starzeniu przez 7 dni NO może skutkować wzrostem stężenia RNS, zbliżonym do optymalnego, określanego jako "drzwi nitrozacyjne". W wyniku tego dochodzi do aktywacji procesów metabolicznych, w tym stymulacji systemu antyoksydacyjnego (wzrostu aktywności GR, GPx-like, wzrostu zawartości glutationu) (**Publikacja 3**), podtrzymania korzystnego stosunku *m*-Tyr/Tyr (**Publikacja 2**) oraz zmian zawartości białek ubikwitynowanych (**Publikacja 4**). Na II i III etapie starzenia (14 i 21 dni zabiegu), mechanizm łagodzenia efektów starzenia przez NO przejawia się wzmożoną aktywacją proteasomu 20S, sprzyjając regulacji zawartości białek karbonylowanych oraz prawdopodobnie nitrowanych **(Publikacja 4)** (Rysunek 5.1). Tym samym, ochronne działanie NO może polegać na przywracaniu proteostazy, kierując do degradacji białka zapasowe (np. prunina), białka, których działanie jest już zbędne w osiach kiełkujących zarodków, lub takie, które utraciły swoją strukturę/funkcję. Istotnym i wciąż otwartym problemem badawczym pozostaje określenie dalszego losu nieodwracalnie zmodyfikowanych fragmentów białek (powstałych podczas proteolizy peptydów) oraz wyjaśnienie ich potencjalnej roli i sposobu wykorzystania w komórkach roślinnych. Prawdopodobnie, mogą one pełnić funkcje sygnałowe, co zostało zasugerowane w przypadku peptydów zawierających grupę karbonylową. Jednakże odpowiedź na to pytanie wymaga dalszych badań.



Rysunek 5.1 Uproszczony schemat przedstawiający udział NO w łagodzeniu negatywnych skutków starzenia nasion na przykładzie zarodków jabłoni. Działanie NO zależy od czasu trwania zabiegu przyspieszonego starzenia i zachodzi kierunkowo.

6. Weryfikacja hipotez

Hipoteza 1.

Starzenie nasion wiąże się ze wzrostem zawartości *m*-Tyr w osiach zarodkowych jabłoni.

Hipoteza zweryfikowana pozytywnie

Hipoteza 2.

Krótkotrwałe traktowanie zarodków jabłoni izolowanych z postarzanych nasion NO ogranicza wzrost zawartości *m*-Tyr w osiach zarodkowych.

Hipoteza zweryfikowana negatywnie

Hipoteza 3.

Krótkotrwałe traktowanie zarodków jabłoni izolowanych z postarzanych nasion NO reguluje przemiany glutationu i wpływa na utrzymanie równowagi GSH/GSSG w osiach zarodkowych.

Hipoteza zweryfikowana pozytywnie

Hipoteza 4.

Krótkotrwałe traktowanie zarodków jabłoni izolowanych z postarzanych nasion NO zmienia jakościowo białka karbonylowane i nitrowane izolowane z osi zarodkowych.

Hipoteza zweryfikowana pozytywnie

Hipoteza 5.

Krótkotrwałe traktowanie zarodków jabłoni izolowanych z postarzanych nasion NO reguluje degradację białek w osiach zarodkowych.

Hipoteza zweryfikowana pozytywnie

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Review

Toxicity of *meta*-Tyrosine

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Abstract: L-Tyrosine (Tyr) is one of the twenty proteinogenic amino acids and also acts as a precursor for secondary metabolites. Tyr is prone to modifications, especially under conditions of cellular redox imbalance. The oxidation of Tyr precursor phenylalanine leads to the formation of Tyr non-proteinogenic isomers, including *meta*-Tyr (*m*-Tyr), a marker of oxidative stress. The aim of this review is to summarize the current knowledge on *m*-Tyr toxicity. The direct *m*-Tyr mode of action is linked to its incorporation into proteins, resulting in their improper conformation. Furthermore, *m*-Tyr produced by some plants as an allelochemical impacts the growth and development of neighboring organisms. In plants, the direct harmful effect of *m*-Tyr is due to its modification of the proteins structure, whereas its indirect action is linked to the disruption of reactive oxygen and nitrogen species metabolism. In humans, the elevated concentration of *m*-Tyr is characteristic of various diseases and ageing. Indeed, *m*-Tyr is believed to play an important role in cancer physiology. Thus, since, in animal cells, *m*-Tyr is formed directly in response to oxidative stress, whereas, in plants, *m*-Tyr is also synthesized enzymatically and serves as a chemical weapon in plant–plant competition, the general concept of *m*-Tyr role in living organisms should be specified.

Keywords: allelochemical; non-proteinogenic amino acids; reactive oxygen species; cancer; fescue

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1. Introduction

Twenty canonical amino acids (AAs) are the base of the proteins structure in living organisms. Besides them, numerous non-proteinogenic amino acids (NPAAs) are produced in plants [1]. Some of the NPAAs are described as antimetabolites, analogs of proteinogenic AAs, and serve as toxins. The negative effect of a specific NPAA may be removed by the application of its proteinogenic analog.

Tyrosine (Tyr-α-amino-β-(*p*-hydroxyphenyl)propionic acid) belongs to the group of aromatic amino acids (AAAs), and occurs as two optical isomers (enantiomers) L-Tyr and D-Tyr [2]. Most living organisms utilize L-Tyr to form proteins/peptides. The content of this AA in Arabidopsis (*Arabidopsis thaliana* (L.) Henh.) leaves is about 5 pmol mg^{-1} fresh weight (FW) ([3] and citations herein). Tyr is a Phe derivative, with a hydroxyl group located in the *para*- position on the benzyl ring (*p*-Tyr) [4]. In proteins, Tyr residues are often located on the surface and are prone to various post-translational modifications, such as phosphorylation or nitration [5]. In plants, AAAs are precursors for many secondary metabolites belonging to, e.g., groups of flavonoids, tannins or lignins [6], and alkaloids [6,7]. Although the main precursor for lignins biosynthesis is Phe, recent investigations on stiff brome (*Brachypodium distachyon* (L.) P.Beauv.) revealed that the synthesis of these compounds directly from Tyr is also possible [8,9]. As Tyr is implicated in the plastoquinones biosynthesis, its deficiency may disturb the electron transport chain in photosystems [4,10]. In plants, the increased concentration of Tyr is observed during the reaction against biotic stresses. The Tyr content in shimbillo (*Inga umbellifera* (Vahl) Steud. ex DC) expanding leaves is about 6.6 to 10% of the dry weight (DW), although, in mature leaves, it decreases to only 0.05% of the DW [11]. Herbivore attacks on this tropical

legume tree increased the Tyr concentration in the leaves [12]. In response to herbivores, Coley et al. [13] correlated the Tyr accumulation in the young leaves of genus *Inga* to the overexpression of the genes encoding the enzymes of Tyr biosynthesis.

In sorgo (*Sorghum bicolor* L. Moench.), Tyr is a precursor in the synthesis of dhurrin, a cyanogenic glucoside, which has a defensive role against herbivores [14]. The toxicity of Tyr (mixture of L-Tyr and D-Tyr isomers) at elevated concentrations was confirmed for insects [11]. The adddition of the Tyr racemate (at the level of 4%) into the diet of the tobacco budworm (*Heliothis virescens*) completely inhibited larvae growth [11]. Ten times higher than the physiological concentration of L-Tyr in plasma led to severe brain damage and DNA damage in Wistar rats (*Rattus norvegicus domestica*), possibly because of an oxidative stress [15]. Such a toxic concentration of L-Tyr is observed in patients suffering from tyrosinemia type II [15].

The *de novo* synthesis of Tyr occurs only in plants and microbes. Chorismate, the final product of the shikimate pathway, is a precursor for Phe and Tyr biosynthesis in plants, bacteria, and fungi ([16] and references herein). In plants, chorismate mutase (CM) converts chorismate into prephenate, the main substrate for these AAs' biosynthesis (Figure 1). Then, prephenate may be quickly transformed into arogenate in the reaction catalyzed by prephenate aminotransferase (PPA-AT), and further to Tyr by arogenate dehydrogenase (ADH). Another possible pathway of Tyr biosynthesis is the alteration of prephenate, in the presence of nicotinamide adenine dinucleotide phosphate ($NADP^+$), in the reaction catalyzed by prephenate dehydrogenase (PDH). The product, *p*-hydroxyphenylpyruvate (HPP), undergoes transamination into Tyr in the reaction catalyzed by Tyr aminotransferase (Tyr-AT) [4]. In plants, Phe and Tyr are synthesized mostly in plastids, and most likely exported to the cytosol via plastid located AAA-transporters [7,17]. Animals obtain Tyr from the diet; it has been demonstrated that Phe may be converted to *p*-Tyr in the reaction catalyzed by Phe hydroxylase [18].

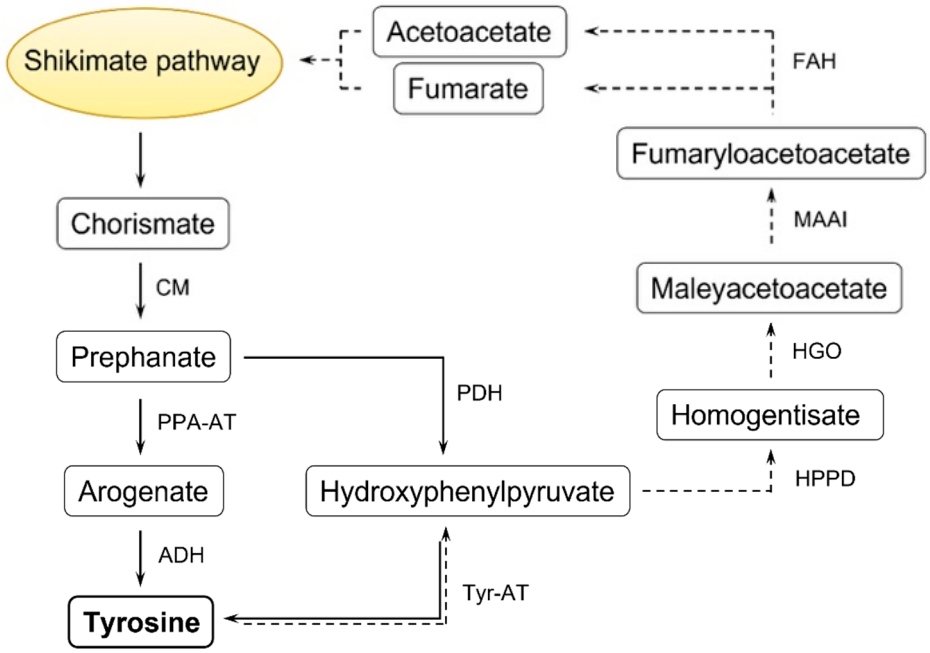

Figure 1. Tyr synthesis (solid line) and Tyr catabolism (dashed line). Enzymes: chorismate mutase (CM), prephenate aminotransferase (PPA-AT), arogenate dehydrogenase (ADH), prephenate dehydrogenase (PDH), Tyr aminotransferase (Tyr-AT), 4-hydroxyphenylpyruvate dioxygenase (HPPD), homogentisate 1,2-dioxygenase (HGO), maleylacetoacetate isomerase (MAAI), fumarylacetoacetate hydrolase (FAH). Substrates or products: *p*-hydroxyphenylpyruvate (HPP), homogentisate (HGA). According to [4] with some modifications.

The catabolism of Tyr is a multi-step process occurring mostly under stress conditions and senescence. It is suggested that it takes place in the cytosol ([3] and citations herein). The degradation of Tyr starts through its transamination into HPP by Tyr-AT (Figure 1). Next, the 4-hydroxyphenylpyruvate dioxygenase (HPPD) converts HPP to homogentisate (HGA), a precursor for plastoquinones or vitamin E [19]. The formation of HGA in the AAs' degradation pathway is followed by the conversion into maleylacetoacetate by homogentisate 1,2-dioxygenase (HGO). The role of maleylacetoacetate isomerase (MAAI) is to produce fumarylacetoacetate—the next intermediate of Tyr catabolism. The degradation of Tyr in the oxidative pathway ends with the release of the acetoacetate and fumarate from fumarylacetoacetate in the reaction catalyzed by fumarylacetoacetate hydrolase (FAH) [4]. Further, these compounds can be reintegrated into the shikimate pathway (Figure 1).

2. Tyrosine Structural Isomers: *meta-*, *ortho-*, *para*-Tyr

Depending on the location of a hydroxyl group in the benzyl ring, three structural Tyr isomers are described: (i) *para*-(*p*-Tyr), which is the most common product of metabolic reactions integrated into proteins; and (ii) *meta*-(*m*-Tyr) and (iii) *ortho*-(*o*-Tyr), which are both products of the oxidation of Phe, known also as markers of oxidative stress [18] (Figure 2). Although the *o*-Tyr has been proven to have a deteriorative effect in animal cells [20], its toxicity in plants is still not fully described.

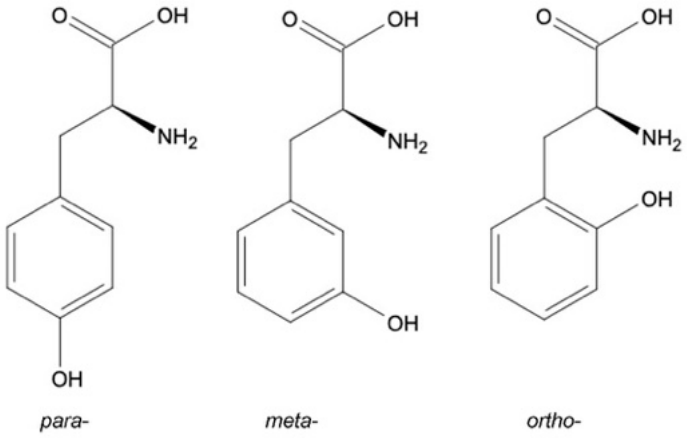

Figure 2. Structural isomers of Tyr: *para*-Tyr, *meta*-Tyr, *ortho*-Tyr.

Various biotic and abiotic stressors lead to the induction of oxidative stress in plants [21]. Oxidative stress is an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and molecular damage [22]. Under the conditions of ROS over-accumulation, *m*-Tyr and *o*-Tyr may be formed non-enzymatically via Phe hydroxylation or oxidation. The hydroxyl radical attack on the Phe ring initiates a two-step process, producing the highly reactive hydroxyphenylalanine radical intermediate. The formation of a stable isomer from the intermediate terminates the reaction. The mechanism of the stable end product generation may be the result of one of three possible mechanisms: abstraction, oxygenation, or disproportionation ([18] and citations herein). Tyr non-proteinogenic isomers can also emerge during the exposure of Phe to highly energetic radiation or in the reaction with peroxynitrite ($ONOO^-$), a compound formed from the superoxide anion (O^{2-}) and nitric oxide (NO). The hydroxylation of Phe in the presence of $ONOO^-$ occurs partially via hydroxyl radicals formation [23].

3. Fescue as the Biological Source of *m*-Tyr

The genus of fescue (*Festuca* L.) includes about 450 species around the world [24]. Of agronomical importance are mainly fine leaf fescues (*Festuca rubra* L. spp. rubra, *Festuca rubra* L. spp. trichophylla Gaud., and spp. littoralis (Meyer) Auquiz), fescues forming clumps (*Festuca rubra* L. spp. commutata Gaud.), and sheep fescue (*Festuca ovina* L.) [25].

Fine leaf fescues were used as golf grasslands in the 16th century and are still typical for golf courses, sports fields, and lawns in temperate climate regions of North and South America and Europe [24]. The name "festuca" comes from the old Latin name "grass weed". Fescues' popularity is due to their high resistance to drought and low nitrogen demand; they do not require intensive care, while low sensitivity to lead allows their use nearby the communication routes [24]. Fescue grasslands do not contain other species characteristic for grassland habitats e.g., clover (*Trifolium* L. sp.), dandelion (*Taraxacum* F.H. Wigg. sp.), or daisies (*Bellis* L. sp.). This is because most of the fescues produce allelopathic compounds that inhibit the growth and development of neighboring plants. The use of fescue to reduce excess weeds in crops was intensively studied in the second half of the XX century. The water extracts of the dried shoots and roots of reed fescue (*Festuca arundinacea* Schreb.) inhibited the growth of black cabbage (*Brassica nigra* L.) [26] and negatively affected the seed germination, growth, and development of seedlings and the yield of trefoil (*Lotus corniculatus* L.) [27]. The application of an aqueous extract of fescue leaves resulted in the reduction of the yield of red clover (*Trifolium pratensis* L.) [28]. At the beginning of the 21st century, 78 species of fescue were examined, among which the seven most strongly limiting the weed infestation in field conditions were selected [25]. Further laboratory analyses have shown that the allelopathic potential of fescues corresponds to their root exudates. Fescues' shoot extract inhibited the root growth of cress seedlings (*Lepidium sativum* L.) in 40%, while the root exudates limited the growth in 70% [25]. The main fescues' root exudates component is *m*-Tyr, and the highest content of *m*-Tyr was recorded in the exudates of *F. rubra* spp. commutata and *F. rubra* spp. rubra [25]. The content of *m*-Tyr in the roots of fescue seedlings (about 6500 pmol mg^{-1} FW) was about 10 times higher than in leaves (590 pmol mg^{-1} FW). In dry seeds, *m*-Tyr was present at a much lower concentration, about 24 pmol mg^{-1} FW [29]. In fescues, epidermal cells of the root apex are responsible for the synthesis and release of exudates containing *m*-Tyr into the environment [25]. Fescues tolerate the presence of *m*-Tyr in the tissues possibly through its accumulation in intracellular or intercellular spaces [30]. The half-life of *m*-Tyr is rather short; it is estimated that, in filter paper bioassays, the *m*-Tyr half-life is less than 2 days and, in soil bioassays, less than 24 h [31].

Additionally, *m*-Tyr was detected in the donkey-tail spurge (*Euphorbia myrsinites* L.), but this plant does not exudate this NPAA into the environment [29]. As a secondary metabolite, *m*-Tyr is produced by some bacteria and may be a component of antibiotics, for example, pactamycin [32].

Contrary to the spontaneous formation under high ROS concentration, in fescues and donkey-tail spurge, *m*-Tyr is produced via enzymatic pathways by the transamination of *m*-hydroxyphenylpyruvate or the hydroxylation of Phe, respectively [33].

4. Mode of Action of *m*-Tyr

The direct mode of action linked to *m*-Tyr toxicity is its incorporation into the proteins, which alters physiological functionality [34]. In *Escherichia coli,* the incorporation occurs through the binding of *m*-Tyr to the $tRNA^{Phe}$ [35]. As was shown for bacteria and human cells, the cytoplasmic or mitochondrial aminoacyl-tRNA synthetases are prone to catalyzing the binding of $tRNA^{Phe}$ with *m*-Tyr [36]; thus, Tyr isomers at higher concentrations compete with Phe for $tRNA^{Phe}$ ([37] and references herein).

The incorporation of *m*-Tyr into plant proteins was also demonstrated for 5-days-old arabidopsis seedlings [38]. The authors demonstrated that application of 10 µM *m*-Tyr reduced chlorophyll content, altered organellar biogenesis, and decreased photosynthesis and respiration rates.

As was mentioned, the toxicity of *m*-Tyr might be overcome by the Phe application. The recovery effect was described for tomato (*Solanum lycopersicum* L.) seedlings [39] and confirmed for arabidopsis [38]. The mechanism of the recovery effect is most probably based on the competition between Phe and *m*-Tyr [29]. The resistance to *m*-Tyr observed in arabidopsis mutants was linked to the accumulation of free Phe in leaves due to the

overexpression of the genes encoding ADH, one of the enzymes of the Phe synthesis pathway [40].

In tomato seedlings, the mechanism of toxicity of *m*-Tyr is linked to altered ROS metabolism [39,41]. The treatment of tomato seedlings with *m*-Tyr led to the overproduction of ROS and the accumulation of carbonylated proteins ([39,41] and citations herein). Carbonyl groups in proteins are considered as markers of oxidative stress and ageing [42]. Similar to *m*-Tyr, the mode of action has been proven for another NPAA–canavanine (L-2-amino-4-guanidooxy-butanoic acid–CAN). This arginine analog, found in some Fabaceae plants, is considered as a toxic compound, protecting plants against herbivores ([43] and references herein). The direct mode of action of CAN is similar to *m*-Tyr; CAN is incorporated into proteins producing aberrant molecules [44]. Moreover, CAN supplementation into the growing medium of tomato seedlings stimulated ROS accumulation in roots and increased the level of carbonylated proteins [45]. Furthermore, *m*-Tyr decreased the enzymatic and non-enzymatic antioxidant activity in the roots of tomato seedlings [39] and decreased the generation of $ONOO^-$ [46]. This indicates, that besides the alteration of the ROS metabolism, *m*-Tyr has an impact on the reactive nitrogen species (RNS) content [41,46]. In plants, the biological action of RNS is linked to post-translational protein modifications, including Tyr nitration. The formation of 3-nitro-tyrosine (3-NT) occurs through the addition of a nitro group ($-NO_2$) in the *ortho* position of the Tyr ring via covalent binding [47]. Such modified, nitrated proteins lose their function [48,49]. There are no data indicating the possibility of the prevention of free *p*-Tyr nitration due to the *m*-Tyr formation. May *m*-Tyr incorporated into the protein structure also be nitrated? Does it physiologically matter? These questions need further investigation.

Duke [30] suggested that the possible mechanism of *m*-Tyr toxicity in plant cells is that *m*-Tyr could be converted into dihydroxyphenylalanine (L-DOPA), a compound of physiological meaning. The currently described action of *m*-Tyr in plants, observed after the application of synthetic DL *m*-Tyr, has been summarized in Table 1.

Table 1. Action of *m*-Tyr in various plants. Information on the methods/analytical procedure used in the experiments is given in parentheses.

Plant Material, *m*-Tyr Concentration	Effect of *m*-Tyr Application	References
Lettuce (*Lactuca sativa* L.) (DL-*m*-Tyr: 10–320 μM)	Inhibition of root growth (filter paper bioassays, soil bioassays)	[29,31]
Tomato (*Solanum lycopersicum* L.) (DL-*m*-Tyr: 50–250 μM)	Inhibition of root growth (filter paper bioassays) Accumulation of ROS (spectrophotometry) Increased emission of NO (fluorimetry) Modification of enzymatic and nonenzymatic cellular antioxidant system (spectrophotometry) Increased protein carbonylation level and protein nitration level (ELISA)	[39,46]
Arabidopsis (*Arabidopsis thaliana* ecotype Columbia (Col-0)) (DL-*m*-Tyr: 2.5–320 μM, according to personal information)	Inhibition of root growth and decreased leaves number (agar plate bioassays) Decreased total chlorophyll content (spectrophotometry), aberration in chloroplast ultrastructure (TEM), lowered photosynthesis rate (Clark-type electrode) Modification of mitochondria structure (TEM), lowered respiration rate (Clark-type electrode)	[38]

The inhibition of the growth of *Sacchromyces cerevisiae* after the addition of *m*-Tyr into the growing media was observed [35]. In *Escherichia coli*, *m*-Tyr altered a major part of the proteome as misfolded proteins crowded and formed aggregates [50]. To prevent this kind of damage, bacteria have a protective mechanism against mischarged AA. The activity of some tRNA synthetases is regulated by the quality control system that hydrolyzes the invalid AA before or after binding with the tRNA [50]. In *Escherichia coli* cells lacking the $tRNA^{Phe}$ synthetase quality control, the exposure of *m*-Tyr led to protein aggregates accumulation. This was followed by the up-regulation of genes involved in the unfolded protein stress response [50].

The mode of action of *m*-Tyr in animals is mostly based on the incorporation of this NPAA into the proteins. This phenomenon was confirmed by Gurer-Orhan et al. [34] using radiolabeled *m*-Tyr added into the culture of hamster ovary cells. L-*m*-Tyr (0.2 mM) inhibited colony formation by 30% [34]. The simultaneous application of *m*-Tyr (0.2 mM) and Phe (1 mM) completely preserved the ability to proliferate. In humans, the elevated concentration of this Tyr isomer occurs in neurodegenerative diseases and diseases associated with oxidative stress and/or ageing: diabetes, artherosclerosis, and others ([18] and references herein) (Table 2). Moreover, *m*-Tyr can play a significant role in the cancer cells in animals. The concomitant tumor resistance is the phenomenon of the inhibition of secondary tumor implants or metastasis development in hosts that are already affected by the primary tumor [51]. Indeed, *m*-Tyr and *o*-Tyr were found to be factors leading to that resistance as they were discovered in the serum of tumor-bearing mice (*Mus musculus*). Administration of these NPAAs inhibited the growth of tumors in the murine models of cancer [52]. As was discussed, while secondary tumors are inhibited by circulating *m*-Tyr, the primary tumor microenvironment is protected by an accumulation of AA with counteracting properties (i.e. Phe) [53]. The primary tumor affected ROS generation, resulting in the increased *m*-Tyr and *o*-Tyr content [52]. This effect was observed in different human tumor types (prostate tumor, lung anaplastic, and nasopharyngeal carcinoma), where the application of *m*-Tyr led to the inhibition of cancer proliferation [54]. The treatment of cancer cells with *m*-Tyr induced the autophagy; however, the application of Phe reversed the toxic effect of *m*-Tyr on secondary cancer growth [52,54]. Studies on the key role of non-proteinogenic Tyr isomers in the mechanism of concomitant tumor resistance have shown an antiproliferative and anti-metastaic effect of *m*-Tyr [52–54]. These findings may be useful in the prevention of tumor metastasis. Moreover, *m*-Tyr can stop the growth of cells from tumor fragments that could have remained post-surgery. Importantly, for the therapeutic use of *m*-Tyr, no evidence of toxic effects of this NPAA at the concentration used for humans was found. After the oral application of 28 µmole *m*-Tyr kg^{-1} given to adults, the majority of the load was metabolized to 3-hydroxymandelic acid, 4-dihydroxyphenylacetic acid, and 3-hydroxyphenylacetic acid, detected in urine. In this experiment, no side effects have been observed [55]. Various examples of the *m*-Tyr mode of action in animals are presented in Table 2.

As was mentioned, exogeneous Phe might overcome the toxic effect of *m*-Tyr as the pool of free Phe in cells increases. An increased Phe level may be achieved by the inhibition of the activity of Phe hydroxylase (the enzyme responsible for Phe catabolism). On the other hand, the degradation of Tyr isomers may occur by the higher activity of Tyr-AT—the first enzyme in the Tyr catabolism pathway ([18] and citations herein). It was revealed that oxidative stress promotes the up-regulation of genes coding Tyr-AT in *Caenorhabditis elegans* [56]. The authors discussed that it might be the first step in the response against *m*-Tyr toxicity.

Table 2. Examples of pathophysiological effects related to the presence or application of *m*-Tyr in animal cells. Information on the methods/analytical procedure used in the experiments is given in parentheses.

Tissue/Biological Material	Level of *m*-Tyr	Physiological Effect Linked to *m*-Tyr and Accompanied Biochemical Changes	References
Human lenses	20.3 nmol g^{-1} protein	Cataract related with the lower content of soluble proteins (spectrophotometry) and Phe (HPLC)	[57]
Cerebrospinal fluid of newborn infants	20.5 nM	Hypoxic ischemic encephalopathy related with the increased content of ascorbic acid (HPLC), the higher *ortho*-Tyr/Phe and *m*-Tyr/Phe ratio (GC/MS), and the detection of non-protein-bound iron (spectrophotometry)	[58]
Human synovial fluid	0.5–3.5 µM	Rheumatoid arthritis. Determination of hydroxyradical attack on Phe (HPLC)	[59]
Human tumors (prostate, murine mammary carcinomas) in mice	* Injection of *m*-Tyr (67 mg kg^{-1} day^{-1})	Inhibition of the implantation of metastases (all treated and control mice were killed and metastases were counted)	[54]
T-lymphoma in BALB/c mice	* Injection of *m*-Tyr (0.2 mL of 500 µg mL^{-1} *m*-Tyr, daily for 5 days)	Decrease of tumor volume (histopathologic studies), the low expression of proliferation marker Ki-67 protein (immunostaining)	[52]
TF-1 erythroblast cell culture	* Treatment with *m*-Tyr (20 mg L^{-1})	Inhibition of cells proliferation (Bürker cell counting chamber) Induction of erythropoietin resistance (erythropoietin-response, cell counting)	[60,61]

* no information about used optical isomers.

5. Summary

In animal cells, *m*-Tyr is formed as a product of Phe oxidation and thus is commonly accepted as a marker of the oxidative stress accompanying various pathological processes and diseases. In plants, besides Phe oxidation, *m*-Tyr is synthesized enzymatically, predominantly as a chemical weapon necessary for successful competition with neighbors in the environment. Despite the direct action of *m*-Tyr based on its incorporation into the protein structure, which is characteristic for both animal and plant tissues, there are several proofs that, in plants, *m*-Tyr alters the ROS and RNS metabolism [39,41].

Based on several data, the question as to why plants are more sensitive to *m*-Tyr than animals arose. According to previously published data, the Phe content in the leaves of arabidopsis is around 13.6 nmol g^{-1} FW [62], whereas the concentration of this AA in human skeletal muscles is about 2000-fold higher—around 46 µmol g^{-1} FW [63]. Such a difference in the Phe concentration may partially explain the *m*-Tyr toxicity in plants' organs due to the limitation of the endogenous Phe necessary for the recovery effect. On the other hand, it may justify the existence of various enzymatic pathways of *m*-Tyr biosynthesis in plants.

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Nitric oxide–an antidote to seed aging modifies *meta*-tyrosine content and expression of aging-linked genes in apple embryos

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Short-term (3 h) treatment of embryos isolated from accelerated aged apple seeds (*Malus domestica* Borkh.) with nitric oxide (NO) partially reduced the effects of aging. The study aimed to investigate the impact of the short-term NO treatment of embryos isolated from apple seeds subjected to accelerated aging on the expression of genes potentially linked to the regulation of seed aging. Apple seeds were artificially aged for 7, 14, or 21 days. Then, the embryos were isolated from the seeds, treated with NO, and cultured for 48 h. Progression of seed aging was associated with the decreased transcript levels of most of the analyzed genes (*Lea1*, *Lea2a*, *Lea4*, *Hsp70b*, *Hsp20a*, *Hsp20b*, *ClpB1*, *ClpB4*, *Cpn60a*, *Cpn60b*, *Raptor*, and *Saur*). The role of NO in the mitigation of seed aging depended on the duration of the aging. After 7 and 14 days of seed aging, a decreased expression of genes potentially associated with the promotion of aging (*Tor*, *Raptor*, *Saur*) was noted. NO-dependent regulation of seed aging was associated with the stimulation of the expression of genes encoding chaperones and proteins involved in the repair of damaged proteins. After NO application, the greatest upregulation of *ClpB*, *Pimt* was noted in the embryos isolated from seeds subjected to 7-day long accelerated aging, *Hsp70b*, *Hsp70c*, and *Cpn* in the embryos of seeds aged for 14 days, and *Lea2a* in the embryos of seeds after 21 days of aging. We also demonstrated the increased *meta*-tyrosine concentration depending or in respect the progression of artificial aging, and the NO-induced increased phenylalanine content in seeds artificially aged for 21 days. In the NO-treated embryos of seeds aged for 7 and 21 days, the level of tyrosine was almost doubled compared to the aged tissue. Our data confirmed the usage of *meta*-tyrosine as a marker of seed aging and indicated that the increased *meta*-tyrosine/tyrosine ratio could be related to the loss of seed viability.

KEYWORDS

apple seeds, Hsp, Lea, ROS, Saur, Tor, artificial aging

Introduction

Seed aging is a natural process of great impact from an ecological, agronomical, and economical point of view, thus understanding its regulation is still of great interest and importance. The beneficial effect of NO on the maintenance of seed vigor during aging was recently reviewed by Ciacka et al. (2020). The application of NO or its donors before implementation of the aging protocol for elm (*Ulmus pumila* L.) seeds activated the protection mechanisms that prevented the reduction of vigor in aged seeds (He et al., 2018). NO treatment of oat (*Avena sativa* L.) seeds after accelerated aging also improved their quality (Mao et al., 2018).

Apple (*Malus domestica* Borkh.) seeds belong to the *orthodox* type, and seeds of this type are rather insensitive to environmental factors, and, generally, age slowly. Apple seeds are characterized by a deep embryonic dormancy, which is removed by 3 months-long cold stratifications (4–5°C) (Lewak, 2011). This process is accompanied by an increased emission of NO from the embryonic axes (Dębska et al., 2013), and in a laboratory may be replaced by a short-term (3 h) fumigation of isolated embryos with NO (Gniazdowska et al., 2010). Embryos isolated from cold stratified apple seeds germinate fast and developing seedlings are of typical features (Lewak, 2011; Dębska et al., 2013). In contrast, warm stratification of apple seeds (25°C) did not lead to dormancy removal. The NO emission from the axes of warm stratified seeds was lower in comparison to the NO emission from the axes isolated from cold stratified seeds (Dębska et al., 2013). Conditions of warm stratification are similar to those described in accelerated aging protocols. Our previously published data have shown that keeping dormant apple seeds in wet sand at 33°C for 14 and 21 days may be successfully used to achieve material for studies of seed artificial aging (Ciacka et al., 2022). We have proven that NO may be regarded as a remedy against oxidative damages in the apple seeds undergoing accelerated aging (Ciacka et al., 2022).

Oxidative damages due to a cellular redox imbalance are at the center of the free radical theory of aging (Corbineau, 2012; Kurek et al., 2019), thus, a balanced reactive oxygen species (ROS) metabolism is crucial for the preservation of seed vigor. In the embryos of aged apple seeds, a stimulation of the total antioxidant capacity after NO fumigation was demonstrated. It was accompanied by increased transcript levels of genes encoding enzymatic antioxidants, resulting in the protection of the embryonic tissue against overaccumulation of ROS (Ciacka et al., 2022). In addition, we have analyzed in detail many aspects of ROS action in seeds during artificial aging and its changes in response to NO treatment (Ciacka et al., 2022). Thus, in the current research, we have decided to measure the content of *meta*-tyrosine (*m*-Tyr), the structural Tyr isomer, which is a product of the oxidation of phenylalanine (Phe). It may serve as a marker of oxidative stress (Ipson and Fisher, 2016; Tyminski et al., 2021 and references therein). One of the toxic actions of *m*-Tyr in plants is its impact on ROS and reactive nitrogen species (RNS) metabolism (Krasuska et al., 2017; Andrzejczak et al., 2018). The higher concentration of *m*-Tyr is typical for patients suffering from neurodegenerative diseases and diseases associated with oxidative stress and/or aging (Tyminski et al., 2021 and references therein). Consequently, we suspect that the prolonged artificial aging of apple seeds could result in the accumulation of *m*-Tyr in the embryonic axes, and this effect could be altered by the treatment with NO.

Seed quality and viability may be estimated by the analysis of the expression of genes encoding proteins belonging to Heat shock proteins (Hsp) and Late embryogenesis abundant proteins (Lea) (Smolikova et al., 2021). Higher levels of Hsp seem to be necessary for the extension of the seed's life span and the improvement of seedlings' resistance to a variety of stresses (Kaur et al., 2015; Thakur, 2020). Hsp is a molecular chaperone, involved in protein folding and stability maintenance, as well as in protein protection against oxidative damage (Job et al., 2005). Arabidopsis seeds with an enhanced accumulation of Hsp were characterized by an improved tolerance to aging (Prieto-Dapena et al., 2006). The casein lytic proteinase/heat shock proteins 100 (Clp/Hsp100) are chaperones that may alter the formation of protein aggregates in an ATP-depending manner (Lee et al., 2006). Remodeling properties of Clp are related to the changes in the activity of protein complexes, unfolding protein for its degradation, or facilitating the refolding of protein aggregates. In plants, one of the best-studied proteins from this family is cytosolic ClpB. The ClpB/Hsp100 expression is regulated by heat stress and developmental signals (Lee et al., 2006). Rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), maize (*Zea mays* L.), and Chinese mustard [*Brassica juncea* L.(Czern.)] seeds constitutively accumulate high levels of ClpB (Mishra and Grover, 2016).

Chaperonins (Cpn, known also as Hsp60) regulate protein folding, assembly, and translocation (Ishikawa et al., 2003). Hsp60 proteins interact with histidine kinase and participate in phytohormonal signaling and resistance to stresses (Nagaraju et al., 2021 and references therein).

Lea proteins are known as the key elements in sustaining seed viability (Smolikova et al., 2021 and references therein). A decreased content of Lea2 proteins was noticed as beech (*Fagus sylvatica* L.) seeds storage was prolonged (Kalemba and Pukacka, 2008). After 8 years of seed storage, a 2-fold decrease in the content of Lea2 in embryonic axes, in comparison to that in seeds stored for only 1 year, was observed. In contrast, the content of Lea in beech seeds increased during seed maturation in correlation to tissue dehydration (Kalemba and Pukacka, 2014). Lea is stable in a wide range of temperatures. During seed dehydration, these proteins act as chaperons, stabilizing the structure of other proteins and cell membranes, in case of protein damages that promote their refolding (Smolikova et al., 2021 and references therein).

Natural or accelerated aging of organisms or organs, e.g., seeds result in a conversion of L-aspartyl or asparaginyl residues to abnormal isoaspartyl (isoAsp) residues (Mudgett et al., 1997; Sano et al., 2016), and lead to the loss of proteins' function. L-Isoaspartyl *O*-methyltransferase (Pimt) repairs these abnormal residues. The overexpression of genes encoding Pimt led to the decrease in isoAsp residue accumulation in seed proteins and increased seed longevity, while the decreased Pimt level resulted in the loss of seed viability (Ogeì et al., 2008; Rajjou and Debeaujon, 2008 and references therein; Verma et al., 2013). Thus, Pimt-mediated protein repair is important for the regulation of seeds' lifespan.

The target of rapamycin–Tor is an evolutionary conserved Ser/Thr protein kinase playing the important role in the control of cell proliferation and growth in Eukaryotes (Xiong and Sheen, 2014; Kravchenko et al., 2015). In plants, Tor signaling is engaged in the regulation of embryogenesis, meristem activation, root development and growth, flowering, and lifespan determination. Its action in seeds is under the control of phytohormones involved in the regulation of seed germination (Salem et al., 2017; Salem and Giavalisco, 2019). In plants, Tor interacts with two proteins: regulatory associated partner of Tor (Raptor) and lethal with sec-thirteen protein 8 (Lst8), forming the TorC1 (Moreau et al., 2012; Kravchenko et al., 2015; Salem et al., 2017; Salem and Giavalisco, 2019). Tor is suggested to control ribosomal biogenesis, polysome accumulation, and various protein translational processes, as well as rRNA transcription (Ben-Shem et al., 2011; Xiong and Sheen, 2014), especially important during prolonged seed imbibition, and early seedling growth. In addition, in seeds, TOR regulates β-oxidation of stored lipids and oils (Xiong et al., 2013; Xiong and Sheen, 2014).

Small auxin-up RNA (*Saur*) is the largest family of early auxin-responsive genes in higher plants. In Arabidopsis, its number is above 80 (Zhang et al., 2021). *Saur* genes have been shown to play roles in diverse processes of plant growth, development, and stress responses (high temperature, drought, or salt). Overexpression of *AtSaur49* was linked to the promotion of leaf senescence (Zhang et al., 2021 and references therein).

As indicated by many authors, seed aging is accompanied by changes in the content of proteins mentioned above (Rajjou et al., 2008). These proteins are regarded as markers of seed longevity, because they are involved in protein folding, stabilizing, and repairing protein structure (Lea, Hsp, Pimt), and are potentially related to autophagy regulation (Tor, Raptor, and Saur). Thus, the expression of genes: *Lea*, *Hsp*, *Pimt*, *Tor*, *Raptor*, and *Saur* were at the center of our interest related to NO-dependent restoration of apple seeds viability after 7 to 21 days of accelerated aging. Furthermore, we analyzed the content of *m*-Tyr, its proteinogenic analog—Phe, and the total concentration of its structural isomer—Tyr. Determination of *m*-Tyr in the apple embryos may confirm the universal harmfulness of this non-proteinogenic amino acid in Eukaryotes correlated to the destabilization of ROS metabolism during aging.

Materials and methods

Plant material

As a biological material apple (*Malus domestica* Borkh. cv. Antonówka) seeds subjected to the accelerated aging protocol according to Ciacka et al. (2022) were used. The ripened apple fruits were obtained from the WIKPOL fruit producer (Dąbrowa, Poland). Seeds after isolation from apple fruits were dried and stored in dark glass containers at 5°C to preserve dormancy.

Dry apple seeds (100 seeds) were placed in a Petri dish (Ø 15 cm) filled with 300 g of sterile quartz sand (sand volume 180 ml), and moistured with 110 ml of sterile, distilled water. Accelerated aging was performed at 33°C (in Nahita incubator, 636 Plus) for 7, 14, and 21 days in 4 independent replications (each replication consisted of 8–10 Petri dishes). After aging, seeds were removed from the sand, rinsed with water, and the embryos were isolated. The embryos, treated with NO, were shortly fumigated with vapors of a solution of acidified nitrite [14.5 mM $NaNO_2$ (Chempur 117926907), 0.2 M HCl] for exactly 3 h as described by Ciacka et al. (2022). After NO fumigation, the embryos were rinsed in distilled water. The non-treated embryos (the control, embryos isolated from seeds only subjected to accelerated aging) and the embryos isolated from seeds subjected to aging and shortly treated with NO (NO treatment) were placed in glass Petri dishes on filter paper moistened with distilled water for 48 h in a growth chamber (Sanyo MLR-35OH) in 25/20°C day/night, 12/12 h photoperiod, and with the light intensity of 100 μmol PAR m^{-2} s^{-1}. After 48 h of the culture, the embryonic axes (50 embryonic axes corresponding to approximately 50 mg FW per sample) were isolated and used for experiments.

The experimental model was presented in Ciacka et al. (2022).

Determination of amino acids concentration

Determination of Tyr, Phe, and *m*-Tyr concentration in the embryonic axes isolated from the embryos of aged apple seeds (the control) and the embryos treated with NO (NO treatment) after isolation from seeds subjected to accelerated aging was performed as described by Du et al. (2004) with modifications. The experiment was conducted with HPLC-grade chemicals.

For samples derivatization, 1 mM of the 6-aminoquinolyl-hydroxysuccinimidyl carbamate (AQC) (Cayman Chemical 30877) dissolved in acetonitrile (HPLC grade, Poch 102644151) was used.

The embryonic axes (50 mg) were homogenized in 100% methanol (HPLC grade, Poch 621995156) (0.5 mL) with the addition of 2 μl of the internal standard 0.1 mM 5-methyl-DL-tryptophan (Sigma M0534). Homogenized samples were vigorously mixed and centrifuged at 20,000 *g* for 10 min at room temperature. Supernatants were collected and dried under vacuum (Eppendorf concentrator plus) to remove methanol. The obtained samples were dissolved in 100 μl of acetonitrile (HPLC grade).

Before separation, 20 μl of samples were mixed well with 70 μl of 0.2 M borate buffer pH 8.8 (sodium tetraborate, Sigma B9876; boric acid, Sigma 31146) and 10 μl of 1 mM AQC, and incubated for 10 min at 55°C in darkness.

After derivatization, 20 μl of the sample was injected onto the Bionacom Velocity LPH C18 column (4.6 × 150; 3 mm) that was held in a thermostat set at 30°C. For peak detection, the FP-2020/2025 Intelligent Fluorescence Detector (JASCO) was used (E_x 250 nm, E_m 395 nm). The mobile phase flow rate was set at 1 ml min^{-1}. As mobile phase A, 0.15 M acetate buffer pH 6.15 (sodium acetate, Chempur 118056403; acetic acid, Poch BA8760114) with 0.033% (v/v) triethylamine (TEA, Sigma T0886) was used. Acetonitrile was used as mobile phase B.

The following gradient program was used for samples separation: 0–10 min 84–80% A, 10–21 min 80–70% A, 21–25 min 70–60% A, 25–27 min 60–50% A, 27–30 min 50–20% A, 30–31 min 20–10% A, 31–32 min 10–30% A, 32–35 min 30–60% A, and 37–37 min 60–84% A.

As standards, L-Tyr (J&K 185061), L-Phe (Sigma P17008), and *m*-Tyr (J&K 263747) were used. Standard amino acids were mixed with 0.2 M borate buffer pH 8.8, derivatized with 1 mM AQC, and separated under the conditions described above.

The analysis was performed using three biological repetitions, in three technical replications. The concentration of amino acids was presented as pmol g^{-1} FW.

Analysis of genes expression

Total RNA was isolated from approximately 50 mg of embryonic axes, using RNAzol® RT, according to Andryka-Dudek et al. (2019). After treatment with DNAse I (Thermo Scientific EN0523), 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. The finished product was diluted 3.5 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, 1 μl cDNA, 1 μl primer, and 4 μl sterile H_2O). Specific primers were designed based on nucleotide sequences available in the National Center for Biotechnological Information (NCBI) and the Genome Database for Rosaceae[1] (**Supplementary Table 1**). As an effect of using the BLASTP algorithm (NCBI), the sequences of the encoded proteins were aligned with Arabidopsis proteins. The name of the corresponding protein in Arabidopsis and the reference number in the Uniprot database can be found in **Supplementary Table 1**.

The expression levels were normalized using two reference genes and calculated using the method described by Vandesompele et al. (2002) and Hellemans et al. (2007). The experiments were done in three biological replicates, in three technical repetitions.

Statistics

The data were analyzed using the Statistica13 Software. All data were obtained from at least three independent experiments with at least three repetitions each and presented as mean values ± SD. For gene expression analysis, the significant differences were evaluated using Student's *t*-test (**Figures 2–6**), or after one-way ANOVA, homogenous groups were evaluated using Tukey's test (**Supplementary Figure 2**). For the results of *m*-Tyr, Phe, and Tyr content and *m*-Tyr-Tyr ratio **Figure 1**, two-way ANOVA was used to determine the statistically significant differences between the means. The homogeneous groups were obtained by Duncan's test. In the supplement, the results of the content of amino acids in the axes of embryos after NO treatment expressed as a percentage of the control (the embryos of aged seeds) were analyzed using Student's *t*-test (**Supplementary Figure 1**).

Results

Seed aging increased the *m*-Tyr level in the embryonic axes and nitric oxide modified the content of *m*-Tyr, Phe, Tyr, and *m*-Tyr/Tyr ratios in the embryonic axes of aged seeds

After 7 days of apple seeds' accelerated aging, the *m*-Tyr concentration was approximately 200 pmol g^{-1} FW in the embryonic axes and did not change as aging lasted until 14 days. Treatment of these embryos (after seeds aging for 7 and 14 days) with NO resulted in an increase in the

1 https://www.rosaceae.org/

10.3389/fpls.2022.929245

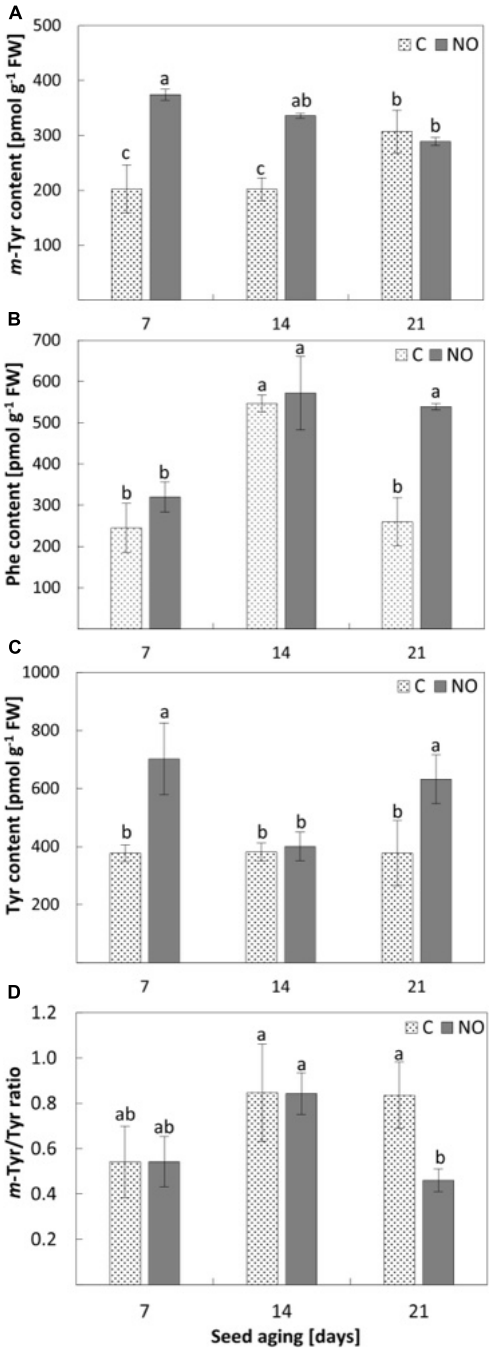

FIGURE 1
The content of *m*-Tyr **(A)**, Phe **(B)**, Tyr **(C)**, and the *m*-Tyr/Tyr ratio **(D)** in the axes of apple seeds subjected to accelerated aging for 7, 14, and 21 days (C) and in the axes of apple embryos fumigated with NO after seeds accelerated aging (NO). Values are average ± SD of 3 independent repetitions. Letters indicate the significant differences determined by Duncan's test ($P < 0.05$).

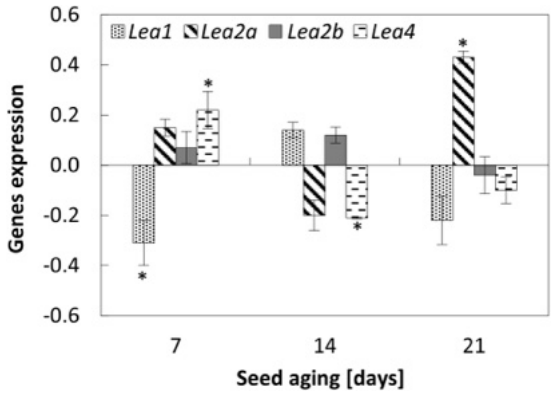

FIGURE 2
NO-dependent changes in the expression of *Lea1*, *Lea2a*, *Lea2b*, and *Lea4* in the embryonic axes of apple seeds subjected to accelerated aging for 7, 14, and 21 days. Values are average ± SD of 3 repetitions. Asterisks indicate significant differences between the treated sample compared to the control sample, obtained by the Student's *t*-test ($P < 0.05$).

level of *m*-Tyr by 85 and 65%, respectively. Prolongation of seeds aging (until 21 days) increased the content of *m*-Tyr by approximately 50% compared to 7 and 14 days aged seeds. Fumigation of the embryos isolated from 21 days aged seeds with NO did not change the *m*-Tyr level in the axes (Figure 1A).

After 7 days of seed aging, the content of Phe was around 300 pmol g^{-1} FW, regardless of NO application (Figure 1B). The level of this amino acid increased by 80% in the axes of apple seeds subjected to accelerated aging for 14 days and the value of this parameter did not change after the embryos' treatment with NO. After 21 days of seed aging, the content of Phe was similar to that observed in the embryonic axes of seeds aged for the shortest period (7 days). Fumigation of these embryos with NO increased the content of Phe in the axes more than twice, to the level observed in the axes of seeds aged for 14 days (Figure 1B).

The total content of Tyr in the embryonic axes of aged seeds remained constant irrespectively of the duration of aging (Figure 1C). NO application increased the level of this amino acid in seeds aged for the shortest (7 days) and the longest (21 days) period by approximately 85 and 65%, respectively. In the axes of embryos isolated from seeds aged for 14 days, fumigation with NO did not influence Tyr content (Figure 1B).

The highest value of the *m*-Tyr/Tyr ratio was characteristic for the axes of embryos isolated from seeds aged for 14 days (regardless of the embryos' treatment with NO) and 21 days (non-treated with NO) (Figure 1D). The embryonic axes of seeds aged for 7 days differed in the mean value of the ratio in comparison to the tissue aged for 14 and 21 days, but this difference was not statistically significant. NO fumigation did not change the value of the *m*-Tyr/Tyr ratio in the axes of embryos of seeds aged for a week or two. While, after 21 days of seeds aging, NO application resulted in a 2-fold

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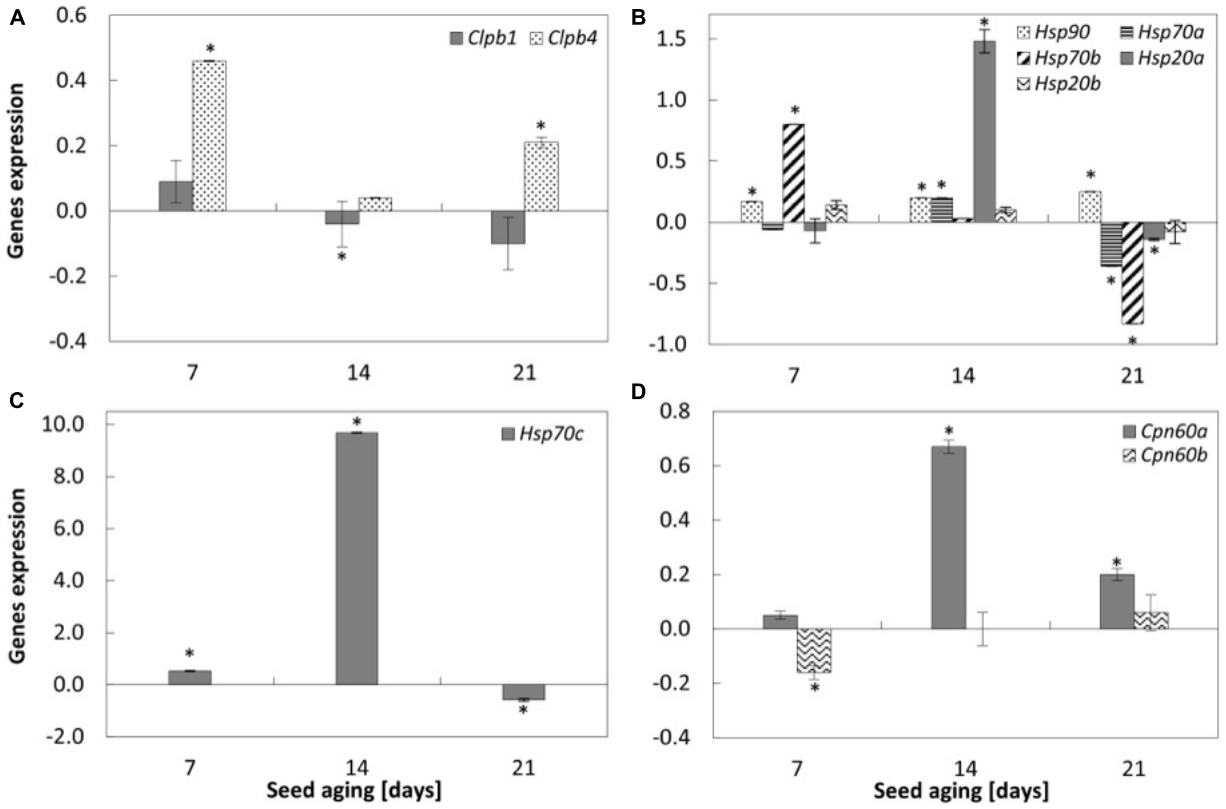

FIGURE 3
NO-induced modifications in the expression of *ClpB1* and *ClpB4* **(A)**, *Hsp90*, *Hsp70a*, *Hsp70b*, *Hsp20a*, *Hsp20b* **(B)**, *Hsp70c* **(C)**, *Cpn60a* and *Cpn60b* **(D)** in the embryonic axes of apple seeds subjected to accelerated aging for 7, 14, and 21 days. Values are average ± SD of 3 repetitions. Asterisks indicate significant differences between the treated sample compared to the control sample, obtained by the Student's *t*-test ($P < 0.05$).

decrease in the value of the *m*-Tyr/Tyr ratio compared to the control (Figure 1D).

Nitric oxide modified the expression of *Lea* in the embryonic axes of aged seeds

Accelerated aging of apple seeds resulted in the decreased transcript levels of *Lea1*, *Lea2a*, and *Lea4* in the embryonic axes. Only for the transcript level of *Lea2b* fluctuations with the minimum on the 14th day of aging was noticed (Supplementary Figure 2).

After 7 days of accelerated aging, the embryos' fumigation with NO increased the expression of gene encoding Lea4 (Figure 2). In the axes of these embryos, the decrease in *Lea1* and no changes in *Lea2a* and *Lea2b* expression levels were noticed. After 14 days of seed artificial aging, NO application altered only the expression of *Lea4*, which was downregulated. In the axes of apple embryos isolated from seeds aged for 21 days, NO treatment led to the upregulation of *Lea2a* (Figure 2), while the expression of other genes did not differ.

Nitric oxide upregulated the expression of *Hsp* in the embryonic axes of aged seeds

Accelerated aging of apple seeds resulted in a gradual decrease in *Clpb1* and *Clpb4* transcript levels in the embryonic axes (Supplementary Figure 2).

Nitric oxide fumigation of apple embryos isolated from aged seeds changed the expression of one of the two studied genes encoding proteins belonging to the Clp/Hsp100 family. The increase in the transcript levels of *ClpB4* was noticed in the embryonic axes of seeds aged 7 and 21 days (Figure 3A).

Progression of accelerated aging of apple seeds resulted in the decrease in the transcript levels of most of the analyzed genes encoding Hsp proteins. The downregulation of *Hsp70b*, *Hsp20a*, *Hsp20a*, and *Hsp20b* was observed (Supplementary Figure 2).

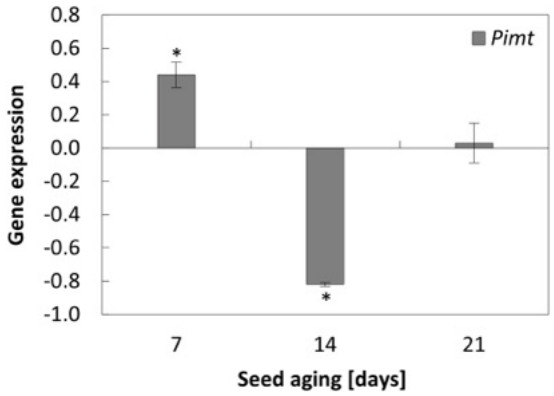

FIGURE 4
NO-dependent alterations in the expression of *Pimt* in the embryonic axes of apple seeds subjected to accelerated aging for 7, 14, and 21 days. Values are average ± SD of 3 repetitions. Asterisks indicate significant differences between the treated sample compared to the control sample, obtained by the Student's *t*-test ($P < 0.05$).

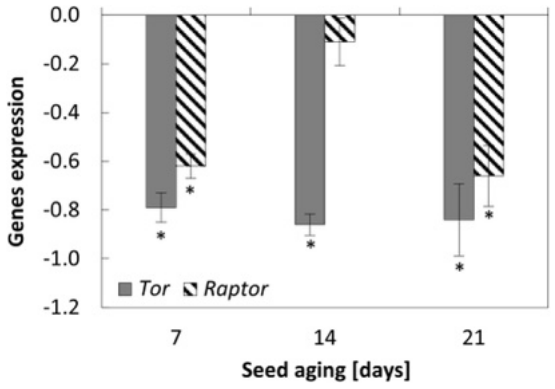

FIGURE 5
NO-dependent alterations in the expression of *Tor* and *Raptor* in the embryonic axes of apple seeds subjected to accelerated aging for 7, 14, and 21 days. Values are average ± SD of 3 repetitions. Asterisks indicate significant differences between the treated sample compared to the control sample, obtained by the Student's *t*-test ($P < 0.05$).

Transcript levels of *Hsp90* and *Hsp70a* were stable irrespectively of the duration of the aging.

Nitric oxide treatment of the embryos isolated from seeds aged for 7 days resulted in the increase in the expression of *Hsp90*, *Hsp70b*, and *Hsp70c* (**Figures 3B,C**), while the transcript levels of *Hsp70a*, *Hsp20a*, and *Hsp20b* did not change. After 14 days of seed aging, NO upregulated the expression of most of these genes, especially *Hsp70c* (**Figures 3B,C**). In the axes of seeds subjected to accelerated aging for 21 days and after being exposed to NO, a decrease in the expression of *Hsp70a,b,c*, and *Hsp20a* was observed. The transcript level of *Hsp90* was upregulated, while the expression of *Hsp20b* did not differ (**Figure 3B**).

Accelerated aging of apple seeds decreased the transcript levels of both analyzed genes (*Cpn60a* and *Cpn60b*), known also as *Hsp60*, encoding chaperonins (**Supplementary Figure 2**). Although the greatest reduction in the transcript level was observed for 14-day-long aged seeds (**Supplementary Figure 2**). As a result of NO treatment, *Cpn60a* was upregulated in the axes of apple embryos of seeds subjected to accelerated aging for 14 and 21 days, while the expression of *Cpn60b* decreased after 7 days of seed aging (**Figure 3D**).

Nitric oxide modified the expression of *Pimt* as seed aging lasted up to 14 days

Accelerated aging of apple seeds resulted in the fluctuation of *Pimt* transcript levels in the embryonic axes. Its maximum was noticed after 14 days of seed aging, while prolongation of the aging resulted in the downregulation of the gene, which expression was even lower than in the axes of 7-day-aged seeds (**Supplementary Figure 2**).

The NO-dependent alterations in the transcript level of *Pimt* were characteristic for the axes of apple embryos isolated from seeds aged for 7 and 14 days (**Figure 4**). When the duration of the aging procedure was shorter, the expression of *Pimt* was upregulated. After 14 days of aging, NO application decreased the transcript level of *Pimt* (**Figure 4**).

Nitric oxide downregulated the expression of genes encoding elements of Tor complex in the embryonic axes of aged seeds

Accelerated aging of the seeds for 14 and 21 days decreased the transcript level of *Raptor*, while the changes of *Tor* transcript level exhibited the fluctuation with the minimum at the 14th day (**Supplementary Figure 2**).

Nitric oxide application resulted in the decrease in the expression of *Tor* and *Raptor* in the axes of apple seeds after 7 and 21 days of aging. After 14 days of aging, NO treatment led to the decrease in the transcript level of *Tor*, while the expression of *Raptor* did not differ (**Figure 5**).

Nitric oxide changed the expression of *Saur* when seed aging lasted for at least 14 days

In the axes of seeds subjected to accelerated aging protocol, a decrease in the expression of *Saur* was observed. Its

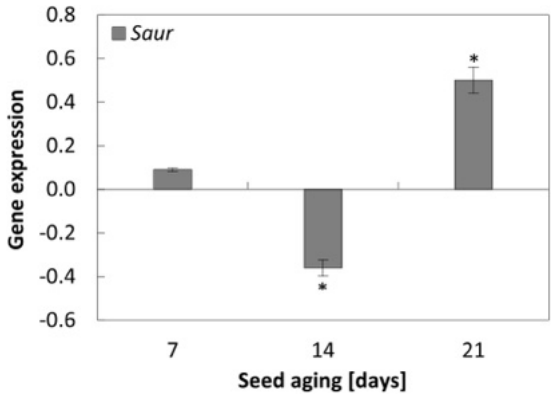

FIGURE 6
NO-dependent changes in the expression of *Saur* in the embryonic axes of apple seeds subjected to accelerated aging for 7, 14, and 21 days. Values are average ± SD of 3 repetitions. Asterisks indicate significant differences between the treated sample compared to the control sample, obtained by the Student's *t*-test ($P < 0.05$).

transcript level was similar after 14 and 21 days of aging (**Supplementary Figure 2**).

The changes in the expression of *Saur* after NO treatment were noticed when seeds were aged for 14 and 21 days. In the axes of apple seeds aged for 2 weeks, the transcript level was downregulated, while after 21 days, the increase in its expression was observed after embryos' fumigation with NO (**Figure 6**).

Discussion

Recently, the conditions for the accelerated aging of apple seeds have been established (Ciacka et al., 2022). Embryos of apple seeds placed at 33°C and 65% humidity for more than 14 days exhibited the visible symptoms of aging, while after 40 days, more than 70% of seeds died (Ciacka et al., 2022; **Figures 7**, **8**). One week of accelerated aging resulted in the partial removal of the dormancy of apple seeds. It can be assumed, that during 7-day aging, the apple seeds undergo imbibition, but these conditions do not provide the overall metabolic changes required for the entire dormancy breakage. These embryos need a long period of imbibition (the culture) to accomplish germination. After 7 days of accelerated aging, they germinated in 60% after 1 week of the culture (Ciacka et al., 2022; **Figure 7**). Based on previous results, we regarded this stage as the early phase of apple seeds' artificial aging. After 48 h of the culture under conditions favorable for germination, the embryonic axes of seeds aged for 7, 14, and 21 days do not elongate nor bent gravitropically (**Figure 7**). In our experiments, we used the axes of apple embryos isolated from aged seeds and shortly treated with NO. To detect the long-term effect of NO application, it was necessary to culture embryos for 48 h under favorable conditions (**Figure 7**).

The role of NO in delaying the negative effects of seed aging is partially linked to the modulation of ROS metabolism (He et al., 2018; Mao et al., 2018; Ciacka et al., 2022). Stable molecular markers pointing to the progression of seed aging are needed to determine seed quality. In human tissue, *m*-Tyr is regarded as such an indicator, because its accumulation corresponds to the induction of oxidative stress (Ipson and Fisher, 2016). *m*-Tyr content increased as accelerated aging was prolonged and in the embryonic axes of apple seeds subjected to 21 days of aging was approximately twice higher than in the embryonic axes of seeds aged for 7 or 14 days. It corresponded to the increase in malondialdehyde content (Ciacka et al., 2022) and pointed at the oxidative stress, induced during seed aging, thus, confirming the universality of *m*-Tyr as a marker of aging in living organisms. Supplementation of young tomato seedlings with *m*-Tyr (50 μM) resulted in an increased ROS/RNS level in the roots (Krasuska et al., 2017; Andrzejczak et al., 2018). Thus, the elevated level of this non-proteinogenic amino acid may, in part, explain the increased content of ROS in aged embryos (Dębska et al., 2013; Ciacka et al., 2022). The *m*-Tyr is an antimetabolite of Phe, and both amino acids are competitors for $tRNA^{Phe}$ (Bertin et al., 2007; Rodgers et al., 2017). Phe at high concentration protects the tissue against the toxic action of *m*-Tyr, lowering its incorporation into protein structures. The recovery effects of Phe were demonstrated for tomato (Andrzejczak et al., 2018) and Arabidopsis (Zer et al., 2020) plants supplemented with *m*-Tyr. Thus, the fluctuation in the levels of Phe in the embryos of aged apple seeds (non-treated with NO) indicates that one of the explanations for the cellular deterioration observed after 21-day artificial aging could be the disruption of the Phe/*m*-Tyr level, favoring *m*-Tyr accumulation. The embryos isolated from seeds, aged for 21 days and treated with NO, were characterized by a similar *m*-Tyr level to that in the control seeds but with a 2-fold higher content of Phe. Moreover, in these embryos, the ratio of *m*-Tyr to Tyr was lower, mostly due to the increased content of the total Tyr. The high free Tyr level induced by NO may act *via* the intracellular amino acids' isoform competition, mitigating advanced aging processes. After 7 and 14 days of accelerated aging of apple seeds in the axes of NO-treated embryos, the *m*-Tyr content was higher compared to the aged seeds, while Phe was at a similar level (**Supplementary Figure 1**). Therefore, it can be proposed that NO, by the stimulation of peroxynitrite ($ONOO^-$) formation, leads to Phe oxidation, resulting in the accumulation of *m*-Tyr. Surprisingly, after NO treatment, the decrease in the Phe content in the axes of 7 and 14 days aged seeds was not detected. It may be due to the putative increase in protein degradation, stimulated by NO (Krasuska et al., 2014), especially that the increased Tyr content after 7 days of aging was observed. The increased degradation of proteins

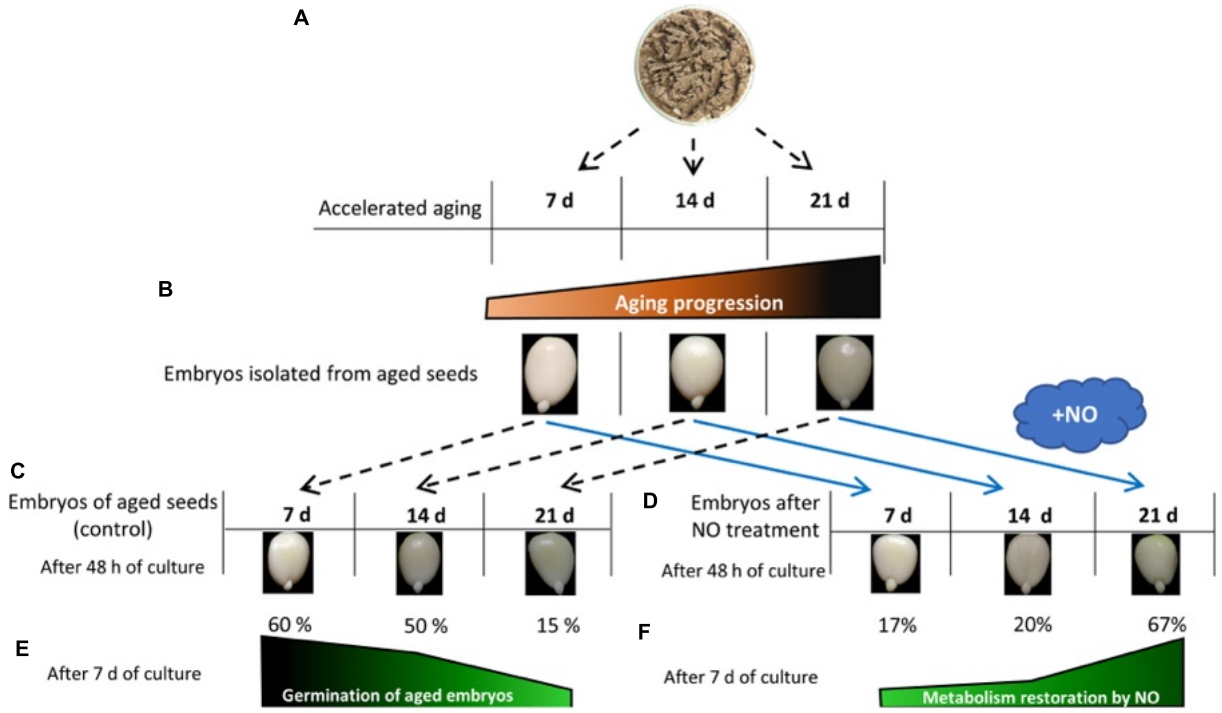

FIGURE 7
The diagram describes the basic features of the biological material and the scheme of the experiment. Apple seeds were subjected to accelerated aging for 7, 14, or 21 days **(A)**. After seed aging embryos were isolated **(B)**. The typical embryos isolated from aged seeds [control **(C)**] and the typical NO-treated embryos after 48 h of culture are shown panel **(D)**. Progression of seed aging was linked to the gradual decrease in germination rate of the embryos after 7 and 14 days, and a drop-down in the germination of the embryos as the aging lasted for 21 days **(E)**. NO acted as a remedy against aging by the increase in germination rate of the embryos isolated from aged seeds [metabolism restoration **(F)**]. Metabolism restoration (%) was calculated based on the germination rate of the embryos after NO fumigation according to data presented by Ciacka et al. (2022). Embryos isolated from aged seeds and after NO treatment were viable (as indicated by the TTC test) as described by Ciacka et al. (2022).

during advanced aging may also be the reason for *m*-Tyr release from oxidized proteins. Moreover, it was suggested that the transcriptional coordination of Phe, and not the Tyr biosynthesis with its upstream and downstream pathways, likely supports the preferred synthesis of Phe over Tyr in plants (Schenck and Maeda, 2018); therefore, NO action could restore this regularity. The direct or non-direct impact of NO on Phe metabolism in the aspect of *m*-Tyr accumulation needs further investigation.

According to previous data, 7 days of accelerated aging of apple seeds did not induce any significant, visible damages (Ciacka et al., 2022; **Figure 7**). Additionally, NO fumigation of the embryos isolated from seeds aged for 7 days has slightly accelerated the germination rate and enhanced the percentage of the properly grown seedlings (Ciacka et al., 2022; **Figure 7**). In the axes of these embryos cultured for 48 h, only a few investigated genes were upregulated (*Lea4*, *ClpB4*, *Hsp90*, *Hsp70b*, and *Pimt*). Proteins encoded by these genes are responsible for repair processes and protein stabilization, which could be necessary for the correction of damages, which take place during dehydration at the seed maturation stage and rehydration in imbibition.

The impact of NO on seed longevity maintenance could be explained by different mechanisms of its action, depending on the duration of accelerated aging and the biochemical state of the seeds (**Figure 8**). This assumption is based on the abundance of the transcripts encoding enzymatic antioxidants after 21 days of seed aging (Ciacka et al., 2022). In addition, the duration of apple seed accelerated aging treatment and experimental time points used in our study were selected to match the pattern of seed viability decrease vs. time described by Walters et al. (2010). Seed aging is linked to a sigmoidal dependence between viability and storage time. The unspecified duration of seed viability (the early aging phase of no apparent changes) is followed by the rapid viability loss—a mortality phase. Furthermore, seed longevity depends on metabolism (the degree of oxidation of cellular molecules and accumulation of oxidants), strictly linked to seed moisture (Walters et al., 2010) and temperature. The imbibition at 33°C from 7 to 40 days differentiates the degree of apple embryo tissue hydration and physiological activity. So,

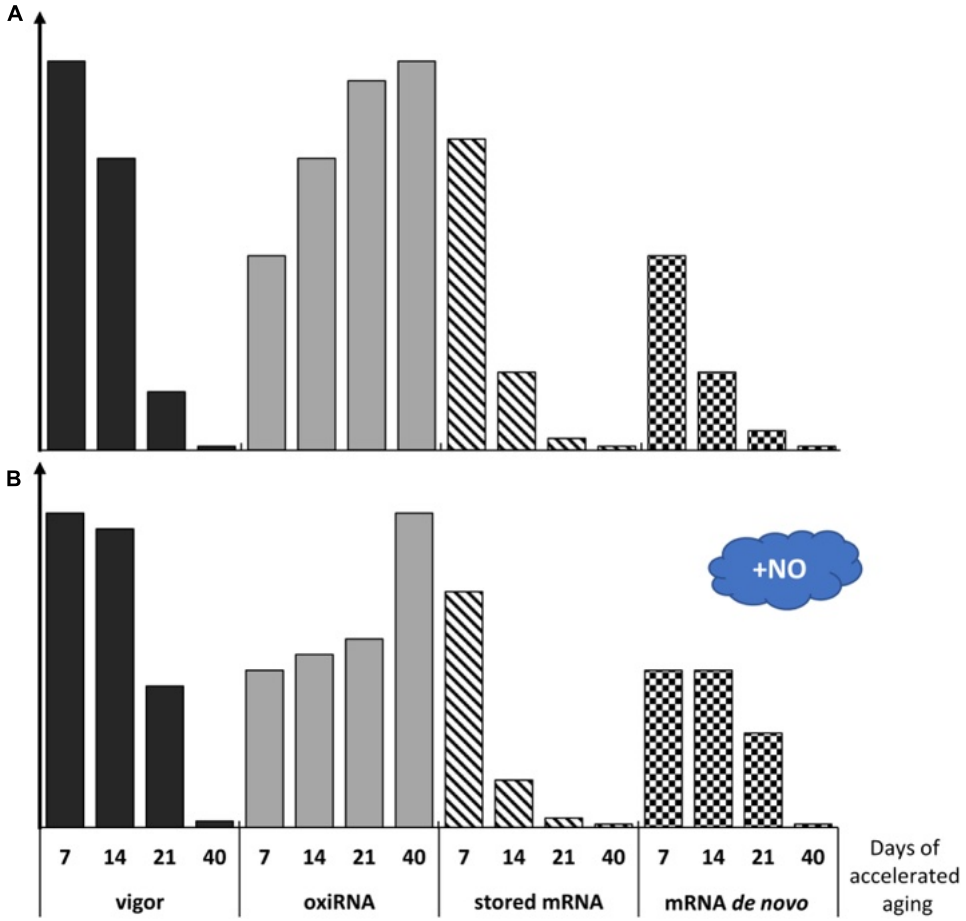

FIGURE 8
The figure describes the vigor of apple embryos in dependence on the progression of seed accelerated aging concerning the changes in the level of the oxidized RNA (oxiRNA), stored mRNA, and newly synthesized RNA (mRNA *de novo*) in the control embryos, non-treated with NO **(A)**, and the embryos shortly fumigated with NO **(B)**. The height of the bars filled with color (vigor-black and oxiRNA-gray) is proposed according to data by Ciacka et al. (2022). The height of the bars filled with sloping lines–stored mRNA, is estimated according to Zhao et al. (2020a, 2020b). The height of the checkered bars–mRNA *de novo* is hypothetical.

metabolically, embryos isolated after each time point should be considered separately and detected alterations should not be viewed linearly.

The asymptomatic phase of seed aging (corresponding probably to 7 days of apple seeds aging) is hard to determine if based only on seed germination tests (Zhao et al., 2020a,b). It has been proposed that stored mRNA (the amount and quality) may serve as an indicator of the progression of seed aging (Zhao et al., 2020a,b). The authors using Arabidopsis seeds indicated that stored mRNA degraded at a constant rate over the aging time. However, there is no information about the metabolic use of stored mRNA in artificially aged seeds as they are subjected to imbibition. The degradation of stored mRNA may also be linked to the rate of its oxidation.

Since our work concerns alterations in the transcript levels of the genes related to seed aging, we propose a hypothetical model describing changes in vigor, stored mRNA level, newly synthesized mRNA (mRNA *de novo*), and oxidized RNA (oxiRNA) in the embryonic axes of apple seeds subjected to accelerated aging for 7–40 days (**Figure 8A**). We suggest that until the 7th day of accelerated aging, embryos remind in the asymptomatic phase, and thus, are characterized by only minor changes in metabolism and quality. A decline in embryo vigor observed after 14 days of accelerated aging corresponds to rapid mortality and leads to death at the time point of 40 days (**Figure 8A**).

Continuing the investigation of the molecular action of NO during seed aging, we suggest that NO prolongs the asymptomatic phase and shallows the mortality phase (**Figure 8B**), which is related to the alterations in the expression of genes potentially involved in the regulation of seed longevity.

Lea proteins influence seed longevity by participation in the formation of the glassy state. The accelerated aging conditions promote the loss of the glassy state of the embryo and

enhance seed deterioration (Hundertmark et al., 2011). Lea proteins accumulate during seed maturation, as well as in vegetative tissues in response to water deficit (Liu et al., 2011). In plants, there is a large number of genes encoding Lea (Kalemba and Pukacka, 2008; Leprince et al., 2016). Based on the sequence similarity and properties, Lea is divided into 7 groups (Leprince et al., 2016). Lea of the 2nd group is known as dehydrins, whereas proteins from the 4th and 5th groups are possibly involved in the membrane stability maintenance. During water loss, Lea proteins act as chaperons, prevent protein aggregation, and modulate ROS formation, acting as scavengers of transitional metal ions, e.g., Fe^{3+} (Alsheikh et al., 2003; Liu et al., 2011). After synthesis during seed maturation, Lea is transported to different cellular organelles and membranes to protect and stabilize molecules. During seed imbibition, most of the mRNA of *Lea* is gradually degraded (Kalemba and Pukacka, 2007 and references therein). The protective role of Lea during seed maturation is well-known, whereas there is no evidence concerning their role in the relief of the effect of degenerative processes related to seed aging. The seeds obtained from Arabidopsis lines with a significant reduction in *Lea* expression stored at 35°C and 75% relative humidity (the glassy state of the cytoplasm is likely no longer exist) exerted the reduced viability (Hundertmark et al., 2011). The authors proposed that some Lea proteins play a protective role even in seeds that lost their glassy state, thus, the decrease in the level of dehydrins affects seed storability. In our experiment, the transcript levels of *Lea1*, *Lea2a*, and *Lea4* decreased in response to seed aging, which corresponds well to the low germination rate (**Figure 7**). It is also proposed that Lea exhibits different functions depending on the cellular water status (Leprince et al., 2016). In beech seeds that have been stored at −10°C and 8–9% moisture content, an increased level of Lea was noted, especially in the embryonic axes (Kalemba and Pukacka, 2008). NO-fumigated apple embryos isolated from seeds aged for 21 days were characterized by the highest abundance of *Lea2a*, and it was not observed in the axes of seeds subjected to the shorter period of accelerated aging. During seed maturation, the accumulation of stored mRNAs or long-lived mRNAs (encoding, e.g., 12S seed storage proteins and dehydrins) was detected. These mRNAs were used in protein translation during the early phases of germination (Rajjou et al., 2004; Sano et al., 2020) and probably in germinating embryos isolated from the seeds in the asymptomatic phase of aging (**Figure 8**). If we assume that 48 h of the embryo's culture corresponds to the early germination stage, especially after 7 and 14 days of accelerated aging, NO may promote the translation of Lea proteins (Lea1 or Lea4) from stored mRNA. After NO treatment, transcript levels of these genes decreased. The prolongation of apple seeds' accelerated aging favors ROS formation (Ciacka et al., 2022; **Figure 8**). Thus, the NO-induced expression of the specific *Lea* could be a protective mechanism against ROS overaccumulation. Such Lea may serve as free radicals modulators by binding metal ions (Liu et al., 2011).

Besides Lea, Hsp is another protein engaged in plant stress responses. Depending on their molecular weight, these proteins are divided into six classes: Hsp100 (Clp), Hsp90, Hsp70, Hsp60 (Cpn), Hsp40, and small Hsp (sHsp) (Kalemba and Pukacka, 2007; Kumar et al., 2020). They are accumulated at the late stage of seed maturation, and during germination, their abundance decreases constantly. The decreased transcript levels of genes encoding Hsp and ClpB proteins were observed as apple seed aging was in progress (**Supplementary Figure 2**). The protective role of Hsp is related to their chaperone function. They participate in the resolubilization of protein aggregates and assist in the folding of nascent polypeptides (Mayer and Bukau, 2005; Kumar et al., 2020). Hsp is accumulated in plant tissue under high temperatures. The increase in sHsp level was found in the embryos of dry maize seeds artificially aged at 50°C (Xin et al., 2011) and in the embryos of dry wheat and barley seeds subjected to 40°C (Dell'Aquila et al., 1998). Our experiments have demonstrated the diverse impact of NO-treatment on the level of various *Hsp* transcripts, depending on the progression of seeds aging. In the axes of embryos isolated from seeds aged for 14 days, as the result of NO treatment connected with the increase in the embryos' vigor, the upregulation of *Hsp70a*, *Hsp70c*, and *Hsp20a* was detected; whereas after 21 days of aging, the treatment of the embryos with NO led to a decrease in the expression of these genes, as well as *Hsp70b*. In turn, for these embryos, a slight increase in the level of *ClpB4* was noted. ClpB proteins, known as Hsp100, are involved in the maintenance of protein structure. Probably, they took part in the dissolution of cytosolic or nuclear protein aggregates formed during heat stress (Hong and Vierling, 2001 and references therein). The study using the Arabidopsis mutant characterized by the complete absence of the Hsp101 showed that plant growth, as well as seed germination, was not affected by the lack of this protein (Hong and Vierling, 2001). Authors suggested that Hsp101 activity is not critical in the absence of stress, although the biosynthesis of Hsp101 is regulated during seed development and the proteins are stored in dry seeds. In contrast, the mutation related to the reduction of the Hsp101 level led to the decrease in the thermotolerance of germinating Arabidopsis seeds. This fact emphasized the importance of Hsp101 to seed biology during stress conditions, probably due to their chaperone, and the putative translational regulatory functions (Hong and Vierling, 2001). The role of Hsp101 in the germination of Arabidopsis seeds subjected to heat stress was also proven by Queitsch et al. (2000).

The mode of action of NO in the regulation of viability of apple embryos isolated from seeds aged for 14 and 21 days was associated with the increase in the expression of both *Hsp90* and *Cpn60a*. However, the NO-dependent upregulation of *Cpn60a* in the axes of the seeds aged for 21 days, in

comparison to the control, was not so noticeable as for the seeds aged for 14 days. The viability of NO-treated embryos of 21-day aged seeds is not so high as embryos of 14-day aged seeds (**Figure 7**). The germinability of NO-treated embryos after 21 days of aging was only 50% of that after 14 days of aging (Ciacka et al., 2022). As aging was prolonged up to 21 days, the NO-dependent increase in the expression of *ClpB* was accompanied by the decrease in the expression of *Hsp70a* and *Hsp70b*. Therefore, we may assume that the mechanism of regulation of protein structure by Hsp is insufficient in this tissue in comparison to the NO-treated embryos isolated from seeds aged for 14 days, especially since the disaggregation of proteins may require the cooperation of Hsp100, Hsp70, and Hsp40 homologs (Queitsch et al., 2000 and references therein). The enhancement of the level of transcripts encoding protein "protectors" (Hsp100 or Hsp60), after NO application, especially in the earlier stage of aging, may prevent the putative accumulation of protein aggregates that may occur as a result of oxidative stress (Ciacka et al., 2020).

The aging of chickpea (*Cicer arietinum* L.) seeds increased the level of isoaspartyl residues in proteins and decreased the Pimt activity (Verma et al., 2013). The enhancement of vigor and longevity of chickpea seed were connected with the increased expression of *CaPimt* (Verma et al., 2013). Similarly, the increased expression of *Pimt1* was important in the preservation of vigor and extended longevity of Arabidopsis seeds, subjected to the controlled deterioration treatment (40°C, 82% relative humidity) (Ogeì et al., 2008). In this experiment, the highest transcript level of *Pimt* was characteristic for the axes of 14-day aged apple seeds. It corresponds to the relatively high germination rate of these embryos (**Figure 7**), suggesting also the activation of some repair mechanisms at this stage of aging progression. The most profitable effect of NO action on apple embryo viability was confirmed by the higher level of *Pimt* in 7-day aged seeds when the aging-induced degradation of cellular components is not so advanced. The decrease in expression of *Pimt* was characteristic of the axes of NO-treated embryos of seeds aged for 14 days, which can be explained by the drop-down of stored mRNA as the aging process was in progression (**Figure 8**). The lowest transcript level of *Pimt* was observed in the axes of 21-day-aged seeds. In these embryos, NO did not alter the gene expression, which may be reflected in the worst germinability.

In the last 5 years, the number of information regarding Tor activity in plant physiology has increased. In plants, Tor is involved in the control of mRNA translation, protein synthesis, and life span determination (Quilichini et al., 2019). The treatment of wheat (*Triticum aestivum* L.) seeds with rapamycin or torin1 (the inhibitors of Tor activity) resulted in the inhibition of germination under favorable conditions (Smailov et al., 2020). The authors proposed that the Tor signaling pathway is involved in the GA-dependent synthesis of α-amylase and following seed germination. As was demonstrated for Arabidopsis, Raptor, the essential element of Tor machinery, also regulates the undisturbed seed germination. The delay in Arabidopsis seed germination was observed for mutants with a loss of function in *raptor1B* (Salem et al., 2018). The authors indicated that Raptor1B is a positive regulator of seed germination (examined under favorable conditions), and is involved in the maintenance of hormonal and nutritional balance. Delayed germination of *raptor1b* seeds (the knockout mutant) was accompanied by an increase in the level of phytohormones: ABA and auxin (Salem and Giavalisco, 2019). Inhibition of seed germination in *Raptor* mutants corresponded to the lowering transcript level of *Raptor* in the axes of aged apple seeds, in which viability and germination were reduced (**Figure 7**).

Regardless of the duration of apple seeds aging, the NO-dependent downregulation in *Tor* and *Raptor* (except for seeds aged for 14 days) expression was observed. As mentioned above, in seeds, the role of Tor is related to the regulation of seed germination, especially at the level of the transition from heterotrophic to photoautotrophic growth (McCready et al., 2020). Regardless of the duration of seed aging and the embryos' treatment with NO, after 2 days of the culture, apple embryos do not differ visually (**Figure 7**). As mentioned in the first part of the discussion, these embryos are characterized by short, not bent embryonic axes, which means they are not at the stage of the transition into seedlings. Therefore, we were not surprised by the decrease in *Tor* and *Raptor* expression after embryos' treatment with NO. The upregulation of *Lea4*, *ClpB4*, *Hsp90*, *Hsp70b*, or *Pimt* seems to be the priority because reparation of some damages that occurred during, e.g., seed rehydration is required. Thus, the removal of damages is a critical point to follow the completion of germination and seedling development. Going on further, NO may even prevent the accumulation of *Tor* and *Raptor* to lower proliferation activity and minimize the damage. Whether the *Tor* and *Raptor* transcripts belong to stored mRNA and NO regulates its utility, needs additional experiments.

In plants, Tor acts as a negative regulator of autophagy, the process which is necessary to recycle cytoplasmic contents under stress conditions or during senescence/aging (Liu and Bassham, 2010). An increasing number of studies have proven the selective autophagic-dependent degradation of various cell components (Su et al., 2020 and references therein), which may prevent early aging under stress conditions. In the axes of NO-treated apple embryos, the reduced levels of *Tor* (after 14 and 21 days of aging) and *Raptor* (after 21 days of aging) may promote autophagy events. Autophagy can be induced by various pathways, one of which is related to ROS (Pérez-Pérez et al., 2012). Alteration in Tor activity is also dependent on the cellular ROS level. The decrease in Tor activity in

Arabidopsis seedlings under oxidative stress conditions was noted (Ahn et al., 2019). However, the studies conducted on Arabidopsis mutants with the downregulation of *Tor* by RNA interference have shown that NADPH oxidase (involved in ROS – $O_2^{\bullet -}$ generation) inhibitors did not block the constitutive autophagy in this plant (Liu and Bassham, 2010). Previous research on aged apple seeds has shown the NO-dependent stimulation of the expression of genes encoding NADPH oxidase homologs (especially in the axes of embryos isolated from seeds aged for 21 days) (Ciacka et al., 2022). Therefore, we may assume that the autophagy in this tissue can be regulated *via* various pathways depending on the progression of tissue aging, but further research is needed to confirm this statement.

The Tor-dependent regulation of autophagy in aged apple seeds may also be controlled by auxin. After 14 days, the mechanism of NO action in the mitigation of the seed aging effect is also connected with the decrease in *Saur* (the auxin response gene) expression. *Saur* analyzed in our experiment, encodes Saur36 protein in Arabidopsis. According to the Uniprot database, this protein is responsible for the regulation of seed germination (**Supplementary Table 1**). Moreover, Saur36 functions as a positive regulator of leaf senescence and may mediate auxin-induced leaf senescence (**Supplementary Table 1**). After 14 days of aging of apple seeds, the lower expression of *Saur* in the axes of NO-treated embryos, compared to the control, may indicate the decrease in auxin level in this tissue. It has been shown that auxin triggers Tor activation (Schepetilnikov et al., 2013). It suggests that this phytohormone is connected with the negative regulation of autophagy. It depends on the type of stress affecting plant tissue (Pu et al., 2017). In the experiment with Arabidopsis mutant with *Tor* overexpression, the addition of auxin–1-naphthaleneacetic acid (NAA) inhibited autophagy during nutrient deficiency, salt, or osmotic stress, but not, e.g., during oxidative stress. Moreover, NAA treatment was unable to block autophagy induced by a Tor inhibitor or by a mutation in the *raptor1B* (the knockout line), indicating that auxin is upstream of Tor in the regulation of autophagy (Pu et al., 2017). It means that autophagy can be controlled *via* Tor-dependent and -independent pathways in plant tissue, and the Tor signaling pathway requires auxin as its regulator. Therefore, in the apple embryos of aged seeds, NO-treatment did not only impair the expression of genes encoding elements of the Tor complex, but it could also affect Tor activity through the regulation of auxin level. The direct impact of NO on Tor activity (*via* nitration/*S*-nitrosation of TorC1 components) or indirect [*via* nutrient availability (Pu et al., 2017)] needs further investigation. The possible regulation of autophagy by NO and the extension of seed longevity *via* TorC1 activity modulation also needs to be experimentally demonstrated. Taking all the above together, in non-stress conditions, proteins encoded by *Tor* and *Raptor* promote seed germination and seedling development, while when the seeds are aged, it is necessary to temporarily lower the level of TorC1 elements to recycle cell components and/or repair the oxidative damage.

Conclusion

Prolonged conditions of accelerated aging resulted in visible symptoms of apple seeds aging and a drastic decrease in their germinability. Our current study pointed to the universal usage of *m*-Tyr as a marker of cellular aging, although it should be mentioned that more studies on Phe metabolism are necessary to fill the knowledge on the role of these amino acids in the regulation of aging processes. We have demonstrated that NO may be used as a remedy against some deteriorations occurring in seeds during artificial aging. The beneficial effect of NO on the restoration of proper metabolism of seeds was observed predominantly in the tissue, in which no severe damages were induced by prolonged high moisture and high temperature. The upregulation of genes encoding some Lea or Hsp after NO treatment of embryos of aged seeds explained the involvement of this RNS in the preservation of seed longevity, which is strengthened by the downregulation of *Tor* and *Raptor*. Alterations in the transcript levels of the genes indicate a putative important role of NO in the regulation of aging processes in seeds, but they cannot be directly related to the activity of the protein. These data point to the requirement for further analysis of the level and activity of individual proteins - the products of the analyzed genes. The regulatory action of NO in seed aging may be linked to its reactivity with RNA (nitration and oxidation). Recently, we have demonstrated that NO fumigation of apple embryos isolated from seeds subjected to accelerated aging has modified RNA nitration and oxidation level (Ciacka et al., 2022), although we have no data on which specific transcripts have been modified. It cannot be ruled out that the downregulation of some of the genes analyzed in this experiment was due to such NO-direct regulation. The differences in transcript levels after NO application in the embryos after 14 vs. 21 days of aging and 14 vs. 7 days of aging may be related to the variant use of stored mRNA (degradation vs. metabolic use), as well as dissimilar rate of newly synthesized mRNA. We propose that NO application may stimulate the use of stored mRNA, lower the oxidation level of RNA, and stimulate synthesis *de novo* of mRNA (**Figure 8**).

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE207591, GSE207591.

Author contributions

KC: conceptualization, methodology, investigation, data curation, writing – original draft preparation, and writing – review and editing. UK: conceptualization, writing – review and editing, and funding. MT: investigation and writing—editing. AW: investigation. AG: writing – review and editing. All authors have read and approved the final version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.929245/full#supplementary-material

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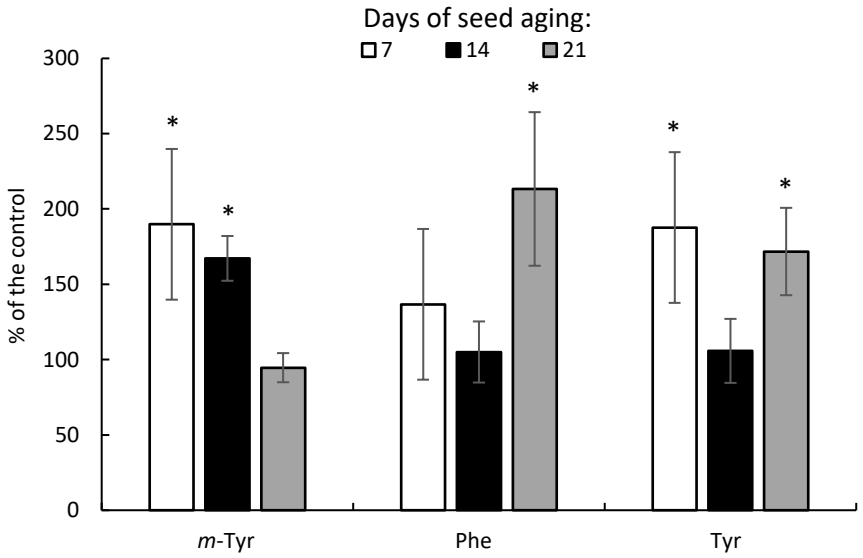
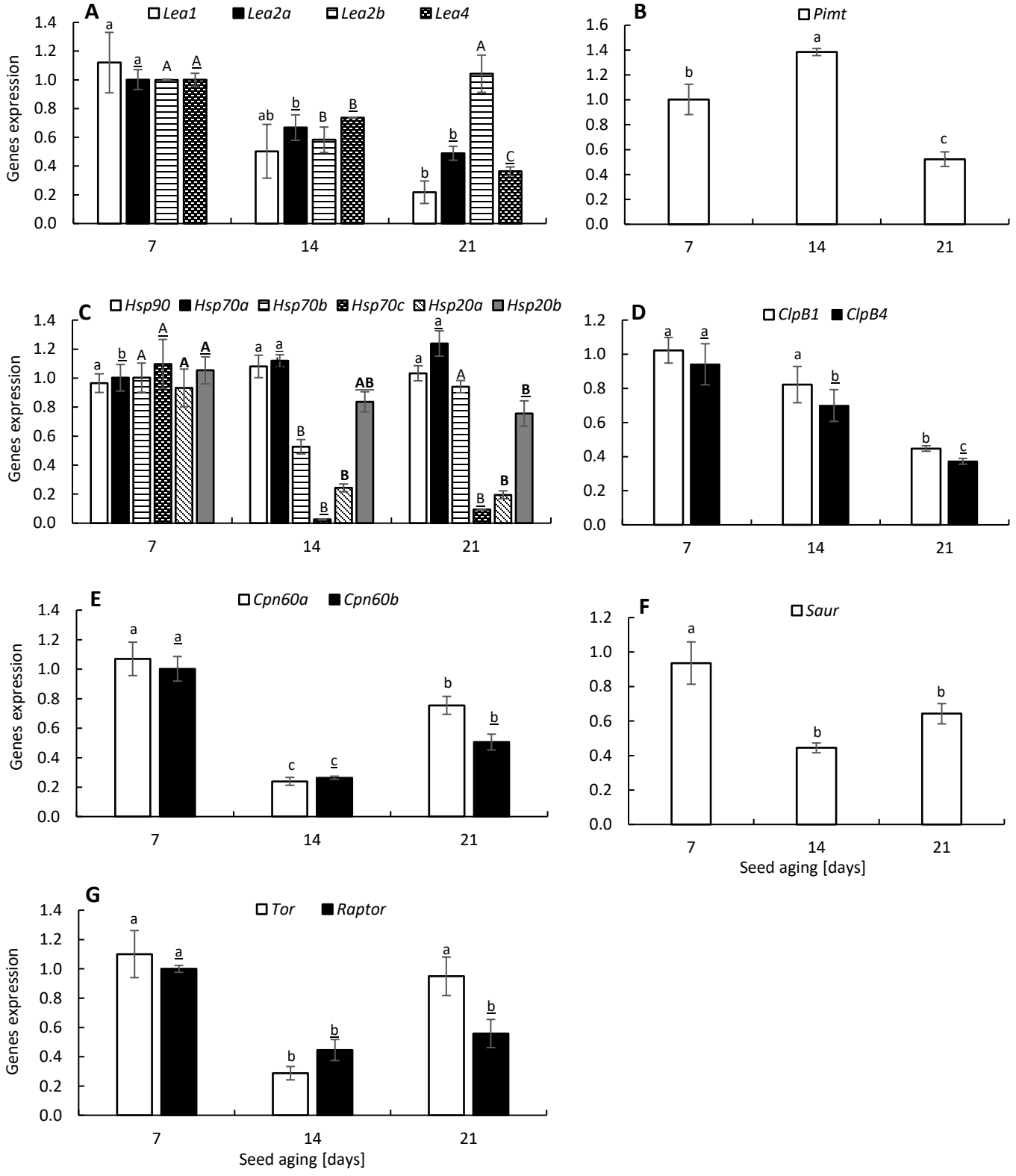


Supplemantary Figure 1. Changes in the content of *m*-Tyr, Phe and Tyr in the axes of embryos after NO treatment expressed as a percentage of the control (content of the amino acids in the axes of aged seeds). Values are average ± SD of 3 repetitions. Asterisks indicate significant differences determined by Student's t-test ($P<0.05$).



Supplemantary Figure 2. Changes in the expression of *Lea1*, *Lea2a*, *Lea2b*, *Lea4* (**A**) *Pimt* (**B**) *Hsp90*, *Hsp70a*, *Hsp70b*, *Hsp70c*, *Hsp20a*, *Hsp20b* (**C**), *ClpB1* and *ClpB4* (***D***), *Cpn60a* and *Cpn60b* (**E**) *Saur* (**F**) *Tor* and *Raptor* (**G**) in the embryonic axes of apple seeds subjected to accelerated aging for 7, 14, and 21 days. Values are average ± SD of 3 repetitions. After one-way ANOVA, homogenous groups were evaluated using the Tukey's test (P<0.05) and signed as a-c (*Lea1*, *Pimt*, *Hsp90*, *ClpB1*, *Cpn60a*, *Saur*, *Tor*), a-c (*Lea2a*, *Hsp70a*, *ClpB4*, *Cpn60b*, *Raptor*), A-B (*Lea2b*, *Hsp70b*), A-C (*Lea4*, *Hsp70c*), **A-B** (*Hsp20a*) **A-B** (*Hsp20b*).

Supplementary Table 1. List of primers. The name of the corresponding protein in Arabidopsis and its reference no. in the Uniprot database were marked using Asterisk (*)

Gene symbol	Primer sequence 5'-3'	Encoded protein	Gene ID (GDR, NCBI)
Tor	F:ATCTGGTGGAGCAGCTTTGC R:TCGTTATACCGTTCTGCATCAC	Target of rapamycin/ *Serine/threonine-protein kinase TOR Q9FR53	MD10G1112000 MDP0000177219 MDP0000258439
Raptor	F:TGAATGCTGTTGTGGATTGG R:GCTACAATCTGATGAAGAAGG	TOR binding protein/ *Regulatory-associated protein of TOR 1 Q93YQ1	MDP0000061332 MDP0000061330 MDP0000209431
Saur	F:CATGTCAAGGCTGCTCACCTGG R:CCAACCGCTTGTAACCCGAC	Small auxin up-regulated RNA/ *Auxin-responsive protein SAUR36 O22150	MDP0000807487
Pimt	F:GATGGTGGATCATTTGAAGAG R:CTATCAATAGTCTCCATCACTTCAG	Protein l-isoaspartyl methyltransferase/ *Protein-L-isoaspartate O-methyltransferase 1 Q42539	MDP0000314934 MDP0000299692
Lea1	F:CACCAGATGTCCGCACTTC R:ACCCGTGTTGGTCCCAATA	Late-embryogenesis abundant protein/ *Late embryogenesis abundant protein 46 Q9FG31	MDP0000850643 MDP0000187047
Lea2a	F:TCCACTACTCCTCCACC R:ATATCCACAGCGGCGTC	Late-embryogenesis abundant protein/ *WHy domain-containing protein A0A384KR80	MDP0000808291 MDP0000385140
Lea2b	F:GGATCAAGTACGGCGAGTCCAG R:ACGACTTGGCGGACAGTGT	Late-embryogenesis abundant protein/ *LEA_2 domain-containing protein A0A178VWF6	MDP0000281672 MDP0000552607 MDP0000282151
Lea4	F:GTCGATGATGAAGGCAGTGAC R:GCCCTTCTCTTGAACCTGACT	Late-embryogenesis abundant protein/ *Late embryogenesis abundant protein ECP63 Q9SKP0	MDP0000183836 MDP0000183375 MDP0000305723
ClpB1	F:GAGACGGTTGGACCAGAACA R:CCACTACTCTCTTATGCAACCT	Caseinolytic peptidase B protein homolog/ *Chaperone protein ClpB1 P42730	MDP0000755970 MDP0000217508 MDP0000303015 MDP0000637620
ClpB4	F:GCAGTTCTCTCAGACAGATACATTAC R:TCGGTCTACCTCATCCAACT	Caseinolytic peptidase B protein homolog/ *Chaperone protein ClpB4, mitochondrial Q8VYJ7	MDP0000242288 MDP0000304292 MDP0000304623
Hsp90	F:TTCCAGGCCGAGATCAAC R:GAAAGATCTCCTTGTTGCTGTA	Heat shock proteins 90 kDa/ *Heat shock protein 90-1 P27323	MDP0000254260 MDP0000303430
Hsp70a	F:TTGTCGGGTCAGGGTCTAG R:AGCATTGCACATTGAAGAGCG	Heat shock protein 70 kDa/ *Heat shock 70 kDa protein 16 Q9SAB1	MDP0000682297 MDP0000295388 MDP0000277804 MDP0000279175 MDP0000779762
Hsp70b	F:ATCACCAACGACAAGGGCA R:CTTTGCCTCCACCTTCTTCT	Heat shock protein 70 kDa/ *Heat shock 70 kDa protein 1 P22953	MDP0000322220

Hsp70c	F:CAATGATGCTCAGAGACAGG R:ACCACCAAGATCAAATACAGC	Heat shock protein 70 kDa/ *Heat shock 70 kDa protein 10, mitochondrial Q9LDZ0	MDP0000416692
Cpn60a	F:ACATCTGAAGAAATAGCTCAGG R:CATTCCTTCAACGACCTCCAA	Chaperonin 60 kDa/ *Chaperonin CPN60, mitochondrial P29197	MDP0000859313 MDP0000235765
Cpn60b	F:GCAGAGCATTGATGATAAGCAC R:GTGTTCCCATCAGCAACAGTA	Chaperonin 60 kDa/ *Chaperonin CPN60-like 2, mitochondrial Q93ZM7	MDP0000185591
Hsp20a	F:GAGAGGAAGAGGGAGCAGGA R:AGCCGAAACCTCCTCGTGAAC	Heat shock protein 20 kDa/ *17.6 kDa class I heat shock protein 3 P13853	MDP0000412799
Hsp20b	F:TCCAGGTGGAGGACGACAA R:CACAAACTTCCTCATGAACTTGC	Heat shock protein 20 kDa/ *17.6 kDa class II heat shock protein P29830	MDP0000294594 MDP0000700383 MDP0000418416
Sar1	F: TTGATTTGGGCGGGCATCAGATTG R: TCATCAGAGAGGAGAGCATCCAGC	Small GTP-binding protein	MD04G1194800
Pdi	F: TGCTGTACACAGCCAACGAT R: CATCTTTAGCGGCGTTATCCTTG	Protein disulfide isomerase	XM_008344461.2

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Ciacka Katarzyna, **Tyminski Marcin**, Wal Agnieszka, Gniazdowska Agnieszka, Krasuska Urszula. 2022. Nitric oxide-an antidote to seed aging modifies *meta*-tyrosine content and expression of aging-linked genes in apple embryos. Frontiers in Plant Science 13: 929245. https://doi.org/10.3389/fpls.2022.929245

mój indywidualny udział w jej powstaniu obejmował współuczestnictwo w opracowaniu koncepcji pracy, opracowanie metodyki badań, sprawowanie merytorycznej opieki nad prowadzonymi badaniami oraz oznaczenie ekspresji genów. Analizowałam uzyskane wyniki oraz przygotowałam pierwszą wersję manuskryptu. Uczestniczyłam w redakcji i korekcie manuskryptu oraz pełniłam rolę autorki korespondencyjnej.

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mój indywidualny udział w jej powstaniu obejmował przygotowanie materiału badawczego oraz przeprowadzenie doświadczeń oznaczenia zawartości aminokwasów (*meta*-tyrozyny, tyrozyny, fenyloalaniny). Brałem udział w przygotowaniu publikacji, redakcji i korekcie manuskryptu.

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mój indywidualny udział w jej powstaniu polegał na uczestnictwie w oznaczeniu ekspresji wybranych genów.

Swój wkład oceniam na 5%

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mój indywidualny udział w jej powstaniu obejmował sprawowanie opieki podczas przygotowywania publikacji oraz redakcję i korektę tekstu manuskryptu.

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ORIGINAL ARTICLE

Reactive nitrogen species act as the enhancers of glutathione pool in embryonic axes of apple seeds subjected to accelerated ageing

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Abstract

Main conclusion **Reactive nitrogen species mitigate the deteriorative effect of accelerated seed ageing by affecting the glutathione concentration and activities of GR and GPX-like.**

Abstract The treatment of apple (*Malus domestica* Borkh.) embryos isolated from accelerated aged seeds with nitric oxide-derived compounds increases their vigour and is linked to the alleviation of the negative effect of excessive oxidation processes. Reduced form of glutathione (GSH) is involved in the maintenance of redox potential. Glutathione peroxidase-like (GPX-like) uses GSH and converts it to oxidised form (GSSG), while glutathione reductase (GR) reduces GSSG into GSH. The aim of this work was to investigate the impact of the short-time NOx treatment of embryos isolated from apple seeds subjected to accelerated ageing on glutathione-related parameters. Apple seeds were subjected to accelerated ageing for 7, 14 or 21 days. Isolated embryos were shortly treated with NOx and cultured for 48 h. During ageing, in the axes of apple embryos, GSH and GSSG levels as well as half-cell reduction potential remained stable, while GR and GPX-like activities decreased. However, the positive effect of NOx in the vigour preservation of embryos isolated from prolonged aged seeds is linked to the increased total glutathione pool, and above all, higher GSH content. Moreover, NOx increased the level of transcripts encoding GPX-like and stimulated enzymatic activity. The obtained results indicate that high seed vigour related to the mode of action of NO and its derivatives is closely linked to the maintenance of higher GSH levels.

Keywords Nitric oxide derivatives · Glutathione peroxidase-like · Glutathione reductase · Reactive oxygen species · Redox potential

Abbreviations

GPX-like	Glutathione peroxidase-like
GR	Glutathione reductase
GSH	Reduced form of glutathione
GSSG	Oxidised form of glutathione
HNO	Nitroxyl
NOx	Nitric oxide derivatives
$ONOO^-$	Peroxynitrite
RNS	Reactive nitrogen species
ROS	Reactive oxygen species

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Introduction

Excess reactive oxygen species (ROS), in conjunction with the defective antioxidant system, are factors contributing to ageing (Kurek et al. 2019). Seed ageing is a time-dependent, progressive physiological process that generates various economic and ecological problems. Changing and unpredictable environmental conditions fuel the ever-growing interest in the issue of oxidative stress in plants. Thus, the topic linked to seed ageing should be constantly expanded with new aspects.

ROS and reactive nitrogen species (RNS), including nitric oxide (NO), are regulatory compounds implicating seed physiology (Bailly 2019; Ciacka et al. 2022b). Considering

the complex chemistry and reactivity of molecules derived from the NO radical form ($^{\bullet}$NO), the term "RNS" is limited. Some reactive species formed from NO are compounds that do not possess nitrogen in their structure, e.g. $CO_3^{\bullet-}$ (Hughes 2008; Möller et al. 2019). Therefore, for the purposes of this publication, according to the biochemistry of NO, we will consider RNS as NO and related species/compounds (Hughes 2008). In the presence of oxygen and/or superoxide anion ($O_2^{\bullet-}$), NO forms derivatives, including peroxynitrite ($ONOO^-$) and its protonated form — peroxynitrous acid (ONOOH) (Stamler et al. 1992 and citations therein). The hydrophobic environment accelerates the reaction of NO with oxygen, resulting in the formation of toxic nitrogen dioxide (NO_2), which, may be further converted to dinitrogen trioxide (N_2O_3) in the presence of NO. Recently, the presence of nitroxyl (HNO), a signalling molecule, was confirmed in living cells, in the model plant Arabidopsis (*Arabidopsis thaliana* (L.) Heynh.) (Arasimowicz-Jelonek et al. 2022). Depending on the structure and the number of electrons in the molecule, some RNS act as oxidising, nitrosating, or nitrating agents, which may react with various molecules, such as amines and thiols (Hughes 2008; Möller et al. 2019). Thus, the physical properties of NO, such as its small size, high diffusion rate, and lipophilicity, together with electron-dependent reactivity, make NO a molecule significantly involved in metabolism, acting as a protector or oxidiser (Stamler et al. 1992; Hughes 2008).

The deteriorative impact of overaccumulated ROS on seed vigour is well described (Kurek et al. 2019). ROS are responsible for the irreversible oxidation of essential cellular components such as proteins and nucleic acids. This is of particular importance since both proteins and RNA are accumulated in the seed's embryo and used in the first stages of germination (Bailly 2019; Ciacka et al. 2020, 2022b). To date, the RNS action during seed deterioration has not been fully explored. The "nitrosative door" model presents the bimodal action of RNS in seeds (Krasuska and Gniazdowska 2012; Krasuska et al. 2015; Ciacka et al. 2022a). The final molecular effect (positive or negative) of RNS that react with proteins, fatty acids and nucleic acids depends on the concentration of these reactive compounds. The physiological state of the organism is also relevant in this consideration (Ciacka et al. 2022a). Data on the biological action of NO/RNS in the improvement of the quality of aged seeds are rather scarce. The reduction of the negative effect of accelerated ageing of elm (*Ulmus pumila* L.) seeds was achieved using NO donors application before ageing treatment (He et al. 2018). Similarly, the use of NO donors after the artificial ageing of oat (*Avena sativa* L.) seeds enhanced their vigour (Mao et al. 2018). Prolonged warm stratification at 25 °C (conditions consistent with accelerated ageing) of apple (*Malus domestica* Borkh.) seeds resulted in a decrease in their vigour associated with embryo deterioration and a reduction in NO emissions from the embryonic axes (Dębska et al. 2013). Short-term fumigation with NOx of apple embryos isolated from aged seeds improved their germination in comparison to the control (non-treated embryos) (Ciacka et al. 2022b). ROS, a part of a cellular signalling system, are divided into free radicals e.g. superoxide anion ($O_2^{\bullet-}$), generally characterised by higher reactivity and instability, and non-radical molecules like hydrogen peroxide (H_2O_2) with lower reactivity and longer half-life (Mittler 2017; Considine and Foyer 2021). These compounds regulate the growth and development of plants (Considine and Foyer 2021). ROS influence the cell components directly, and/or are involved in oxidation–reduction (redox) reaction that enables sensing and adaptation to stress conditions. The maintenance of the cellular ROS balance is crucial for proper metabolism. Therefore, the content of each highly reactive molecule of physiological importance must be carefully controlled (the maintenance of homeostasis).

The conditions of low water content in the mature seeds are not conducive to enzymatic activity, so any repair mechanisms are related to the presence of non-enzymatic molecules, which are usually regulators of the redox potential. An important part of the "redox hub" — the core linking the cellular energy system and the net of signalling pathways (Foyer and Noctor 2011) are compounds with cysteinyl (Cys) residues. The biological activity of thiol groups (–SH) in the side chain of Cys depends on the chemical properties of sulphur atoms, which play a key role in reversible redox reactions together with redox signalling (Considine and Foyer 2021). Proteins containing Cys are prone to multiple posttranslational modifications (thiol-based oxidative PTMs) such as glutathionylation or *S*-nitrosation, which can affect the metabolism of various molecules such as glutathione or nicotinamide adenine dinucleotide phosphate (NADPH) (Corpas et al. 2022).

The reduced form of glutathione (GSH: γ-glutamyl cysteinyl glycine), the crucial element of a non-enzymatic, ROS-modulating system, is a water-soluble, low-molecular-weight thiol. As an electron donor, this evolutionary old molecule stabilises free radicals and thus participates in the preservation of a cellular redox state (Kranner et al. 2006; Noctor et al. 2012). Glutathione, located in the cytosol, nucleus, mitochondria and chloroplasts, is a vital compound for seeds (Cairns et al. 2006; Diaz-Vivancos et al. 2015), especially since orthodox seeds may lose ascorbic acid (another non-enzymatic antioxidant compound) during the desiccation phase, and the amount of which in dry seeds is minimal (Tommasi et al. 1999). GSH is the most abundant non-protein thiol in aerobic organisms, as its concentration can be measured even in mM (Noctor et al. 2012), which indicates its unique value. The oxidised form of GSH is GSSG. By controlling ROS content, GSH acts as a regulatory molecule responsible for cell fate (Diaz-Vivancos et al. 2015). The standard redox potential of glutathione in pH 7 is

− 240 mV (Noctor et al. 2012). Thus, the half-cell reduction potential ($E_{GSSG/2GSH}$) may serve as a marker of the current physiological state of the cells. Elevated values of this indicator point to the stress conditions or ageing progress. The values range between − 180 and − 160 mV is linked to the initiation of irreversible lethal alterations of the cell (Kranner et al. 2006).

ROS detoxification leads to GSH oxidation and GSSG formation. Such conversion of glutathione may be enzymatically stimulated via the activity of the heme-free thiol peroxidases — glutathione peroxidase (GPX; EC 1.11.1.9) (Pei et al. 2023). GPX utilises GSH and/or other reducing equivalents (Ursini et al. 1995). Depending on the amino-acid sequence, substrate specificity and the differences in the tissue expression, there are eight members of the mammalian GPX family. GPX1 location is the cytosol, GPX2 is the gastro-intestinal enzyme, GPX3 is presented in the plasma, GPX4 scavenges the phospholipid hydroperoxides, and seleno-independent GPX5 is located in the epididymis (Chu 1994; Drevet 2000). GPX6 is only expressed in the human body. The active site of GPX5 and GPX7-GPX8 lacks selenium Cys (SeCys) and is replaced by Cys (Pei et al. 2023). In plants, GPX-like enzymes are not SeCys proteins. There is no clear scientific evidence for the existence of a UGA codon (opal codon) in the gene sequences, responsible for the insertion of a SeCys (Herbette et al. 2002 and citations therein). However, the occurrence of the selenium-dependent GPX in plants cannot be excluded (Fu et al. 2002). The presence of Cys in plants' GPX-like active site lowers its peroxidase activity compared to the animal SeCys isoforms (Herbette et al. 2002 and citations therein). Nevertheless, GPXs-like were localised in various cellular compartments of Arabidopsis (cytosol, mitochondria, chloroplasts, peroxisomes, and apoplast) (Rodriguez Milla et al. 2003), which may prove their special role in plant cells. In plants, alterations in the GPX-like activity as a reaction to the stress factors have been shown (Fu et al. 2002). Moreover, higher activity of this enzyme during seed dormancy alleviation and germination was demonstrated (Krasuska and Gniazdowska 2012; Ciacka et al. 2022a).

The enzyme responsible for the recycling of GSSG to GSH and the maintenance of the high level of GSH in cells is glutathione reductase (GR, EC 1.6.4.2). This enzyme is mostly located in the cellular compartments of high electron flux, i.e. plastids or mitochondria (Couto et al. 2016). GR is a flavoprotein oxidoreductase, which uses flavin adenine dinucleotide (FAD) and NADPH as cofactors that serve as an electron donor to reduce GSSG into GSH (Kranner et al. 2006; Noctor et al. 2012). Increased expression of GR is noted during stress conditions, as was observed for various plant species (Gill et al. 2013). Moreover, it was demonstrated, that apple seed dormancy breakage was accompanied by higher GR activity (Krasuska and Gniazdowska 2012; Ciacka et al. 2022a).

Apple seeds are characterised by deep dormancy, and as they belong to the *orthodox* type, they are subjected to intensive desiccation during seed maturation (Lewak 2011). Due to the low water content, the state of very low metabolic activity inhibits germination and maintains high seed vigour but does not entirely prevent ageing. Slowly progressing deterioration processes of *orthodox* seeds force the use of different laboratory protocols to speed up ageing. However, the effects of such procedures do not fully reflect the changes occurring in seeds during their long-term storage, e.g. in seed banks (Roach et al. 2018). The advantage of using ageing protocols is to fasten the appearance of metabolic alterations resulting in the accumulation of toxic compounds. This is somewhat of a compromise in the ageing research, especially useful for *orthodox* seeds. For apple seeds, the protocol of accelerated ageing was described in detail by Ciacka et al. (2022b).

As we demonstrated previously, apple embryos isolated from aged seeds, shortly treated with NOx, were characterised by a higher germination rate and a higher total free radicals removal capacity. NOx application implicated ROS metabolism, lowering the level of total oxidised RNA (Ciacka et al. 2022b). Furthermore, NOx fumigation of embryos of artificially aged apple seeds increased the level of phenylalanine and tyrosine, mitigating the accumulation of *meta*-tyrosine, a non-protein amino acid, whose level increased in a highly oxidative environment (Ciacka et al. 2022c). Such treated, aged embryos were also characterised by alterations in the expression of genes associated with the regulation of degenerative processes (Ciacka et al. 2022c, b). The results indicate that NO may be a molecule that improves the quality of aged seeds. This effect is most likely related to the modulatory role of NO on ROS metabolism.

NO as a "remedy" against seed ageing, regulates the detrimental effect of ROS accumulation. Taking into account that GSH prevents redox imbalance during oxidative stress, it is valuable to combine the role of NO as a molecule that mitigates the effects of seed ageing with glutathione metabolism. Thus, in the current work, we focussed on the influence of NOx on GSH and GSSG content, GSH:GSSG ratio and values of $E_{GSSG/2GSH}$ in the axes of apple embryos isolated from seeds subjected to artificial ageing for 7, 14, and 21 days, cultured for 48 h under optimal conditions. We analysed transcript levels of genes encoding GPX-like and GR, together with the enzymatic activity. The aim of our work was to link the beneficial role of NO in the maintenance of seed vigour of aged *orthodox* seeds with a multifaceted compound — glutathione. Publications on the participation of the glutathione system in *orthodox* seeds, especially in terms of NO impact on seed ageing are unique.

Materials and methods

Plant material

Experiments were conducted on axes of apple (*Malus domestica* Borkh. cv. Antonówka) embryos isolated from seeds (Fig. 1) subjected to accelerated ageing treatment, as described in Ciacka et al. (2022b). Fully ripe apple fruits were acquired from the same local orchard WIKPOL (Dąbrowa, Poland), harvested in 2022 and 2023. The seeds were manually isolated and dried out on air at room temperature. Before use, dried seeds were stored in a glass container at 5 °C for dormancy preservation. To assess seed quality, germination tests were performed each year on new seed material.

The procedure of accelerated ageing was carried out as follows: seeds were placed in glass Petri dishes (100 seeds per dish ⌀ 15 cm), mixed with sterile sand, and watered. The humidity was set to 50%. Seeds were stored at 35 °C for 7, 14 and 21 days. During the process, seeds were thoroughly stirred two times a week. After the ageing treatment, seeds were washed from sand, and seed coats were removed.

Isolated apple embryos were subjected to short (3 h) treatment with NOx. Embryos (50 per container) were placed in a sealed glass container (0.5 L) and fumigated with gaseous NOx produced in a reaction of 14.5 mM $NaNO_2$ with 0.2 M HCl (Yamasaki 2000; Ciacka et al. 2022b). After the procedure, embryos were rinsed with distilled water. To assess whether a given seed ageing treatment produces similar effects, germination tests of control and NOx-treated embryos isolated from aged seeds were performed.

Embryos treated with NOx and those non-treated (the control, embryos of aged seeds) were placed in Petri dishes filled with moistened filter paper for 48 h in a growth chamber (Sanyo MLR-35OH) at 25/20 °C day/night, a 12/12 h photoperiod, the light intensity of 100 µmol PAR m^{-2} s^{-1}. After 48 h, the embryonic axes were isolated from the embryos and used in the experiments.

Germination test

Apple embryos isolated from aged seeds, non-treated (the control) and NOx-treated (NO) were placed in Petri dishes (15 embryos per dish ⌀ 9 cm) filled with moistened filter paper with water. In addition, aged embryos were placed in Petri dishes on filter paper moistened with 1 mM GSH water solution. Embryo culture was performed in a growth chamber (Sanyo MLR-35OH, in conditions described above) for 7 days. Every day, observations of germinating embryos were made. The number of germinated embryos (with gravitropic bending of the embryonic root), the uniformity of seedling cotyledons' greening, and the seedlings' general phenotype were noted. The 1 mM solution of GSH was changed every 2 days. Germination tests were repeated three times.

Measurement of GSH and GSSG content

For the measurement of GSH and GSSG concentration, the method of separation with the reversed-phase HPLC and fluorescence detector was used. The procedure was conducted according to Kranner (1998) with modifications.

Approximately, 30–35 embryonic axes (30 mg) were grounded in liquid nitrogen. The obtained tissue powder was transferred to the Eppendorf probes and homogenised with 100 mM HCl, 2% (w/v) PVPP and 1 mM EDTA. Then, the samples were vigorously vortexed, incubated at RT for 20 min and centrifuged (4 °C, 12,000 *g*, 20 min).

Determination of total glutathione content was conducted by mixing 20 µl of obtained supernatant with 30 µl of 200 mM CHES (Sigma-Aldrich, C8210) buffer (pH 9.4) and 5 µl of 3 mM DTT (Roth, 6908.1), followed by 1-h incubation at RT. For derivatisation, 3.4 µl of 15 mM monobromobimane (MBBr; Sigma, 69,898) was added. The reaction was carried on for 15 min at RT in darkness until it was terminated by the addition of 3.4 µl of 20% acetic acid. Into the HPLC system, 10 µl of the sample was injected.

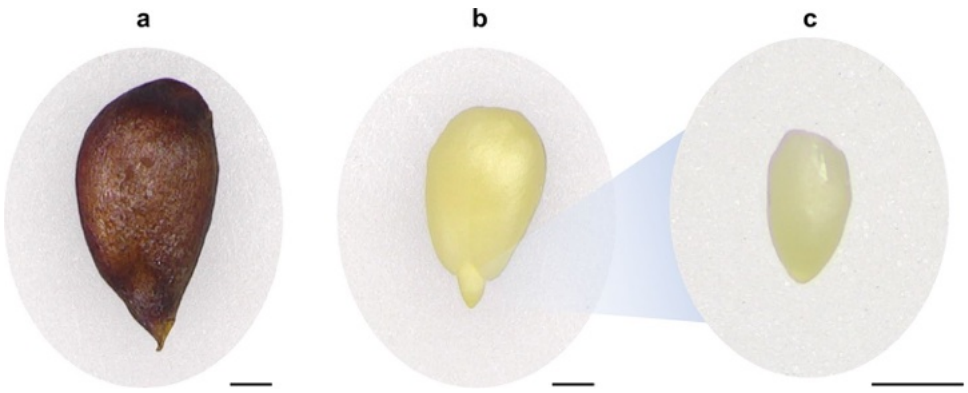

Fig. 1 Representative photos of the experimental material. Apple seed (**a**), apple embryo (**b**), isolated apple embryonic axis (**c**). Bar = 1 mm

Measurement of GSSG concentration was performed by mixing 133 µl of the obtained sample with 10 µl of 50 mM N-ethylmaleimide (NEM, Acros organics, 2928) and 200 µl of 200 mM CHES. To discard the NEM residues, the mixture was combined with 350 µl of toluene (Sigma-Aldrich, 203-625-9) and properly vortexed for 30 s. After the separation of phases, the toluene phase was disposed of. Removal of excess NEM was performed 6 times. Then, 50 µl of the sample was mixed with 5 µl of 3 mM DTT and incubated for 1 h at RT in darkness. Derivatisation of samples was performed as described above.

Separation of bromine derivatives was performed with Bionacom Velocity C18 LPH (4.6 * 150; 3 µm) column held in the oven set at 35 °C. For peak detection, the FP-2020/2025 Intelligent Fluorescence Detector (JASCO) (Ex 390 nm; Em 478 nm) was used. As mobile phases, 0.25% acetic acid containing 5% methanol, adjusted to pH 3.9 with 5 M NaOH (A) and 100% methanol (B) were used.

The gradient program used for sample separation: 0–5 min 80% A, 5–30 min 80–75% A, 30–38 min 75–70% A, 38–45 min 70–0% A, and 45–50 min 0–80% A. The flow of the mobile phase was set at 1 ml min^{-1}.

The standard curve was prepared with GSH as a standard and processed as described above. Measurements were done in four biological replicates, each in two technical repetitions. Total glutathione pool, GSH and GSSG content were presented as nmol g^{-1} FW.

Glutathione half-cell reduction potential

$E_{GSSG/2GSH}$ (in mV) was calculated based on the Nernst equation (Kranner et al. 2006; Morscher et al. 2015) as described in Ciacka et al. (2019):

$$E_{GSSG/2GSH} = -E^{0\prime} - RT\,[nF]^{-1}1n\,[GSH^2GSSG^{-1}]$$

where R is the gas constant (8.314 J K^{-1} mol^{-1}); T is the temperature in K during ageing (35 °C); n is the number of transferred electrons ($2GSH \rightarrow GSSG + 2H^+ + 2e^-$); F is the Faraday constant 9.6485 10^4 C mol^{-1}; E0′ is the standard half-cell reduction potential of glutathione (− 0.240 V). As for glutathione measurement, the calculations were done in four biological replicates, each in two technical repetitions.

Measurement of GPX-like activity

The determination of GPX-like activity was performed according to Flohe and Günzler (1984) with modifications described in Krasuska and Gniazdowska (2012). Apple embryonic axes (25 mg) were homogenised in 50 mM K-phosphate buffer (pH 7.5), with 0.1% triton X-100, 5 mM DTT (Sigma, D0632), 5% (w/v) glycerol, 0.1% protease inhibitor cocktail (Sigma, P9599) and 2% (w/v) PVPP on ice. The sample was centrifuged at 13,000 g, 4 °C for 10 min and the supernatant was desalted using concentrator PES, 3 K MWCO (Thermo Scientific) at 13,000 *g*, 4 °C for 20 min. The obtained desalted supernatant (25 µl) was incubated with 125 µl of 50 mM K-phosphate buffer (pH 7.5), 25 µl 0.1 M aminotriazole, 25 µl of 2.5 mM GSH and 2.5 U of GR (Sigma G3664) for 10 min at 30 °C. Then, to the mixture, 12.5 µl of 3% H_2O_2 and 25 µl 2 mM β-NADPH (Sigma-Aldrich, N5130) were added.

The activity of GPX-like was measured as the decline in the absorbance at 340 nm. The measurement was performed with the microplate reader (Clariostar Plus, BMG LabTech). The GPX-like activity was expressed as nmol NADPH min^{-1} mg^{-1} protein. Experiments were done in three biological replicates, in three technical repetitions.

Measurement of GR activity

The activity of GR was determined as described by Esterbauer and Grill (1978) with modifications. Apple embryonic axes (25 mg were homogenised in 50 mM K-phosphate buffer pH 7.5 containing 5 mM DTT, 5% (w/v) glycerol, 0.1% (w/v) triton X-100, 0,1% (v/v) protease inhibitor cocktail and 2% (w/v) PVPP on ice. Then, the sample was vortexed, centrifuged (13,000 g, 4 °C for 10 min) and the supernatant was desalted with centrifugal concentrator PES, 3 K MWCO (Thermo Scientific) at 13,000 *g*, 4 °C for 20 min.

Protein extract (40 µl) was added to 175 µl of K-phosphate buffer pH 7.5 and 25 µl of 5 mM GSSG (Sigma G-6654). Then, the mixture was incubated at 30 °C for 10 min. The reaction was induced by 25 µl of 2 mM NADPH and was carried out at 30 °C for 5 min. The activity of GR was measured as the decline in absorbance at 340 nm (microplate reader Clariostar Plus, BMG LabTech). Simultaneously, the reaction was held without GSSG addition. GR activity was expressed as nmol NADPH min^{-1} mg^{-1} protein. Experiments were conducted in three biological replicates, in three technical repetitions.

Measurement of protein concentration

For calculations of enzymatic activity, the protein concentration in the apple embryonic axes was determined using a Bradford reagent (Bradford 1976). The standard curve was prepared with serum bovine albumin (BSA — Sigma, A7030) as a standard.

Gene expression analysis

The measurement of gene expression in the axes of apple embryos was done using Bio-Rad CFX Connect™ Real-Time PCR Detection System Total RNA was isolated using RNAzol® RT (Sigma, R4533), according to the

manufacturer's guidelines. 200 ng of total RNA was taken for cDNA synthesis. The cDNA was synthesised using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, #K1622) in a total volume of 10 µl. The final product was diluted 3.5 times. 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, 1 µl cDNA, 1 µl primer, 4 µl H_2O). Specific primers were designed based on nucleotide sequences available in the GenBank database on the National Center of Biotechnological Information (NCBI) and in the Genome Database for Rosaceae (https://www.rosaceae.org/) (Table S1). Gene names were defined based on similarity to the sequences of Arabidopsis genes.

Expression levels were normalised using two reference genes and calculated using the method described by Vandesompele et al. (2002) and Hellemans et al. (2007). Experiments were done in three biological replicates, in three technical repetitions.

Statistics

Data were analysed using the Statistica13 Software. All data were obtained in at least 3 independent experiments with at least 2 repetitions each and presented as mean values ± SD. After two-way ANOVA, homogenous groups were evaluated using the Duncan's test. For the gene expression analysis, the *t* test was used for group comparison.

Results

Ageing effect of apple seeds was mitigated by the treatment with NOx or GSH

Germination test of embryos isolated from aged seeds revealed numerous alterations in embryo/seedling morphology (Fig. 2). The process of ageing resulted in deterioration of seed vigour, which was manifested by rotten embryos, or non-germinated (with inhibited axes' elongation) embryos with green cotyledons, or seedlings with only one green cotyledon. The detrimental effect of ageing was intensified as the procedure was prolonged (14 and 21 days). NOx fumigation led to an improvement in the vitality of developing seedlings. Although not reversed, the ageing effect was mitigated by NOx treatment, especially after 14 and 21 days of ageing. Seedlings developed from embryos subjected to GSH water concentration, similarly to those treated with NOx exhibited less morphological abnormalities and did not manifest such tissue degeneration as the control (Fig. 2).

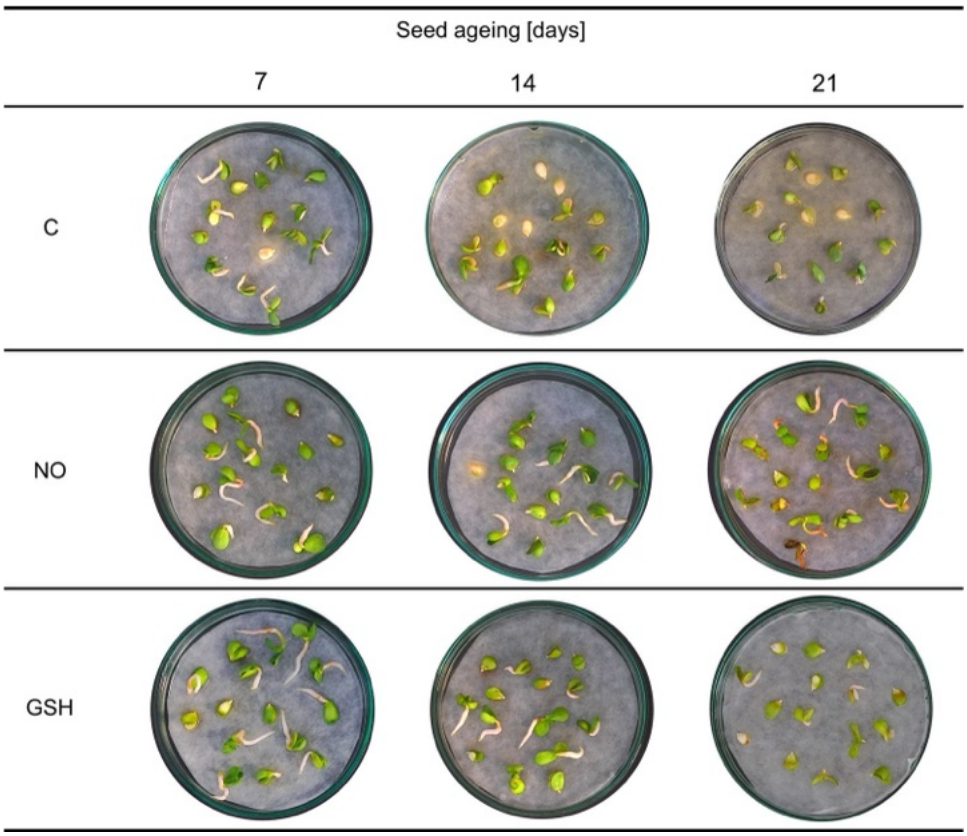

Fig. 2 Representative photos of apple seedlings developing from germinating embryos isolated from seeds subjected to accelerated ageing for 7, 14 and 21 days, after 7 days of the culture under optimal conditions (detailed description in the text). Control embryos (C), embryos briefly treated with NOx (NO) and embryos germinating in the presence of 1 mM GSH (GSH). Germinating tests were conducted three times

NOx and GSH enhanced the germination of embryos isolated from aged apple seeds

Apple embryos isolated from seeds subjected to 7-day accelerated ageing after 7 days of the culture germinated in approx. 60% (Fig. 3). NOx treatment significantly increased the germination of these embryos, up to 70%. Using a GSH solution, only a slight and insignificant increase in germination was observed (65%).

Extending the duration of ageing (14 and 21 days — control) led to a reduction in the number of germinated embryos to 40% and 30%, respectively (Fig. 3). Fumigation with NOx as well as GSH application improved the germination of embryos isolated from seeds aged for 14 days by 40%. After 21 days of ageing, both embryo treatments resulted in an increase in germination by 60%.

NOx affected the level of glutathione after the shortest and the longest duration of seed ageing

The concentration of GSH in the axes isolated from embryos of apple seeds subjected to ageing for 7 days, after 48 h of embryo culture, was approximately 445 nmol g^{-1} FW and did not change significantly during prolonged ageing (Fig. 4a). Treatment of the embryos with NOx resulted in an increase in GSH content in the embryonic axes isolated from seeds aged for 7 and 21 days by 22% and 56%, respectively.

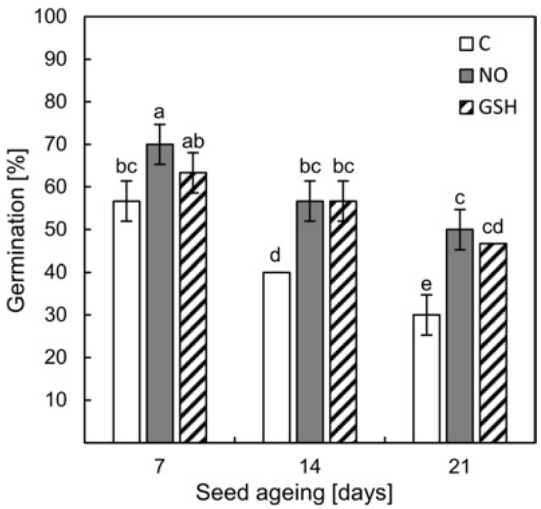

Fig. 3 Germination of apple embryos isolated from seeds subjected to accelerated ageing for 7, 14 and 21 days, after 7 days of the culture under optimal conditions (detailed description in the text). Control embryos (C), embryos briefly treated with NOx (NO) and embryos germinating in the presence of 1 mM GSH (GSH). Values are an average of three biological repetitions ± SD. The homogeneous groups were labelled with lowercase letters, according to the Duncan's post-hoc test ($P < 0.05$)

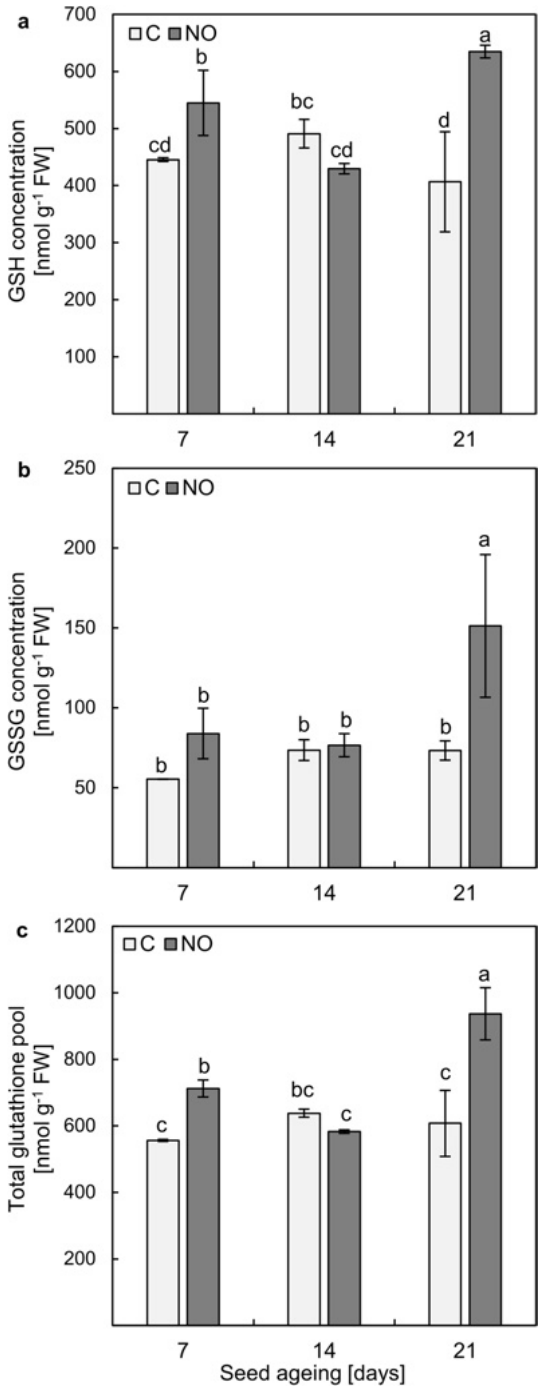

Fig. 4 The concentration of GSH (**a**), GSSG (**b**) and total glutathione pool (**c**) in the axes of apple embryos isolated from seeds subjected to ageing for 7, 14 and 21 days (C—control) or the embryos isolated from aged seeds and treated with NOx. Values are an average of four biological repetitions ± SD. The homogeneous groups were labelled with lowercase letters, according to the Duncan's post hoc test ($P < 0.05$)

No significant changes in GSH concentration were observed in the axes of NOx-fumigated embryos isolated from seeds aged for 14 days in comparison to the control.

In the embryonic axes, the level of GSSG did not differ regardless of how long the seeds were aged (Fig. 4b). Treatment of the embryos with NOx led to a slight increase in GSSG concentration after 7 days of ageing, but the result was not statistically significant. NOx stimulated the accumulation of GSSG only in the axes of embryos of seeds aged for the longest time. In this tissue, GSSG concentration increased 2 times in comparison to the control.

The concentration of the glutathione (both reduced and oxidised—the total glutathione pool) was similar regardless of the duration of seed ageing (Fig. 4c). NOx treatment changed the level of the total glutathione pool after the shortest and longest time of seed ageing. The level of total glutathione in the axes of NOx-treated embryos of seeds aged for 7 days increased by approximately 30% compared to the control. The most pronounced effect of NOx treatment was observed in the embryonic axes of seeds after 21 days of ageing, resulting in a 54% change.

The GSH:GSSG ratio and $EGSSG_{/2GSH}$ did not significantly differ after fumigation of aged apple embryos with NOx

Despite the slight changes in the average values of GSH:GSSG ratio and $E_{GSSG/2GSH}$ in the axes of embryos of aged seeds, treated with NOx or not, statistical analysis did not reveal significant differences (Table 1).

NOx improved GR and GPX activity in the embryonic axes of seeds subjected to accelerated ageing

The accelerated ageing resulted in a decrease in GR activity in the embryonic axes (Fig. 5a). In the axes of seeds aged for 7 days, the activity was 23 nmol min^{-1} mg^{-1} protein. After 14 days of ageing, it decreased by more than twice and remained at the same low level after 21 days of the procedure. NOx treatment increased GR activity in the axes of seeds aged for 7 and 14 days. The enzymatic activity was higher by 21% and 160%, respectively. The stimulatory

Table 1 The GSH:GSSG ratio and half-cell reduction potential in the axes of apple embryos isolated from seeds subjected to ageing for 7, 14 and 21 days (C—control) or the embryos isolated from aged seeds and treated with NOx

		Seed ageing [days]		
		7	**14**	**21**
GSH:GSSG ratio	**C**	8 ± 0.1 a	6.71± 0.93 ab	5.5 ± 0.6 ab
	NO	6.7 ± 1.9 ab	5.6 ± 0.6 ab	4.4 ± 1.4 b
$E_{GSSG/2GSH}$ [mV]	**C**	-256.9 ± 0.2 a	-253.9 ± 0 a	-250.7 ± 2.9 a
	NO	-256.8 ± 5.3 a	-251.7 ± 1.8 a	-256.1 ± 0.5 a

Values are an average of four biological repetitions ± SD. The homogeneous groups were labelled with lowercase letters, according to Duncan's *post-hoc* test ($P < 0.05$).

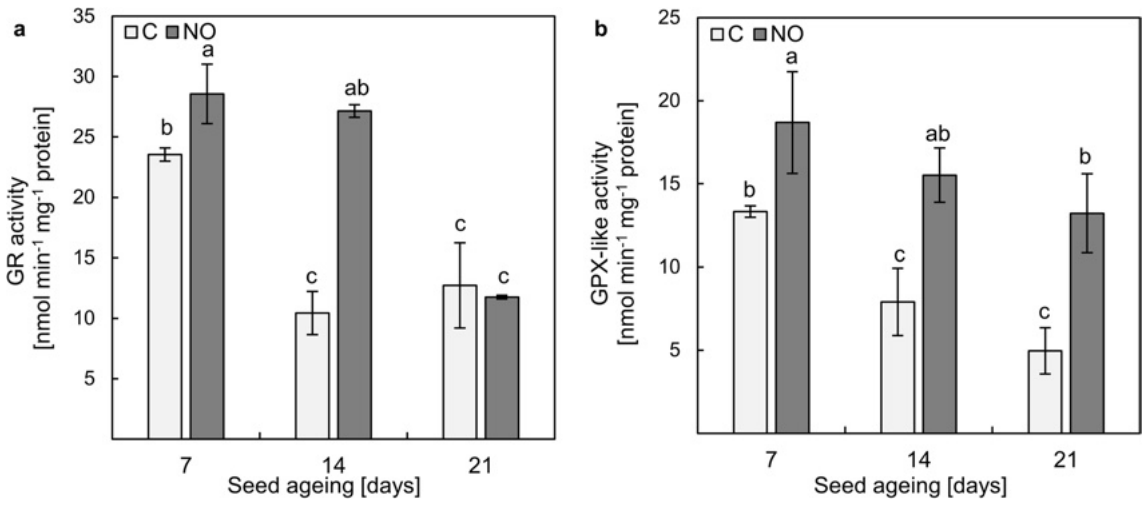

Fig. 5 The activity of GR (**a**) and GPX-like (**b**) in the axes of apple embryos isolated from seeds subjected to ageing for 7, 14 and 21 days (C—control) or the embryos isolated from aged seeds and treated with NOx. Values are an average of three biological repetitions ± SD. The homogeneous groups were labelled with lowercase letters, according to the Duncan's post hoc test ($P < 0.05$)

effect of NOx application on GR activity was not observed in the axes of seeds aged for 21 days (Fig. 5a).

In the embryonic axes, the activity of GPX-like decreased as a result of prolongation of the duration of ageing (Fig. 5b). After 7 days of ageing, the enzyme activity was 13 nmol min^{-1} mg^{-1} protein and it decreased by almost 41% after 14 days of ageing. After the longest ageing time, GPX-like activity did not differ from that in the tissue after 14 days of ageing. NOx treatment increased GPX-like activity regardless of the duration of seed ageing (Fig. 5b). In comparison to the controls, after 7 days of ageing, the enzyme activity was higher by 25%, after 14 days of ageing it was doubled, and after 21 days of seed ageing increased almost 3 times.

NOx upregulated the expression of *GR* and *GPX* genes in the embryonic axes of aged apple seeds

The analysis of *GR* transcript levels concerned genes encoding the enzyme localised in the cytoplasm (*GRc*) and plastids (*GRp*) (Fig. 6a). Treatment of 7-day-aged embryos with NOx resulted in the downregulation of both analysed genes. After 14 days of ageing, NOx did not affect the transcript level of *GRp*; however, the level of *GRc* was upregulated by 0.17 units in comparison to the control. In the axes of seeds aged for 21 days, NOx increased *GRc* and *GRp* transcript levels by 0.6 and 0.2 units, respectively.

The transcript levels of genes encoding GPX-like (*GPX2*, *GPX6*, *GPX7*, *GPX8*) were analysed (Fig. 6b). Fumigation of 7-day-aged embryos with NOx resulted in the upregulation of only *GPX2*. The changes in transcript levels of other analysed genes were not significant. In the embryonic axes of seeds subjected to ageing for 14 days, NOx increased the expression of *GPX6*, *GPX7* and *GPX8*. After 21 days of ageing, as a result of NOx exposition, the upregulation of *GPX2*, *GPX6*, and *GPX8* was observed.

Discussion

The rate of ageing of the *orthodox*-type seeds depends on the plant species and storage or environmental conditions. Factors conducive to dormancy preservation, like very slow metabolism resulting from low water content in tissues, allow such seeds to sustain vigour at a high level for a long time. Various molecular mechanisms associated with deep dormancy maintenance or induction of germination of apple seeds have already been well described in the literature (Lewak 2011; Dębska et al. 2013; Ciacka et al. 2019, 2022a). Thus, this experimental material was used in the investigation of the biology of the typical *orthodox* seeds in the context of seed ageing. We established a laboratory protocol for accelerated ageing. The effectiveness of the procedure was confirmed by the viability test (TTC test) and the germination test of isolated embryos (Ciacka et al. 2022b). Prolonged accelerated ageing (21 days) of apple seeds resulted in considerable viability loss and even death of almost 90% of isolated embryos after 40 days of seed ageing. We also confirmed the beneficial effect of short-term treatment with NOx of apple embryos isolated from

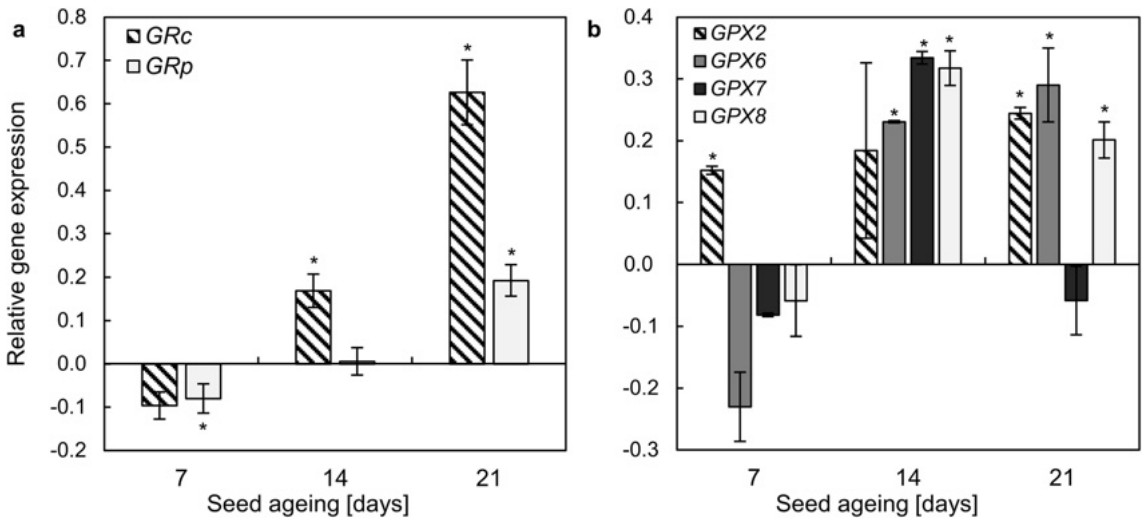

Fig. 6 Changes in the expression of genes encoding GR homologues: *GRc* and *GRp* (**a**) and GPX: *GPX2*, *GPX6*, *GPX7*, *GPX8* (**b**) induced by NOx in the axes of apple embryos isolated from seeds subjected to ageing for 7, 14 and 21 days. Values are an average of three biological repetitions ± SD. Asterisks indicate significant differences between the treated sample compared to the control sample, obtained by the Student's *t* test ($P < 0.05$)

aged seeds, which stimulated total antioxidant activity in the embryonic axes (Ciacka et al. 2022b). RNS-dependent mitigation of the negative outcomes of seed ageing persisted until the 21st day of accelerated ageing and was reflected in a higher germination rate of embryos and undisturbed growth of seedlings, compared to the aged and non-treated control. The results of our previous experiments indicate that the effect of exogenous NOx on apple embryo vigour improvement depends on the seed ageing duration. The same applies to the results presented in this manuscript, as shown in Figs. 2 and 3.

We analysed the impact of NOx application after 48 h of the treatment, while the embryos were cultured on distilled water, under optimal conditions for germination. Based on the obtained results, we concluded that this point of time of the experiment is optimal for observation of the symptoms of disturbance in embryo metabolism as a consequence of seed ageing. In parallel, the RNS-dependent recovery effect and NO-related metabolic alterations in the tissue subjected to ageing were also noticed at this measurement point. As was mentioned in the Introduction section, RNS and other reactive NO derivatives are characterised by a high capacity to alter the structure and function of various functional molecules of cells. These changes include, among others, nitration (tyrosine or tryptophan residues) or *S*-nitrosation, mainly of proteins or peptides with -SH groups (Möller et al. 2019; Ciacka et al. 2022b). Besides the biochemical importance of $ONOO^-$ in living systems, newly confirmed in plant cells, HNO react with thiol-containing proteins/peptides (Arasimowicz-Jelonek et al. 2022). Moreover, this molecule is involved in the regulation of the ageing process, as was demonstrated for Arabidopsis nitroxyl-pre-treated leaves, characterised by lower accumulation of senescence-associated gene transcripts. The kinetics of HNO depend on the redox status of the cell, and reductive conditions promote the formation of this NO derivative (Arasimowicz-Jelonek et al. 2022).

In this work, we focussed on the exogenous RNS impact on glutathione (GSH and GSSG) pool and metabolism in the axes of aged apple embryos, which is responsible for maintaining cell viability, redox signalling and various biochemical processes. GSH is engaged in the regulation of the thiol-disulphide status of the proteins that affect their function. This may especially concern redox-sensitive Cys residues in proteins and peptides, which have low pKa values (3–6) and are known as acidic thiols. Such thiols are highly susceptible to ROS and/or RNS attacks (Diaz-Vivancos et al. 2015). Data on the use of GSH to improve the quality of aged seeds are limited. The positive impact of the application of GSH on the germination of aged *Elymus sibiricus* (L.) seeds was noted (Yan et al. 2017). In addition, artificially aged oat seed primed with 1 mM GSH was characterised by a higher germination rate in comparison to the non-primed control (Xia et al. 2020). In our study, we compared the effect of 1 mM GSH application on the germination of embryos isolated from aged seeds to the germination of the NOx-treated embryos isolated from aged apple seeds. The most beneficial effect was noted for embryos isolated from seeds subjected to artificial ageing for 14 and 21 days. Both the percentage of germinated embryos (Fig. 3) and typical seedling growth (Fig. 2) in the presence of 1 mM GSH or after NOx treatment were similar. The seedlings were characterised by the cotyledons' greening and the embryonic root's growth. Therefore, this result allows us to assume that the effects of seed ageing may be mitigated by the overlapping actions of both NOx and GSH.

The oxidation of GSH during seed ageing is recognised. The alterations in GSH and GSSG levels were described for tomato (*Solanum lycopersicum* L.) seeds undergoing long-term storage or artificial ageing (De Vos et al. 1994). Artificial ageing of tomato seeds was accompanied by a marked loss of GSH and an increase in GSSG content. The decline in GSH concentration was observed for barley (*Hordeum vulgare* L.) seeds undergoing ageing under seed bank conditions and controlled deterioration treatment (CDT) — the type of artificial ageing protocol (Roach et al. 2018). The decrease in GSH and the increase in GSSG were also noted for aged pea (*Pisum sativum* L.) seeds (Kranner et al. 2006). The extension of the duration of CDT (21 days) of sunflower (*Helianthus annuus* L.) embryos (dormant and non-dormant), regardless of the O_2 concentration in the environment, was accompanied by the decrease in total glutathione content, with a simultaneous increase in GSSG level in the embryonic axes (Morscher et al. 2015). In the case of apple embryos isolated from seeds artificially aged for 7, 14 and 21 days, after 48 h of the embryos culture, no particularly significant changes in the GSH content (Fig. 4a), and only a slight increase in GSSG level after 14 and 21 days of seed ageing (Fig. 4b) were observed. It has been noted that the higher GSSG content occurs in aged seeds of different plant species before their imbibition as a consequence of increased ROS levels and intensified oxidation reactions (Kranner et al. 2006; Morscher et al. 2015; He et al. 2018). During the imbibition of aged and non-aged tomato seeds, a serious decrease in GSSG content was demonstrated. In parallel, the significant increase in GSH content in non-aged tomato seeds, after 3 days of imbibition, was linked to the visible symptoms of germination (De Vos et al. 1994). Although short-term NOx treatment of the embryos isolated from 14-day-aged apple seeds did not alter GSH and GSSG levels in comparison to the control (Fig. 4a, b), it increased the GSH level (Fig. 4a) as well as the total glutathione pool (Fig. 4c) after the shortest and the longest time of ageing. The increase in the GSH content in such tissue may point to the induction of the germination process. Especially that, the enhanced GSH level was reached by fully non-dormant,

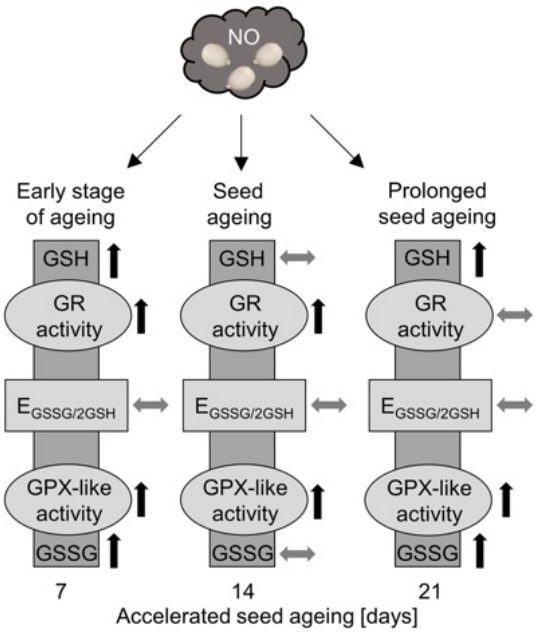

Fig. 7 The role of NO and NO derivatives in embryo vigour maintenance by alteration of glutathione metabolism in the axes of embryos of aged apple seeds. The ageing process was divided into three phases. The state of an embryo isolated from 7-day-aged seeds is considered an early stage of ageing; after 14 days of accelerated ageing, symptoms of the deterioration processes are observed; prolonged seed ageing refers to advanced detrimental events

ready-to-germinate apple embryos (Ciacka et al. 2022a). Moreover, short-term (3 h) treatment of dormant non-aged apple embryos with NOx resulted in stimulation of their germination (Gniazdowska et al. 2010). The slight decrease in GSH content in the axes of NOx-fumigated apple embryos isolated from seeds aged for 14 days confirms our observations regarding the variability of RNS action depending on the duration of the seed ageing treatment (Fig. 7) (Ciacka et al. 2022b). One probable explanation is the possibility of *S*-nitrosoglutathione (GSNO) formation, which acts as an intracellular NO reservoir and transporter (Corpas et al. 2013). The change in GSNO content was analysed in the axes of apple embryos during the transition from the dormant to the non-dormant stage, revealing an increased level of this compound characteristic of embryos capable of germination (Ciacka et al. 2019). In elm seeds, the use of NO donors before applying the ageing protocol increased the level of the *S*-nitrosated group (He et al. 2018). In desiccated *recalcitrant Antiaris toxicaria* (Pars.) Lesch. seeds that were NO fumigated, intensified *S*-nitrosation of antioxidant enzymes was noted (Bai et al. 2011). This effect was linked to higher germination rate. It can be assumed that *S*-nitrosation is a universal NO mechanism in improving seed quality. Seed ageing is connected with ROS accumulation; therefore, the measurement of GSNO concentration and the level of *S*-nitrosated proteins in the experimental model described in this work have to be considered in future studies.

The elevated glutathione pool (GSH + GSSG) (Fig. 4c) may indicate a higher antioxidant capacity and intensified total metabolic activity of the cells of the aged apple embryonic axes as a result of the RNS-dependent recovery effect. NOx treatment of elm seeds, before exposing seeds to accelerated ageing increased the GSH:GSSG ratio, compared to the aged (non-treated) control, and a decrease in value of the parameter was obtained when NO scavenger (c-PTIO) was used (He et al. 2018).

We also analysed $E_{GSSG/2GSH}$ as a viability marker (Table 1). After 24 days of CDT of sunflower dormant and non-dormant embryos characterised by decreased germination capacity up to 50% and 60%, respectively, the $E_{GSSG/2GSH}$ reached approx. − 150 mV (Morscher et al. 2015). Although the ageing of apple seeds was also associated with reduced germination capacity of isolated embryos and an increased number of rotten embryos (after 7 days of the culture), the embryos (both non-treated and NOx-treated) remained viable after 48 h of their culture (Ciacka et al. 2022b). Additionally, short-term NOx treatment of apple embryos isolated from aged seeds resulted in a higher percentage of germinated embryos and their higher viability (Ciacka et al. 2022b). The values of $E_{GSSG/2GSH}$ in the axes of aged apple embryos non-treated and shortly treated with NOx remained constant and reached a low value of approx. − 250 mV (Table 1). Thus, the obtained results for $E_{GSSG/2GSH}$ confirmed the results of the TTC test for such embryos (Ciacka et al. 2022b).

The stimulatory effect of NOx treatment on non-aged, dormant apple embryos on GR and GPX-like activity has been demonstrated, and an increase in activities of these enzymes was correlated with the progression of germination sensu *stricte* (Krasuska and Gniazdowska 2012). Accelerated ageing of apple seeds resulted in decreased GR activity (Fig. 5a). Short-term NOx treatment of apple embryos isolated from seeds aged for 7 and 14 days stimulated GR activity in the embryonic axes after 48 h of the embryos' imbibition. The increase in GR activity was also obtained in aged oat seeds as a result of sodium nitroprusside (NO donor) treatment (Mao et al. 2018). Despite higher levels of transcripts encoding GR in the axes of NOx-treated embryos isolated from apple seeds subjected to accelerated ageing for 21 days, the activity was comparable to the control (Fig. 5a). This result was accompanied by a relatively high level of GSSG in this tissue (Fig. 4b). During the artificial ageing of oat seeds, up to the 40th day of seed ageing, GR activity decreased and finally on the 42nd day of the ageing increased, reaching the level of GR activity in non-aged seeds. In parallel, GSH and GSSG contents decreased gradually, but for GSSG the downward trend was more considerable (Sun et al. 2022). This was reflected in

the gradual increase in the GSH:GSSG ratio during the prolongation of seed artificial ageing (Sun et al. 2022). As GR depends on the availability of NADPH, we propose that after prolonged conditions of higher temperature and humidity, progressive metabolic changes may be related to the depletion of this nucleotide. However, regardless of the lower GR activity recorded, GSH content was elevated (Figs. 4a, 5a), which may suggest an enhanced GSH biosynthesis. After the ageing of NO pre-treated elm seeds, the upregulation of the expression of genes encoding enzymes of glutathione formation (γ-glutamylcysteine synthetase and glutathione synthetase) was noticed (He et al. 2018). Moreover, priming the aged oat seeds with 1 mM GSH stimulated GR activity (Xia et al. 2020), indicating that increased GSH levels may have a positive effect on various antioxidant pathways in aged tissues.

As in the case of GR activity, the artificial ageing of apple seeds was associated with a decrease in GPX-like activity in the isolated embryonic axes. Moreover, NOx treatment of apple embryos isolated from these seeds had a stimulatory effect on GPX-like activity (Fig. 5b). In the embryonic axes of sunflower non-dormant and dormant embryos subjected to the CDT, GR activity slightly decreased but only under high O_2 conditions (Morscher et al. 2015). Such conditions also implicated GPX-like activity, which was lower, compared to the GPX-like activity measured in the axes of embryos subjected to CDT under ambient O_2. The ageing of sunflower embryos under ambient O_2 increased the activity of GPX-like in comparison to the non-aged tissue (Morscher et al. 2015).

The alterations in the enzymatic activity, especially in the case of seeds, should be considered together with changes in transcript levels encoding these enzymes. Accelerated ageing is linked to the lower expression of transcripts encoding various proteins of the "repair system" (Ciacka et al. 2022b). Mature pea seeds subjected to accelerated ageing were characterised by lower transcript abundance of *GRc* and *GRp* (Yao et al. 2012). The authors suggested that ageing triggered the degradation of seed-stored transcripts, and proposed that this process also induced inhibition of de novo mRNA synthesis for these genes (Yao et al. 2012). NOx treatment of apple embryos isolated from aged seeds altered the levels of GR transcripts (Fig. 6a). After 7 days of ageing (and 48 h of the culture), NOx fumigation lowered levels of *GRp* compared to the control (Fig. 6a). However, a significant increase in *GRp* and *GRc* transcripts was observed after 14 and 21 days of seed ageing. The same trend was noted for GPX-like genes (especially for *GPX6* and *GPX8*) (Fig. 6b). NOx treatment of elm seeds also led to the higher expression of *GPX*, which may be the explanation for the alterations in GSH content during ageing (He et al. 2018). We propose that NOx supplementation of embryos from the 14th day of seed accelerated ageing influences (directly or indirectly) the transcript levels and expressions of encoding enzymes, resulting in a balanced metabolism of GSH, and maintaining the adequate GSH:GSSG ratio.

GR as well as GPX-like, supported by glutathione, are the essence of the repair machinery during the progression of cellular deterioration. GPX-like enzymes are involved in the detoxification of lipid hydroperoxides and other reactive molecules, and, as non-heme-containing proteins are less sensitive to hydrogen cyanide (HCN) or RNS (Krasuska and Gniazdowska 2012). This is of great importance, especially in the case of apple seeds, in which cyanogenic compounds are metabolised (Krasuska et al. 2023). GPX-like is also considered an enzyme involved in H_2O_2 signal transduction (rather than in its scavenging); therefore, it acts as a regulator of the expression of ROS detoxification-related genes in response to different stress conditions (Bela et al. 2015). Together with GSH, GPX-like is engaged in the regulation of cellular redox homeostasis. Ageing of *orthodox* seeds disturbs redox balance; hence, RNS may be considered as compounds that preserve a more reductive environment of the cells of embryonic axes. Moreover, we have already proposed that the effect of NO depends on the duration of apple seed accelerated ageing (Ciacka et al. 2022b). Thus, we suggest that after 14 days of the ageing of apple seeds, NOx application stimulates GR and GPX-like activities and probably promotes the further conversion of GSH, which, however, requires verification. After 21 days of accelerated ageing, metabolic changes resulting from the progressive deterioration process, alter the NO modulatory effect (stimulation of GSH synthesis).

Summary and perspectives

This research emphasises the importance of the cooperation between glutathione and antioxidant enzymes of glutathione metabolism in maintaining seed vigour. Despite the constant GSH, GSSG levels and GSH:GSSG ratio, and the relatively low value of $E_{GSSG/2GSH}$ in embryonic axes isolated from seeds subjected to artificial ageing, embryos exhibit diminished vigour while developing seedlings possess degraded tissues. No changes in the value of the glutathione-related parameters are the manifestation of the decrease in GPX-like and GR activities, which may result in impairment of ROS homeostasis in aged tissue.

Moreover, the results confirm the earlier conjecture that the anti-ageing effects of RNS depend on the degree of tissue deterioration (Fig. 7). Up to the 14th day of ageing, NOx application stimulated the activity of GPX-like, accompanied by the simultaneous increase in GR activity, which prevents toxic GSSG accumulation. However, after extending the ageing protocol to 21 days, embryo fumigation with NOx provided potential support for ROS scavenging through

increased GPX-like activity. Nevertheless, in this tissue, despite the upregulation of gene expression, no increase in GR activity (likely due to NADPH depletion) led to the accumulation of GSSG. Taking into account the previous studies, the beneficial effects of RNS against seed ageing have a time limit in the application of the treatment. Exceeding the limit synonymous with the advanced ageing processes, entailing irreversible cellular changes results in RNS no longer functioning as molecules exhibiting advantageous properties and even could promote tissue deterioration.

In addition, the conclusion we can draw is that the action of glutathione in the preservation of seed vigour should be considered comprehensively by analysing the entire glutathione metabolism. Considering that the findings from the research indicate additional potential applications for glutathione, an intriguing aspect for exploration would involve investigating the other pathways through which glutathione is engaged. This includes its potential role as a reservoir for NO or its involvement in reactions related to protein glutathionylation. This should be the subject of further investigation.

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Data availability Data are available under reasonable request to the corresponding author.

Declarations

Conflict of interest The authors have no conflict of interest to declare.

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Table S1 List of primers

Gene symbol	Primer sequence 5'-3'	Encoded protein	Gene ID (NCBI/ GDR)
Sar1	F: TTGATTTGGGCGGGCATCAGATTG R: TCATCAGAGAGGAGAGCATCCAGC	small GTP-binding protein	MD04G1194800
Pdi	F: TGCTGTACACAGCCAACGAT R: CATCTTTAGCGGCGTTATCCTTG	protein disulfide isomerase	XM_008344461.2
MdGRc	F:CTCTGTTATCATGTCGAGGAAGATGC R:ATCTGGAGGCACGGACACCA	cytoplasmic glutathione reductase	XM_008382372.3
MdGRp	F: CCAAAGGTTGTGGCTCCTGA R:ACATGCTGCATCTGTGCTCT	plastidic glutathione reductase	XM_008395622.2
MdGPX2	F:GTGGTTTAACACAATCCAACTACAAGG R: GCAAGGAAACGCCAAAATCTCAAA	glutathione peroxidase	MD04G1034400
MdGPX6	F: TTCCCGGCAGATCCTTTTGTGGT R: CTAGCCATTGTACGATTCAATCCG	glutathione peroxidase	XM_008394342.3
MdGPX7	F: GTTGAGAGATACCCACCAACGAC R: TCAAGCTGCGACCAGTTTC	glutathione peroxidase	MD08G1055100 MD15G1042100
MdGPX8	F: TTTGGTGATGAGGAACCGGG R: GGGACCAGCATTATCACCGT	glutathione peroxidase	XM_008385761.3

Warszawa, 3.06.2026

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Rada dyscypliny Nauki Biologiczne
Szkoły Głównej Gospodarstwa Wiejskiego w Warszawie

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Tyminski Marcin, Ciacka Katarzyna, Krasuska Urszula. 2024. Reactive nitrogen species act as the enhancers of glutathione pool in embryonic axes of apple seeds subjected to accelerated ageing. Planta 260: 51. https://doi.org/10.1007/s00425-024-04472-5

mój indywidualny udział w jej powstaniu polegał na przygotowaniu materiału badawczego, przeprowadzeniu doświadczeń: testów kiełkowania, oznaczenia zawartości glutationu zredukowanego i utlenionego, oznaczenia aktywności enzymatycznej i analizy ekspresji genów. Brałem udział w opracowaniu metodyki wykorzystanej w pracy i w analizie otrzymanych wyników. Współprzygotowałem pierwszą wersję manuskryptu, brałem udział w jego późniejszej redakcji i korekcie, a także przygotowałem rysunki i schematy zawarte w pracy. Pełniłem rolę autora korespondencyjnego.

Swój wkład oceniam na 65%

Marcin Tymiński

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mój indywidualny udział w jej powstaniu obejmował sprawowanie merytorycznej opieki podczas przeprowadzania doświadczeń i przygotowania publikacji oraz redakcję i korektę manuskryptu.

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mój indywidualny udział w jej powstaniu obejmował opracowanie koncepcji pracy, sprawowanie merytorycznej opieki nad przygotowaniem publikacji, współprzygotowanie pierwszej wersji manuskryptu oraz jego późniejszą korektę i redakcję. Zapewniłam finansowanie prowadzonych badań.

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NO treatment of embryos isolated from artificially aged apple (*Malus domestica* Borkh.) seeds modifies the pattern of nitrated and carbonylated proteins

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ABSTRACT

Accelerated ageing of orthodox seeds of apple (*Malus domestica* Borkh.), resulting in loss of vigour, is associated with the overaccumulation of reactive oxygen species (ROS) and the depletion of reactive nitrogen species (RNS). Nitric oxide (NO) mitigates the detrimental effects of seed ageing. The aim of this work was to study the effects of NO treatment on the profile of proteins modified by ROS (carbonylated) and RNS (nitrated), in embryonic axes isolated from apple seeds artificially aged for 7, 14, and 21 days. We linked ageing-related deterioration in seeds with the activity of the protein quality control system: proteolytic degradation, ubiquitin-dependent and independent (20S) proteasome pathways. Various storage proteins underwent both carbonylation and nitration, which may be related to their signalling role. Most of the carbonylated proteins belonged to the late embryogenesis abundant (LEA) family. The carbonylation of heat shock protein (HSP) and seed biotin-containing protein (SBP) was also noted. Nearly all identified nitrated proteins were associated with energy generation, suggesting their impairment in seed ageing. NO treatment enhanced the activity of 20S proteasome in aged embryonic axes, revealing its role in protein degradation. Thus, we assume that NO mitigates the negative effects of seed ageing by partially alleviating embryo dormancy, activating its metabolism and stimulating the protein quality control system.

1. Introduction

Seeds are organs that play a crucial function in plant ontogenesis, enabling to spread of species, and thus have a pivotal role in biodiversity conservation and ecological restoration. The maintenance of seed viability depends on internal, molecular mechanisms and external conditions, hence, seed lifespan is genetically imprinted and influenced by environmental factors (Rajjou et al., 2008). Seeds are classified into two types: *orthodox* (generally characterised by longer longevity due to high desiccation resistance) and recalcitrant (sensitive to deep tissue dehydration) (Ciacka et al., 2020a, 2020b; Rajjou et al., 2008; Sano et al., 2016). The main purpose of the global research on the problem of seed ageing is to reduce biological and economic losses, as seeds are not only a sowing material, but also a food for humans and animals. Seeds accumulate proteins, which are rapidly degraded during seed germination (and used as a nitrogen source) or participate in metabolic pathways in post-germination phases (Job et al., 2005).

Seed metabolism (also during the deep dormancy stage) is susceptible to the action of regulatory molecules, specifically reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Bailly, 2019; Ciacka et al., 2022b). Modulators of RONS (ROS and RNS) content, especially non-enzymatic antioxidants, play a crucial role in seed vigour preservation (Tyminski et al., 2024). ROS, particularly free radicals like hydroxyl radical ($^{\bullet}OH$) or superoxide anion ($O_2^{\bullet-}$), react with nucleic acids, proteins, lipids, and sugars. Hydrogen peroxide (H_2O_2) is less reactive and serves predominantly as a signalling molecule (Ciacka et al., 2022a; Møller et al., 2011). Some oxidative modifications of proteins are reversible under physiological conditions (e.g., sulfenylation), and some are not (e.g., carbonylation) (Ciacka et al., 2020b). Oxi-derivatisation, as posttranslational modification (PTM) of proteins, may involve dozens of reactions of variable importance to the cell (Ciacka et al., 2020b; Møller et al., 2011). The incorporation of carbonyls into the structure of proteins, lipids, and nucleic acids defines carbonylation (Dalle-Donne et al., 2006, 2003). Increased protein carbonylation is commonly recognised

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as the consequence of ROS imbalance, and is linked to ageing of various organisms, including seeds (Ciacka et al., 2020b; Levine, 2002; Levine and Stadtman, 2001; Rajjou et al., 2008). Such conditions correlate with lower germination capacity and shorter storability in seed banks (Kalemba and Pukacka, 2014; Rajjou et al., 2008). Despite its irreversibility, protein carbonylation is not a random process and affects specific proteins, which have also been identified in plants (Johansson et al., 2004; Kristensen et al., 2004). The best described pathways leading to protein carbonylation are: direct ROS reaction with the residues of proline (Pro), arginine (Arg), lysine (Lys) and threonine (Thr), metal-catalysed oxidation (MCO) attack on these amino acids residues, the adduction of advanced glycation end products (AGEs), and the incorporation of products of lipid peroxidation (Ciacka et al., 2020b). AGEs are non-enzymatically formed in the presence of reducing sugars, e.g., glucose and ROS. This reaction may be particularly relevant for artificially aged seeds, whose metabolism is disturbed. Various carbonylated proteins have been identified in mature, germinating, and aged seeds (Job et al., 2005; Kalemba and Pukacka, 2014; Rajjou et al., 2008).

The radical form of NO ($^{\bullet}NO$) reacts with other radicals and forms RNS (Hughes, 2008; Möller et al., 2019). The nitration agent, peroxynitrite ($ONOO^-$), is the product of the reaction of $^{\bullet}NO$ and $O_2^{\bullet-}$ and exists also in the protonated form as peroxynitrous acid (ONOOH) (Stamler et al., 1992). Other RNS are nitrogen dioxide (NO_2), dinitrogen trioxide (N_2O_3), and nitroxyl (HNO), signalling molecules also present in plants (Arasimowicz-Jelonek et al., 2022). Depending on the reactivity, RNS participate in oxidation, nitrosation and nitration of various biomolecules (Hughes, 2008; Möller et al., 2019). The function of NO as ROS level modulator depends on its properties: small size, high diffusion rate, and lipophilicity, together with electron-dependent reactivity (Hughes, 2008; Stamler et al., 1992). A stable, NO-related protein modification is nitration, which has been investigated in plants (Lozano-Juste et al., 2011). Stress-induced conditions are linked to the alterations in the content of nitrated proteins. Similarly, to protein carbonylation, nitration is an irreversible modification under physiological conditions. The formation of 3-nitro-tyrosine (3-NT) requires the reaction of substitution of hydrogen by the nitro group ($–NO_2$) at the *ortho* position of a phenolic ring of the target tyrosine (Tyr) (Bayden et al., 2011). The properties of 3-NT are different from those of Tyr. For example, 3-NT is about 50 % charged at physiological pH, whereas Tyr is neutral. This results in changes to the structure and function or spatial interactions of the target protein (Abello et al., 2009). Nitration of proteins is not random, even though Tyr is mildly hydrophilic and often surface-exposed in proteins (Abello et al., 2009).

Reserve mobilisation and degradation of irreversibly modified proteins that lose their function require active repair mechanisms based on rapid labelling and decomposition (protein quality control). Among numerous proteolytic enzymes, proteases belonging to cysteine proteases, serine proteases, aspartic proteases, metalloproteases, and dipeptidases were identified in seeds (Tan-Wilson and Wilson, 2012). The activity of proteases, especially those related to the hydrolysis of storage proteins, is associated with the initiation of metabolism, i.e. during seed imbibition. The covalent binding of ubiquitin (Ub) to the target protein leads to its degradation by the 26S proteasome, an ATP-dependent process (Sadanandom et al., 2012). Besides the activity of the 26S proteasome, aberrant proteins are targeted to the 20S proteasome *via* Ub-independent proteolysis (Kumar Deshmukh et al., 2019; Kurepa and Smalle, 2008). The link between carbonylated protein degradation and proteasome 20S activity has been demonstrated (Kästle and Grune, 2011).

Climate change and weather anomalies disturb seed physiological processes. Thus, the environmental conditions affect the ability of seeds to remain dormant (or germinate) at the appropriate time or influence the rate of ageing. Maintaining the dormancy state for a long time is important for seed banks to preserve biodiversity (Arc et al., 2011).

Orthodox-type apple (*Malus domestica* Borkh.) seeds undergo deep embryonic dormancy, linked to the low metabolic activity reinforced by the extremely low water content (Lewak, 2011). Breakage of apple seed dormancy is achieved after short-term fumigation of isolated embryos with NO (Gniazdowska et al., 2010, 2007), which enhanced utilisation of storage proteins (Krasuska et al., 2016), and stimulated autotrophy of the seedlings (Krasuska et al., 2015). Deep dormancy favours the longevity maintenance for an extended period. Therefore, *orthodox* seeds require specific treatment protocols, which accelerate ageing (Ciacka et al., 2022a). Short-term fumigation of embryos isolated from aged seeds with NO delayed and reduced the negative effect of the ageing treatment, although it was limited to a specific time frame, up to 21 days (Ciacka et al., 2022a, 2022b; Tyminski et al., 2024). Three weeks of artificial ageing of apple seeds can be considered a threshold, beyond which rapid and irreversible deterioration occurs, which may be referred to as the critical node phase (Yin et al., 2017). Using this model, we have demonstrated that seed ageing is accompanied by nitro-oxidative stress as a result of the excess of ROS and the RNS deficiency. As previously proposed, the key role of NO in maintaining the viability of aged apple seeds may be associated with the regulation of ROS homeostasis through the enhancement of antioxidant capacity (Ciacka et al., 2022a). As a consequence of metabolism restoration (by NO), repair mechanisms are switched on.

The RONS imbalance causes an irreversible mark on the protein structure (carbonylation and nitration). The aim of our study was to identify the markers of these alterations that could create a pattern of seed ageing progression depending on the phase of ageing.

Despite the importance of the topic and the increasing understanding of protein carbonylation and nitration, data related to these modifications in seeds undergoing ageing are scarce. There are no information linking these two PTMs in seeds subjected to accelerated ageing. We have demonstrated that NO plays a significant role in improving the quality of aged seeds (Ciacka et al., 2022a); thus, we continue our research on the topic. We wanted to identify the specific protein targets of carbonylation and nitration (differentiating proteins that create a nitro-oxidative code) during the progression of artificial ageing of apple seeds. Additionally, we analysed the NO impact on vigour maintenance of aged apple seeds by investigating proteolytic activities, level of Ub-tagged proteins, and proteasome 20S activity.

2. Materials and methods

2.1. Plant material

Embryonic axes isolated from apple (*Malus domestica* Borkh., cv. Antonówka) seeds subjected to artificial ageing were used for the experiments. Seeds were obtained from apple fruits acquired from the local orchard WIKPOL (Dąbrówka, Poland). Isolated, room-temperature dried seeds were stored at 5 °C until further use.

2.2. Artificial ageing procedure

The artificial ageing procedure was conducted according to Ciacka et al. (2022a). Seeds were placed in glass Petri dishes, mixed with sterile sand and distilled water (50 % humidity), at 35 °C for 7, 14, and 21 days. Then, a set of the embryos was shortly (3 h) fumigated with NO as follows: embryos were placed in a sealed container (500 mL) and treated with gaseous NO, released from the reaction of 14.5 mM $NaNO_2$ (5 mL) with 0.2 M HCl (5 mL) (Ciacka et al., 2022a; Yamasaki, 2000). After treatment, the embryos were washed with distilled water and placed on Petri dishes on watered filter paper and cultured in a growth chamber (Sanyo MLR-35OH) for 48 h at 25/20 °C day/night, a 12/12 h photoperiod, and a light intensity of 100 μmol PAR m^{-2} s^{-1}. As the control, non-treated with NO embryos isolated from artificially aged seeds were used.

After 48 h of embryo culture, embryonic axes were isolated and deep-frozen until the experiments were conducted.

2.3. Proteolytic activity measurement

The proteolytic activity was measured according to Pande et al. (2006), with some modifications. The azocasein was used as a substrate for the reaction. Embryonic axes of apple seeds (50 mg) were homogenised using a mortar and pestle, on ice, with 0.5 mL of a cold mixture containing 0.1 M potassium phosphate buffer (pH 7.0), 5 mM dithiothreitol (DTT), 150 mM NaCl, and 2 % PVPP. Homogenate was centrifuged (12,000 *g*, 4 °C, 10 min), and the supernatant was collected and used for the analysis.

The proteolytic activity was assessed under two distinct conditions: the 0.1 M acetate buffer, pH 5.4, or the 0.1 M Tris-HCl, pH 8.8. The reaction was carried out using a homogenate containing 300 μg of protein, mixed with the above-described buffers to obtain 200 μL of the final solution. Protein concentration was measured according to Bradford (1976). Then, 200 μL of 1 % azocasein (dissolved in 1.5 % ethanol and 0.1 M Tris-HCl, pH 8.8 or 0.1 M acetate buffer pH 5.4) was added. The mixture was incubated for 1 h at 37 °C, and the reaction was stopped using 100 % trichloroacetic acid (TCA). After 5 min incubation at RT, samples were centrifuged (12,000 *g*, RT, 5 min), and the obtained supernatants were neutralised with 2 M NaOH. The absorbance was measured at 440 nm using a microplate reader (ClarioStar Plus).

One unit of protease activity was defined as 0.1 absorbance change at 37 °C. The proteolytic activity was expressed as U per mg protein. Measurements were done in 3 biological repetitions.

2.4. Total soluble protein degradation measurement

The measurement of protein degradation was performed as follows: apple embryonic axes were homogenised on ice, with cold buffer containing 0.1 M potassium phosphate buffer (pH 7.0), 5 mM DTT, 150 mM NaCl and 2 % PVPP. After centrifugation (12,000 *g*, 4 °C, 10 min), the protein concentration in the resulting supernatant was measured using Bradford reagent as described below. The sample was aliquoted into two tubes, each containing 20 μl of the sample. To one of the tubes, 1 μL of protease inhibitors (Sigma-Aldrich, P9599) diluted 1:1000 in 0.1 M potassium phosphate buffer (pH 7.0) was added. The ingredients of the protease inhibitors cocktail were presented in Table S1. Samples were incubated for 3 h at 37 °C, followed by repeated protein concentration measurement.

The difference in protein concentration, for samples before and after incubation, was calculated and expressed as a percentage of the initial concentration. Hence, this factor expresses the percentage of degraded protein. The experiment was performed in 3 biological repetitions.

2.5. Protein extraction and purification

The total protein extraction from embryonic axes was performed in a multi-phased procedure, according to Wu et al. (2014) with modifications. The obtained protein pool was used for the identification of carbonylated and nitrated proteins separated with two-dimensional electrophoresis (2DE) and for immunoblotting experiments.

Embryonic axes (100 mg) were homogenised in liquid nitrogen using a mortar and pestle. The obtained tissue powder was moved into the Eppendorf tube and dissolved in 1.5 mL of cold 10 % TCA in 80 % acetone (v/v) with 10 mM DTT. Samples were vigorously vortexed and sonicated for 20 min. Then, samples were centrifuged (15,000 *g*, 4 °C, 5 min), the supernatant was discarded, and the pellet was dissolved in 1 mL of extraction mixture. The sonification, centrifugation and dissolvement were conducted two more times. The obtained pellet was dissolved in 80 % acetone (v/v) with 10 mM DTT, vortexed, sonicated for 20 min, and centrifuged (15,000 *g*, 4 °C, 5 min). The supernatant was discarded, and the sample pellet was dried in the fume hood. The next step was the extraction of proteins with the SDS buffer.

The SDS-buffer extraction was performed as follows: dried protein pellet was dissolved in 1.5 mL of SDS buffer containing 1 % SDS (w/v), 0.15 M Tris-HCl pH 8.8, 0.1 M DTT, 1 mM EDTA, 2 mM PMSF and protease inhibitors (1:1000; Sigma-Aldrich P9599). Samples were vortexed and sonicated (20 min), followed by 1 h incubation at RT with mild shaking. Then, samples were centrifuged (15,000 *g*, RT, 10 min), and the supernatant was collected and subjected to protein precipitation as described below.

For protein precipitation, the liquid samples were mixed with cold 0.1 M ammonium acetate in methanol (five times the volume of the precipitated sample) and incubated at −20 °C for 2 h. Then, the sample was centrifuged (10,000 *g*, 4 °C, 15 min), and the acquired pellet was dissolved in 2 mL of cold 0.2 M ammonium acetate, vortexed and sonicated (10 min). The mixture was centrifuged, and the pellet was dissolved in 1 mL of 80 % (v/v) acetone, vortexed, sonicated (10 min), and centrifuged once more. The final pellet containing proteins was dried under a fume hood and used for the experiments.

2.6. Two-dimensional electrophoresis (2DE)

Obtained total protein precipitates were dissolved in rehydration buffer for isoelectric focusing (IEF) containing 7 M urea, 2 M thiourea, and 4 % (w/v) CHAPS.

For IEF, the prepared protein samples were mixed with 20 mM DTT, 0.5 % (v/v) ampholytes (Bio-Lyte 3/10, 40 %; BioRad 163–1113), 1 % (w/v) bromophenol blue (the final volume of the mixture was 125 μL), and loaded onto 7 cm linear IPG strips (pH 3–10, BioRad 1632000). After the 12 h of rehydration process, the isoelectric focusing was performed (20 000 V, Bio-Rad PROTEAN i12 IEF Cell). Then, the protein samples loaded onto strips were reduced by gentle agitation in equilibration buffer (6 M urea, 30 % (w/v) glycerol, 2 % (w/v) SDS in 0.05 M Tris-HCl buffer pH 8.8) with 1 % (w/v) DTT for 10 min. Sample alkylation was performed by strips agitation in equilibration buffer with 4 % (w/v) iodoacetamide (Sigma, I6125) for 10 min. Next, prepared strips were sealed on top of a 3 % stacking polyacrylamide gel with 0.5 % (w/v) agarose in 0.1 M Tris-HCl, pH 6.8, containing 0.001 % (w/v) bromophenol and resolved in a 10 % gel.

SDS-PAGE electrophoresis was carried out in a Mini-Protean system (Bio-Rad) at 100 V. Gels, after electrophoresis, were stained overnight in Coomassie blue (0.25 % G-250, 0.25 % R-250), followed by destaining in 30 % (v/v) methanol in water with 5 % (v/v) acetic acid. Gels were scanned with the ChemiDoc XRS+Imaging System (Bio-Rad).

2.7. Identification of carbonylated proteins

Carbonylated proteins were identified according to Gietler et al. (2017) with modifications using the above-described total protein precipitate. Protein samples, containing 200 μg of protein, were separated using 2DE (as described above), obtaining two identical gels for each sample. Then, proteins in one gel set were labelled with 10 mM dinitrophenylhydrazine (DNPH) in 2.5 M HCl by incubation for 30 min at RT (Hawkins et al., 2009). The excess reagent was removed by repeatedly incubating the gels in a 1:1 (v/v) ethanol:ethyl acetate solution for 20 min, followed by incubation in water for an additional 20 min. The total washing time was 2 h.

Detection of DNP-labelled proteins was conducted using the immunoblotting technique. Proteins were electrotransferred overnight to nitrocellulose membrane (Amersham Protran 0.2 μm, 10600009) using the Bio-Rad wet blotting system at a constant voltage of 16 V. Then, membranes were blocked for 2 h with non-fat dry milk in Tris-buffered saline with Tween 20 (TBST). After blocking, membranes were washed three times with TBST and incubated for 1 h with anti-DNP monoclonal antibodies (1:60000; Sigma A2831) conjugated with alkaline phosphatase. Subsequently, visualisation of carbonylated proteins was performed by the addition of 0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$, 0.2 mM NBT, and 0.21 mM 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

The remaining gels, after 2DE, were stained with Coomassie blue as

described above. Obtained membranes and corresponding gels were digitised, and protein spots were analysed with PDQuest 8.1 software (Bio-Rad).

All protein spots that were clearly visible on the gel and corresponded with the immunoblot were cut out from the gels and used for mass spectrometry protein identification. Moreover, the densitometry comparison was conducted on membranes. Membranes containing proteins from the NO-treated sample were compared with the control for each artificial ageing period (7, 14, 21 days) separately. The intensity of protein spots was investigated. Protein spots were considered differentially abundant if they showed at least a 2-fold difference or if they were present in only one condition (e.g., detected as carbonylated in the NO-treated sample but absent in the control).

2.8. Identification of nitrated proteins

Nitrated proteins purification was conducted by immunoprecipitation, according to Staszek et al. (2019), using the above-described total protein precipitate. Proteins were dissolved in 500 μL of 0.1 M Tris-HCl pH 7.0 with 5 M urea, followed by protein concentration measurement. For the immunoprecipitation, 1 mg of protein was used, to which 2 μL of anti-nitrotyrosine antibodies (Agrisera AS10 706) were added. The protein sample was incubated overnight at 4 °C. Then, the 5 μL of Protein G (Thermo Scientific 22851) was added, according to the producer's manual, and the sample was incubated for 6 h on ice with mild shaking. The sample was centrifuged (2500 *g*, 4 °C, 3 min), pellet was suspended in TBS (0.1 M Tris-HCl with 0.15-M NaCl, pH 8.0) and incubated overnight at 4 °C. After centrifugation (2500 *g*, 4 °C, 3 min) pellet was dissolved in 200 μL of IEF rehydration buffer (as described above), from which 121 μL was used for IEF.

IEF was performed at 15,000 V. Proteins were then separated by SDS-PAGE, stained with Coomassie and scanned as described above. Protein profiles of the NO-treated sample were compared with the control for each artificial ageing period (7, 14, 21 days) separately with previously described software. Protein spots were considered differentially abundant if they showed at least a 2-fold intensity difference. All nitrated protein spots, marked by the PDQuest software, that were clearly visible on the gel were cut out and used for mass spectrometry identification.

2.9. Mass spectrometry identification of proteins

Selected spots were cut out from the gel, destained in a mixture of 50 mM ammonium bicarbonate and acetonitrile (ACN) (60 %/40 %), dehydrated with ACN and dried in a speed vac. Then, the sample was overnight digested with 20 μL of sequencing-grade modified trypsin (5 ng μL^{-1}) (Promega V511B). Peptides were extracted by incubating gels with water for 20 min, and then with 5 % (v/v) formic acid. After each step, supernatant was collected, and gels were dehydrated with ACN. Supernatants were pooled, and the mixture was dried in the speed vac. Subsequently, peptides were dissolved in 6 μL of 30 % (v/v) ACN with 0.1 % (v/v) trifluoroacetic acid (TFA).

Peptide sample (1 μL) was mixed with 0.5 μL of the matrix solution (10 mg α-Cyano-4-hydroxycinnamic acid – CHCA in 500 μL of 70 % (v/v) ACN, 28.4 % (v/v) water, 1.6 % (v/v) TFA). Then, 1 μL of the mixture was spotted on the steel target plate (Kratos AXIMA DE1580TA). The mass spectra were acquired in a positive ion reflectron mode with a MALDI-TOF-TOF mass spectrometer (Shimadzu AXIMA PERFORMANCE). For protein identification, the Peptide Mass Fingerprint (PMF) method with a MASCOT search system (Matrix Science) was used (Perkins et al., 1999). The searches were performed using trypsin as a digestive enzyme, with one missed cleavage allowed, and two modifications set: carboxymethylation of cysteine (fixed) and oxidation of methionine (variable). The peptide tolerance was set at 0.4 Da. Mass spectrometry identification of nitrated proteins was performed similarly, except for adding nitration of tyrosine as a variable modification during the MASCOT PMF search. Protein identification was performed using the National Center for Biotechnology Information nr database (nr NCBI 31.01.2025, 860583640 sequences; 328366298390 residues) within *Malus* taxa. Scores higher than 65 indicate significant homology ($P > 0.05$).

2.10. Immunodetection of ubiquitinated proteins

Detection of proteins conjugated with Ub was performed with the immunoblotting technique using the above-described total protein precipitate. Proteins were dissolved in 500 μL of 0.1 M Tris-HCl pH 7.0 with 5 M urea, followed by a determination of protein concentration. Then, protein samples containing 7.5 μg of protein were dissolved in sample buffer (63 mM Tris-HCl, pH 6.8, 1 % (w/v) SDS, 10 % (w/v) glycerol, 0.01 % (w/v) bromophenol blue, and 20 mM DTT), and denatured for 10 min at 95 °C. Proteins were separated during the SDS-PAGE electrophoresis in a 10 % gel in a Mini-Protean apparatus (Bio-Rad). During the next step, proteins were electrotransferred to nitrocellulose membranes (Amersham Protran 0.2 μm; 10600009) in the 2 h procedure at 90 V. Membranes were blocked overnight in non-fat milk in TBST. The incubation with anti-Ub antibodies (1:1000) (Agrisera AS08 307 A) was carried out overnight at 4 °C. The anti-rabbit IgG conjugated with alkaline phosphatase (Sigma-Aldrich A3687, 1:30000) were used as secondary antibodies. Visualisation of AP-immunolabelled membranes was performed as described above.

Images of representative immunoblot bands were shown after picture processing in Image Lab Software (Bio-Rad). The total band's intensity was calculated in the software and was expressed in arbitrary units (AU). For clarity and ease of data interpretation, the results are presented as values normalised by dividing by 100,000,000. The experiment was done with 3 biological replications with 2 technical repetitions. The original immunoblots were provided in S2.

2.11. Proteasome 20S chymotrypsin-like protease activity measurement

The activity of the 20S proteasome was performed using the Proteasome 20S Activity Assay Kit (Sigma-Aldrich, MAK172) according to the manufacturer's manual.

Apple seeds' embryonic axes (20 mg) were homogenised on ice with a mortar and pestle in 0.3 mL of homogenization buffer (0.05 M Tris-HCl pH 7.5, 0.05 mM 2-mercaptoethanol, 10 mM $MgCl_2$ and 2 % (w/v) PVPP. Homogenate was incubated for 30 min at 4 °C, followed by centrifugation (15,000 *g*, 4 °C, 30 min). In the obtained supernatant, the protein concentration was measured. For the analysis, 25 μL of protein sample (containing 70 μg of protein) was mixed with 25 μL of a kit working solution consisting of an LLVY-R110 probe. R110 is a fluorescent compound released during probe cleavage by the proteasome. The mixture was incubated at 37 °C for 1 h, and the fluorescence was recorded using a microplate reader (excitation 490 nm, emission 525 nm, ClarioStar Plus). The relative proteasome activity was expressed in AU, calculated by comparing each sample with a 7-day-aged control. The experiment was done in 3 biological replications with 3 technical repetitions.

2.12. Protein measurement

In the above-described experiments, the protein concentration in the apple embryonic axes was determined spectrophotometrically using a Bradford reagent (Bradford, 1976) at 595 nm (ClarioStar Plus). The standard curve was prepared with bovine serum albumin (BSA – Sigma, A7030) as a standard.

2.13. Statistical analysis

Data were analysed using the Statistica13 Software. All data were obtained in at least 3 independent experiments with at least 2–3 repetitions each and presented as mean values ± SD. After two-way ANOVA,

homogeneous groups were evaluated using Duncan's test. For the analysis of total Ub-conjugated protein band intensity and proteasome activity, NO-treated samples were compared with the corresponding control using Student's *t*-test.

3. Results

3.1. Proteolytic activity in embryonic axes is not affected by NO

The proteolytic activity in embryonic axes of aged seeds, measured at acidic pH, was around 10.5 mg^{-1} protein and did not change as the ageing progressed (Fig. 1A). Statistically significant difference, a 10 % increase, was measured only in 7-day aged axes isolated from embryos treated with NO. This treatment led to a decrease in the proteolytic activity as ageing progressed; however, the values were not significantly different from those of the control (Fig. 1A).

Proteolytic activity, measured at pH 8.8, was lower compared to that measured at pH 5.4, as overall it did not exceed 5 mg^{-1} protein (Fig. 1B). The lowest value was noted for axes isolated from seeds aged for 7 days (approximately 3.5 mg^{-1} protein). After 14 and 21 days of ageing, the proteolytic activity in the control tissue increased up to 4.6 mg^{-1} protein. For up to 14 days, the proteolytic activity in axes of embryos treated with NO remained at a constant level, comparable with 7-day aged control (Fig. 1B). The longest period of ageing resulted in the highest proteolytic activity in NO-treated axes; however, this value did not differ from the control (Fig. 1B).

3.2. NO impacts total soluble protein concentration and protein degradation

Total soluble protein concentration did not differ in axes of embryos subjected to artificial ageing and was approximately 55–60 μg protein mg^{-1} FW for the whole experiment (Table 1). Fumigation with NO led to a slight, non-significant decrease in protein concentration in axes of 14-day-aged embryos. The lowest value, significantly different from the control, was determined for axes of NO-treated embryos after 21 days of ageing (below 50 μg protein mg^{-1} FW, Table 1).

Protein degradation in embryonic axes, after 3 h incubation of the sole crude extract, was rising with the progression of ageing, reaching 42 % after 21 days (Table 1). Treatment of embryos with NO resulted in an additional increase in that parameter; however, only for the 14-day aged axes, the change was statistically significant. The highest value (46 %) was noted for axes of 21-day aged embryos treated with NO.

Incubating crude extract with a protease inhibitor cocktail resulted in a low protein degradation factor. It did not significantly change in embryonic axes regardless of seed ageing duration (Table 1) and was approximately 4.5 %. The only significant change was noted in the embryonic axes of seeds aged for the longest period and treated with NO. This was the highest value, as it reached 15 % (Table 1).

3.3. NO treatment alters protein carbonylation profile in embryonic axes of aged seeds

Analysis of the immunoblots of 2DE protein profiles revealed various changes in protein carbonylation patterns over a prolonged ageing procedure (Figs. 2–4). In general, after 21 days of ageing, fewer carbonylated protein spots were observed, in comparison with 7 and 14 days of ageing. Clearly visible on gels, protein spots selected by the analysis software, were cut out and analysed with MALDI TOF-TOF MS. We also detected multiple carbonylated spots on immunoblots that were not visible on gels and therefore were not analysed (marked with circles on Figs. 2–4).

After 7 days of ageing, 26 various protein spots were detected as carbonylated on immunoblots (including those invisible on gels, Fig. 2). At least a 2-fold increase in spot intensity in samples treated with NO was observed for 6 protein spots, among which seed biotin-containing protein SBP65 (Fig. 2, spot no. 2), senescence-associated protein AAF, chlorolplastic-like (Fig. 2, spot no. 5), prunin 1 Pru du 6.0101-like (Fig. 2, spot no. 8), and em protein H5 (Fig. 2, spot no. 17) were identified (Table 2). Moreover, treatment of 7-day aged embryos with NO resulted in carbonylation of 17.1 kDa class II heat shock protein-like (Fig. 2, Table 2, spot no 11), as this spot was not detected in the control. Simultaneously, spot no. 16 (Fig. 2, Table 2) appeared on immunoblots after NO treatment; however, the protein was not identified. The other carbonylated proteins that showed no change in intensity include late embryogenesis abundant protein ECP63-like isoform X2 (Fig. 2, Table 2, spot no. 4), em-like protein GEA1 (Fig. 2, Table 2, spots no. 13, 14) and late embryogenesis abundant protein 2-like (Fig. 2, Table 2, spot no. 15).

NO impacted carbonylated proteins profile of 14-day aged axes, as was visualised on immunoblot (Fig. 3). Increased spot intensity was detected for spots identified as prunin 1 Pru du 6.0101-like (Fig. 3, Table 2, spots no. 3, 7), senescence-associated protein AAF, chlorolplastic-like (Fig. 3, Table 2, spot no. 5) or em-like protein GEA1 (Fig. 3, Table 2, spot no. 9). Decreased spot intensity was observed for late embryogenesis abundant protein ECP63-like isoform X2 (Fig. 3, Table 2, spot no. 4). For spot no. 2 (Fig. 3, Table 2), identified as seed biotin-containing protein SBP65, no intensity change was detected. In this spot, another protein was identified; moreover, it was only detected in samples aged for 14 days (3-ketoacyl-CoA synthase 5-like, Fig. 3, Table 2, spot no. 2). Although not identified, based on their membrane localisation, it is likely that spot no. 10 (Fig. 3) corresponds to em-like

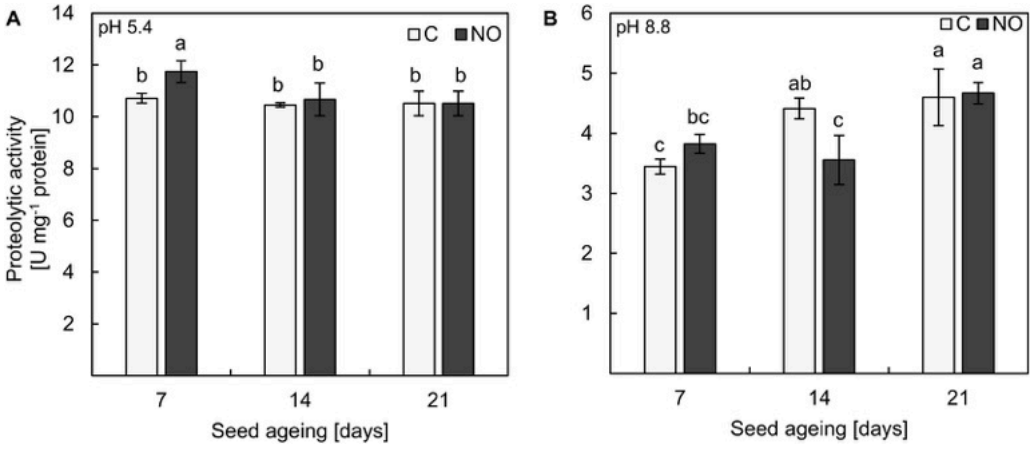

Fig. 1. Proteolytic activity measured at pH 5.4 (**A**) and 8.8 (**B**) in axes of apple embryos artificially aged for 7, 14 and 21 days (C) or aged and treated with NO (NO). Values are the average of 3 biological repetitions. Letters indicate the homogeneous groups identified according to Duncan's post hoc test ($P < 0.05$).

Table 1
Total soluble protein concentration and protein degradation factor in samples after 3 h of incubation with or without protease inhibitor cocktail in embryonic axes of non-treated (C) or NO-treated (NO) apple embryos isolated from seeds aged for 7, 14, 21 days

	Seed ageing [days]					
	7		14		21	
	C	NO	C	NO	C	NO
Protein concentration [μg protein mg^{-1} FW]	54.2 ± 4.5 ab	57.6 ± 0.8 ab	62.0 ± 7.1 a	54.7 ± 0.5 ab	59.5 ± 0.9 a	49.8 ± 0.6 b
Protein degradation after incubation without protease inhibitor cocktail [%]	28.3 ± 3.0 d	32.2 ± 0.6 cd	30.3 ± 4.3 d	37.5 ± 0.4 bc	42.0 ± 0.3 ab	45.9 ± 1.3 a
Protein degradation after incubation with protease inhibitor cocktail [%]	4.6 ± 0.3 b	4.4 ± 2.2 b	4.3 ± 2.8 b	4.6 ± 3.4 b	0.0 ± 0.0 b	14.6 ± 0.5 a

Letters (a-d) indicate homogeneous groups that were assessed using Duncan's test at $P \leq 0.05$.

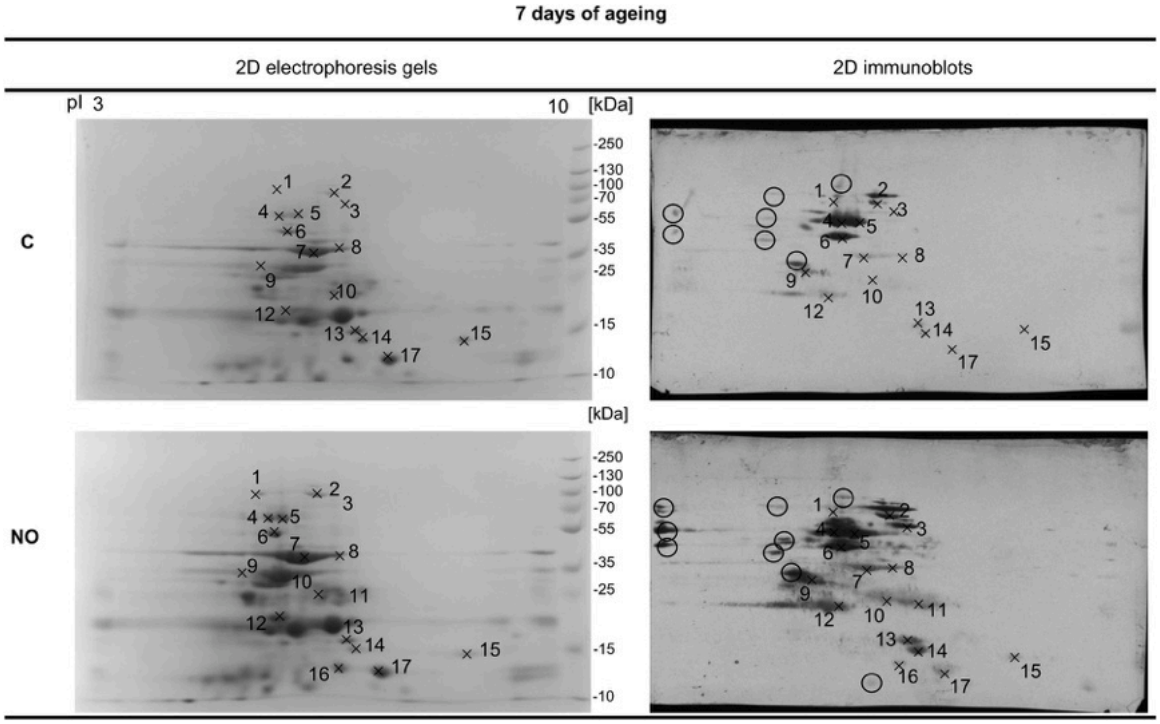

Fig. 2. Profile of carbonylated proteins, after the 2DE of 7-day aged embryonic axes' protein extract (C) or extract from axes of embryos treated with NO (NO). The figure consists of representative photos of gels after 2DE and immunodetection of carbonylated proteins (WesternBlot). Numbers correspond to the proteins identified by mass spectrometry (Table 2). Carbonylated protein spots detected on immunoblots and invisible on gels were labelled with circles.

protein GEA1, spot no. 11 to em protein H5, and spot no. 12 to late embryogenesis abundant protein 2-like.

The least number of carbonylated proteins was detected in the samples artificially aged for 21 days (Fig. 4, Table 2). For those embryos, fumigation with NO resulted in a decrease in the intensity of most of the detected carbonylated proteins, including: seed biotin-containing protein SBP65 (Fig. 4, Table 2, spots no. 2, 3), late embryogenesis abundant protein ECP63-like isoform X2 (Fig. 4, Table 2, spot no. 4) and prunin 1 Pru du 6.0101-like (Fig. 4, Table 2, spots no. 5, 7).

3.4. Nitrated protein patterns are not changed after treatment of apple embryos with NO

Treatment with NO did not lead to the emergence of additional nitrated proteins, in comparison with respective controls (Fig. 5, Table 3). All clearly visible protein spots, selected by the analysis software, were cut out of gels and analysed with MALDI TOF-TOF MS.

Five proteins were detected as nitrated in each of the analysed gels: serpin-ZX-like (Fig. 5, spots no. 4, 18, 34), L-lactate dehydrogenase B-like (Fig. 5, spots no. 6, 20, 21, 36, 37), hypothetical protein DVH24_025610 (Fig. 5, spots no. 11, 26, 27, 41) and phosphopantetheine adenylyltransferase-like (Fig. 5, spots no. 12, 13, 28, 29, 42) (Table 3). Moreover, Prunin 1 Pru du 6.0101-like was noted as the most abundant nitrated protein (Fig. 5, spots no. 2, 3, 14, 17, 30, 32, 33) (Table 3). Based on the location of the spots identified as prunin (Fig. 5, spots no. 14 and 30), it is likely that they represent protein fragments, as they appear on the gel well below the theoretical molecular mass of the full-length protein.

In the protein profile of 7-day aged axes, 14 various protein spots were recognised, among which, 7 proteins were identified (Table 3). In addition to the previously mentioned proteins, mitochondrial ATP synthase subunit beta (Fig. 5, spot no. 1), and E3 ubiquitin-protein ligase CCNB1IP1 homolog isoform X2 (Fig. 5, spot no. 14) were detected. These proteins were also identified in samples from embryos aged for 14 days (Fig. 5, spots no. 15 and 30, respectively). It is likely that in 21-day aged samples, spot 31 (Fig. 5) corresponds to ATP synthase and spot 43 (Fig. 5) to E3 ubiquitin-protein ligase; however, they were not identified. Treatment of apple embryos, that were isolated from seeds aged for 7 days, with NO, resulted in an at least 2-fold increase in the intensity of protein spots no. 5, 9, 10 (Fig. 5, Table 3, proteins unidentified) and a

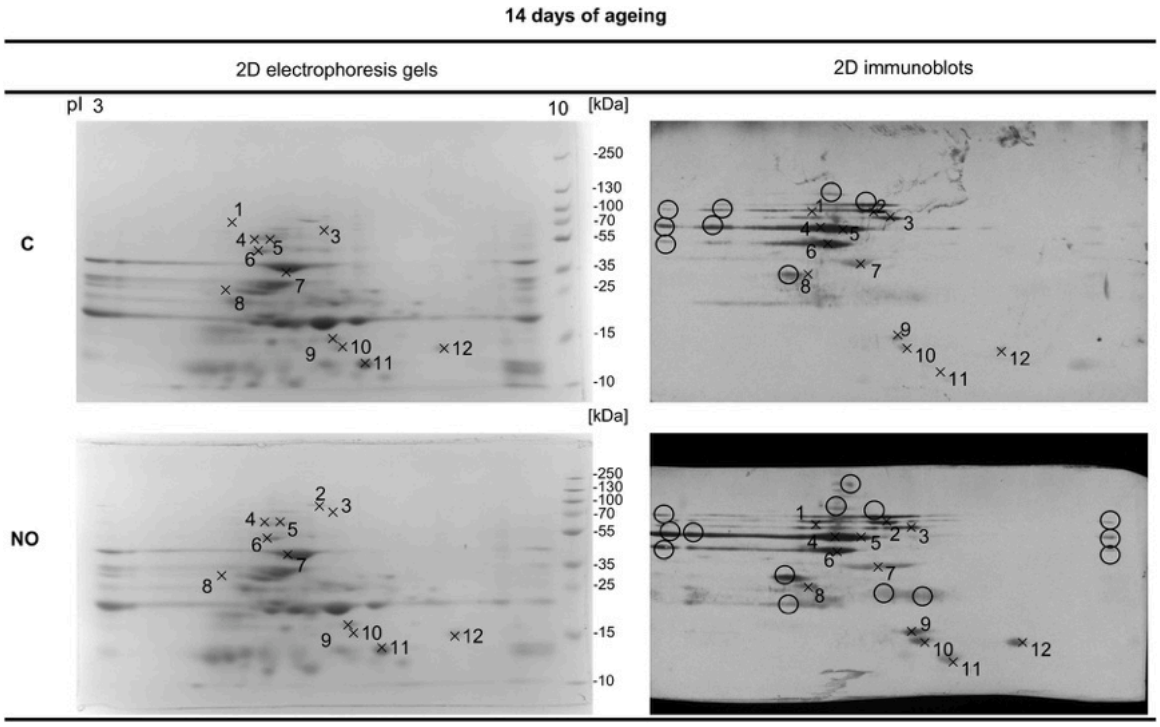

Fig. 3. Profile of carbonylated proteins, after the 2DE of 14-day aged embryonic axes' protein extract (C) or extract from axes of embryos treated with NO (NO). The figure consists of representative photos of gels after 2DE and immunodetection of carbonylated proteins (WesternBlot). Numbers correspond to the proteins identified by mass spectrometry (Table 2). Carbonylated protein spots detected on immunoblots and invisible on gels were labelled with circles.

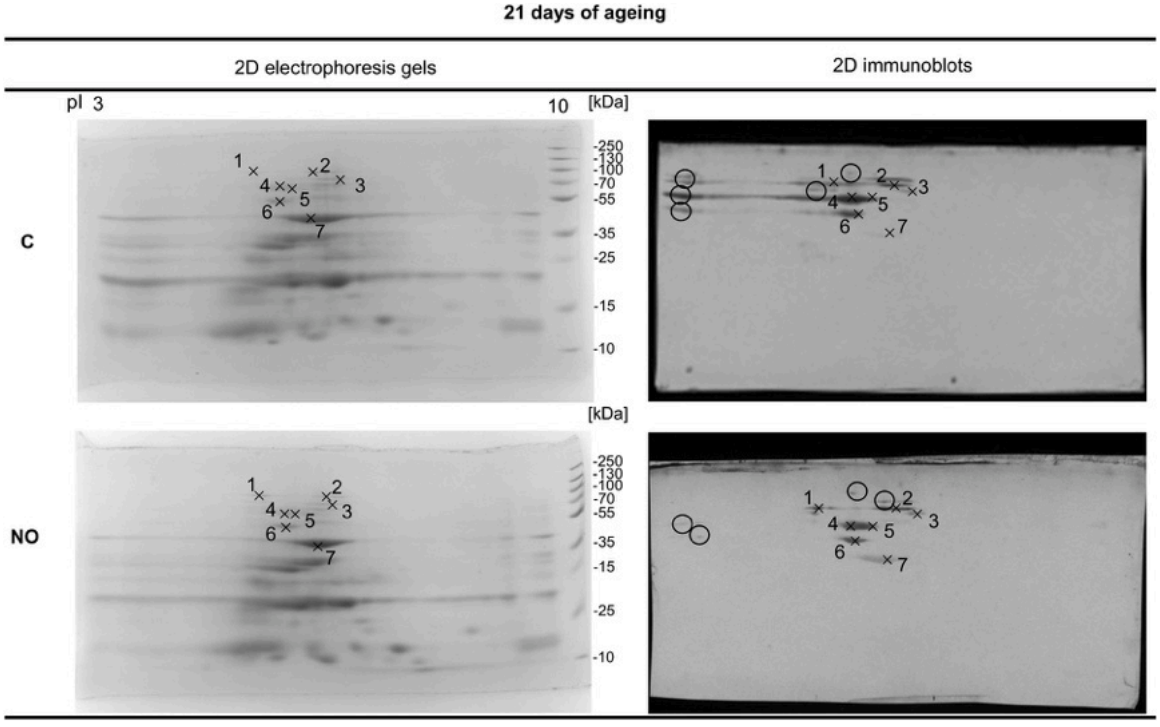

Fig. 4. Profile of carbonylated proteins, after the 2DE of 21-day aged embryonic axes' protein extract (C) or extract from axes of embryos treated with NO (NO). The figure consists of representative photos of gels after 2DE and immunodetection of carbonylated proteins (WesternBlot). Numbers correspond to the proteins identified by mass spectrometry (Table 2). Carbonylated protein spots detected on immunoblots and invisible on gels were labelled with circles.

decrease in the intensity of spot no. 11 (Fig. 5, Table 3, hypothetical protein DVH24_025610) in comparison with control.

The analysis of the nitrated protein profile of embryonic axes from seeds artificially aged for 14 days revealed 17 protein spots (Table 3). Most had been reported previously; however, enolase (Fig. 5, Table 3, spot no. 16) was specific to this ageing period. Similarly, the 17.1 kDa class II heat shock protein-like was detected only after 14 days of ageing (Fig. 5, Table 3, spot no. 23). According to the protein spot localisation

Table 2
Mass spectrometry identification of carbonylated proteins in the axes of embryos isolated from seeds aged for 7, 14 and 21 days and treated with NO. Arrows indicate a minimum twofold change in spot intensity in the NO-treated sample in comparison with the control on analysed immunoblots. Additionally, spots corresponding to the proteins that are not carbonylated in the control were indicated with an asterisk (*)

Seed ageing [days]	Spot no	Protein name	NCBI accession number	Theoretical protein mass [kDa]	Mascot score	Change
7	1	Not identified				↑
	3					↑
	6					*
	9					
	10					
	12					
	16					
	2	seed biotin-containing protein SBP65 [*Malus domestica*]	XP_017179412.3	81.3	114	↑
	4	late embryogenesis abundant protein ECP63-like isoform X2 [*Malus domestica*]	XP_070660990.1	54.6	89	
	5	senescence-associated protein AAF, chlorolplastic-like [*Malus sylvestris*]	XP_050134254.1	51.2	74	↑
	7	prunin 1 Pru du 6.0101-like [*Malus sylvestris*]	XP_050145447.1	65.6	100	↑
	8				118	
	11	17.1 kDa class II heat shock protein-like [*Malus domestica*]	XP_008377809.3	26.1	83	*
	13	em-like protein GEA1 [*Malus sylvestris*]	XP_050116036.1	17.7	80	
	14				80	
	15	late embryogenesis abundant protein 2-like [*Malus domestica*]	XP_008390149.3	16	88	
	17	em protein H5 [*Malus domestica*]	XP_008362293.1	10	78	↑
14	1	Not identified				
	6					
	8					↑
	10					
	11					
	12					
	2	seed biotin-containing protein SBP65 [*Malus domestica*]	XP_017179412.3	81.3	70	
	2	3-ketoacyl-CoA synthase 5-like [*Malus domestica*]	XP_008346290.2	28.7	90	
	3	prunin 1 Pru du 6.0101-like [*Malus sylvestris*]	XP_050145447.1	65.6	69	↑
	7				98	↑
	4	late embryogenesis abundant protein ECP63-like isoform X2 [*Malus domestica*]	XP_070660990.1	54.6	122	↓
	5	senescence-associated protein AAF, chlorolplastic-like [*Malus sylvestris*]	XP_050134254.1	51.2	90	↑
	9	em-like protein GEA1 [*Malus sylvestris*]	XP_050116036.1	17.7	93	↑
21	1	Not identified				↑
	6					↓
	2	seed biotin-containing protein SBP65 [*Malus domestica*]	XP_017179412.3	81.3	78	↓
	3				78	↓
	4	late embryogenesis abundant protein ECP63-like isoform X2 [*Malus domestica*]	XP_070660990.1	54.6	70	↓
	5	prunin 1 Pru du 6.0101-like [*Malus sylvestris*]	XP_050145447.1	65.6	134	↓
	7				122	
	5	senescence-associated protein AAF, chlorolplastic-like [*Malus sylvestris*]	XP_050134254.1	51.2	127	

Protein spots from samples aged for 7, 14, and 21 days are presented in Figures 2-4, respectively.

on the gel, it is possible that this protein also occurs in samples obtained from 7-day aged seeds (Fig. 5, Table 3, spot no. 8); however, it was not identified. Treatment of embryos aged for 14 days with NO resulted in a decrease in the intensity of vast protein spots. At least a 2-fold reduction was detected in spots identified as Prunin 1 Pru du, L-lactate dehydrogenase, hypothetical protein DVH24_025610 and E3 ubiquitin-protein ligase (Table 3). In contrast, it was noted that the intensity of the spot of ATP synthase rose (Table 3).

Seed ageing for more than 14 days resulted in the detection of 13 nitrated protein spots (Fig. 5, Table 3). Among them, spots identified as Prunin 1 Pru du, L-lactate dehydrogenase and phosphopantetheine adenylyltransferase-like presented higher intensity in NO-treated samples than in control (Table 3).

3.5. Ubiquitinated protein patterns fluctuate in the embryonic axes isolated from aged embryos treated with NO

In analysed samples, a similar ubiquitinated protein pattern was detected, regardless of seed ageing duration or NO treatment (Fig. 6A). Nevertheless, the band intensity varied in the performed immunoblots, reaching the highest value 1.2 AU in embryonic axes of 7-day aged seeds (Fig. 6B). Densitometry analysis of Ub-conjugated protein bands obtained from embryonic axes of seeds artificially aged for 7 days and treated with NO revealed significant, approximately 50 % decrease in intensity in comparison with the control. This level remained stable after seed ageing for 14 days and was not affected by NO treatment. The longest ageing period resulted in the higher intensity of Ub-tagged protein bands (1.1 AU), and it did not change after treatment with NO (Fig. 6B).

3.6. Treatment of embryonic axes with NO improves proteasome activity

A decreasing trend was observed in 20S proteasome activity, measured in axes isolated from aged embryos (Fig. 7). Artificial ageing treatment for longer than 7 days resulted in a decline in the proteasome activity, reaching the lowest value in axes from 21-day aged seed

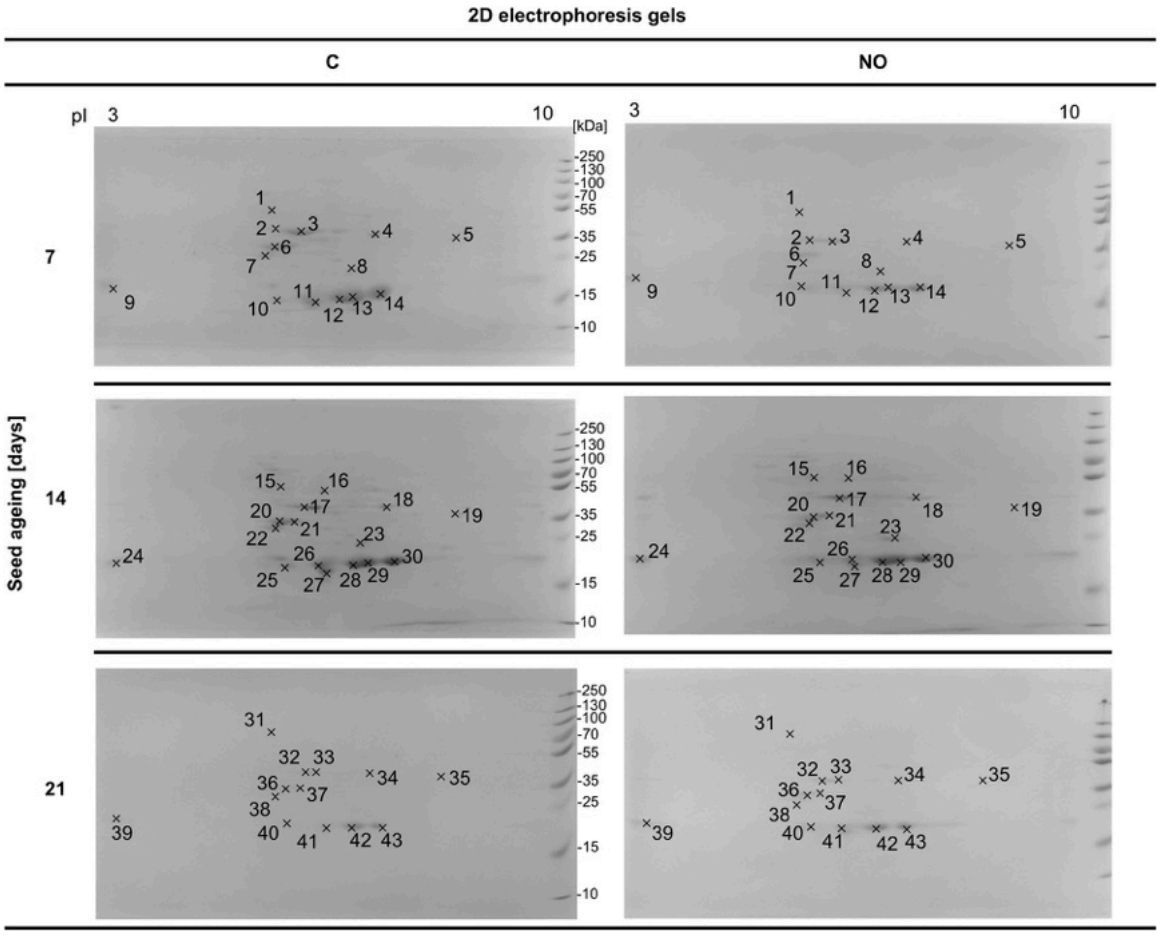

Fig. 5. Representative 2DE profiles of nitrated proteins in axes of apple embryos isolated from seeds aged for 7, 14, and 21 days (C), or aged and treated with NO (NO), after immunoprecipitation with anti-3NT antibodies. Numbers correspond to the proteins identified by mass spectrometry (Table 3).

(0.6 AU, Fig. 7). In general, treatment with NO improved the proteasome activity in the artificially aged embryonic axes. Starting from 14 days of ageing, the activity of the proteasome 20S was significantly higher than that of the control. It was noted that after 21 days of ageing, this value increased in the NO-treated sample by 70 % in comparison with the control, and reached the level observed in 7-day aged embryonic axes (Fig. 7).

4. Discussion

Apple embryos isolated from seeds subjected to artificial ageing for 7, 14, and 21 days deteriorate differently (which is manifested by impaired germination, S3), as we have previously described in detail (Ciacka et al., 2022a). Apple seeds artificial ageing lasting for at least 14 days is required to observe the deleterious effects (Ciacka et al., 2022a; Tyminski et al., 2024). In conditions similar to one applied in this work, apple seeds, subjected to increased temperature in moist sand exhibited a decrease in germination capacity, accompanied by various morphological modifications of the embryos (Dawidowicz-Grzegorzewska, 1989; Dębska et al., 2013). The ultrastructure analyses of such embryos confirmed the maintenance of dormancy state, while elevated temperature and water availability led to the morphological alterations (e.g., damage to the mitochondria structure). The warm treatment of apple seeds also resulted in the partial degradation of storage protein bodies (Dawidowicz-Grzegorzewska, 1989). The author proposed that the observed alterations in protein structure were the result of the increased activity of stored proteases or proteases synthesised *de novo* during seed imbibition. Based on the changes in stored proteins in embryos isolated from warm-treated seeds (Dawidowicz-Grzegorzewska, 1989), we assume that the protein quality control system (e.g., proteolysis) also undergoes significant modifications. Ageing is accompanied by high catabolic activity, including protein degradation (Diaz-Mendoza et al., 2016). In non-aged seeds, the induction of seed metabolism during germination is linked to nutrient remobilisation, which relies on proteolytic activity (Tan-Wilson and Wilson, 2012). Exopeptidases (carboxypeptidases) require acidic pH. The broader range of pH determines the optimum activity of endopeptidases (serine peptidases, cysteine peptidases, aspartic peptidases, and metallopeptidases) (Belozersky et al., 1990; Jones et al., 1996), which initiate the breakdown of storage proteins (Tan-Wilson and Wilson, 2012). Our previous work demonstrated that short-term NO pre-treatment of dormant (non-aged) apple embryos was accompanied by an increase in proteolytic activity in embryonic axes, starting after 24 h of the culture (Krasuska et al., 2014). The observed, higher proteolytic activities in senescent organs, e.g., leaves, are mostly associated with the remobilisation of nutrients (Diaz-Mendoza et al., 2016). In seeds subjected to ageing, the progress in reduction of proteolytic capacity is rather linked to the exhaustion of repair mechanisms.

Artificial ageing of apple seeds was accompanied by constant activity of acidic peptidases in the embryonic axes (Fig. 1A). Even though the seed imbibition under 35 °C does not break dormancy (Dawidowicz-Grzegorzewska, 1989; Lewak, 2011), it favours the activation of metabolism (e.g., the proteolytic activity at pH 8.8), as we observed in the artificially aged seeds (Fig. 1B, Table 1). Utilisation of reserve materials (proteins) while the seeds remain dormant decreases their viability. On the contrary, the application of NO during the early

Table 3
Mass spectrometry identification of nitrated proteins in the axes of embryos isolated from seeds aged for 7, 14 and 21 days and treated with NO. Arrows indicate a minimum twofold change in spot intensity in the NO-treated sample in comparison with the control. Bolded spot numbers indicate the possibility that the spot represents a protein fragment, based on its position on the gel

Seed ageing [days]	Spot no.	Protein name	NCBI accession number	Theoretical mass [kDa]	Mascot score	Change
7	1	ATP synthase subunit beta, mitochondrial [*Malus domestica*]	XP_008339590.2	60.1	110	
	2				72	
	3	prunin 1 Pru du 6.0101-like [*Malus sylvestris*]	XP_050145447.1	65.6	79	
	14				89	
	4	serpin-ZX-like [*Malus domestica*]	XP_008357195.1	42	72	
	5					
	7	Not identified				↑
	8					↑
	9					↑
	10					
	6	L-lactate dehydrogenase B-like [*Malus sylvestris*]	XP_050150638.1	37.6	89	
	11	hypothetical protein DVH24_025610 [*Malus domestica*]	RXH72109.1	26.8	95	↓
	12	phosphopantetheine adenylyltransferase-like isoform X2 [*Malus sylvestris*]	XP_050128066.1	18.4	102	
	13				78	
	14	E3 ubiquitin-protein ligase CCNB1IP1 homolog isoform X2 [*Malus domestica*]	XP_028944950.1	35	76	
	15	ATP synthase subunit beta, mitochondrial [*Malus domestica*]	XP_008339590.2	60.1	110	↑
	16	enolase [*Malus domestica*]	XP_008386927.3	47.9	83	
	17	prunin 1 Pru du 6.0101-like [*Malus sylvestris*]	XP_050145447.1	65.6	95	↓
	30				+	↓
	18	serpin-ZX-like [*Malus domestica*]	XP_008357195.1	42	88	
	19					
14	22	Not identified				↓
	24					↓
	25					
	20	L-lactate dehydrogenase B-like [*Malus sylvestris*]	XP_050150638.1	37.6	74	↓
	21				84	
	23	17.1 kDa class II heat shock protein-like [*Malus domestica*]	XP_008377809.2	26.2	82	
	26	hypothetical protein DVH24_025610 [*Malus domestica*]	RXH72109.1	26.8	84	↓
	27				94	
	28	phosphopantetheine adenylyltransferase-like isoform X2 [*Malus sylvestris*]	XP_050128066.1	18.4	86	
	29				96	
	30	E3 ubiquitin-protein ligase CCNB1IP1 homolog isoform X2 [*Malus domestica*]	XP_028944950.1	35	75	↓
	31					
	35	Not identified				
	38					↓
	39					↑
	40					
	43					
21	32	prunin 1 Pru du 6.0101-like [*Malus sylvestris*]	XP_050145447.1	65.6	110	↑
	33				98	↑
	34	serpin-ZX-like [*Malus domestica*]	XP_008357195.1	42		
	36	L-lactate dehydrogenase B-like [*Malus sylvestris*]	XP_050150638.1	37.6	74	↑
	37				74	
	41	hypothetical protein DVH24_025610 [*Malus domestica*]	RXH72109.1	26.8	75	
	42	phosphopantetheine adenylyltransferase-like isoform X2 [*Malus sylvestris*]	XP_050128066.1	18.4	102	↑

+Identified from MS/MS peptide fragmentation. Parental ions: 1063.60 – score 46 (>39 indicates identity); 1730.59 – score 109 (>41 indicates identity).

stages of seed ageing may alleviate dormancy, resulting in the initiation of germination followed by the utilisation of storage protein for growth and development. Despite the similarities in proteolytic activity in the control and NO-treated embryos of artificially aged seeds, different processes may occur in embryonic axes. Partial breakage of dormancy in aged seeds may change the purpose of protein utilisation to growth transformations and stimulate aberrant protein degradation. This result demonstrates that NO is necessary to mitigate the detrimental effects of seed ageing. Experiments regarding the priming of aged groundnut (*Arachis hypogaea* L.) seeds with 150 μM of sodium nitroprusside (NO donor) before drought stress revealed an increase in total soluble protein concentration (Sepehri and Rouhi, 2016). Although opposite to ours, this result may confirm that NO affects aged seeds in multiple ways. It is also possible that additional mechanisms of the protein control system are induced after NO application. The decrease in endopeptidase activity in the long-term storage (9 years) of beech (*Fagus sylvatica* L.) seeds, compared to the activity analysed in seeds stored for 3 months, was demonstrated (Ratajczak et al., 2015). In our study, prolonged (14 and 21 days) apple seed artificial ageing rather slightly stimulated endopeptidase activity (pH 8.8, Fig. 1B), and we associate it with the constant utilisation of storage proteins.

We also analysed total protein decomposition capacity in the crude extract from apple embryonic axes in the presence or without the addition of protease inhibitor cocktail (inhibitors of serine proteases, aminopeptidases, cysteine proteases, acid proteases, and metalloproteases, Table 1). The decrease in the content of total soluble proteins in axes of NO-treated embryos isolated from seeds aged for 21 days was observed (Table 1), highlighting other possible mechanisms of protein control than sole proteases. This correlates well with the higher activity of 20S proteasome in axes of NO-treated embryos isolated from seeds subjected to 14 and 21 days of ageing (Fig. 7). Additionally, a slightly higher total degradation factor of the crude extract from axes of NO-treated embryos after incubation without protease inhibitors was detected (Table 1). Protease inhibitor cocktail blocks protein

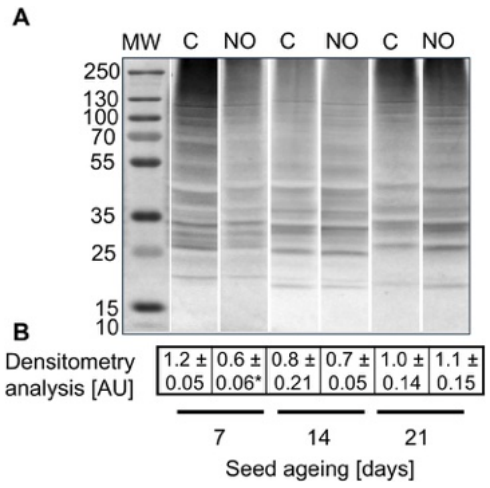

Fig. 6. The pattern of proteins conjugated with ubiquitin (immunoblot) in axes of apple embryos isolated from seeds aged for 7, 14, and 21 days (C), or aged and treated with NO (NO) (**A**). Representative photos were adjusted and presented in this figure. The densitometry analysis in arbitrary units (AU) of the bands intensity of each line (**B**). Values are the average of 3 biological repetitions ± SD. Asterisk indicate values significantly different from the corresponding control according to Student's *t*-test ($P < 0.05$).

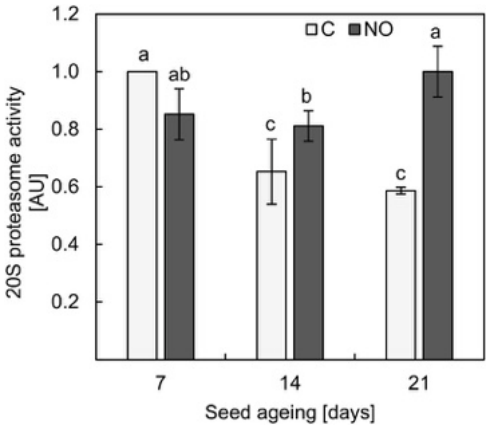

Fig. 7. The Proteasome 20S chymotrypsin-like protease activity measurement in axes of apple embryos isolated from seeds aged for 7, 14, and 21 days (C), or aged and treated with NO (NO). The relative proteasome activity was expressed in arbitrary units (AU), calculated by comparing each sample with a 7-day aged control. Values are the average of 3 biological repetitions. Letters indicate the homogeneous groups identified according to Duncan's post hoc test ($P < 0.05$).

degradation, except for the embryonic axes aged for 21 days and treated with NO (Table 1). Hence, NO during seed artificial ageing may have a beneficial effect on protein control systems at the level of regulation of proteolytic activity and the 20S proteasome. Besides the Ub-26S proteasome pathway, proteins may also be degraded by the core 20S proteasome itself, which does not require ATP and Ub tagging. It is proposed that this pathway is not a marginal, but an independent way of protein decomposition (Kumar Deshmukh et al., 2019). 20S proteasome may be engaged in proteolysis during diverse cellular events, especially oxidative stress; however, the whole mechanism is still undescribed (Kumar Deshmukh et al., 2019). Ub-tagging of proteins participates in phytohormonal signalling and is an important mechanism involved in the control of seed dormancy state. The Ub-26S proteasome system participates in the degradation of abscisic acid (ABA) and gibberellin (GA) signalling components (Chiu et al., 2016; Morris et al., 2011). On the other hand, high temperature (32 °C) and ABA repressed Ub-26S proteasome, and germination of Arabidopsis seeds (Chiu et al., 2016).

In apple axes of embryos treated with NO, isolated from seeds artificially aged for 7 days the Ub-tagged protein profiling demonstrated the decrease in such proteins (Fig. 6). As we mentioned, the conditions of early stage of ageing, together with NO treatment are linked to the dormancy release, and most probably to the involvement of Ub-26S proteasome in this process. However, we assume that prolonged treatment (21 days) of apple seeds rather inhibits Ub-26S proteasome system. The Ub-tagged proteins profile from axes of embryos treated with NO or the control, isolated from seeds subjected to artificial ageing for 21 days, was similar (Fig. 6). Probable impairment of Ub-26S proteasome system is linked to the mitochondrial dysfunction as was observed using apple seeds exposed to elevated temperature (Dawidowicz-Grzegorzewska, 1989), which was likely related to the ATP-deficiency. Similarly, the alteration in mitochondria morphology of Siberian elm (*Ulmus pumila* L.) seeds subjected to artificial ageing was observed (Li et al., 2017). We assume that under conditions of high temperature and water availability (imbibition), protein degradation in apple embryonic axes depends on proteases activity and 20S proteasome systems. This protein control system is linked to the degradation of carbonylated proteins. It was demonstrated for the metastatic melanoma cell line WM451Lu treated with H_2O_2 or UV-A irradiation. Such modified proteins were mostly not tagged with Ub, and degraded *via* the 20S proteasome (Kästle and Grune, 2011).

The increased content of proteins with carbonyl groups was observed in artificially and naturally aged seeds of Arabidopsis (Rajjou et al., 2008), in artificially aged sunflower (*Helianthus annuus* L.) (Morscher et al., 2015), elm (Li et al., 2017), and rice (*Oryza sativa* L.) seeds (Yin et al., 2017). Previously, we observed no differences in the total amount of carbonylated proteins in the axes of NO-treated and untreated embryos (isolated from seeds aged for 7, 14, and 21 days) (Ciacka et al., 2022a). In this work, we focused mainly on proteins whose carbonylation was associated with short-term treatment of aged embryos with NO. Such proteins may indicate the likelihood of being a target of NO action (some proteins are more prone to carbonylation, and some are protected from excessive carbonylation). After 7 days of apple seed ageing, NO treatment of embryos was linked to the carbonylation of class II heat shock protein-like (HSP-like, Fig. 2, Table 2, spot no. 11), not detected in the control (still dormant during this period). These proteins are found in various cellular compartments and play an important role in proteostasis (e.g., prevent aggregation of abnormal proteins, participate in protein folding), especially under oxidative stress conditions (Kalemba and Pukacka, 2008; Sun et al., 2002). Ageing of beech seeds was accompanied by an increase in HSPs levels (Kalemba and Pukacka, 2008). Moreover, proteomic analyses of artificially aged rice seeds indicated that class II cytosolic HSP is an ageing-responsive protein (Kaur et al., 2015). Thus, we assume that NO treatment of embryos after 7 days of apple seed ageing not only alleviates dormancy but also mitigates some symptoms of their deterioration, e.g., via carbonylation of class II cytosolic HSPs.

NO treatment of 7-day aged embryos increased the carbonylation of seed biotin-containing protein (SBP65, Fig. 2, Table 2, spot no. 2), likely to release free biotin for undisturbed germination (Table 2). SBPs are characteristic for seeds and undergo degradation during germination (Alban et al., 2000). The presence of SBPs in mature seeds of pea (*Pisum sativum* L.) was confirmed, and their primary role as free biotin scavengers was suggested (Duval et al., 1994). The mobilisation of SBP was also shown for germinating embryos of non-aged apple seeds, and we proposed that nitration facilitated this process (Krasuska et al., 2016). The carbonylation of SBP65 was noted in apple embryonic axes of NO-treated and control embryos after 14 and 21 days of seed artificial ageing (Fig. 3, spot no. 2, Fig. 4 spots no. 2, 3, Table 2). Thus, we propose that increased free biotin content (before dormancy release) is an additional factor contributing to the acceleration of seed ageing. However, NO treatment of embryos participates in the removal of germination inhibitors (Ciacka et al., 2020a). Thus, the released biotin may be used in various biochemical pathways, enabling the execution of the

ontogenetic seeds' program. In our experiment, the control embryos had only a partially activated metabolism (were still dormant), they couldn't enter further stages of growth and development, at the same time, reserves were depleted, and the deterioration was in progress. After 7 days of apple seed ageing, the carbonylation of senescence-associated protein ARABIDOPSIS A-FIFTEEN (AAF) (chloroplast-like, Figs. 2, 3, 4, Table 2, spots no. 5) was detected. This protein is related to the activity of senescence-associated genes (SAGs). AAF was identified in senescing leaves of sweet potato (*Ipomoea batatas* L. (Lam.)) as SPA15 (Yap et al., 2003). In Arabidopsis, AAF was expressed in early senescent leaves and in tissues with highly proliferative activities, and is also proposed to be involved in the regulation of redox homeostasis (Chen et al., 2012). The higher level of carbonyl groups in this protein (higher intensity of spot) in apple axes after NO treatment of embryos isolated from seeds aged for 7 and 14 days, indicates that this gaseous molecule participates in the mitigation of the negative effect of ageing at the early stages of deterioration. A higher carbonyl group content was also detected for the small hydrophilic em protein H5 after NO treatment of 7-day aged embryos (Fig. 2, Table 2, spot no. 17). This protein is characteristic for seeds and belongs to the Late Embryogenesis Abundant (LEA) 5 super protein family (according to NCBI). Accumulation of LEA is linked to the specific development stage of the plant and reaction to the stress factors, including water deficiency. Such proteins are stored in *orthodox* seeds and enhance tolerance to desiccation during maturation or protect against ROS overaccumulation (Ciacka et al., 2022b; Kalemba and Pukacka, 2008). Moreover, carbonylation of em-like protein GEA1 (Fig. 2, Table 2, spots no. 13, 14) and LEA 2-like (Fig. 2, Table 2, spot no. 15) was noted in samples from 7-day aged embryos, both in control and NO-treated samples. We propose that the increase in ROS concentration (Ciacka et al., 2022a) may promote carbonylation and degradation of these proteins. However, in NO-treated embryos, this was associated with the partial alleviation of dormancy, while in the control, it was associated with the loss of protective properties of LEA proteins. Lower intensity of the carbonylated protein spot identified as LEA ECP63-like protein was observed in NO-treated axes isolated from seeds artificially aged for 14 and 21 days (Figs. 3 and 4, Table 2, spot no. 4). This result is consistent with our previous work related to the analyses of transcript levels of *Lea1*, *Lea2a*, and *Lea4*, as it demonstrated their decrease as apple seeds ageing was prolonged. NO treatment of embryos of apple seeds aged for 21 days increased the abundance of *Lea2a*, which may play a protective, ROS-scavenging role (Ciacka et al., 2022b). The disappearance of dehydrin, belonging to the LEA proteins, during ageing of Arabidopsis seeds was already noted (Rajjou et al., 2008), as well as the decreased level of these proteins during prolonged storage of beech seed (Kalemba and Pukacka, 2014, 2008). Apple seed ageing for 14 days is associated with carbonylation of 3-ketoacyl-CoA synthase 5-like (Fig. 4, Table 2, spot no. 2), which is related to storage lipid metabolism and involved in stress responses (Luo et al., 2024). Apple embryos accumulate lipids as reserve material (Lewak, 2011), hence lipid metabolism disruption may be the key to accelerated ageing.

From the 7th day of artificial ageing of apple seeds, the presence of carbonylated prunin1 (Pru 1) was confirmed (Fig. 2, spots no. 7, 8, Fig. 3, spots no. 3, 7, Fig. 4, spots no. 5, 7, Table 2). This protein is the major component of the 11S storage protein, described in almonds (*Prunus dulcis* L.) (Jin et al., 2009). Carbonylation of reserve proteins during seed maturation was previously demonstrated and discussed (Barba-Espín et al., 2011; Job et al., 2005; Oracz et al., 2007). It is well accepted that this irreversible oxidation facilitates the mobilisation of these proteins during germination, most probably via the 20S proteasome (Job et al., 2005). Carbonylated storage proteins were also identified in naturally aged beech seeds (Kalemba and Pukacka, 2014). Covalent binding of the -NO_2 group to an amino acid residue facilitates protein degradation, and even a single nitration is sufficient to initiate this process via the proteasome, including the 20S proteasome pathways (Gow et al., 1996; Souza et al., 2000). Treatment of isolated proteins with $ONOO^-$ stimulated their degradation (Gow et al., 1996; Grune et al., 2001; Souza et al., 2000). Moreover, modification of proteins by singlet oxygen or $ONOO^-$ is a selective marker that provides their recognition and degradation by the proteasomal system, as was shown *in vitro* and *in vivo* (Grune et al., 2001). The 2DE of nitrated proteins (after immunoprecipitation using 3-NT antibodies) revealed that Pru1 is not only carbonylated but also nitrated during apple seed ageing (Fig. 5, Table 3, spots no. 2, 3, 14, 17, 30, 32, 33). It is not known whether nitration occurs during seed maturation, as is the case for carbonylation (Job et al., 2005).

Artificial ageing of apple seeds was also accompanied by the presence of nitrated serpin-ZX-like (Fig. 5, Table 3, spots no. 4, 18, 34). Serpins are classified as irreversible inhibitors of serine and cysteine proteases (Ferreira et al., 2023). Nitration of serpins is related to the proteolytic activity in apple embryonic axes and may serve as a repair mechanism. No change in serpin spot intensity in the control and after NO-treatment indicates that this modification could occur during seed maturation and does not depend on artificial ageing. Seed ageing for 14 days resulted in the nitration of 17.1 kDa class II heat shock protein-like (HSP-like) (Fig. 5, Table 3, spots no. 23 and probably 8). The accumulation of these proteins is related to the late stage of seed maturation. Previously, the decrease in levels of *Hsp* in the control apple axes of aged seeds was demonstrated. NO treatment of embryos isolated from seeds aged for 14 days upregulated *Hsp70a*, *Hsp70c*, and *Hsp20a* transcript levels (Ciacka et al., 2022b). As the number of transcripts increases, the probability of nitration of these proteins rises, indicating an important positive regulatory mechanism. Artificial ageing of rice seeds was linked to the increase in the abundance of HSP18.2, considered an ageing-responsive protein (Kaur et al., 2015). The enhancement of HSP content was also found in the embryos of artificially aged maize (*Zea mays* L.) seeds (Xin et al., 2011) and in the embryos of aged seeds of wheat (*Triticum aestivum* L.) or barley (*Hordeum vulgare* L.) (Dell'Aquila et al., 1998). Other proteins whose nitration has been confirmed in axes of artificially aged apple seeds include: L-lactate dehydrogenase B-like (involved in anaerobic metabolism, Fig. 5, Table 3, spots no. 6, 20, 21, 36, 37), enolase (key protein in glycolysis pathway, Fig. 5, Table 3, spot no. 16), ATP synthase subunit β (involved in energy metabolism, Fig. 5, Table 3, spots no. 1, 15 and probably 31), mitochondrial hypothetical protein DVH24_025610 (Fig. 5, Table 3, spots no. 11, 26, 27, 41), phosphopantetheine adenylyltransferase-like isoform X2 (the coenzyme A biosynthetic enzyme, Fig. 5, Table 3, spots no. 12, 13, 28, 29, 42), E3 ubiquitin-protein ligase CCNB1IP1 homolog isoform X2 (Fig. 5, Table 3, spots no. 14, 30, and probably 43). Clearly, most of those identified nitrated proteins are functionally associated with energy generation. As was already mentioned, this modification may affect the function of the protein. Thus, it can be assumed that the deteriorative effect of seed ageing is linked with the impaired energy homeostasis resulting from both protein nitration and ROS overaccumulation. Treatment of apple embryos with NO resulted in varying intensities of nitrated protein spots among samples collected from different ageing periods. In some cases, the abundance of a given spot (after 2-DE) was higher in the control than in the NO-treated samples, while in others, the levels were comparable. This suggests that the presence of similar amounts of these nitrated proteins across different stages of apple seed ageing may reflect nitration events occurring during seed maturation, and that the regulation of a given protein's activity could be associated with its subsequent degradation.

5. Conclusion and perspectives

Recognising the beneficial role of NO in improving the quality of aged seeds, including apple seeds, it is important to note its multidirectional effects (Fig. 8). The variability in NO effect, with no linear dependence, was reflected at the metabolomic level (Ciacka et al., 2022a; Tyminski et al., 2024), transcriptomic level (Ciacka et al., 2022b), and the current data confirmed this tendency at the proteomic level (Fig. 8). Protein quality and quantity changes were linked to the

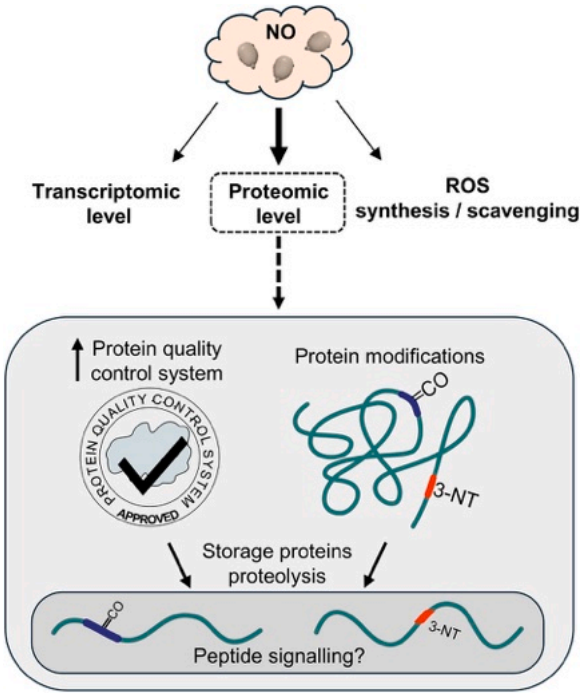

Fig. 8. The role of NO in the mitigation of negative ageing effects in apple embryonic axes at various cellular levels. The transcriptomic level was discussed by Ciacka et al. (2022b), the ROS/antioxidants level was discussed by Ciacka et al. (2022a) and Tyminski et al. (2024). Proteomic level (discussed in this work) represents both the protein quality control system and possible irreversible PTMs: carbonylation (CO groups) and nitration (3-NT group). Modified storage protein (eg. prunin 1), after proteolysis, may release peptides with CO and/or 3-NT groups, which may have a possible signalling.

different activities of protein control systems. In this context, NO may be suggested to influence the protein control mechanisms to maintain proteostasis. This is especially important at the time when seeds enter the late phase of ageing. No specific proteins were clearly identified as particularly carbonylated or nitrated at each stage of apple seed ageing. These modifications affected various proteins, depending on whether negative changes had already occurred or not.

Furthermore, our data raise an important question: why can the storage protein be both carbonylated and nitrated? We suppose that this may be because released fragments with carbonyl or nitro groups, or even the modified amino acids themselves, may act as messengers. This concept is in line with the idea of Møller and Sweetlove (2010). The authors proposed that carbonylated peptides may participate in the signal transduction related to the ROS level in various cellular compartments. Previously, we demonstrated that in germinating, non-aged, apple embryos, the level of nitrated proteins altered, especially of those with higher molecular mass corresponding to storage proteins and SBPs (Krasuska et al., 2016). We concluded that the level of nitrated proteins (most probably reserve proteins) may serve as a stable indicator of dormancy status. Seed storage proteins may also function as ROS scavengers in seeds due to their abundance and high affinity for ROS-mediated PTMs (Nguyen et al., 2015). Whether these proteins would perform RNS scavenger function during seed maturation needs further experimental verification. Interestingly, Tyr nitration is functionally connected with polyubiquitination (León, 2022). Such interconnected modifications were observed in Arabidopsis during inactivation of PYR/PYL/RCAR ABA receptors (Castillo et al., 2015). As was described in detail, proteins with NO-related PTM can undergo additional modifications(León, 2022 and citations therein). Therefore, multiple changes in seed storage proteins (both carbonylation and nitration), and theirs further stability or cellular translocation should be examined.

In this study we managed to link the ROS- and RNS-dependent modifications with seed ageing progress. This novel results are a foundation for future research, possibly not only focusing on *orthodox*-type seeds. Interestingly, such ROS- and RNS-dependent irreversible modifications were related mostly to storage proteins. Rapid degradation of storage proteins is inherent in the biology of seeds whose dormancy has been released. However, the question still remains open what happens to the irreversibly modified fragments of reserve proteins and how they are subsequently utilized in plant cells.

CRediT authorship contribution statement

Marcin Tyminski: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Data curation. **Katarzyna Ciacka:** Writing – review & editing, Methodology. **Pawel Staszek:** Writing – review & editing, Methodology. **Urszula Krasuska:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Agnieszka Gniazdowska:** Writing – review & editing.

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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.2025.112828.

Data availability

Data will be made available on request.

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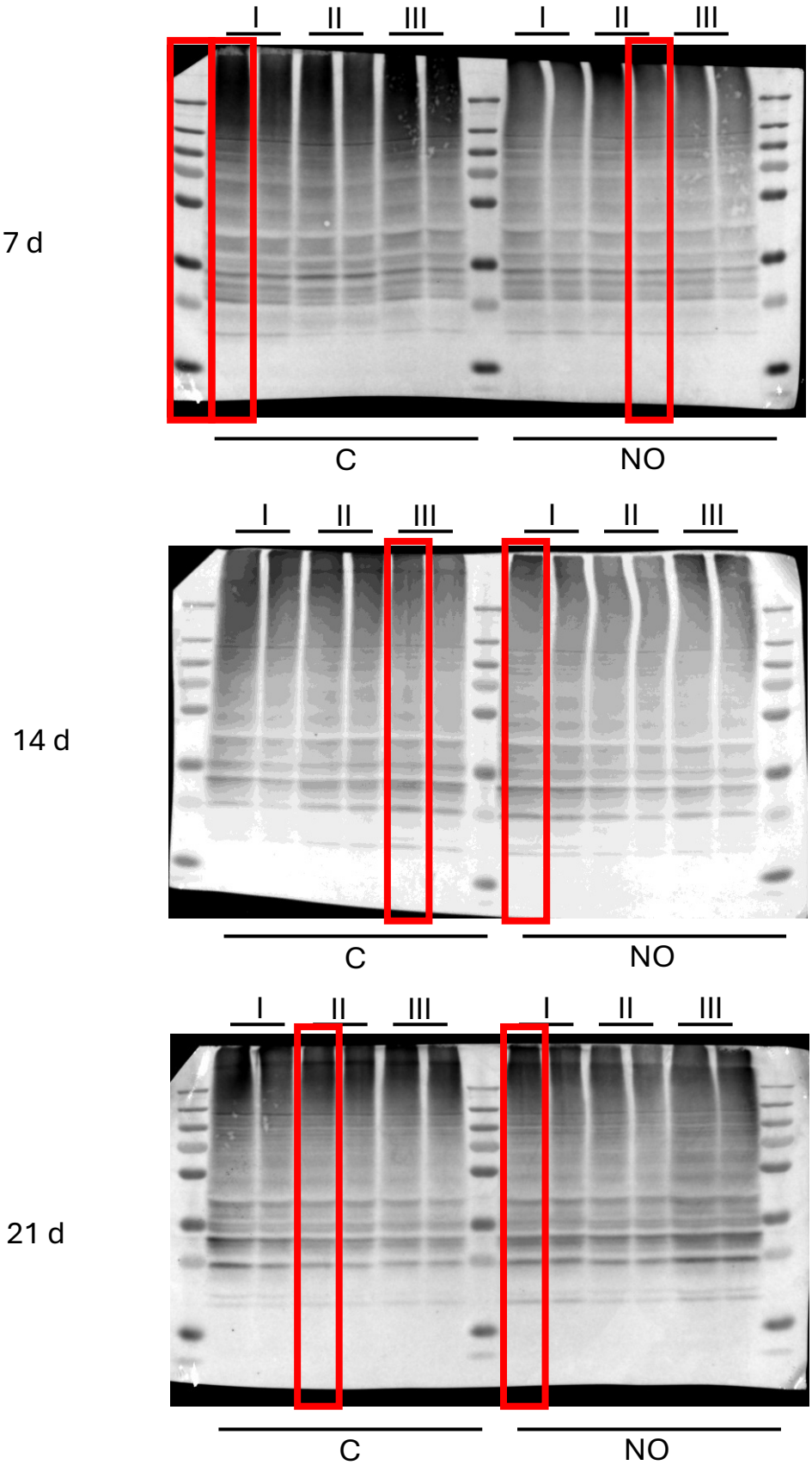
Table S1. Ingredients of Protease Inhibitor Cocktail

Inhibitor	Inhibitor target
AEBSF [4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride]	serine proteases, such as trypsin, chymotrypsin, plasmin, kallikrein and thrombin
Bestatin hydrochloride	aminopeptidases, such as leucine aminopeptidase and alanyl aminopeptidase1-4
E-64 [N-(trans-Epoxysuccinyl)-L-leucine 4-guanidinobutylamide]	cysteine proteases, such as calpain, papain, cathepsin B, and cathepsin L
Leupeptin hemisulfate salt	serine proteases and cysteine proteases, such as plasmin, trypsin, papain, and cathepsin B
Pepstatin A	acid proteases (such as pepsin, renin and cathepsin D) and many microbial aspartic proteases
1,10-phenanthroline	metalloproteases

Data obtained from manual of Protease Inhibitor Coctail (P9599 Sigma-Aldrich)

S2. Immunoblots visualising the pattern of ubiquitin-conjugated proteins in axes of apple embryos isolated from seeds aged for 7, 14, and 21 days (C), or aged and treated with NO (NO), which were used for the construction of Fig. 6. Biological repetitions were indicated by numbers (I-III). The red boxes indicate the pathways that were used in Fig 6.



S3 Germination tests

Apple embryos isolated from aged seeds were subjected to germination tests. Embryos treated with NO, as well as the control, were placed on glass Petri dishes (15 per dish) on wet filter paper. The germination tests were conducted for 7 days in the growth chamber under the conditions described above. Embryos with embryonic roots exhibiting gravitropic bending were noted. Germination tests were performed in 3 independent repetitions.

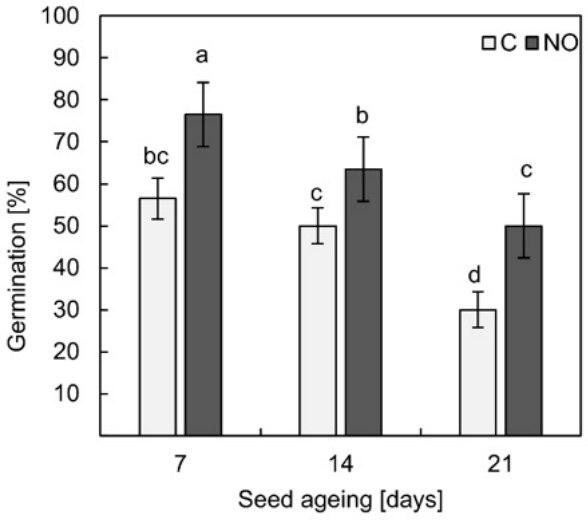
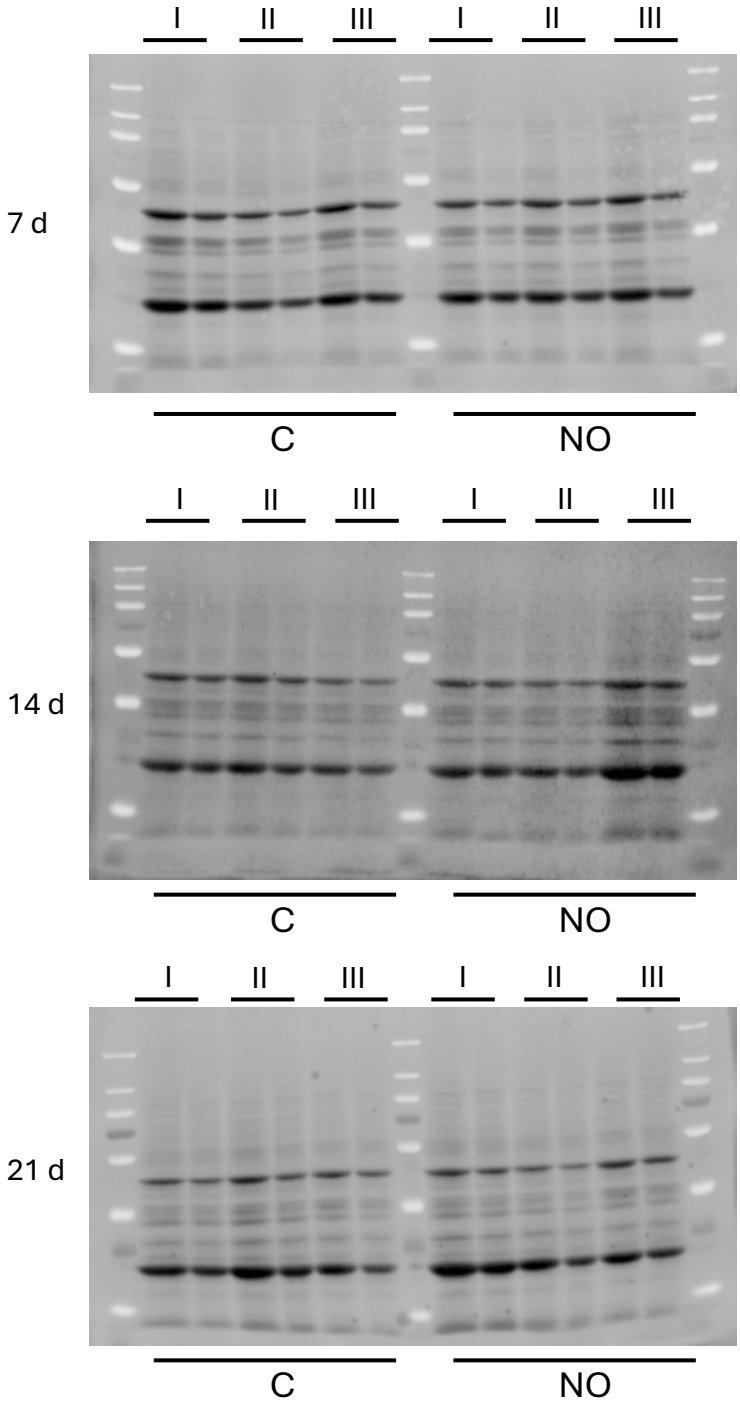


Figure. Germination of apple embryos isolated from seeds subjected to accelerated ageing for 7, 14 and 21 days, after 7 days of culture under optimal conditions. NO-treated embryos are marked as NO, and control embryos as C. Values are the average of 3 biological repetitions ± SD. The homogeneous groups were labelled with letters, according to Duncan's post hoc test ($P < 0.05$).

Nitric oxide improves the germination of artificially aged embryos

Germination tests conducted in this study confirmed the detrimental effect of artificial seed ageing on the vigour of apple embryos. Embryos isolated from seeds aged for 7 days germinated in approximately 55% (Figure). Extension of seed ageing to 14 days resulted in a slight, non-significant decrease in embryo germination (approximately 50%). Fumigation with NO significantly increased germination of those embryos up to 75% and 64%, respectively (Figure). Further deterioration in embryo vigour was noticed after the 21-day ageing procedure. Embryos germinated poorly, by approximately 30%. It was noted that germination of embryos isolated from seeds aged for 21 days and treated with NO was enhanced by approximately 70% in comparison with the control (Figure).

S2. Images of membranes obtained after protein transfer. Total protein content on each membrane was visualized using the Stain-Free technology prior to immunodetection. These membranes were used for immunodetection of ubiquitin-conjugated proteins in axes of apple embryos isolated from seeds aged for 7, 14, and 21 days (C), or aged and treated with NO (NO), which were used for the construction of Fig. 6. Biological repetitions were indicated by numbers (I-III).



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Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy:

Tyminski Marcin, Staszek Pawel, Katarzyna Ciacka, Agnieszka Gniazdowska, Urszula Krasuska. 2026. NO treatment of embryos isolated from artificially aged apple (*Malus domestica* Borkh.) seeds modifies the pattern of nitrated and carbonylated proteins. Plant Science 362: 112828. https://doi.org/10.1016/j.plantsci.2025.112828

mój indywidualny udział w jej powstaniu obejmował przygotowanie materiału badawczego, przeprowadzenie eksperymentów: oznaczenia aktywności proteolitycznej, tempa degradacji białek rozpuszczalnych w wodzie, immunodetekcji białek ubikwitynowanych, oraz określenia aktywności proteasomu 20S. Ponadto dokonałem identyfikacji białek karbonylowanych i nitrowanych. Uczestniczyłem w opracowaniu metodyki badań opisanych w pracy. Byłem odpowiedzialny za analizę wyników i przygotowanie rysunków znajdujących się w pracy. Brałem udział w przygotowaniu pierwszej wersji manuskryptu oraz uczestniczyłem w jego korekcie i redakcji. Pełniłem funkcję autora korespondencyjnego.

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mój indywidualny udział w jej powstaniu obejmował współuczestnictwo w opracowaniu metodyki identyfikacji białek karbonylowanych i nitrowanych, sprawowaniu opieki merytorycznej w trakcie prowadzenia badań oraz redakcję i korektę tekstu manuskryptu.

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mój indywidualny udział w jej powstaniu polegał na przygotowaniu koncepcji pracy, sprawowaniu merytorycznej opieki podczas prowadzonych badań oraz przygotowywania publikacji. Uczestniczyłam w przygotowaniu pierwszej wersji manuskryptu oraz jego redakcji i korekcie. Zapewniłam finansowanie badań opisanych w pracy.

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