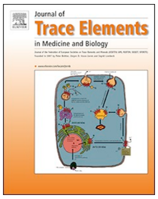
Journal of Trace Elements in Medicine and Biology 55 (2019) 96–106

Contents lists available at ScienceDirect

Journal of Trace Elements in Medicine and Biology

journal homepage: www.elsevier.com/locate/jtemb



Physiology

Senescence is delayed by selenium in oilseed rape plants

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ARTICLE INFO

Keywords:
Selenium
Senescence
Antioxidative defense
Nitric oxide
SAG12-1 gene
Cab gene

ABSTRACT

Leaf senescence is a genetically programmed process that can also be induced by nitrogen (N) deficiency. Although selenium (Se) delays leaf senescence, the underlying mechanisms are still unknown. To explore the mechanisms of Se-mediated delay of leaf senescence, we studied the biochemical and molecular events that occur during developmental and N deficiency-induced senescence. Oilseed rape (*Brassica napus* L.) plants were grown under adequate N (AN, 16 mM) or low N (LN, 4 mM) conditions during the rosette growth stage and treated with Se (15 µg plant^{-1} as Na_2SeO_4) either through roots or leaves for four weeks. Shoot dry matter production was not influenced, while the photosynthetic parameters were improved by Se application in both young and old leaves under both AN and LN conditions. The Se treatment rarely influenced the concentrations of reactive oxygen species (ROS), while it increased the nitric oxide (NO) levels in young and old leaves under both AN and LN conditions. The positive correlation between the NO level and leaf photosynthetic parameters in old leaves of LN plants suggested a role for NO boosting, mediated by Se, in the protection of aging leaves from LN-induced accelerated senescence. This implication was further supported by the clear down-regulation of *SAG12-1* and up-regulation of *Cab*, particularly by root application of Se in old leaves of LN plants. Our results provide the first evidence that Se influences the expression of senescence-associated genes and delays senescence through NO signalling but is independent of the ROS defence system.

1. Introduction

Senescence in plants is an age-dependent programmed degradation process of organs or the entire organism [1,2]. Leaf senescence is the last phase of leaf development, during which cellular macromolecules are degraded and metabolic activity ceases [1,2]. Because one of the earliest features of leaf senescence is the decrease of photosynthesis, the progression of leaf senescence is usually evaluated by changes in the protein and chlorophyll (Chl) contents and photosystem II (PSII) photochemistry [1,2]. Leaf senescence is accompanied by a decrease in the expression of senescence-down-regulated genes (SDGs), *e.g.*, *Cab*, which encodes a Chl *a*-binding protein, and up-regulation of senescence-associated genes (SAGs), *e.g.*, *SAG12-1*, which encodes a cysteine protease [3].

Generation of reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2) and the superoxide radical ($O_2^{\cdot -}$), is a common phenomenon in aging leaves [4]. Reactive oxygen species play an essential role in leaf senescence in two different aspects: signalling and degradation of macromolecules and membrane constituents [5]. Antioxidative enzymes, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and peroxidase (POD), are responsible for scavenging ROS and protecting the membrane from damage [6]. In addition to H_2O_2 and $O_2^{\cdot -}$, nitric oxide (NO), a reactive nitrogen species and signalling molecule, is a key player in the regulation of different developmental processes in plants, including leaf senescence [7]. Nitric oxide was shown to act as a negative regulator of the Chl catabolic pathway and function in the maintenance of the stability of thylakoid membranes during leaf senescence in an NO-deficient *Arabidopsis* mutant [8].

Selenium (Se) is not an essential nutrient for higher plants, but at low concentrations (> ˜20–40 µM for SeVI), it has been considered a beneficial element [9]. In addition to the amelioration of stress (for a review see [10] and [11]), Se-mediated inhibition of senescence progression has been reported in lettuce [12] and soybean [13]. Although this effect has been attributed to the activation of the antioxidative defence system [13], sufficient experimental evidence for the contribution of antioxidative defence to the Se-mediated delay of leaf senescence is lacking. At toxic concentrations, Se influences plant growth

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https://doi.org/10.1016/j.jtemb.2019.06.005
Received 23 January 2019; Received in revised form 26 May 2019; Accepted 11 June 2019

through NO generation [14,15]. Nevertheless, the effect of beneficial low Se concentrations on NO generation and signalling has not been investigated yet.

Nitrogen (N) is an essential macronutrient for plants. An inadequate N supply induces chlorosis and accumulation of ROS [16] and accelerates senescence of leaves [17]. Considering that NO is generated, in part, through nitrate reductase [18], a key enzyme involved in N assimilation [19], NO production can be affected by N supply, and conversely NO appears to regulate nitrate uptake [20]. However, the effect of N deficiency on the production of NO and its role in N deficiency-induced leaf senescence has not been investigated so far.

In oilseed rape (*Brassica napus*), similar to other members of Brassicaceae, maturation of leaves is independent of the reproductive cycle and they senesce following a period of rapid expansion [21]. This provides an opportunity to study leaf senescence even before the bolting and flowering stages. Oilseed rape is a secondary accumulator of Se [22] and is known to have low N use efficiency [23]. One strategy to improve the N use efficiency in oilseed rape would be to delay senescence until seed filling is completed [24]. In a previous work on this species, we observed that leaves of Se-treated plants remained fairly green even one week after detachment [25]. However, the potential effect of Se on delaying N-deficiency-induced senescence has not been investigated so far.

Foliar fertilization is a common approach to supply nutrients to crop plants [26]. Leaf-applied nutrients can enter the leaf either by penetration of the cuticle or *via* the stomatal pathway [27]. Foliar application of Se is increasingly used for biofortification purposes [28,29] because of the higher recovery rates of leaf-applied Se in edible portions of crops than those of soil-applied Se [30] and the disadvantages of soil fertilization, such as excessive Se accumulation with long-term application of Se fertilizer [31]. However, the rate of uptake and assimilation of leaf-applied Se is species- and genotype-specific [31,32], and there are no comparative studies on the efficacy of leaf versus root application of Se on its senescence-delaying effect.

Here, we characterize the effect of N nutritional status and Se treatment on leaf senescence in oilseed rape plants. Our working hypothesis was that Se delays both developmental and N-deficiency-induced senescence through the same mechanisms. Therefore, we investigated the contribution of ROS and NO signalling and reported the expression of two important senescence indicators, *i.e.*, *SAG12-1* and *Cab*, in this species.

2. Material and methods

2.1. Plants culture and treatments

Seeds of oilseed rape (*Brassica napus* L. cv. Hayola) plants provided by the Seed and Plant Improvement Institute (SPII, Karaj, Iran) were surface sterilized using commercial bleach (containing 10% sodium hypochlorite) and germinated on sterile perlite in the dark. One-week-old seedlings were transferred to the light and irrigated with 50% Hoagland nutrient solution (pH 6.0) [33]. All chemicals used for preparation of the nutrient solution were of analytical grade and purchased from Merck (Germany). Two-week-old seedlings were transferred to a 5 L plastic container (5 plants per container) containing washed perlite and irrigated daily at field capacity with nutrient solution or water at intervals. The volume of 100% nutrient solution (which was 500 mL per container on week 1) increased gradually to 1000 mL per container on week 3 and then remained constant until harvest. Plants were grown under controlled environmental conditions with a temperature regime of 25/17 °C day/night, 14/10 h light/dark period, a relative humidity of 50/60% and at a photosynthetic photon flux density (PPFD) of approximately 300 μmol m^{-2} s^{-1} provided by fluorescent lamps.

Four weeks after sowing, two different nitrogen (N) treatments, adequate N (AN, 16 mM) and low N (LN, 4 mM), were started. The N levels were determined by different $Ca(NO_3)_2$ concentrations; to balance the Ca concentration, $CaCl_2$ was added to the low N medium. Simultaneously, selenium (Se) treatments (as Na_2SeO_4, Fluka, Switzerland) were started, and Se was applied either to the roots (+SeR) or to the leaves (+SeL) gradually over 3 weeks. In the Se treatment through roots, the irrigation water supplemented with Se was applied to the pots. A total of 75 μg Se was applied to each container over 3 weeks of treatment. For the leaf application treatment, Se was dissolved in distilled water and leaves were sprayed while keeping them away from direct light. The total volume of Se solution of 150 μg L^{-1} (˜ 2 μM) sprayed during three weeks to each container was 500 mL. Thus, the total Se amount supplied was 15 μg $plant^{-1}$ and similar for the + SeR and + SeL treatments. This Se dose being equivalent to 10.5 g ha^{-1} and 2.25 μg kg^{-1} soil, was chosen on the grounds of previous studies with field-grown (10–60 g ha^{-1}) [28] and pot-grown (2–7 μg kg^{-1} soil) [34] plants.

2.2. Plants harvest

Plants were harvested 8 weeks after sowing, *i.e.*, four weeks after starting different N and Se treatments. Before the harvest, gas exchange and chlorophyll (Chl) fluorescence parameters were determined in the attached leaves. At harvest, whole roots and shoots were excised separately, rinsed with deionized water and blotted dry on filter paper. Plant materials were dried at 65 °C for 2 d, and dry weights (DW) were determined. All analyses were performed in the second youngest and the second oldest leaves. Subsamples (100 mg) taken from the leaves of the specified age were used immediately for or stored in liquid nitrogen until analysis.

2.3. Determination of photochemical and gas exchange parameters

Chl fluorescence parameters were recorded using a portable fluorometer (OSF1, ADC Bioscientific Ltd., UK). The net photosynthetic rate was recorded using a calibrated portable gas exchange system (LCA-4, ADC Bioscientific Ltd., UK) during the light period between 9:00 and 13:00 h under a PPFD of approximately 300 μmol m^{-2} s^{-1} provided by incandescent lamps.

2.4. Determination of leaf Chl and protein

Leaf Chl was extracted in 80% cold acetone in the dark at 4 °C. The absorbance (Abs) was determined at 646 and 663 nm and the concentrations (μg mL^{-1}) of Chl *a* and Chl *b* were determined according to the equations Chl $a = 12.25$ $Abs_{663} - 2.79$ A_{646} and Chl $b = 21.50$ $A_{646} - 5.10$ A_{663} [35] and recalculated on the basis of leaf fresh weight. The soluble protein concentration was determined using Bradford reagent (Sigma-Aldrich, USA) and bovine albumin serum (BSA, Merck, Germany) as standard.

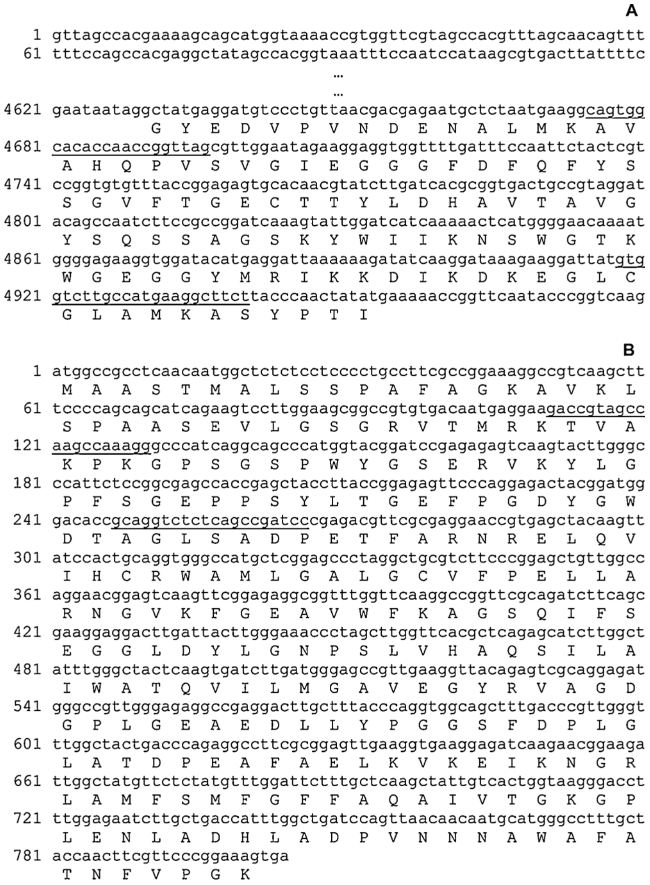
2.5. Determination of enzymes and metabolites of the antioxidative defence system

Total superoxide dismutase (SOD, EC 1.15.1.1) activity was determined on the basis of the reduction of NBT (p-nitro blue tetrazolium chloride, Sigma-Aldrich, USA) and production of blue formazan in the presence of light. After the determination of absorbance at 560 nm, one unit of SOD was calculated as the amount of enzyme that inhibits the reduction of NBT by 50% compared with samples without enzyme [36]. Catalase (CAT, EC 1.11.1.6) activity was assayed by monitoring the decrease in the absorbance of H_2O_2 at 240 nm [37]. The activity of ascorbate peroxidase (APX, EC 1.11.1.11) was quantified by measuring the reduction of the absorbance of ascorbic acid at 290 nm and calculated using an extinction coefficient of 2.8 mM^{-1} cm^{-1} [38]. Peroxidase (POD, EC 1.11.1.7) activity was assayed in a reaction medium containing guaiacol as substrate, the absorbance of the formed tetraguaiacol was determined at 470 nm, and the enzyme activity was calculated using the extinction coefficient of this compound 25.5 $mM^{-1} cm^{-1}$ [37].

The concentration of superoxide radical ($O_2^{\cdot}$–) was determined using its capacity to reduce NBT (250 μM) in medium containing diethyldithiocarbamate (1 mM) to inhibit SOD activity [39]. The concentration of H_2O_2 in the extract obtained with 0.1% (w/v) trichloroacetic acid (Sigma-Aldrich, USA) was determined using potassium iodide (KI, Merck, Germany) at 390 nm (w/v) [40]. The nitric oxide (NO) concentration was determined by the colorimetric method using Griess reagent [41]. After extraction with 100 mM phosphate buffer (pH 7.4) and centrifugation, it was determined in a reagent mixture containing sulphanilamide (Sigma-Aldrich, USA) and *N*-(1- naphthyl) ethylenediamine (NED, Sigma-Aldrich, USA) at 520 nm. The standard curve contained $NaNO_2$ (Merck, Germany) of the range 0–5 μM, and the NO concentrations were calculated as nmol g^{-1} FW [41]. To quantify lipid peroxidation, as an estimate for membrane damage, the amount of malondialdehyde (MDA) was determined spectrophotometrically at 532 nm after its reaction with thiobarbituric acid (Sigma-Aldrich, USA). A calibration curve was created using 1,1,3,3-tetraethoxypropane (Sigma-Aldrich, USA) [42].

2.6. Gene expression analyses

The expression of *B. napus SAG12-1* gene (AF089848.1) (Fig. 1A) and *B. napus Cab* gene (DQ645428.1) (Fig. 1B) were analysed in young leaves of AN and old leaves of LN plants. A modified CTAB-based method was used for total RNA extraction [43]. First-strand cDNA was synthesized from 2 μg of total RNA from each sample using the PrimeScript™ RT reagent kit (Takara Biotechnology, Dalian, China) according to the manufacturer's instructions. Real-time PCR amplification was conducted using SYBR® Premix EX Taq™ II (*Takara Biotechnology*, Dalian, China) on an Eco Real-Time PCR system (Illumina) under the following steps: 1 cycle of 95 °C for 10 min and 40 cycles of 95 °C for 20 s, 63 °C for 30 s and 72 °C for 25 s. The primer sequences were listed in Table A.1. The $2^{-\Delta\Delta CT}$ formula was used for relative gene expression analysis with *β-actin* gene (AF111812.1) as an endogenous control [44].



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                                                                  A
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  61 tttccagccacgaggctatagccacggtaaatttccaatccataagcgtgacttattttc
                                 ...
                                 ...
4621 gaataataggctatgaggatgtccctgttaacgacgagaatgctctaatgaaggcagtgg
               G  Y  E  D  V  P  V  N  D  E  N  A  L  M  K  A  V
4681 cacaccaaccggttagcgttggaatagaaggaggtggttttgatttccaattctactcgt
      A  H  Q  P  V  S  V  G  I  E  G  G  G  F  D  F  Q  F  Y  S
4741 ccggtgtgtttaccggagagtgcacaacgtatcttgatcacgcggtgactgccgtaggat
      S  G  V  F  T  G  E  C  T  T  Y  L  D  H  A  V  T  A  V  G
4801 acagccaatcttccgccggatcaaagtattggatcatcaaaaactcatggggaacaaaat
      Y  S  Q  S  S  A  G  S  K  Y  W  I  I  K  N  S  W  G  T  K
4861 ggggagaaggtggatacatgaggattaaaaagatatcaaggataaagaaggattatgtg
      W  G  E  G  G  Y  M  R  I  K  K  D  I  K  D  K  E  G  L  C
4921 gtcttgccatgaaggcttcttacccaactatatgaaaaaccggttcaatacccggtcaag
      G  L  A  M  K  A  S  Y  P  T  I

                                                                  B
   1 atggccgcctcaacaatggctctctcctcccctgccttcgccggaaaggccgtcaagctt
      M  A  A  S  T  M  A  L  S  S  P  A  F  A  G  K  A  V  K  L
  61 tccccagcagcatcagaagtccttggaagcggccgtgtgacaatgaggaagaccgtagcc
      S  P  A  A  S  E  V  L  G  S  G  R  V  T  M  R  K  T  V  A
 121 aagccaaagggcccatcaggcagcccatggtacggatccgagagagtcaagtacttgggc
      K  P  K  G  P  S  G  S  P  W  Y  G  S  E  R  V  K  Y  L  G
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      P  F  S  G  E  P  P  S  Y  L  T  G  E  F  P  G  D  Y  G  W
 241 gacaccgcaggtctctcagccgatcccgagacgttcgcgaggaaccgtgagctacaagtt
      D  T  A  G  L  S  A  D  P  E  T  F  A  R  N  R  E  L  Q  V
 301 atccactgcaggtgggccatgctcggagccctaggctgcgtcttcccggagctgttggcc
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 361 aggaacggagtcaagttcggagaggcggtttggttcaaggccggttcgcagatcttcagc
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 481 atttgggctactcaagtgatcttgatgggagccgttgaaggttacagagtcgcaggagat
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 541 gggccgttgggagaggccgaggacttgctttacccaggtggcagctttgacccgttgggt
      G  P  L  G  E  A  E  D  L  L  Y  P  G  G  S  F  D  P  L  G
 601 ttggctactgacccagaggccttcgcggagttgaaggtgaaggagatcaagaacggaaga
      L  A  T  D  P  E  A  F  A  E  L  K  V  K  E  I  K  N  G  R
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      L  E  N  L  A  D  H  L  A  D  P  V  N  N  N  A  W  A  F  A
 781 accaacttcgttcccggaaagtga
      T  N  F  V  P  G  K
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Fig. 1. The nucleotide and amino acid sequence of *B. napus* senescence-specific cysteine protease (*SAG12-1*) gene (GenBank accession number: AF089848.1) (A) and the coding and amino acid sequence of *B. napus* chloroplast chlorophyll *a/b* binding protein (*Cab*) gene (GenBank accession number: DQ645428.1) (B). The primers binding sites are underlined.

2.7. Experimental design and statistical analyses

The experiment was undertaken using a completely randomized design with four independent containers as four replicates. Pairwise comparison of means was performed by Tukey's test ($P < 0.05$). Three–way ANOVA was performed for the main effects, including leaf age (L, two levels), nitrogen (N, two levels) and Se treatment (Se, three levels) and their interactions (Table A.2). Correlations between different variables within each leaf and N treatment were calculated by Pearson Correlation (r) using Sigma Stat 3.02. To assign different senescence-related parameters to distinct groups, principal component analysis (PCA) was conducted using Minitab 17.

3. Results

3.1. Plant growth, photosynthesis rate and protein concentration

Dry matter production of the plants decreased under low nitrogen (LN) conditions. The selenium (Se) treatment did not influence shoot dry weight (DW) in either adequate nitrogen (AN) or LN plants. On the contrary, the root application of Se (+SeR) significantly increased the root DW (Fig. 2A). In both N treatments the photosynthesis rate in the leaves declined with their increasing age. The Se treatment increased leaf photosynthesis rate irrespective of leaf age and the N treatment. The effects of the two application modes did not differ except in the old leaves of LN plants where only the + SeR treatment increased the photosynthesis rate (Fig. 2B). As expected, the total leaf protein concentration was lower in LN plants than in AN plants and in the old leaves lower than in the young ones ($P < 0.001$). The Se treatment did not affect the protein concentration in AN plants, whereas + SeR (but not + SeL) elevated it in LN plants (Fig. 2C).

3.2. Leaf Chl concentration and photochemical parameters

The leaf chlorophyll (Chl) concentration decreased under LN conditions, particularly in old leaves. The Se treatment increased the Chl concentration only in old leaves of LN plants (Table 1). The LN treatment decreased the maximum quantum yield of PSII (Fv/Fm) in both young and old leaves. The added Se did not influence Fv/Fm in AN plants but increased this parameter in LN plants, the increase being greater in old leaves. The excitation capture efficiency of PSII (F'v/F'm), by contrast, decreased in both young and old leaves under LN conditions. In the young leaves the Se treatment increased F'v/F'm only under LN conditions, while in old leaves it affected this parameter in both AN and LN plants, +SeL being more effective than + SeR. A significant effect of the LN treatment on the reduction of photochemical quenching (qP) was observed only in old leaves, and the beneficial effect of Se was significant for + SeL. In contrast to qP, non-photochemical quenching (qN) increased under LN conditions in both young and old leaves. The Se treatment further increased this parameter in old leaves; this effect was significant for + SeL (Table 1). The ANOVA showed that the majority of the biophysical and biochemical components of leaf photosynthesis were influenced by all three main factors and their two-way interactions (L × N, L × Se, N × Se) (Table A.2).

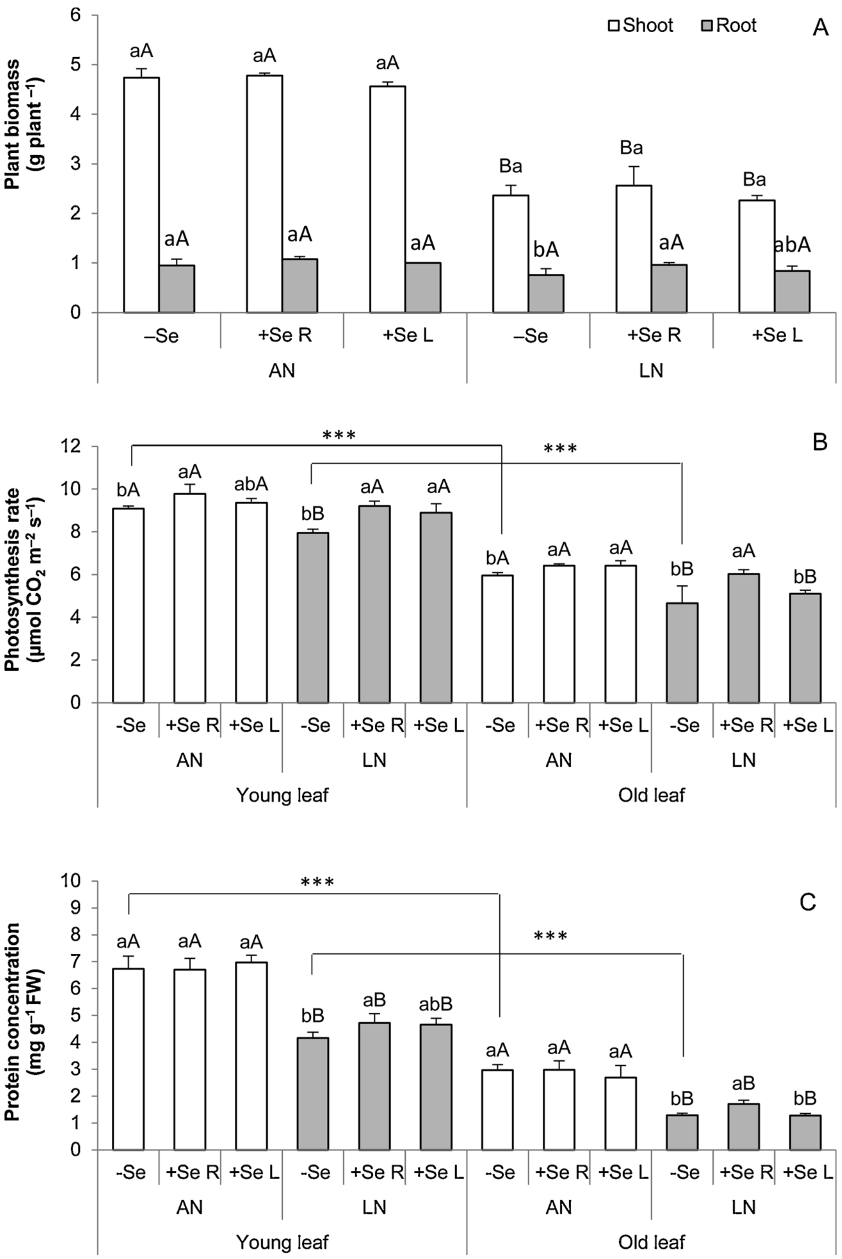

Fig. 2. Plant dry biomass (A), photosynthesis rate (B) and protein concentration (C) in young and old leaves of oilseed rape (*Brassica napus*) plants supplied by adequate nitrogen (AN, 16 mM) or low nitrogen (LN, 4 mM) and treated with selenium (15 µM Se plant^{-1} as Na_2SeO_4) either through roots (+ Se R) or leaves (+ Se L) for four weeks. Significant differences among three Se treatments (within each N level) were indicated by different lower case letters, while different upper case letters indicated significant differences between two N levels (within each Se treatment) ($P < 0.05$). Differences between the young and old leaves in the –Se plants were indicated by an asterisk (*** significant at $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns non-significant).

3.3. Activities of antioxidative enzymes

The activity of SOD was significantly higher in old leaves than young ones and increased under LN conditions. The Se treatment influenced the SOD activity depending on the N treatment, leaf age, and application mode. In young leaves, the + SeR (but not + SeL) treatment increased the SOD activity in AN plants, but slightly decreased it in LN plants. In old leaves, the added Se significantly reduced SOD activity only in AN plants (Fig. 3A). Similar as SOD, the activity of CAT in old leaves was higher than in the young ones, but in contrast to SOD, the CAT activity decreased under LN conditions both in young and old leaves. In young leaves of the AN plants the Se treatment did not affect the CAT activity, while the + SeR treatment decreased it in LN plants. In old leaves, both Se application modes decreased CAT activity in AN plants; for LN plants, this parameter decreased with + SeR and increased with the + SeL treatment (Fig. 3B).

The APX activity increased with aging of the leaves but was not changed under N deficiency conditions. The + SeR treatment significantly decreased APX activity irrespective of leaf age and the N treatment. The effect of + SeL, however, was not significant in young leaves of AN plants (Fig. 3C). The activity of POD was higher in old leaves than young ones and increased under LN conditions, but was not significantly influenced by the Se treatment (Fig. 3D). The ANOVA showed that the activities of all four studied antioxidative enzymes were affected by three main factors and their two-way interactions, except APX and POD which did not show a significant effect of L × Se (Table A.2).

3.4. Concentrations of ROS, NO and MDA

The concentration of $O_2^{\cdot -}$ decreased with aging of the leaves but it accumulated under LN conditions. Selenium did not influence the $O_2^{\cdot -}$ concentration (Fig. 4A). The concentration of H_2O_2, similarly as in the case of $O_2^{\cdot -}$, was higher in the young than in the old leaves of AN plants. The LN treatment increased consistently the H_2O_2 concentration both in the young and old leaves. Selenium did not influence the H_2O_2 level in young or old leaves (Fig. 4B). In contrast to $O_2^{\cdot -}$, the constitutive concentration of NO was higher in the old leaves than in the

Table 1
Leaf chlorophyll (Chl) concentration (mg g^{-1} FW) and Chl fluorescence parameters including maximum quantum yield of PSII (Fv/Fm), excitation capture efficiency of PSII (F'v/F'm), photochemical quenching (qP) and non-photochemical quenching (qN) in young and old leaves of oilseed rape (*Brassica napus*) plants grown under adequate nitrogen (AN, 16 mM) or low nitrogen (LN, 4 mM) supply and treated with selenium (15 µM Se $plant^{-1}$ as Na_2SeO_4) either through roots (+Se R) or leaves (+Se L) for four weeks. Significant differences among three Se treatments (within each N level) were indicated by different lower case letters while different upper case letters indicated significant differences between two N levels (within each Se treatment) ($P < 0.05$).

Treatments		Young leaf				
		Chl *a*+*b*	Fv/Fm	F'v/F'm	qP	qN
AN	–Se	4.78 ± 0.21 ^{aA}	0.84 ± 0.01 ^{aA}	0.83 ± 0.01 ^{aA}	0.89 ± 0.01 ^{aA}	0.10 ± 0.05 ^{aB}
	+Se R	5.04 ± 0.37 ^{aA}	0.85 ± 0.01 ^{aA}	0.85 ± 0.01 ^{aA}	0.88 ± 0.01 ^{aA}	0.11 ± 0.03 ^{aB}
	+Se L	4.75 ± 0.68 ^{aA}	0.84 ± 0.00 ^{aA}	0.84 ± 0.01 ^{aA}	0.88 ± 0.02 ^{aA}	0.10 ± 0.00 ^{aB}
LN	–Se	3.05 ± 0.12 ^{aB}	0.83 ± 0.01 ^{bA}	0.79 ± 0.01 ^{bB}	0.89 ± 0.02 ^{aA}	0.28 ± 0.04 ^{aA}
	+Se R	2.97 ± 0.35 ^{aB}	0.85 ± 0.01 ^{aA}	0.82 ± 0.01 ^{aB}	0.91 ± 0.03 ^{aA}	0.25 ± 0.03 ^{aA}
	+Se L	2.94 ± 0.20 ^{aB}	0.84 ± 0.00 ^{abA}	0.84 ± 0.01 ^{aA}	0.90 ± 0.03 ^{aA}	0.26 ± 0.00 ^{aA}
		Old leaf				
		Chl *a*+*b*	Fv/Fm	F'v/F'm	qP	qN
AN	–Se	3.10 ± 0.16 ^{aA}	0.84 ± 0.01 ^{aA}	0.81 ± 0.00 ^{bA}	0.86 ± 0.01 ^{aA}	0.24 ± 0.03 ^{bB}
	+Se R	2.89 ± 0.14 ^{aA}	0.85 ± 0.01 ^{aA}	0.82 ± 0.01 ^{abA}	0.88 ± 0.01 ^{aA}	0.28 ± 0.00 ^{abB}
	+Se L	2.91 ± 0.35 ^{aA}	0.85 ± 0.01 ^{aA}	0.83 ± 0.01 ^{aB}	0.88 ± 0.02 ^{aA}	0.30 ± 0.03 ^{aB}
LN	–Se	0.90 ± 0.08 ^{bB}	0.69 ± 0.03 ^{cB}	0.60 ± 0.04 ^{cB}	0.79 ± 0.02 ^{bB}	0.32 ± 0.02 ^{bA}
	+Se R	1.63 ± 0.05 ^{aB}	0.80 ± 0.03 ^{bB}	0.78 ± 0.02 ^{bB}	0.80 ± 0.03 ^{abB}	0.33 ± 0.01 ^{abA}
	+Se L	1.60 ± 0.21 ^{aB}	0.86 ± 0.03 ^{aA}	0.86 ± 0.01 ^{aA}	0.84 ± 0.02 ^{aB}	0.37 ± 0.03 ^{aB}

young ones ($P < 0.001$), irrespective of the N and Se treatments. The effect of the LN treatment depended on the leaf age; it increased the NO level in young leaves but decreased it in old leaves. The Se treatment consistently increased the NO concentration in both young and old leaves under both AN and LN conditions. The effect of the two application modes did not differ significantly, except for the young leaves of AN plants which showed a stronger effect of the + SeR treatment.

The LN treatment decreased the MDA concentration in the young leaves but increased it in the old ones. In AN plants, the MDA concentration in the young leaves was higher than in the old ones, but an opposite response was recorded in LN plants. Selenium application did not influence the MDA concentration in AN plants but decreased it in the young and old leaves of LN plants (Fig. 4D). The ANOVA showed that the concentration of $O_2^{\cdot -}$ was connected to leaf age, the N treatment, and all two-way interactions, while the H_2O_2 level was significantly affected by all three main factors, the two-way interactions of L × N and N × Se, and a three-way interaction (*i.e.*, L × N, L × Se, and N × Se). Interestingly, NO was affected by all main factors and their two-way and three-way interactions. MDA was affected only by Se as the main factor and its interaction with leaf age (L × Se) and the N treatment (N × Se) and the three-way interaction (Table A.2).

3.5. Expression of senescence-related genes

The expression of *SAG12-1* was significantly decreased by the + SeR treatment in both young and old leaves. The effect of the + SeL treatment (analysed only in old leaves) was not statistically significant (Fig. 5A). In contrast to *SAG12-1*, the expression of *Cab* was up-regulated by the + SeR treatment in old leaves of LN plants, while the effect of Se was not significant for young leaves of AN plants (Fig. 5B).

The PCA performed by plotting all 15 senescence-related parameters showed that old and young leaves and AN and LN plants fall into distinct clusters; all data of the same age-N nutrition combination clustered together (Fig. A.1). To explore the grouping of various physiological and biochemical parameters with respect to leaf age and N nutrition, the position of all measured parameters was identified in the second PCA (Fig. 6). This result clearly demonstrated that the variation in the senescence indicators (Chl, Fv/Fm, F'v/F'm, qP, qN, and photosynthesis) can be attributed to four important parameters; *i.e.*, CAT, SOD, MDA, and NO (Fig. 6).

4. Discussion

Selenium (Se) ameliorated symptoms of both senescence and nitrogen (N) deficiency in oilseed rape plants. Because senescence and N deficiency share a number of common symptoms such as loss of chloroplastic pigments and proteins, reduction of photosynthetic biochemical reactions, oxidative stress, and lipid peroxidation [17], our results suggest that developmental and N deficiency-induced senescence are delayed by Se, most probably through the same mechanism.

4.1. Effect of N deficiency and the Se treatment on leaf Chl and the photochemical and gas exchange parameters

The photosynthetic CO_2 fixation rate was significantly reduced not only by leaf aging but also by N deficiency. Decreases in the synthesis of several key enzymes involved in the Calvin cycle accompanied by a selective reduction in the transcript levels of Rubisco are a common feature of senescing leaves [45] and have also been reported under N deficiency conditions [46].

The significant reduction of all of the photochemical quenching parameters (Fv/Fm, F'v/F'm and qP) in old leaves of oilseed rape plants indicated excess production of excitation pressure, resulting in a reduction of the Fv/Fm. The impairment of the biochemical reactions of photosynthesis results in saturation and over-reduction of the electron transport chain [47]. Furthermore, a significant increase in qN observed in the leaves of low N (LN) plants implied enhanced utilization of the xanthophyll cycle [48].

The ameliorative effect of Se on leaf photochemical parameters could be related to both stomatal and non-stomatal mechanisms that prevent over-reduction of the electron transport chain. Selenium affects the rate of photosynthesis in different ways, including elevated stomatal conductance, a higher leaf Chl concentration, and improved activity of fructose 1, 4 bisphosphatase and Rubisco [49–51]. After the Se treatment, the photosynthetic rate of LN plants reached that of adequate N (AN) ones in both young and old leaves, suggesting a conspicuous role of Se supplementation in compensation for the effect of N deficiency in oilseed rape plants.

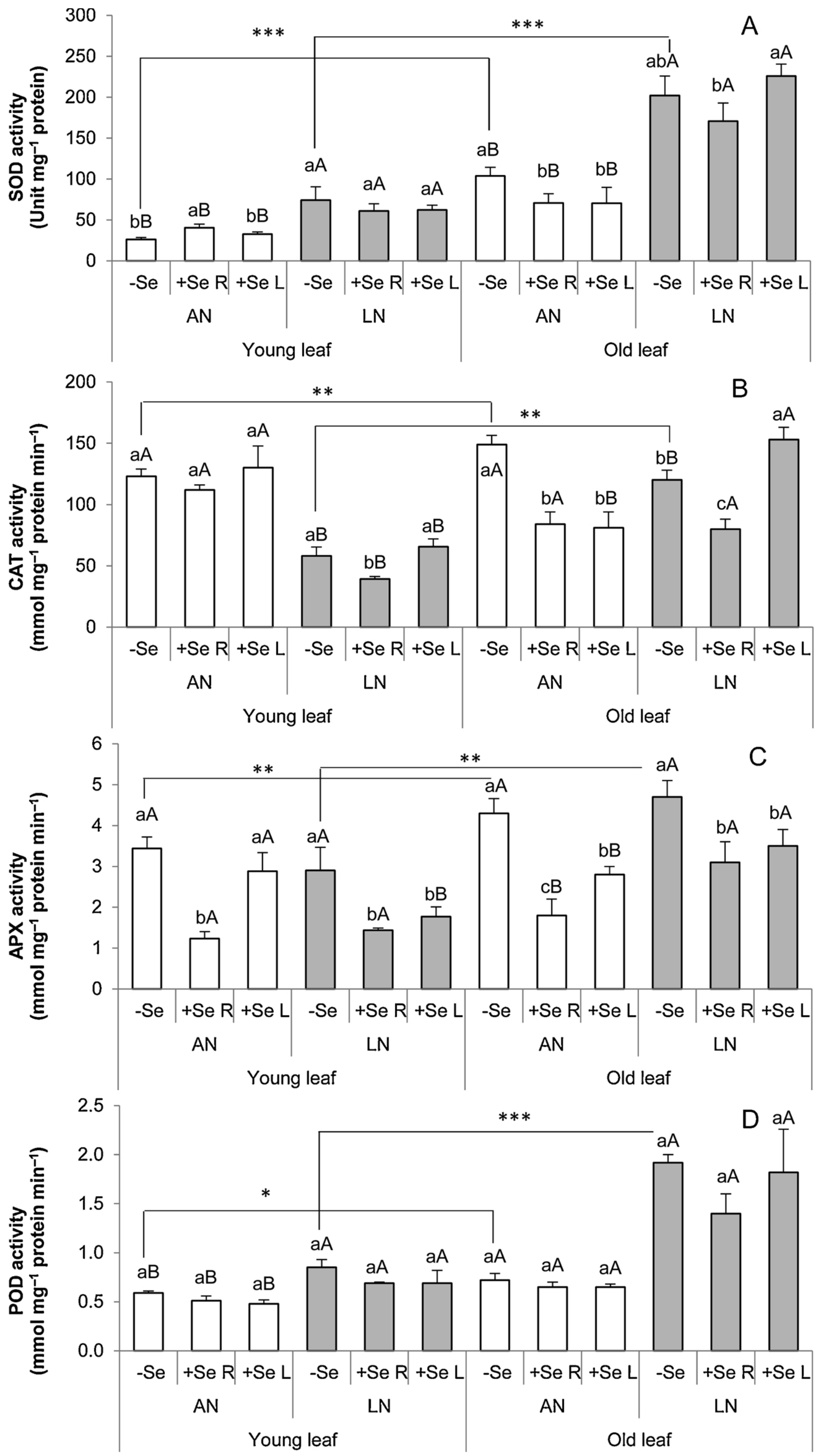

Fig. 3. Activity of superoxide dismutase (SOD) (A), catalase (CAT) (B), ascorbate peroxidase (APX) (C) and peroxidase (POD) (D) in young and old leaves of oilseed rape (*Brassica napus*) plants supplied by adequate nitrogen (AN, 16 mM) or low nitrogen (LN, 4 mM) and treated with selenium (15 µM Se plant^{-1} as Na_2SeO_4) either through roots (+Se R) or leaves (+Se L) for four weeks. The statistical analyses were explained in the legend of Fig. 1.

4.2. Effect of N deficiency and the Se treatment on antioxidative defence components

Consistent elevation of SOD and POD activities under N deficiency conditions is in agreement with observations in other plant species [47] and deficiency in other macronutrients [52]. Saturation of the electron transport chain is the main reason for ROS production and increases the activity of scavenging enzymes in N-deficient leaves [16]. The higher activities of SOD, APX and POD in old leaves compared with young ones were also in agreement with data observed for other species [53].

An increase in the activity of CAT upon leaf senescence, which was similar to the increase in SOD, POD, and APX activities, is inconsistent with reports on *Arabidopsis* [54] and oilseed rape [45] plants. This discrepancy may be due to different whole plant growth stages; our

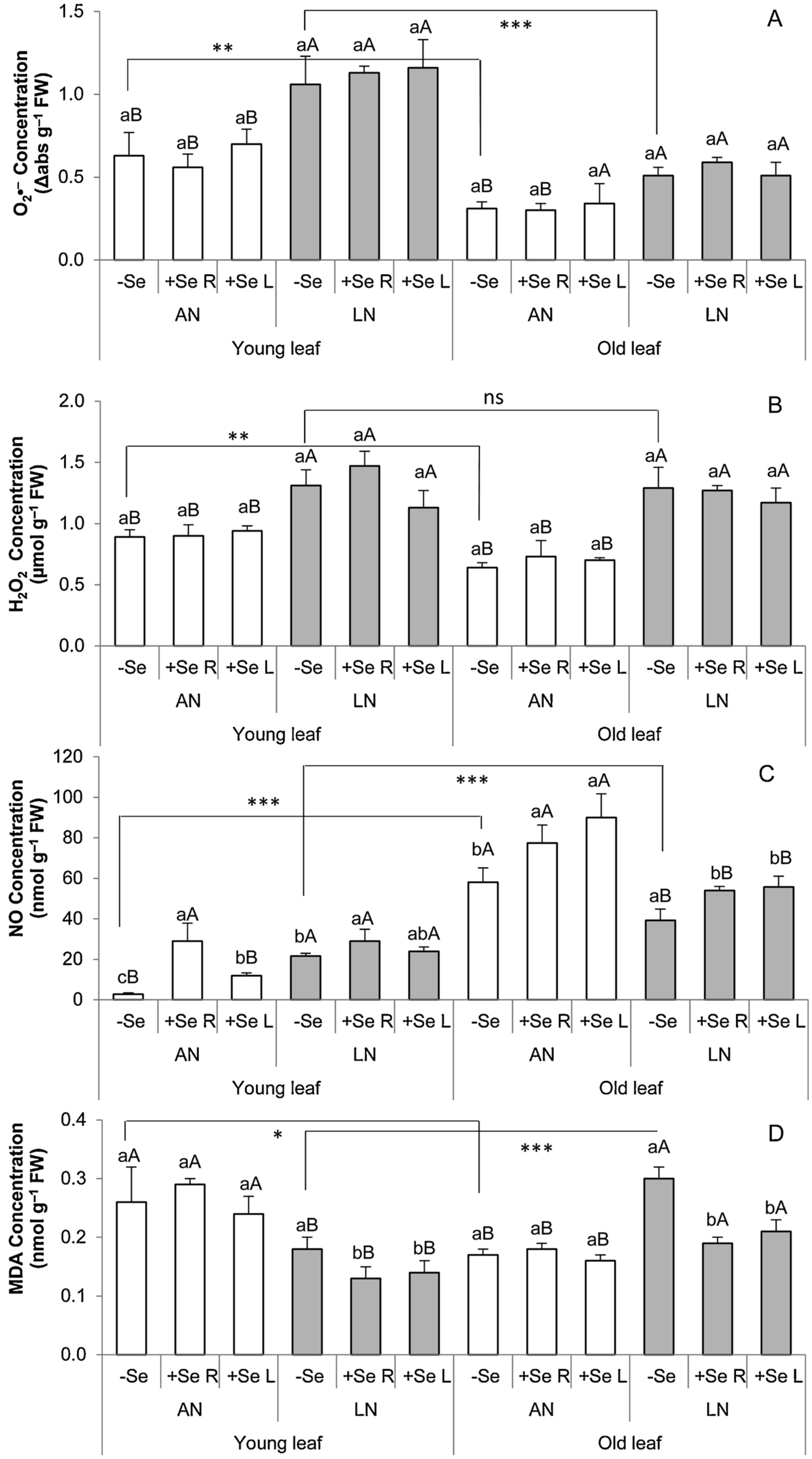

Fig. 4. Concentrations of superoxide anion ($O_2^{\cdot -}$) (A), hydrogen peroxide (H_2O_2) (B), nitric oxide (NO) (C) and malondialdehyde (MDA) (D) in young and old leaves of oilseed rape (*Brassica napus*) plants supplied by adequate nitrogen (AN, 16 mM) or low nitrogen (LN, 4 mM) and treated with selenium (15 µM Se plant^{-1} as Na_2SeO_4) either through roots (+Se R) or leaves (+Se L) for four weeks. The statistical analyses were explained in the legend of Fig. 1.

plants were at the rosette stage, while other authors studied plants at the bolting and early flowering stages. Although there is no information on the effect of the developmental stage of the entire plant on the regulation of antioxidative defence, including CAT, the gene expression profile in aging leaves has been shown to be governed by developmental stage of the entire plant [21]. The reduction of CAT activity upon N starvation observed in young and old leaves, however, was in agreement with that observed in *Arabidopsis*. A significant reduction of CAT activity and protein level quantified by western blot analysis was observed despite the higher mRNA abundance of one Cat isozyme (*cat1*) in N-deficient *Arabidopsis* plants [16]. Our data suggested differential regulation of CAT during developmental senescence and N deficiency. The activity of CAT has been proposed as one of the most important determining components of leaf senescence in *Arabidopsis* [54] and

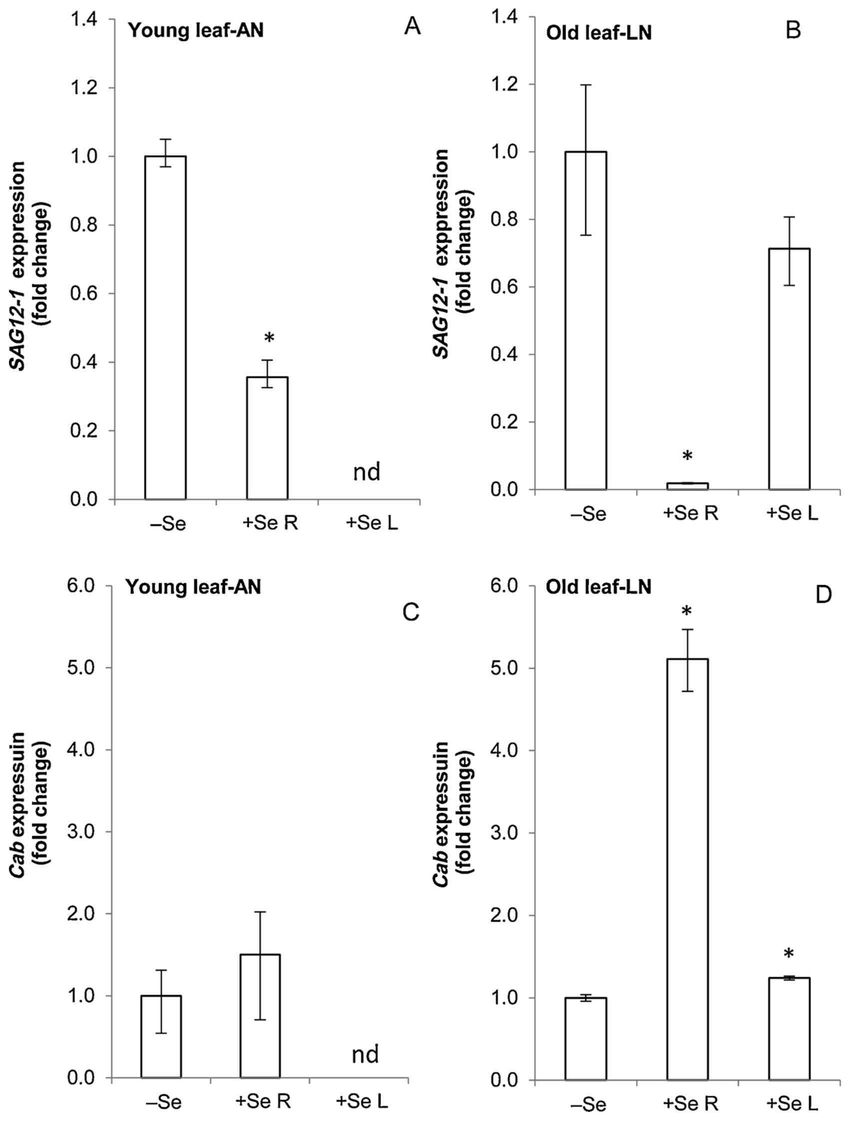

Fig. 5. Relative expression of *SAG12-1* (A, B) and *Cab* (C, D) in the young and old leaves of oilseed rape (*Brassica napus*) plants supplied by adequate nitrogen (AN, 16 mM) or low nitrogen (LN, 4 mM) and treated with selenium (15 μM Se plant^{-1} as Na_2SeO_4) either through roots (+Se R) or leaves (+Se L) for four weeks. nd: not determined. The error bars indicate the upper and lower amounts. Columns in each figure indicated by the asterisks are statistically different from the internal control (–Se) plants ($P < 0.05$).

oilseed rape [45] plants. The PCA analysis indicated that while the other two H_2O_2 scavenging enzymes (APX and POD) were clustered with senescence-related physiological parameters, CAT had a distinct pattern that confirmed the importance of this enzyme in senescence signalling.

Although the activation of antioxidative defence enzymes upon Se treatment has been frequently mentioned as the main mechanism for its ameliorative effect under stress conditions [11], the literature suggests that higher activity of ROS scavenging enzymes is not a common feature for Se-treated plants. In addition to research showing a consistent increase in the capacity of antioxidative defence by Se [55,56], there are also numerous reports that indicate the activation of antioxidative enzymes depend on the Se form (selenite *vs.* selenite) [57], its concentration in the medium [58,59], treatment duration [60], and particularly, its combination with other stresses [61]. In our oilseed rape plants, in most cases, Se either decreased or did not influence the activity of antioxidative enzymes. However, in the young leaves of AN plants Se increased the SOD activity and in the old leaves of LN plants the CAT activity. Selenium did not influence the $O_2^{\cdot}$ and H_2O_2 concentrations. Selenium efficiently protected the plants against deleterious cellular events recorded in LN plants as a significantly lower MDA. However, its effect was likely exerted through mechanisms other than enzymatic ROS scavenging. In contrast to animals and human beings, current molecular knowledge does not confirm the presence of essential selenoproteins, such as glutathione peroxidase (GSH-Px; EC 1.11.1.9), in vascular plants [22]. Selenium may act by increasing glutathione and ascorbate concentrations [51,56,57,62] and is therefore indirectly involved in the redox homeostasis of the cell.

4.3. *Effect of N deficiency and Se treatment on ROS and NO concentrations*

In AN plants the concentration of $O_2^{\cdot -}$ in young leaves was higher than in the old ones ($P < 0.001$) and was accompanied by higher MDA ($P < 0.05$). This result was unexpected because the loss of membrane stability is considered to be a measure of senescence progression [1]. However, this result may suggest that young leaves were exposed to more deleterious concentrations of $O_2^{\cdot -}$ than old leaves. Although ROS serve both as signaling and damaging molecules in senescing leaves [4], the superoxide anion ($O_2^{\cdot -}$) being unstable and having a low permeability [63], exerts a more deleterious effect than H_2O_2 and is likely

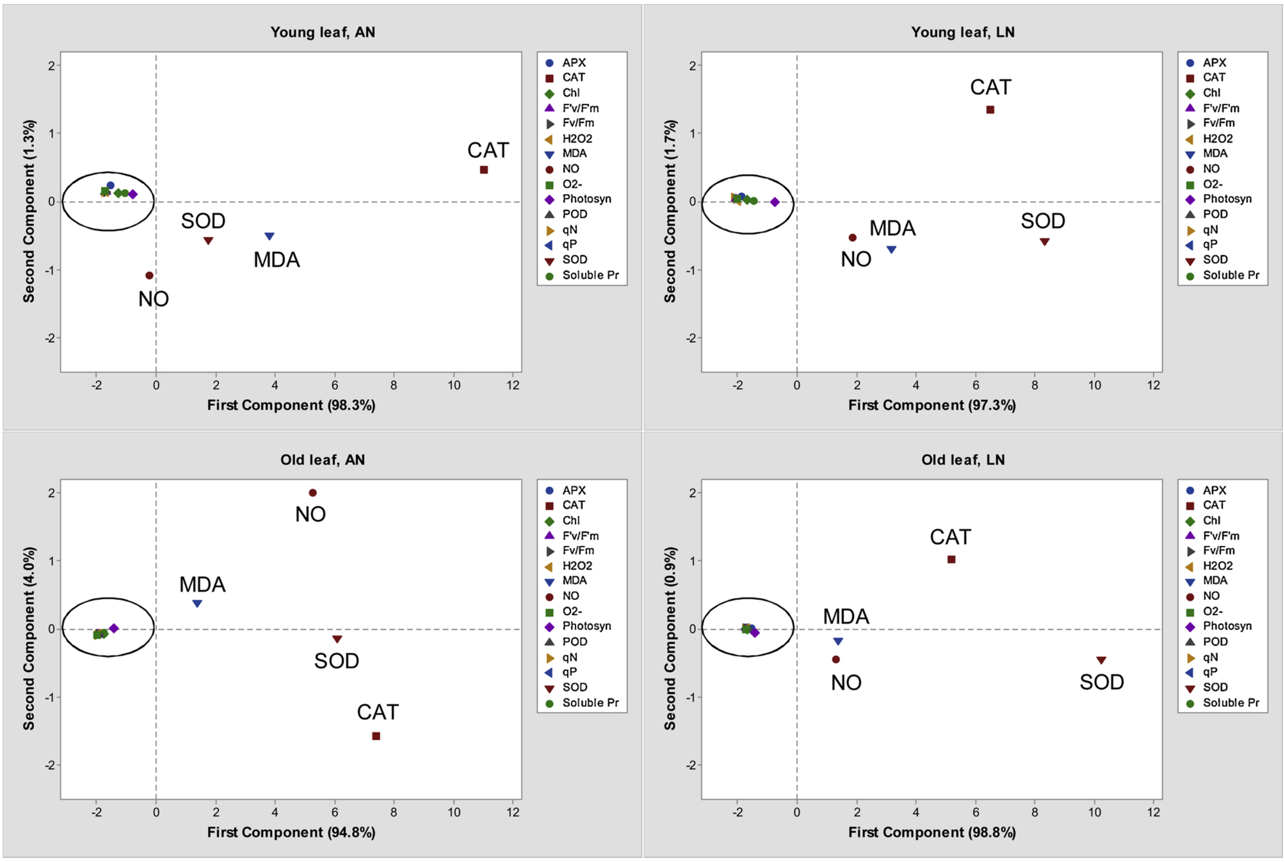

Fig. 6. Principal component analysis of senescence-related parameters in the old and young leaves with different nitrogen (N) treatments.

more relevant in terms of cellular damage than signaling. The $O_2^{\cdot -}$ concentrations were constitutively higher in young leaves that in old ones both in AN and LN plants. This was probably attributable to the lower SOD activity ($P < 0.001$) and/or a higher photosynthetic electron transport rate in young leaves. Superoxide anion in leaves is mainly produced through direct reduction of oxygen in PSI (Mehler reaction) during light-dependent reactions [4].

Under N deficiency conditions and in the absence of an adequate pool of electron acceptors, which is common in N-starved leaves [47,48], accumulation of $O_2^{\cdot -}$ was found in both young and old leaves of our oilseed rape plants. This accumulation occurred despite simultaneous activation of SOD, which suggests that LN plants have insufficient capacity for $O_2^{\cdot -}$ scavenging. MDA data (in the –Se plants) revealed that, in contrast to young leaves, old leaves apparently became more vulnerable to membrane damage under N starvation despite the lower $O_2^{\cdot -}$ concentrations. Taken together, our data do not confirm a cause-effect relationship among ROS, membrane stability, and senescence and raise the question of whether ROS accumulation is required for senescence and/or is a consequence of senescence and aging [64].

It is known that H_2O_2 acts as a signal in early senescence [65]. In *Arabidopsis* and oilseed rape at the flowering stage, H_2O_2 accumulated in the course of leaf senescence in association with a drop in CAT activity [54]. In our rosette plants, by contrast, the H_2O_2 concentration decreased with the increase in leaf age, accompanied by increased CAT activity. Although data on CAT activity was not provided by Bieker et al. [54] for plants at the rosette stage, similar to our work, the H_2O_2 level of rosette plants was lower in old leaves than young ones [54]. Overall, it could be suggested that higher CAT activity in senescing leaves in rosette plants may hinder a H_2O_2 burst and keep its concentration below a critical level. In flowering plants, on the contrary, instead of increasing CAT activity decreases and leads to H_2O_2 accumulation and triggers senescence of the whole plant [54]. H_2O_2 is one of the systemic signals involved in the regulation of plant development, defense, and senescence [4,66].

Reduction of NO emission during natural aging has been reported for *Arabidopsis* in the study of Magalhaes et al. [67] but they did not analyze individual leaves. Cross-regulation between NO signalling and N nutritional status is expected because NO is partly produced through nitrate reductase (NR) [18], which shows lower activity under N deficiency conditions [19]. In comparison to the above findings, the capacity of leaves of our oilseed rape plants to produce NO was expected to decline with their aging and under LN conditions. However, in our oilseed rape plants the endogenous NO level was actually higher in the old leaves than in the young ones, and its response to the LN treatment depended on leaf age. The soluble protein concentration data (Fig. 2C) can be taken as a reliable indicator both of senescence and the N status of an oilseed rape leaf [3]. It may explain the response suggesting the NO level is related to the severity of N deficiency: NO accumulated under mild N deficiency while it was reduced under severe deficiency conditions. To elucidate the role of NO, detailed analysis of its level is required for leaves of different ages, in plants at different growth stages and with different N nutrition statuses. In an elegant work on the proteomics of oilseed rape plants, the individual leaves were analysed in the course of leaf- and whole-plant senescence under different N treatments [68]; however, NO metabolizing or signalling molecules were not considered.

In this study, the Se treatment consistently elevated the NO concentration irrespective of the leaf age and N nutrition in oilseed rape plants. Although the effect of low (beneficial) Se concentrations (< ˜10–20 μM) on NO production and signalling has not been investigated so far, several works reported the effect of toxic Se concentrations (60–460 μM) supplied as selenite (SeIV) on the NO concentration [14,15,69]. This work is the first report that shows that low concentrations of Se act through elevated endogenous NO levels. Likely similarly to the effect of NO donors [70] it protects from oxidative damage and counteracts ROS-mediated programmed cell death.

A significant negative correlation was found between NO and $O_2^{\cdot -}$ ($P < 0.001$) and between NO and H_2O_2 ($P < 0.05$) (Table A. 3). A similar negative correlation between NO and ROS is previously recorded in the NO-deficient *Arabidopsis* mutant during the onset of the senescence phenotype [71]. As a free radical, NO reacts with $O_2^{\cdot -}$ in a diffusion-limited reaction to form peroxynitrite. Moreover, the subsequent fast isomerization of this toxic compound to harmless end products, such as nitrate, represents a possible mechanism to reduce $O_2^{\cdot -}$ levels [71]. Interestingly, significant positive correlations were observed between NO and leaf Chl ($P < 0.001$), Fv/Fm ($P < 0.001$), and F'v/F'm ($P < 0.01$) and a significant negative correlation with MDA ($P < 0.001$) in old leaves of LN plants (Table A. 4). These results confirm that NO plays a major role in the protection of aging leaves against LN-induced senescence acceleration and suggests a mechanism through which Se exerts an anti-senescence effect.

4.4. *Effect of N deficiency and the Se treatment on the expression of SAG12-1 and Cab*

The Se treatment had obvious opposite effects on the expression of *SAG12-1* and *Cab* in old leaves of LN plants. This effect confirms the results of changes in the biochemical markers of senescence and indicates that Se delays senescence through preventing *SAG12-1* and an extended period of *Cab* expression in old leaves in our oilseed rape plants. The effect of Se on the expression of *SAG12-1* and *Cab* was likely mediated by NO in our work. The effect of NO on the expression of these two genes was illustrated in an NO-deficient transgenic plant. In this plant, down-regulation of *Cab* and earlier expression of *SAG12-1* were observed [71]. A strongly accelerated dark-induced senescence was also observed in the *Arabidopsis* mutant impaired in NO production (*Atnos1*) compared with leaves of wild type plants [72].

It is notable that the majority of biochemical and physiological components of senescence in this work responded more to root Se application (+SeR) than leaf Se application (+SeL), except for leaf photochemical parameters, which improved more with + SeL. The superior effect of + SeL on leaf photochemical events could be due to direct assimilation of SeVI into SeIV and even into Se0 in the leaves [32]. Consumption of reducing equivalents in the aforementioned reactions probably ameliorates the deleterious effects of excess excitation energy produced in senescing leaves and under LN conditions.

5. Conclusion

Studying leaf senescence not only contributes to our knowledge of this fundamental developmental process but may also improve the ways of manipulating senescence for agricultural purposes. From both environmental and agronomic points of view, application of effectors to adjust N fertilization is a priority for oilseed rape production. Our study reveals that application of Se could considerably increase the leaf area duration and photosynthesis of plants even with a limited N supply. We showed for the first time that Se delays leaf senescence in a ROS-independent manner but mainly through a NO signalling pathway and up-regulates the genes involved in photosynthetic activity while down-regulating senescence-specific genes.

Declaration of Competing Interest

None.

Funding

This work was supported by the University of Tabriz.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jtemb.2019.06.005.

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