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Selenium delays leaf senescence in oilseed rape plants

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Abstract

Effect of selenium on leaf senescence was studied in oilseed rape plants treated with 10 μM Na_2SeO_4 at a rosette growth stage. In addition to developmental senescence, N deficiency and leaf detachment were used for induction of senescence. Nonphotochemical quenching declined in old leaves as senescence became more advancing but rose progressively in the plants supplied by Se. The total carbohydrate and protein pools decreased with leaf age, while increased by the Se treatment. However, during senescence induced by N deficiency, Se did not change remarkably the C and N metabolism, but delayed senescence mainly through protection of plants from photoinhibitory effects. After detachment, untreated leaves became chlorotic and necrotic, while the Se-treated ones remained fairly green. Our results demonstrated that Se delayed leaf senescence by a maintaining or even improving photochemical activities. During developmental senescence, the Se effect on the extending life span of the leaves was additionally linked to the metabolic regulation of senescence.

Additional key words: leaf photochemistry; nitrate reductase; photosynthetic rate; chlorophyll fluorescence.

Introduction

Senescence is an integral part of plant development that is commonly defined as the sequence of biochemical and physiological events comprising the final stage of development until cell death (Guiboileau *et al.* 2010, Thomas 2013). Leaf senescence is a genetically programmed process and like many other developmental events is regulated by a variety of environmental and autonomous factors. The earliest and most drastic change in plant cellular structures during leaf senescence is the breakdown of the chloroplasts, which contain the photosynthetic machinery of the cell, carry out major biosynthesis, and hold the majority of leaf proteins (Guiboileau *et al.* 2010, Gregersen 2011, Thomas 2013). Leaf senescence is characterized by diminishing photosynthesis due to degradation of both Rubisco and chlorophyll (Chl). As with other types of programmed cell death, leaf senescence is accompanied by expression of senescence-down-regulated genes (SDGs) and upregulation of senescence-associated genes (SAGs) (Guiboileau *et al.* 2010, Thomas 2013).

Leaf senescence can be induced and accelerated by external signals, such as excess light, drought, nutrient deficiency, and wounding. Leaf senescence can be induced by low nutrient, especially low nitrogen (N) supply, probably *via* a tight link to phytohormone control of growth and development of plants (Gregersen 2011). Under inadequate supply of N, the remobilization of this and other nutrients from mature leaves to the young growing leaves accelerates senescence of mature leaves and induces chlorosis (Hörtensteiner and Feller 2002). High light intensities can also promote the senescence process, mainly due to photoinhibition and thermal injuries of the photosynthetic apparatus (Müller *et al.* 2001). Leaf detachment is also a common signal that triggers foliar senescence (Kar *et al.* 1993). Although, there are large differences between the immediate responses of plants to stresses and the developmental senescence program at a level of gene regulation, the different stress impacts appear to funnel into an acceleration of the same execution program such as developmental senescence (Guo and Gan 2012).

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Abbreviations: Car – carotenoids; Chl – chlorophyll; DM – dry mass; ETR – electron transport rate; FM – fresh mass; F_m – maximum fluorescence in dark-adapted leaves; F_m' – maximum fluorescence in light-adapted leaves; F_0 – initial fluorescence in dark-adapted leaves; F_0' – initial fluorescence in light-adapted leaves; F_s – steady-state fluorescence in light-adapted leaves; F_v – variable fluorescence in dark-adapted leaves; F_v/F_m – maximum quantum efficiency of PSII; F_v'/F_m' – excitation capture efficiency of open PSII; MES – 2 (N-morpholino)ethanesulfonic acid; NR – nitrate reductase; NiR – nitrite reductase; P_N – net photosynthetic rate; q_N – nonphotochemical quenching; q_P – photochemical quenching; RC – reaction center; SPAD – soil plant analysis development.
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Selenium (Se) has not been considered as a necessary element for higher plants, but is beneficial for plant growth, particularly under stress conditions (Hajiboland 2012, Feng *et al.* 2013). Se protects plants from various stress factors, particularly drought, salinity, and UV radiation (Hajiboland 2012, Feng *et al.* 2013). The majority of works on the mechanisms of Se effect on plants under stress focused on the antioxidative role of Se. Se activates antioxidative enzymes such as glutathione peroxidase and reduces oxidants and protects membranes from damages caused by reactive oxygen species (Feng *et al.* 2013). There are reports indicating that Se delays senescence, promotes growth of ageing seedlings, prevents Chl degradation, and maintains longer duration of leaf area (Xue *et al.* 2001). Effect of Se on delaying monocarpic senescence and ageing process of leaves has been attributed to the activation of an antioxidative system (Djanaguiraman *et al.* 2005).

Beside elevation of antioxidative defense capacity and amelioration of stress, a series of other physiological effects including activation of photosynthetic enzymes (Owusu-Sekyere *et al.* 2013) and improvement of carbon (C) and N assimilation (Hajiboland and Sadeghzade 2014) has been reported for Se in various plant species. Se stimulates flowering, while delays fruit ripening in oilseed rape plants (Hajiboland and Keivanfar 2012). In nodulated alfalfa plants, Se modifies glutathione redox state in leaves (Hajiboland *et al.* 2015). The broad spectrum of Se effects in plants indeed cannot be explained through simple biochemical and physiological function and attributed to the antioxidative response.

Some detailed studies clearly pinpointed the role of the balance between C and N in the regulation of leaf senescence (Guiboileau *et al.* 2010). Contents of carbohydrates and nitrogenous compounds show distinct changes during leaf senescence, with sugars accumulating, while amino acids declining (Masclaux *et al.* 2000, Wingler *et al.* 2004, 2006). N limitation that induces early leaf senescence often results in sugar accumulation (Hawkesford *et al.* 2012). It is therefore likely that the role of N supply in regulating leaf senescence is at least partly due to effects on sugar metabolism (Palenchar *et al.* 2004, Price *et al.* 2004, Wingler *et al.* 2006). As related to Se, studies are still lacking on leaf C and N assimilation capacity modified by Se during progression of senescence. Considering our previous report on the effect of Se on carbohydrate and nitrogenous metabolite pools (Hajiboland and Sadeghzade 2014), it is plausible that Se-mediated changes in the C and N metabolism may modify the metabolic regulation of leaf senescence.

Oilseed rape (*Brassica napus*) (Brassicaceae) with higher requirement for sulfur than other crops (Weese *et al*. 2015) is considered a secondary accumulator for Se (Terry *et al*. 2000). Likely due to the similarities between sulfur and Se assimilation pathways, this species shows more prominent response to Se supplementation compared to many other species. The objective of this work was to investigate the ability of Se to counteract leaf senescence in oilseed rape. In addition of developmental senescence, N deficiency and leaf detachment were utilized experimentally as an easy way to study the progress of leaf senescence as well as Se effect on this process. The progression of leaf senescence is usually evaluated by different physiological indicators such as leaf yellowing or changes in protein and/or Chl contents. We examined the main metabolic markers in leaves of increasing age by quantification of various C and N metabolites. Our working hypothesis was that Se delays senescence *via* modification of leaf C and N metabolism.

Materials and methods

Plants culture and treatments: Seeds of oilseed rape plants (*Brassica napus* L. cv. RGS) provided by Seed and Plant Improvement Institute (Karaj, Iran) were surface-sterilized with 1% active hypochlorite and germinated in dark. After germination, young seedlings were transferred to the light. One-week-old seedlings were transferred to 2-L plastic pots (two plants in each pot) filled with washed perlite and irrigated with Hoagland nutrient solution or water at field capacity after daily weighing. The volume of 100% nutrient solution was 100 ml L^{-1} per week in the first three weeks and 150 ml L^{-1} per week in the following growth period. Six weeks after preculture (at resetting stage), treatments consisting of control (–Se) and Se treatment (+Se) [10 μg(Na_2SeO_4) per plant] were started. Two weeks after the Se treatment, plants were harvested. Three leaves at distinctly different developmental stages consisting of the second youngest leaf (young leaf), fully developed leaf (middle aged leaf), and the third oldest, still green leaf (old leaf) were subjected to analyses.

For study of N deficiency-induced leaf senescence, different N treatments including adequate (control, 16 mM) and low N supply were started immediately after transferring plants into the pots. Low N treatment was started with 8 mM (for two weeks) followed by 4 mM (for following one week), and finally was applied at 2 mM throughout the further growth period until harvest. Different N contents in the nutrient solution was provided through reduction of $Ca(NO_3)_2$ in the nutrient solution. In order to equilibrate Ca^{2+} concentration between two N treatments, 1.5 mM $CaCl_2$ was added to the low N nutrient solution. Six weeks after beginning of the N treatments (at resetting stage), Se treatments started as control (–Se) and Se treatment (+Se) [10 μg(Na_2SeO_4) per plant]. Two weeks after different Se treatments, plants were harvested. Different analyses were performed in the third oldest, still green leaf in analogy with the old leaf defined in the experiment during developmental senescence.

Plants were grown under controlled environmental

conditions with a temperature regime of 25/18°C day/night, 14/10 h light/dark period, a relative humidity of 50–60%, and at a PPFD of about 300 μmol m^{-2} s^{-1}.

At harvest, fresh mass (FM) and dry mass (DM) of plants (after drying at 70°C for 2 d) were determined. Before harvest, leaf fluorescence and net photosynthetic rate were determined in the attached leaves.

Determination of Chl fluorescence parameters and net photosynthetic rate: Chl fluorescence parameters were recorded using a portable fluorometer (*OSF1, ADC Bioscientific Ltd.,* UK). In the experiment on developmental senescence, three distinct leaves (as described above) were subjected to the measurements at three time intervals all on the same leaf. Initial (F_0), maximum (F_m), variable ($F_v = F_m - F_0$) fluorescence and maximum quantum yield of PSII (F_v/F_m) in dark-adapted leaves and steady-state (F_s) and maximum (F_m') fluorescence in light-adapted leaves were recorded. Calculations were made for $F_0' = F_0/[(F_v/F_m) + (F_0/F_m')]$, $F_v'/F_m' = [(F_m'–F_0')/F_m']$, photochemical quenching, $q_P = [(F_m' – F_s)/(F_m' – F_0')]$, and non-photochemical quenching, $q_N = 1 – [(F_m' – F_0')/(F_m – F_0)]$ (Genty *et al.* 1989). Net photosynthetic rate (P_N) 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 photon flux density of about 300 μmol m^{-2} s^{-1}. For Chl fluorescence and photosynthesis analyses, an average of four records from different parts of each individual leaf was considered for each replicate.

Biochemical determinations: Leaf concentrations of Chl *a*, Chl *b*, and Car were determined according to Lichtenthaller and Wellburn (1983). Flavonoids and anthocyanins were extracted and determined according to the methods described elsewhere (Hajiboland and Keivanfar 2012). Nonstructural carbohydrates were determined using anthrone-sulfuric acid and iodine-HCl solution for soluble sugars and starch, respectively (Yemm and Willis 1954). *In vivo* nitrate reductase (NR, E.C. 1.6.6.1) activity was assayed using the method described by Jaworski (1971). Leaf blades were placed in 25 mM potassium phosphate buffer (pH 7.2) containing 25 mM KNO_3 as incubation buffer and infiltrated using vacuum (80 kPa). After 5 h, the vacuum was released and the samples were incubated at 30°C in darkness for 1 h, then placed in a boiling water bath to stop the NR activity. The resulting nitrite was determined spectrophotometrically (*Specord 200, Analytic Jena*, Germany) at 540 nm. Activity of NR was calculated from a standard curve produced with $NaNO_2$ (*Merck*) and expressed as μmol (NO_2) mg^{-1}(protein) s^{-1}. Determination of nitrate and nitrite was performed according to the method described elsewhere (Hajiboland and Sadeghzade 2014). Soluble protein concentration was determined using a commercial reagent (*Bradford reagent, Sigma*, St. Louis, USA) and bovine albumin serum *(BSA, Merck,* Germany) as standard. Content of total free α-amino acids was assayed using a ninhydrin colorimetric method (Yemm *et al.* 1955) with glycine (*Merck,* Germany) as standard.

Experiment with detached leaves: Six weeks after preculture, plants were divided into two groups; the first group was pretreated with four concentrations of Se (0, 2, 10, and 20 μM) for one week, while the second group kept untreated. Thereafter, the second youngest, fully expanded leaf from each plant was detached and placed in Petri dishes (8 cm in diameter). The leaves of pretreated plants were immersed in distilled water buffered by MES (2-(N-morpholino) ethanesulfonic acid) at 0.1 mM (pH 5.7), while leaves of the second group were immersed in the Na_2SeO_4 solution (pH 5.7) containing four different concentrations of Se (0, 2, 10, and 20 μM). Petri dishes were placed under three different light intensities including 100, 200, and 300 μmol(photon) m^{-2} s^{-1} in a growth chamber. Relative Chl content (SPAD unit) was determined daily using a Chl meter (*SPAD 502, Minolta Co. Ltd.*, Osaka, Japan) and Chl fluorescence parameters were determined every three days as described above.

Experimental design and statistical analyses: Experiment was undertaken in a complete randomized block design with four independent replications. Statistical analyses were carried out using *Sigma Stat 3.5* software (*Systat Software Inc.,* USA). Data were analyzed using one-way analysis of variance (*ANOVA*) and the comparison of means were performed by *Tukey*'s test ($p<0.05$).

Results

Plant DM only slightly increased by the Se treatment, while low N supply led to a significant reduction of shoot and root DM under both +Se and –Se conditions (Fig. 1S, *supplement available online*).

Effect of senescence and Se on the leaf pigment concentrations: Leaf concentrations of Chl *a*, Chl *b*, Car, and flavonoids decreased with increasing leaf age slightly or significantly. Anthocyanin concentration, in contrast, was higher in the middle aged and old leaves compared to the young leaf (Table 1S, *supplement available online*). Influence of Se on the leaf pigment concentrations depended mainly on the pigment and leaf developmental stage. The most prominent effect was observed for Chl *a* that increased significantly by Se, irrespective of the leaf age. Effect of Se on Chl *b* and flavonoids concentrations was significant only in the old leaf. Car concentration was slightly lower in +Se plants in the young and middle aged leaves, while significantly increased in the old leaf by the Se treatment. Anthocyanin concentrations markedly

decreased by Se, up to 14, 22, and 2 fold in the young, middle aged, and old leaves, respectively (Table 1S).

The third oldest leaf showed an earlier response to N deficiency. In this leaf, concentrations of Chl *a*, Chl *b*, flavonoids, and anthocynains (but not Car) were slightly lower in N-deficient compared to N-sufficient plants. In the N-sufficient plants, the Se treatment caused a significant increase of leaf pigments with the exception of anthocyanins; this pigment was lower in +Se plants than that of –Se ones. Similar response to Se was observed in N-deficient plants, however, these effects were mainly insignificant except for Car and anthocynains (Table 2S, *supplement available online*).

Effect of senescence and Se on Chl fluorescence parameters: Maximum photochemical efficiency of PSII (F_v/F_m), excitation capture efficiency of open PSII (F_v'/F_m'), photochemical quenching (q_P), and electron transport rate (ETR) all declined in the leaves of different age during three subsequent time periods (Fig. 1). The extent of reduction, however, depended largely on the leaf developmental stage. More prominent declines were observed in the middle aged and old leaves compared to the young leaf. Decreasing trend in the F_v/F_m, F_v'/F_m', q_P, and ETR was also detected during three subsequent time periods in +Se plants, however, the values in +Se plants were consistently higher than that in –Se plants particularly in the middle aged and old leaves at the last time point. In contrast to other Chl fluorescence parameters, nonphotochemical quenching (q_N) in the young and middle aged leaves increased during the three subsequent time periods and +Se plants had lower q_N compared with –Se ones. In the old leaves, however, q_N decreased during the three time periods. In +Se plants, in addition to an increasing trend of q_N during time, q_N values at all time points were significantly higher than that of –Se counterparts (Fig. 1).

Under N-deficiency conditions, significant reduction was observed in F_v/F_m and F_v'/F_m'. Except for q_N, the Se treatment caused a considerable rise in all Chl fluorescence parameters in both N-sufficient and N-deficient plants, however, its effect on the elevation of F_v/F_m, F_v'/F_m', and ETR was more prominent in N-deficient compared to N-sufficient plants. In contrast to other parameters, q_N was significantly higher in N-deficient plants and increased further upon the Se treatment (Fig. 2).

Effect of senescence and Se on net photosynthesis and NR activity: Net assimilation rate (P_N) decreased from the young to the old leaves. The Se treatment elevated leaf P_N; this effect was significant only in the young and middle aged leaves. NR activity was lower in the middle aged and old leaves in comparison to the young leaves. The Se treatment increased NR activity only in the young leaves. In the middle aged and old leaves, NR activity rather decreased upon the Se treatment (Fig. 3).

Low N plants had lower P_N and Se treatment increased it. Effect of Se on the elevation of P_N, however, was significant only in the N-sufficient plants. As expected, a drastic decrease in NR activity was observed under low N conditions. The Se treatment decreased NR activity in the N-sufficient plants, while did not change it in the N-deficient ones (Fig. 4).

Similar trends were found for NR activity when presented on FM basis, except for a significant increase observed in the N-deficient plants upon the Se treatment (Table 3S, *supplement available online*).

Effect of senescence and Se on C and N metabolites: Soluble sugars and starch concentrations showed a progressive decrease with increasing leaf age. The Se treatment caused an increase in these parameters in the leaves of different age. Concentrations of total free amino acids were highest in the young leaves and declined up to seven fold in the middle aged leaves. In the old leaves, amino acid pool slightly increased but remained lower than that in the young leaves. In contrast to amino acids, soluble protein concentrations were only slightly affected by a leaf developmental stage. The Se treatment decreased amino acid concentrations only in the young leaves, while increased protein concentrations; this effect was significant only in the old leaves. Leaf nitrate concentrations increased with progression of leaf senescence, while nitrite concentrations were lower in the middle aged and old leaves compared to the young leaves. The Se treatment decreased slightly nitrate concentrations, irrespective of the leaf age. Nitrite concentrations, however, decreased by the Se treatment in the young leaf, remained unchanged in the middle aged leaf, but increased significantly in the old leaf (Table 1).

Low N supply to plants resulted in significantly higher soluble sugar concentrations; this effect for starch was not statistically significant. Significant effect of the Se treatment on the soluble sugars and starch concentrations was observed only in the N-sufficient plants. Amino acid concentrations, however, were significantly affected neither by N supply nor Se treatments. By contrast, soluble protein concentrations decreased slightly in response to low N supply. The Se treatment increased a soluble protein content, however, this effect was significant only in the N-sufficient plants. Nitrate concentrations were significantly lower in the N-deficient plants, while nitrite concentration increased by about 3.3 fold under these conditions. The Se treatment did not change nitrate concentrations significantly. In contrast, Se increased nitrite concentrations in the N-sufficient plants and decreased it in the N-deficient ones (Table 2).

Effect of Se on the progression of senescence in the detached leaves: Loss of Chl was visually detected three days after detachment of leaves. This was well reflected in the reduction of SPAD values under all applied light conditions in the absence of Se (Fig. 5). F_v/F_m values were also reduced after detachment during the two weeks of a

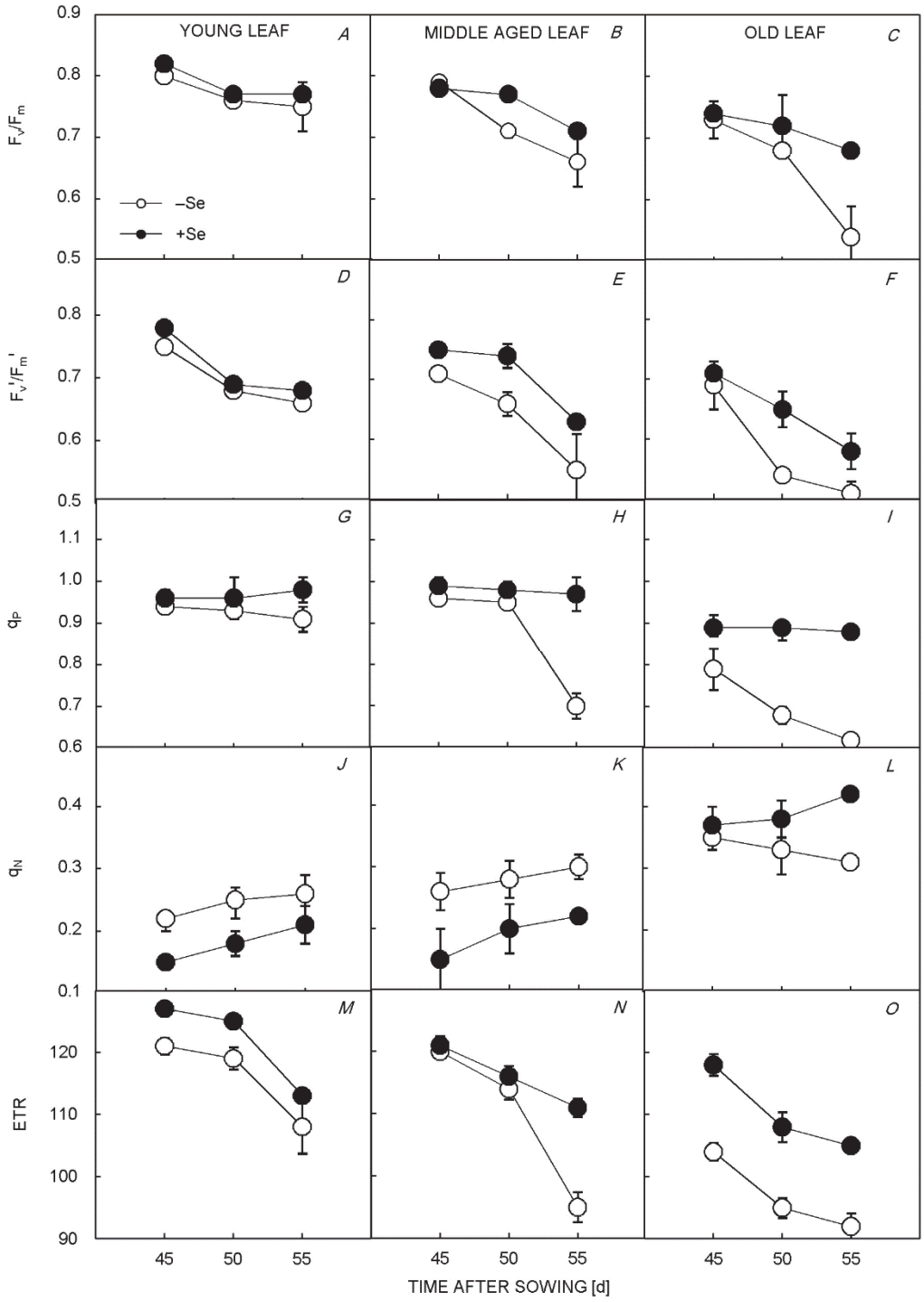

Fig. 1. Chlorophyll fluorescence parameters including maximum photochemical efficiency of PSII (F_v/F_m), excitation capture efficiency of open PSII (F_v'/F_m'), photochemical quenching (q_P), nonphotochemical quenching (q_N), and electron transport rate (ETR) in the second youngest leaf (young leaf), fully developed leaf (middle aged leaf), and the third oldest, still green leaf (old leaf) in oilseed rape plants grown without (–Se) or with 20 μM Na_2SeO_4 (+Se) at 45, 50, and 55 days after sowing (4, 9, and 14 days after Se treatment). Data are means ± SD (n = 4).

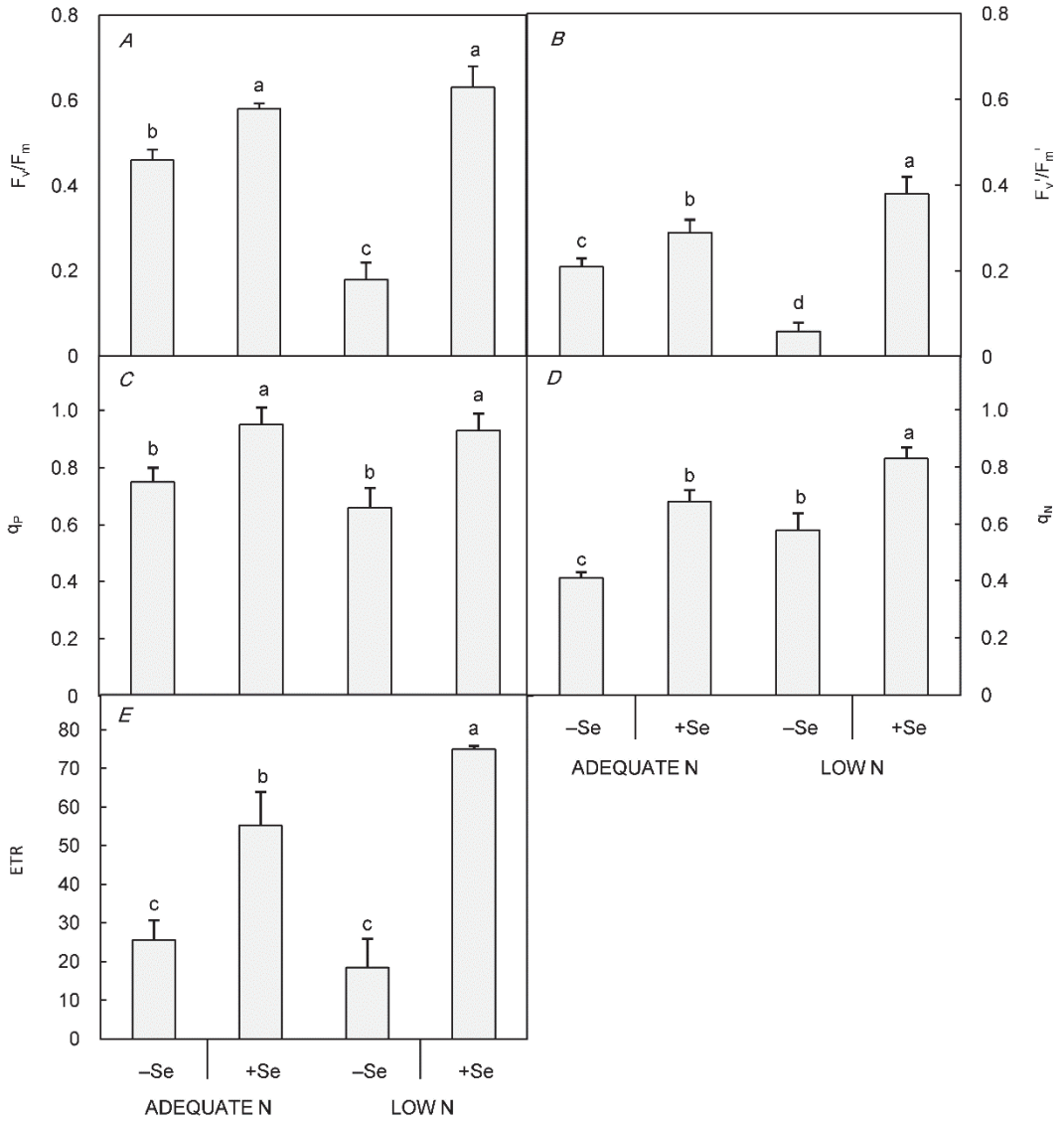

Fig. 2. Chlorophyll fluorescence parameters including maximum photochemical efficiency of PSII (F_v/F_m), excitation capture efficiency of open PSII (F_v'/F_m'), photochemical quenching (q_P), nonphotochemical quenching (q_N), and electron transport rate (ETR) in the third oldest, still green leaf of oilseed rape plants grown at adequate or low N supply without (–Se) or with 20 μM Na_2SeO_4 (+Se). Data are means ± SD (n = 4). Bars indicated by *the same letter* were not statistically significant (p<0.05).

Table 1. Concentration of soluble sugars, starch, total free α-amino acids, soluble proteins, nitrate, and nitrite in the second youngest leaf (young leaf), fully developed leaf (middle aged leaf), and the third oldest, still green leaf (old leaf) in oilseed rape plants grown without (–Se) or with 20 μM Na_2SeO_4 (+Se). Data are mean ± SD (n = 4). Difference between data of each row followed by the same letter was not statistically significant (p<0.05).

Parameter	Young leaf		Middle aged leaf		Old leaf	
	–Se	+Se	–Se	+Se	–Se	+Se
Soluble sugars [mg g^{-1}(FM)]	28.2 ± 5.41^{ab}	34.8 ± 6.82^{a}	9.0 ± 1.09^{cd}	25.3 ± 1.20^{b}	5.3 ± 2.12^{d}	15.5 ±1.72^{c}
Starch [mg g^{-1}(FM)]	2.95 ± 0.93^{a}	1.55 ± 0.18^{b}	1.11 ± 0.80^{b}	0.62 ± 0.06^{b}	0.69 ± 0.19^{b}	1.49 ± 0.42^{b}
Amino acids [μmol g^{-1}(FM)]	26.2 ± 2.50^{a}	14.9 ± 2.32^{b}	3.8 ± 1.34^{c}	4.9 ± 1.25^{c}	6.46 ± 1.96^{c}	5.02 ± 0.84^{c}
Soluble protein [mg g^{-1}(FM)]	4.48 ± 1.23^{bc}	5.58 ± 1.13^{b}	3.16 ± 0.96^{c}	4.52 ± 0.14^{bc}	2.86 ± 0.76^{c}	9.10 ± 1.25^{a}
Nitrate [μmol g^{-1}(FM)]	2.14 ± 0.58^{c}	1.96 ± 0.97^{c}	5.00 ± 0.89^{ab}	4.40 ± 0.59^{b}	6.98 ± 1.8^{a}	4.70 ± 0.78^{ab}
Nitrite [nmol g^{-1}(FM)]	11.9 ± 2.55^{a}	7.84 ± 1.59^{b}	4.96 ± 0.95^{b}	5.42 ± 0.18^{b}	6.67 ± 1.20^{b}	11.7 ± 2.29^{a}

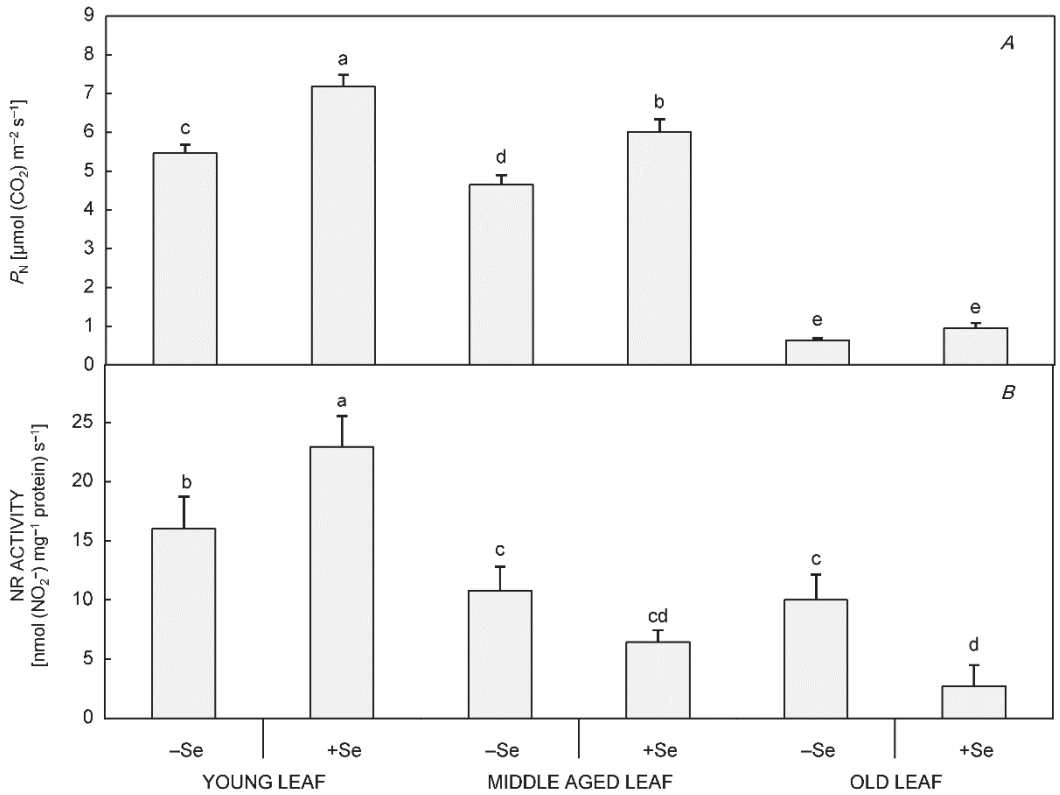

Fig. 3. Net photosynthetic rate (P_N) and activity of nitrate reductase (NR) in the second youngest leaf (young leaf), fully developed leaf (middle aged leaf), and the third oldest, still green leaf (old leaf) in oilseed rape plants grown without (–Se) or with 20 µM Na_2SeO_4 (+Se). Data are means ± SD (n = 4). Bars indicated by *the same letter* were not statistically significant (p<0.05).

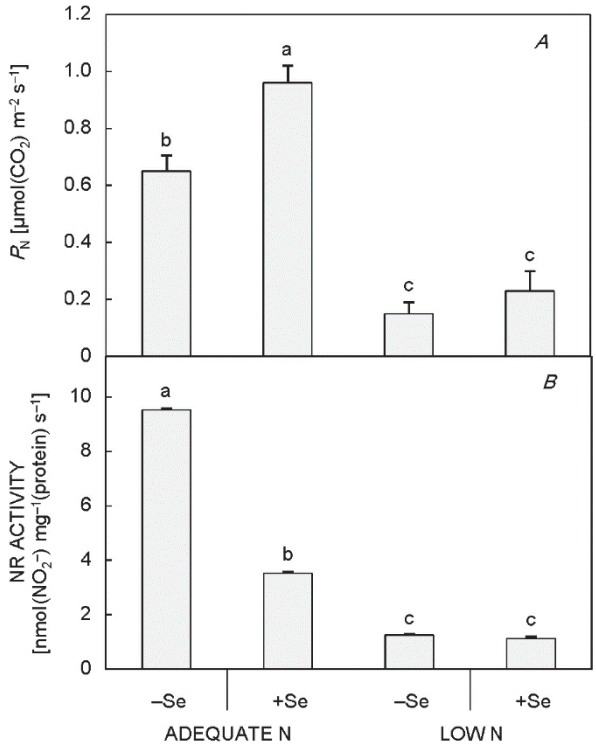

Fig. 4. Net photosynthetic rate (P_N) and activity of nitrate reductase (NR) in the third oldest, still green leaf of oilseed rape plants grown at adequate or low N supply without (–Se) or with 20 µM Na_2SeO_4 (+Se). Data are means ± SD (n = 4). Bars indicated by *the same letter* were not statistically significant (p<0.05).

Table 2. Concentrations of soluble sugars, starch, total free α-amino acids, soluble proteins, nitrate, and nitrite in the third oldest, still green leaf (old leaf) of oilseed rape plants grown at adequate or low N supply without (–Se) or with 20 μM Na_2SeO_4 (+Se). Data are mean ± SD (*n = 4*). Difference between data of each row followed by the same letter was not statistically significant (p<0.05).

Parameter	Adequate N		Low N	
	–Se	+Se	–Se	+Se
Soluble sugars [mg g^{-1}(FM)]	4.28 ± 1.14^{c}	10.80 ± 1.26^{b}	13.2 ± 2.3^{ab}	15.04 ± 2.8^{a}
Starch [mg g^{-1}(FM)]	0.72 ± 0.11^{c}	1.32 ± 0.09^{a}	0.91 ± 0.17^{bc}	1.16 ± 0.16^{ab}
Amino acids [μmol g^{-1}(FM)]	7.49 ± 2.5^{a}	6.02 ±0.32^{a}	7.27 ± 1.91^{a}	8.98 ± 1.48^{a}
Soluble protein [mg g^{-1}(FM)]	5.90 ± 1.52^{b}	10.02 ± 2.17^{a}	3.23 ± 1.06^{b}	5.65 ± 1.52^{b}
Nitrate [μmol g^{-1}(FM)]	7.15 ± 1.30^{a}	5.02 ± 1.92^{a}	1.57 ± 0.71^{b}	1.31 ± 0.90^{b}
Nitrite [nmol g^{-1}(FM)]	5.62 ± 1.4^{c}	12.1 ± 1.11^{b}	18.8 ± 2.01^{a}	11.7 ± 2.95^{b}

monitoring period. Significantly higher F_v/F_m and SPAD values were found in both the Se-treated and pretreated leaves and effect of Se became more prominent towards the end of experimental period, particularly under the highest light intensity applied during the experiment [300 μmol(photon) m^{-2} s^{-1}] (Fig. 5).

At the end of experiment, detached leaves turned yellow or became even necrotic, while the Se-treated leaves remain fairly green without any sign of damage even under higher light intensity two weeks after detachment (Fig. 6) (*see* also Fig. 2S and Fig. 3S, *supplements available online*).

Discussion

Leaf pigments as changed during senescence and modified by Se: Reduction of leaf Chl concentrations was obvious in both developmental and N-starvation-induced senescence in this work. The progression of leaf senescence is most commonly monitored as Chl degradation (Zimmermann and Zentgraf 2005, Thomas 2013). The process of catabolic reactions during senescence in the chloroplasts is characterized by loss of photosynthetic pigments, lipids, proteins of thylakoids, and soluble proteins of stroma (Zimmermann and Zentgraf 2005). Since N constitutes 1.5–2% of plant dry mass and is the key component of many important macromolecules, such as proteins and Chl, reduction of Chl synthesis and content is one of the first symptoms of N deficiency (Hörtensteiner and Feller 2002). In addition to Chl, Car concentrations were also lower in both developmental and N-starvation-induced senescence in this work. Chl, Car, and leaf proteins exhibit simultaneous loss during induction of senescence (Biswal 1995, Merzlyak and Solovchenko 2002) that implies a common point in the mechanism of their degradation (Biswal 1995). The Se treatment increased leaf Chl, particularly Chl *a* concentrations, in the leaves of different developmental stages, albeit its effect in the N-deficient leaves was not statistically significant. Se may increase Chl synthesis and/or prevent its degradation *via* its protecting effect against oxidative damages (Feng *et al.* 2013) and/or delaying destructive reactions associated with senescence (discussed below). Chl *a*/*b* decreased in both developmental and N-deficiency-induced senescence in this work (data not shown). Such a decrease in the photosynthetic pigment stoichiometry indicates relative stability of Chl *b* and has been reported for dark-induced and natural senescence (Biswal 1995). The Se treatment, interestingly, increased this ratio in the leaves of different developmental stages as well as in N-deficient plants (data not shown). An increase in Chl *a*/*b* suggests the improvement in the stability of reaction center II (RCII) complexes (Müller *et al.* 2001) by Se treatment. Retention of β-carotene that is known to be predominantly located in RCII complex is correlated with stability of RCII (Biswal 1995). Accordingly, Se-mediated increase in Car concentrations observed in this work could maintain RCII structurally intact and/or improve its stability during senescence and contributes at least partly to the higher F_v/F_m observed in +Se plants, particularly in the old leaves (*see* below). Car play a photoprotective role not only by quenching $^1O_2$ directly, but also *via* xanthophyll cycle involved in thermal dissipation of excess excitation energy (Ramel *et al.* 2012).

Anthocyanin (but not flavonoids) concentrations increased during leaf ageing in this work. A protective role for anthocynains as "sunscreen" and as scavengers of reactive oxygen species has been suggested for young, expanding leaves (Hoch *et al.* 2001). A direct association between anthocyanin production and the period of increased vulnerability to photoinhibition during senescence confirms that anthocyanins may perform a photoprotective role in senescing leaves (Hoch *et al.* 2001, Anderson and Jordheim 2006). In contrast to developmental senescence, in N-deficiency-induced senescence, anthocyanin concentrations decreased which was observed not only in the old but also in the young and middle aged leaves (data not shown for the latter leaves). In *Arabidopsis*, in contrast, accumulation of abundant anthocyanins is one of responses to N limitation (Peng *et al.* 2008). The Se treatment resulted in significant reduction of anthocynin concentrations. Because Se diminished sensitivity to photoinhibitory conditions (*see* below), there

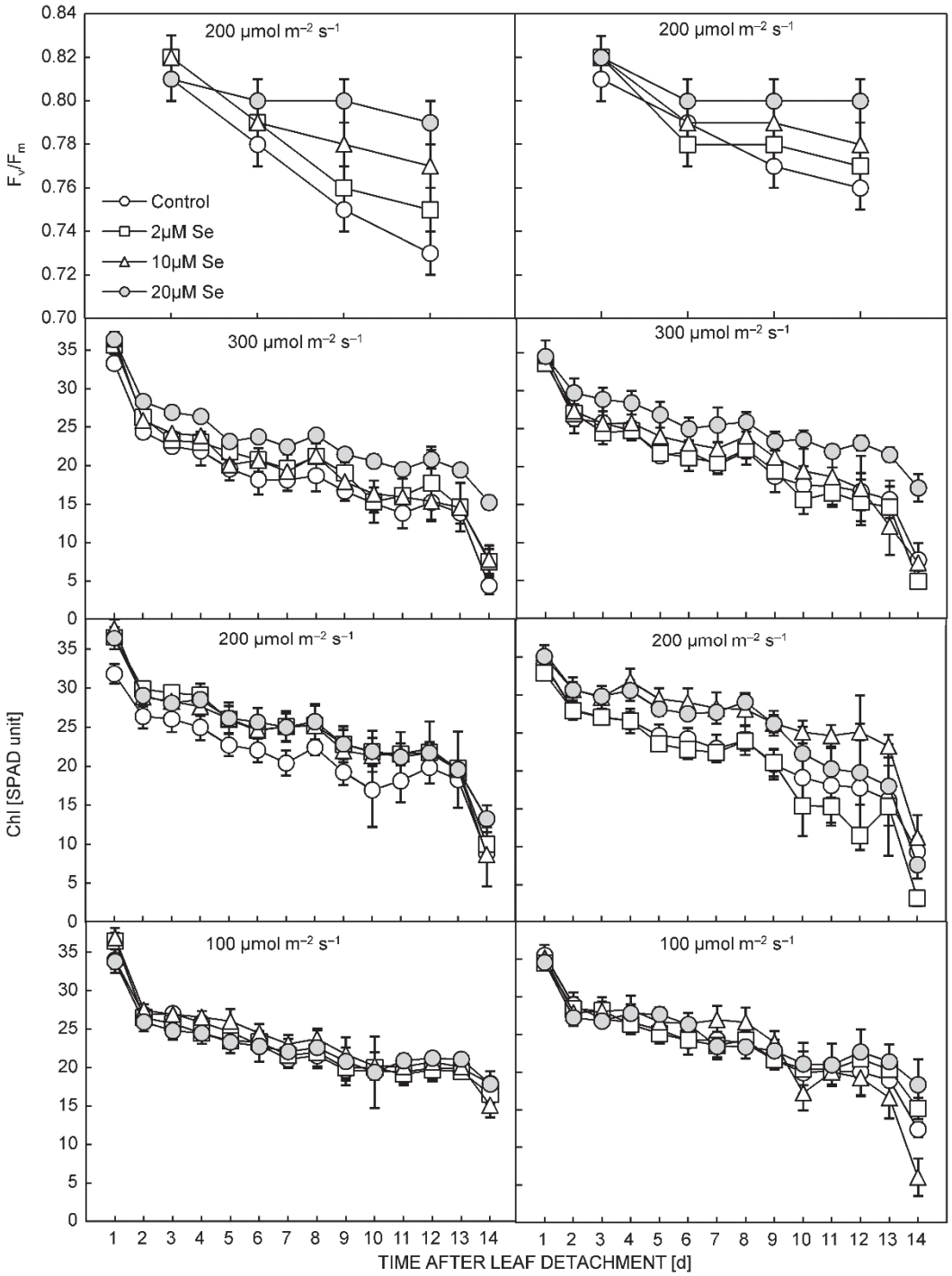

Fig. 5. Changes in the maximum photochemical efficiency of PSII (F_v/F_m) and SPAD values in the fully expanded leaves during two weeks after detachment in oilseed rape plants either pretreated one week before detachment (*left panels*) or treated during detachment (*right panels*) with 2, 10, and 20 µM Na_2SeO_4 under three distinct light intensities [300, 200, and 100 µmol(photon) m^{-2} s^{-1}]. Data are means ± SD (n = 4).

was likely no need for additional photoprotection by anthocyanins in the Se-treated leaves. In this context, up to 22-fold reduction of anthocyanins concentrations upon the Se treatment may results in channeling the phenylpropanoid metabolic flux from anthocyanin synthesis to the carbohydrate pathway that may help to use efficiently carbohydrates for building cellular structures and for producing energy in the senescing leaves.

Chl fluorescence parameters as changed during senescence and modified by Se: F_v/F_m, F_v'/F_m', and ETR all declined over the three subsequent time periods in the

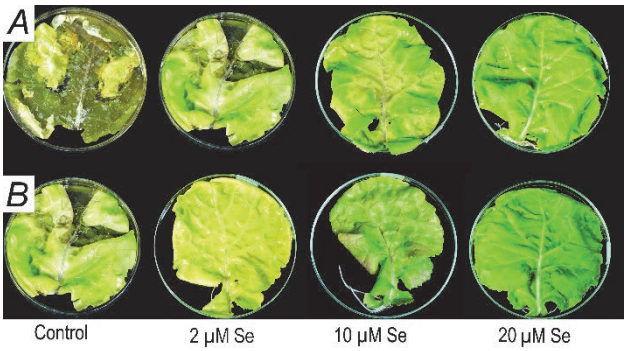

Fig. 6. Chlorosis and necrosis in the fully expanded leaves of oilseed rape plants treated with 2, 10, and 20 μM Na_2SeO_4 under high [300 μmol(photon) m^{-2} s^{-1}], *A*) or low [100 μmol(photon) m^{-2} s^{-1}], *B*) light intensities two weeks after detachment.

leaves of different developmental stages. Similar results for these parameters were reported in wheat plants under field conditions (Lu *et al.* 2001). In the young and middle aged leaves, F_v/F_m, F_v'/F_m', and ETR already started declining before the onset of senescence, which was detectable as a visible leaf yellowing, indicating that photosynthetic light reactions were reduced even before considerable decline of the Chl content. In *Arabidopsis*, ETR were reduced before full leaf expansion and even before reduction of the Chl content (Wingler *et al.* 2004). Decline of F_v'/F_m' and ETR in the young and particularly middle aged leaves were more prominent compared to F_v/F_m suggesting that F_v'/F_m' and ETR are highly sensitive indices for detection of leaf senescence at earlier stages in oilseed rape plants.

A much more steep reduction of F_v/F_m in the old leaf is an indication of an increased susceptibility to photoinhibition that was observed in other plant species and attributed to the concurrent senescence-related Chl loss (Lu *et al.* 2001). Several senescence-related physiological changes have been linked to increased susceptibility to photoinhibition. A marked reduction in the capacity to repair PSII reaction centers during senescence (Hoch *et al.* 2001), deteriorations in protective mechanisms, *e.g.*, xanthophyll cycle pigments (Biswal 1995), as it was confirmed in this work by reduction of Car, and finally a greater decline in C reduction relative to light-harvesting capacity (Wingler *et al.* 2004) appears to be widespread phenomena during leaf senescence. Protective processes, such as nonphotochemical quenching, play a prominent role in preventing damage during senescence (Hoch *et al.* 2001, Lu *et al.* 2001, Wingler *et al.* 2004). In maize and wheat, nonphotochemical quenching increases in combination with an accumulation of Car (Lu and Zhang 1998, Lu *et al.* 2001). In the oilseed rape plants of this work, q_N values tended to increase over three subsequent time points in the young and middle aged leaves, while declined in the old leaves. These data suggest that young and middle aged leaves were able to dissipate efficiently the excess excitation energy, while the limited capacity for heat dissipation in the old leaves may in turn lead to photooxidative damage and accelerated senescence at the final stages of development. Similar response was observed in *Arabidopsis*. The nonphotochemical quenching values in the rosette leaves in this species initially increased, while declined during the final stage of development (Wingler *et al.* 2004).

The most prominent effect of N deficiency on the leaf photochemical activity (measured in the old leaves) was observed on the F_v/F_m and F_v'/F_m' as it represented about 61 and 71% reduction under these conditions, respectively. This was much greater than that observed in developmental senescence, *i.e.*, 28 and 22%, when data (of the last time point) in the young leaves was compared with that in the old leaves. Nitrogen is the main component of chloroplast structures and proteins, thus, N deficiency may hamper synthesis of several structural and functional proteins in the reaction centers.

In the Se-treated plants, F_v/F_m, F_v'/F_m', q_P, and ETR tented to be higher and the difference between +Se and –Se plants became more prominent with increasing leaf developmental stage and in a defined leaf towards the end of experimental period. These data suggested that Se affects photochemical reactions not only *via* an increase in the number of functional PSII centers reflected in the higher F_v/F_m, but also through elevation of efficiency of PSII reaction centers for electron capture and transfer as well as quenching by subsequent chemical reactions. This effect of Se may be mediated at least partly, by activation of dark reactions (*see* below). The Se treatment, interestingly, diminished q_N in the young and middle aged leaf likely due an improvement of PSII efficiency in energy capture and better utilization of excitation energy in biochemical reactions, *i.e.*, increased q_P. In contrast, q_N in the old leaf was higher in the Se-treated plants. It implied that protection of plants against excess excitation energy contributes to Se-mediated delay of leaf senescence mostly at the late stages of development. Similar effect of Se was observed in N-deficient plants for F_v/F_m, F_v'/F_m', q_P, and ETR. Effect of Se on the elevation of q_N during both developmental and N deficiency-induced senescence occurred in parallel with higher Car concentrations in the Se-treated plants. Slightly higher nonphotochemical quenching upon low N conditions has been observed in *Arabidopsis* (Wingler *et al.* 2004). However, increase of q_N in the young and middle aged leaves, but the decline in the old leaf in this work was not correlated with the changes in the Car concentrations that decreased continuously with leaf ageing.

Leaf C and N metabolism as changed during senescence and modified by Se: A hallmark of leaf senescence is the termination of photoassimilation (Koeslin-Findeklee *et al.* 2015). In this work, a decline in the soluble carbohydrate pools during progression of senescence and increase by Se treatment were all well correlated ($r = 0.80$; $p<0.05$) with the concomitant changes in the P_N.

Reduction of nonstructural carbohydrates concentrations and P_N with the increasing leaf developmental stage is attributable to several parallel occurring mechanisms including reduction of Chl, destruction of chloroplasts, and degradation of stromal proteins, particularly Rubisco (Zimmermann and Zentgraf 2005). As related to Se-mediated stimulation of P_N, various mechanisms could be involved, such as elevation of stomatal conductances (Hajiboland and Sedeghzade 2014) and an increased activity of fructose-1,6-bisphosphatase (Owusu-Sekyere *et al.* 2013). In a proteomics study of Se effect in apple leaves, upregulation of three Rubisco large subunits, one PSI reaction center subunit II, and oxygen-evolving enhancer protein 1 have been reported (Ning *et al.* 2013). Se-mediated increase of soluble carbohydrates in the leaves of different age was not observed in N-deficient plants in the old as well as young and middle aged leaves (data were not shown for the latter leaves). It indicates that Se treatment could not enhance C metabolism when N was limited and was not able to compensate for low N supply to the chloroplasts in this regard. Reduction of NR activity with increasing leaf developmental stage has been previously reported by some authors (Masclaux-Daubresse *et al.* 2002, Hawkesford *et al.* 2012). NR activity is an important control point in N assimilation pathway and its activity is governed mainly by light, nitrate, glutamine, minor amino acids, plant hormones, particularly cytokinins, glutathione redox state, and sugars (Stitt *et al.* 2002, Kopriva and Rennenberg 2004, Koeslin-Findeklee *et al.* 2015). The NR activation state is not affected by leaf ageing and the changes in the NR activity during senescence is attributable to the decrease in the steady state level of the transcripts that is correlated with the level of functional NR protein (Masclaux *et al.* 2000). Decline in the NR activity here was expectedly accompanied by nitrate accumulation during progression of senescence that may even play a role as osmoticum in the old leaves (Hawkesford *et al.* 2012). Reduction of nitrite concentration under these conditions, however, was likely the consequence of lower inhibition of nitrite reductase (NiR) compared with NR activity.

The Se treatment elevated NR activity in the young leaf in this work. Considering the above mentioned mechanisms for regulation of NR activity, several potential mechanisms could be suggested for Se-mediated elevation of NR activity. Modifications in the content of minor amino acids (Lee *et al.* 2005) and in the glutathione redox state (Hajiboland *et al.* 2015) by Se and increase in carbohydrate pool as observed in this work could play roles in the Se-mediated enhancement of NR activity. The Se treatment may also influence the phytohormone homeostasis in the leaves and modify senescence-regulated signaling pathways. In this context, Se-mediated decline in the NR activity in the middle aged and old leaves may be related to a modification of signaling events and/or inactivation of metabolic pathways in the senescent leaves that contribute in the young leaves to the Se-mediated elevation of NR activity. More detailed works are required to unravel the mechanisms for changes occurring in the process of ageing in these pathways resulting in even downregulation of NR activity by Se.

Amino acids and soluble protein pools declined during progression of leaf senescence albeit soluble proteins responded to much lesser extent than the amino acids. These changes were highly correlated with correspondingly lower NR activity ($r = 0.99$; $p<0.05$). Under N deficiency conditions, sugars were accumulated in the leaves as the consequence of reduction in demand for C skeletons for synthesis of amino acids and proteins (Hawkesford *et al.* 2012). Unexpectedly, amino acids and soluble protein concentrations did not change significantly by low N supply likely because of greater reduction of biomass than the amounts of these N compounds. Nitrate, but not nitrite concentration, in contrast, responded directly to the level of N supply. Nitrite accumulation in the N-deficient plants was likely the consequence of lower availability of reducing equivalents in the leaves that was well reflected in the significant declines in the leaf photochemical activity under these conditions. The Se treatment caused an increase in the soluble protein concentrations, this effect was much greater in the old compared to young and middle aged leaves. However, a 3.2 fold increase of proteins in the old leaves was not associated with higher NR activity which was even reduced by the Se treatment. This may suggest that in the old leaves, nitrate assimilation was not elevated by Se, while incorporation of amino acids into proteins was enhanced and/or protein degradation was prevented. Taken together, concentration data of different C and N metabolites suggested that soluble sugars, amino acids, and nitrate were reliable metabolic indicators for developmental senescence in the leaves at a rosette growth stage of oilseed rape plants in this work.

Several authors suggested that leaf senescence is triggered by higher availability of C relative to N (Ono *et al*. 1996, Masclaux *et al*. 2000, Masclaux-Daubresse *et al*. 2002). A continuous decline in carbohydrates during progression of senescence in this work seems to contrast with the role of carbohydrate accumulation in triggering senescence. In tobacco, a detailed analysis of carbohydrate contents along the main plant axis indicated that glucose, fructose, sucrose, and starch concentrations declined from the most youngest leaves to the lower leaves, then increased, and reached the maximum value at a particular leaf age followed by a strong fall characteristics for a late senescence (Masclaux *et al.* 2000). Similarly, in *Arabidopsis* rosette leaves, the changes in the glucose, fructose, sucrose, and starch contents formed bell-shaped curves during a period of 20–70 d after sowing (Diaz *et al.* 2005). We suggest that differences in the stage of senescence analyzed in our work was the main cause for this contrastive results and our data indeed reflected the changes occurring typically during the late senescence.

There is evidence that senescence is regulated by the

C–N balance in leaves (Ono *et al*. 1996; Masclaux *et al*. 2000, Masclaux-Daubresse *et al*. 2002, Palenchar *et al.* 2004, Price *et al.* 2004). The Se treatment in this work caused a significant rise of the soluble C metabolite pools, particularly in the senescing leaves, however, it was balanced with a concurrent increase of N compounds. In a previous report, we showed higher carbohydrate and protein concentrations in Se-treated alfalfa plants, but the C/N ratios remained constant (Owusu-Sekyere *et al.* 2013). A parallel rise of C and N metabolism implied likely that not only Se favors energy metabolism and encounters progressive reduction of photosynthetic capacity in senescing leaves, but it compensates for degradation of plastid components as well, *i.e*. by an increased protein synthesis. Such evidence suggests that *via* an enhanced C and N metabolism, Se prevents cessation of leaf metabolism and hinders progression of destructive events during senescence. During N-starvation-induced senescence, however, effect of Se on C and N metabolism was not prominent as that in developmental senescence and Se effect was confined to the protection of leaf photochemical activity and/or maintaining it at a constant level during progression of senescence.

Effect of Se on senescence of detached leaves: Chl content (SPAD unit) started declining immediately after leaf detachment, irrespective of light conditions. However, this reduction occurred more slowly in the presence of Se compared with the control leaves and the difference between –Se and +Se leaves became bigger towards the end of experimental period. F_v/F_m suggested that higher light intensity accelerated considerably senescence of detached leaves in this work most likely as the consequence of a greater risk of photodamge (Kar *et al.* 1993). The effect of higher light intensity in triggering senescence *via* an earlier accumulation of hexose (Wingler *et al.* 2004) could not be also excluded. Protective effect of Se against photo-oxidative damages has been reported for intact plants under UV stress and has been attributed to the increasing activities of antioxidative enzymes (Xue and Hartikainen 2000).

Taken together our results suggest that Se delayed leaf senescence *via* protection of leaf photochemical activities and interactions with metabolic regulation of senescence. Effect of antioxidative defense system was not examined in our work and the relative contribution of this mechanism could not be evaluated in the three senescence types studied in this work.

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