horticultura brasileira

Research

STAGNARI, F; GALIENI, A; D'EGIDIO, S; PAGNANI, G; FICCADENTI, N; PISANTE, M. 2018. Defoliation and S nutrition on radish: growth, polyphenols and antiradical activity. *Horticultura Brasileira* 36: 313-319. DOI - http://dx.doi.org/10.1590/S0102-053620180305

Defoliation and S nutrition on radish: growth, polyphenols and antiradical activity

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ABSTRACT

Experiments were carried out to study patterns of artificial defoliation in radish in combination with sulphur (S) fertilization, to evaluate the contribution of younger and older leaves on plant growth and phenolics accumulation in storage roots. Biomass accumulation and partitioning were related to leaf age, magnitude and timing of the clipping treatments. Older leaves increased biomass production and translocation into the storage organ; besides, they induced higher accumulation of phenolic compounds compared to the younger leaves. The highest S fertilization rate (120 kg ha^{-1}) significantly enhanced the polyphenols accumulation, as well as the antiradical activity. The modulation of S inputs in combination with slightly induced stress from defoliation could effectively enhance the concentration of some important phytochemicals, providing higher nutritionally improved vegetables, without affecting yield.

Keywords: *Raphanus sativus*, biomass partitioning, phenolic compounds, sulphur fertilization.

RESUMO

Desfolha e nutrição do S no rabanete: crescimento, polifenóis e atividade antiradical

Foram realizados experimentos para estudar padrões de desfolha artificial em rabanete em combinação com adubação com enxofre (S), para avaliar a contribuição de folhas jovens e velhas no crescimento de plantas e acúmulo de compostos fenólicos em raízes de armazenamento. O acúmulo e particionamento da biomassa foram relacionados à idade, magnitude e época do corte dos tratamentos. As folhas mais velhas aumentaram a produção de biomassa e a translocação para o órgão de armazenamento, além de induzirem maior acúmulo de compostos fenólicos em relação às folhas mais jovens. A maior taxa de fertilização (120 kg ha^{-1} de S) aumentou significativamente o acúmulo de polifenóis, bem como a atividade anti-radical. A modulação dos insumos de S em combinação com a tensão induzida pela desfolha poderia efetivamente aumentar a concentração de alguns fitoquímicos importantes, fornecendo vegetais melhorados nutricionalmente, sem afetar o rendimento.

Palavras-chave: *Raphanus sativus*, partição de biomasa, compostos fenólicos, fertilização com enxofre.

Received on August 31, 2017; accepted on May 28, 2018

Radish (*Raphanus sativus*), a root crop belonging to the *Brassicaceae* family, is grown in many parts of the world due to its short growth period and high nutritious value (Akram *et al*., 2015), i.e. source of antioxidant compounds (Kim *et al*., 2013). Like in other crops, the occurring of biotic and abiotic stressful conditions lead to morphological, physiological and biochemical changes which, in turn, can affect the chemical composition of storage organs (Stagnari *et al*., 2017). Among abiotic stress, plants defoliation, as result of pest attack, diseases or consequence of agricultural practices (Severino *et al*., 2010), is known to determine impairment of the photosynthetic apparatus, with general reduction in plant growth and yield. Furthermore, modifications in partitioning of assimilate between the different organs for the re-establishment of the source capacity can occur, with an increased sink strength in the developing leaves at the expense of the stems or roots (Iqbal *et al*., 2012). However, the effects are dependent on defoliation timing, vegetative or reproductive stages, younger or older leaf tissues (Iqbal *et al*., 2012).

Plants oppose to mechanical stress also through chemical and/or physical defenses, which involve the activation of secondary metabolism to maintain the balance with reactive oxygen species (ROS) (Anjum *et al*., 2012). In *Brassicaceae* family, phenols constitute one of the most common and widespread groups of defensive compounds, which play a major role against herbivores attacks and microbiological infection (Jahangir *et al*., 2009). Biosynthesis of secondary metabolites is sharply influenced by nutrient availability as well sulphur (S), for example, is essential in the biosynthesis of secondary metabolites in *Brassicaceae*. S fertilization has been demonstrated to significantly enhance several major plant stress-defense (Anjum *et al*., 2012) as in *Brassica napus* (Weese *et al*., 2015), *Brassica olaracea* var. *italica* (Naguib *et al*., 2012) and *Brassica rapa* subsp. *sylvestris* (De Pascale *et al*., 2007).

To date, studies investigating the

combined effect of leaf defoliation and sulphur availability on biomass production and partitioning as well as on phytochemicals concentration, are missing for this species. Therefore, several patterns of artificial leaves clipping in radish plants, supplied with different rates of S fertilizer, have been applied. The primary objective of the study was to evaluate the specific contribution of the younger leaves (i.e. new formed leaves), and older leaves (i.e. leaves already formed) on radish growth, yield, morphology and phenolics accumulation in storage roots, as well as any synergistic effect of S in the chemical compositions of storage organs.

MATERIAL AND METHODS

Under the greenhouse of Agronomy and Crop Sciences Research and Education Centre, University of Teramo, Italy (42°53'N, 13°55'E, altitude 15 m), experiments were carried out from May 9 to June 12, 2013. The environmental conditions were constantly monitored during crop growing cycle with temperature, humidity and photosynthetically active radiation (PAR) sensors connected to a data logger (EM50 Data Collection System, Decagon Devices Inc., Pullman, WA, USA). Maximum, minimum and mean air temperature and mean air relative humidity were 30.5°C, 5.5°C, 18.3°C and 0.67, respectively; the mean of PAR transmitted to the canopy was 652.7 µmol $m^{-2}s^{-1}$ (maximum PAR values: 1525.3 µmol $m^{-2}s^{-1}$).

Seeds of radish (cv. Bostella, Vilmorin Italia S.r.l., Argelato (Bo), Italy) were sown on a nursery potting soil (Humin substrat N3, Neuhaus, Klasmann-Deilmann, Geeste, Germany) and transplanted ten days after sowing into 9 cm diameter plastic pots, at a density of 1 plant per pot. Pots were filled with sphagnum peat moss, perlite and vermiculite at the ratio of 2:1:1.5. At transplanting, plants were fertilized with a NPK fertilizer blend 10-10-10 (Cifoumic 10-10-10, Cifo S.r.l.,Bologna, BO, Italy) at the dose of 120 kg ha^{-1}. Pots were manually irrigated with tap water. On a factorial randomized block design, two experimental factors, i.e. defoliation treatments and sulfur fertilization rates, were applied, as main and secondary factors, respectively. Starting 4 days after transplanting (DAT), the defoliation treatments were carried out on both younger, i.e. new formed leaves (YouD treatments) and older leaves, i.e. leaves already formed (OldD treatments) during the crop cycle, which was divided into 4 experimental periods (4 days) of scalar defoliations (4-DD). We obtained plants with the first 2, 4 and 6 leaves (3YouD, 2YouD and 1YouD) as well as plants with only new formed leaves after defoliation (3OldD, 2OldD, and 1OldD), as shown in Figure 1. Two controls, fully-defoliated (4OldD) and un-defoliated (0YouD) throughout the whole experimental period, were also included. Defoliation treatments were not performed on cotyledonary leaves. At 4 DAT, for each defoliation treatment, three sulfur fertilization rates (0, 60 and 120 kg ha^{-1} of S) were applied using Zolfo Manica Granulare (Manica S.p.A., Rovereto (TN), Italy). Each treatment was replicated three times and each replication consisted in 15 pots.

Three plants per each experimental unit were sampled at 8 and 20 DAT and separated into leaf and storage roots for fresh (FW) and dry weight (DW) determinations after oven-drying at 80°C, until constant weight. Leaf areas (LA, cm^2) and specific leaf area (SLA, cm^2 g^{-1}) were obtained by photocopying leaves of each plant and by acquiring the images with ImageJ (image analysis software, National Institutes of Health, Bethesda, MD, USA). The net assimilation rate, i.e. the amount of increasing storage roots DW per unit leaf area per day (NAR, mg cm^{-2} DAT^{-1}), was calculated as previously reported (Hunt *et al.*, 2002). Storage roots diameter (cm) was measured both longitudinally (D) and transversely (d), using a caliber and the ratio D/d was determined.

Three additional plants per experimental unit were separated into leaf and storage roots and stored at -20°C until being processed for analytical determinations of total polyphenols content (TPC) and radical scavenging activity, performed on FW basis. Radish samples were cut into small pieces and thoroughly mixed. A sub-sample was treated with methanol (1:5 w/v) and homogenized with the T-25 Ultra-Turrax (IKA-LAB, Seneco S.r.l., Italy), then sonicated with Sonis 4 for 1 h in a cooled water bath. The extracts were centrifuged for 10 min at 15000 rpm. Supernatant was filtered in a 20 µm pore size PTFE membrane and transferred to a vial. TPC was assessed using the Folin-Ciocalteu phenol reagent method (Singleton & Rossi, 1965). The TPC was expressed as µg of gallic acid equivalents (GAE) g^{-1} storage root FW.

The radical scavenging activity was performed as previously described by Re *et al.* (1999) and results were expressed as µmol of Trolox equivalents (TE) per g storage root FW.

The spectrophotometer used was a UV/VIS (Lambda Bio20, Perkin-Elmer, Waltham, MA). With the exception of ABTS and Trolox, all the chemicals reagents and standards used for chemical analysis were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Data referring to storage roots dry weight and diameter, as well as to leaves dry weight, NAR, LA and SLA are shown as mean of three independent replicates ± standard errors. TPC and TEAC data were subjected to two-way analysis of variance (ANOVA) using the R software (R Core Team, 2017). If the ANOVA detected significant differences, means separation was conducted through the Tukey's honestly significant difference (HSD) test.

RESULTS AND DISCUSSION

The effect of defoliation on growth of radish leaves and storage roots depended on its magnitude and timing as well as on the age of the involved leaves (Figures 2A and 2B). Besides, sulphur fertilization did not induce any significant differences regardless of the defoliation treatments. As expected, the wider the defoliation period, the smaller the leaf DW (Figure 2A). OldD treatments always gave significantly lower aerial DW than YouD, indicating

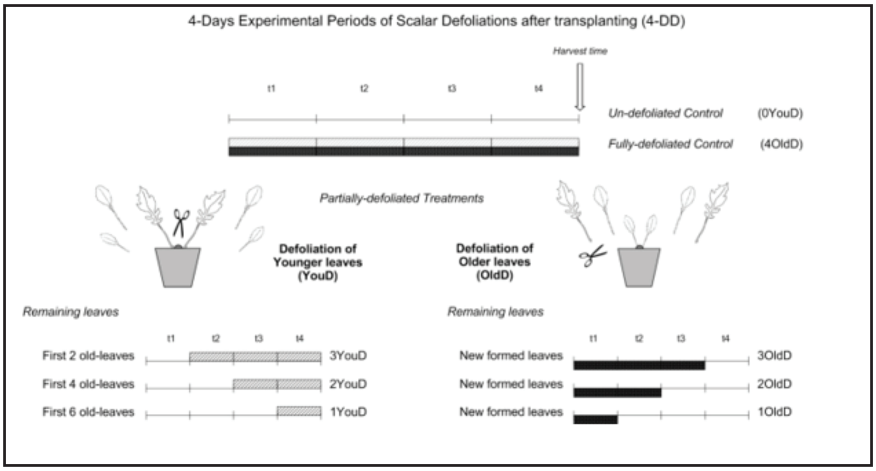

Figure 1. Scheme of the defoliation treatments performed on younger (YouD treatments) and older leaves (OldD treatments) of radish plants. Crop cycle was divided into 4-days experimental periods of scalar defoliations (4-DD) after transplanting; for the partially-defoliated treatments, artificial leaves clipping was carried out for 1, 2 and 3 4-DD, in order to obtain plants with the first 2, 4 and 6 older leaves (3YouD, 2YouD and 1YouD) and plants with the new formed younger leaves after defoliation (3OldD, 2OldD, and 1OldD). A fully-defoliated (4OldD) and un-defoliated (0YouD) controls over the whole crop cycle were also added. Teramo, University of Teramo, Italy, 2013.

a poor ability of radish to replace the lost leaf biomass, already after only 1-period of early scalar defoliation (0.41, 0.37 and 0.25 g plant^{-1} DW on average for un-defoliated Control, 1YouD and 1OldD treatments, respectively). These results were inconsistent with previous findings on castor plants (Severino *et al.*, 2010), revealing that specie-specific differences are probably due to the duration of growing cycles. A similar trend was also observed in terms of leaf area (Figure 3A). The reduction of the photosynthesizing area strongly lowered storage roots DW, reaching values of 71% in the fully-defoliated Control (1.9 g plant^{-1} DW, on average) (Figure 2B). The older leaves were more effective to obtain high yield (see YouD vs. OldD yield values), as also confirmed by NAR data (Figure 2C). YouD treatments indeed induced greater biomass located to storage organ per unit leaf area (at 2-periods of scalar defoliation: 1.36 vs 1.04 mg cm^{-2} DAT^{-1} on average for 2YouD and Control, respectively; at 3-periods of scalar defoliation: 2.64 mg cm^{-2} DAT^{-1} on average for 3YouD).The effect of S fertilization was negligible. Similar behaviors have already been observed (van Den Boogaard *et al.*, 2001) and are probably explained with higher photosynthetic rate, i.e. increased light interception per unit leaf area, as defoliation reduces internal shading (Li *et al.*, 2012), or with a better stomatal conductance of the older leaves (Syvertsen, 1994). Photosynthesis in plants is a normal compensatory response to mechanical damage and it is probably related to less light energy dissipation as heat (Li *et al.*, 2012). OldD treatments decreased NAR index as defoliation periods went on, independently by sulphur treatments (1.22, 0.83 and 0.64 mg cm^{-2} DAT^{-1} on average for 1OldD, 2OldD and 3OldD, respectively). In this case, radish restored the sink-source balance through an increase in the demand of energy for regrowth of damaged organs (Poorter *et al.*, 2012), especially after intense defoliation, requiring largest amount of investment (Liu *et al.*, 2007). This was confirmed by the significantly higher values of the leaf' relative growth rate (RGR_{leaf}, data not shown) of OldD (108 vs 25 mg g^{-1} DAT^{-1}, respectively, averaged over S and defoliation periods). Furthermore, OldD treatments reduced SLA values (Figure 3B) probably due to the lower leaf thickness of the new-formed leaves, thus improving the ability to intercept light more efficiently. Sulphur significantly enhanced the leaf area and, consequently, the SLA values of un-defoliated Control (LA: 113 vs. 189 cm^2 plant^{-1} for 0 and 120 kg S ha^{-1}, respectively; SLA: 228 vs. 464 cm^2 g^{-1} plant^{-1} for 0 and 120 kg ha^{-1} of S, respectively) confirming previous findings (Siddiqui *et al.*, 2012), indicating a role of S application in alleviating the adverse effect of salt stress on growth traits in *Brassica juncea*.

Interestingly, defoliation also altered the storage roots morphology, i.e. longitudinally vs. transversely diameter ratio (D/d) (Figure 3C).

Clipping all the leaves (fully-defoliated Control) reduced the polyphenols concentration in radish storage roots by 23.6% (Table 1), while the effect of partially-defoliated treatments strictly depended on S fertilization (see the significance at $p<0.01$ of the interaction) (Table 1). In general, defoliation enhanced polyphenol concentration only at the highest S fertilization rate (120 kg ha^{-1} of S) and at 1- or 2-periods of scalar defoliation (1YouD, 2YouD, 1OldD and 2OldD treatments). The excessive impairment of photosynthetic apparatus has probably lowered phenolic biosynthesis which result commonly stimulated by mechanical stress (War *et al.*, 2012; Borges *et al.*, 2017). The ascorbate-glutathione (AsA-GSH) pathway has been extensively evidenced to efficiently remove/metabolize ROS and/or their reaction products (Anjum *et al.*, 2012). Since sulphur play a key role as the major modulator of GSH-mediated control of plant stress tolerance, S fertilization could have positively influenced the biosynthesis of phenolic compounds in radish plants in accordance with previous studies on different *Brassicaceae* species (De Pascale *et al.*, 2007; Naguib *et al.*, 2012). At the highest S rates 1YouD treatment showed significantly higher TPC values than 1OldD, confirming the greater role of older leaves in polyphenol biosynthesis (Sousa *et al.*, 2008). Anyway, it is important to specify that the youngest plant tissues are generally characterized by higher secondary metabolites content (Meldau *et al.*, 2012). In our experimental conditions, we also found a similar trend in terms of antiradical activity, highly correlated with TPC ($r^2 = 0.93$) (Stagnari

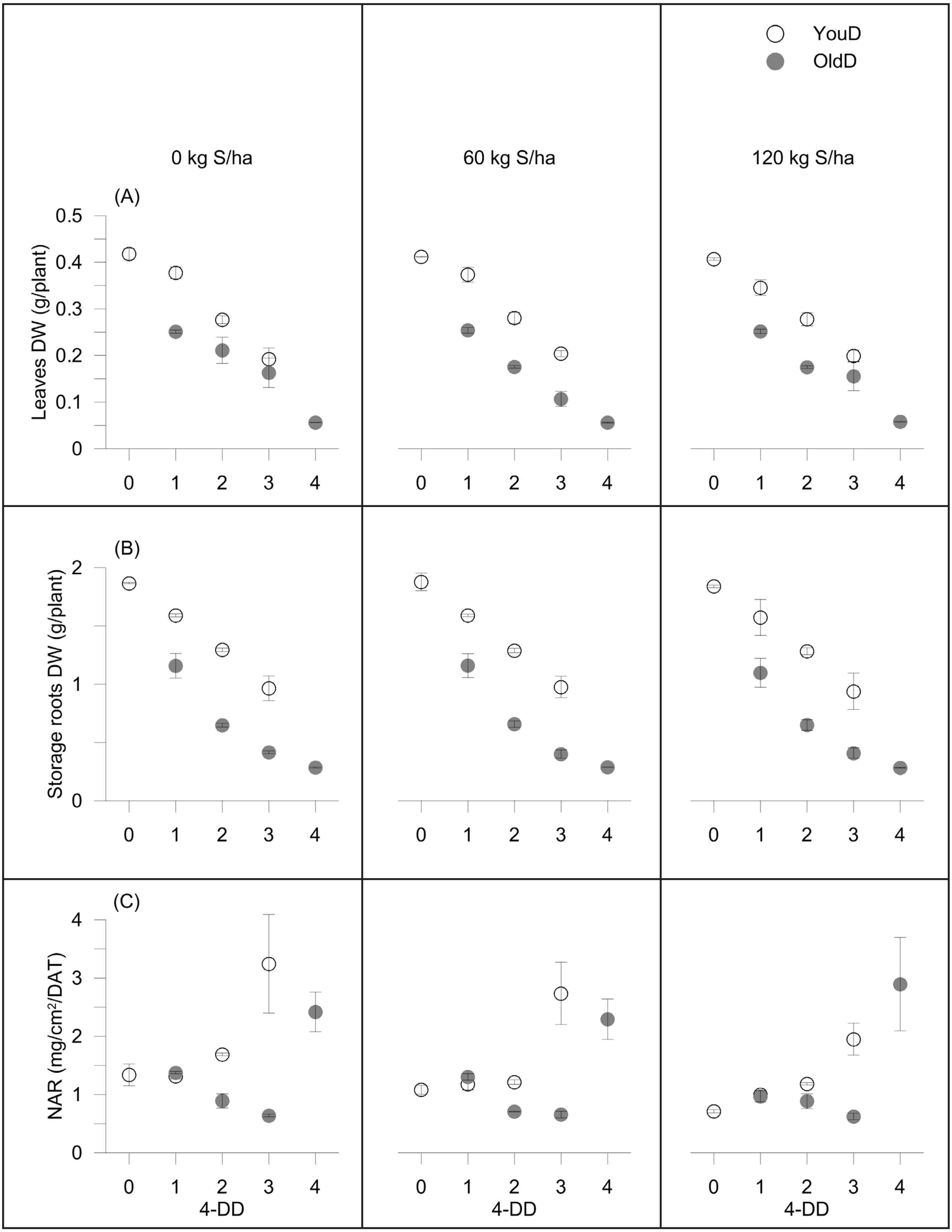

Figure 2. (A), leaves dry weight (g plant^{-1} DW), (B), storage roots dry weight (g plant^{-1} DW) and (C), net assimilation rate (NAR, mg cm^{-2} DAT^{-1}), as recorded for radish plants subjected to 0, 1, 2, 3 and 4 periods of scalar defoliation (4-DD) (as explained in detail in Figure 1) and three S fertilization rates (0, 60 and 120 kg ha^{-1} of S). Average values ± standard errors are depicted. Empty symbols: defoliation of younger leaves (YouD treatments); fully symbols: defoliation of older leaves (OldD treatments). Teramo, University of Teramo, Italy, 2013.

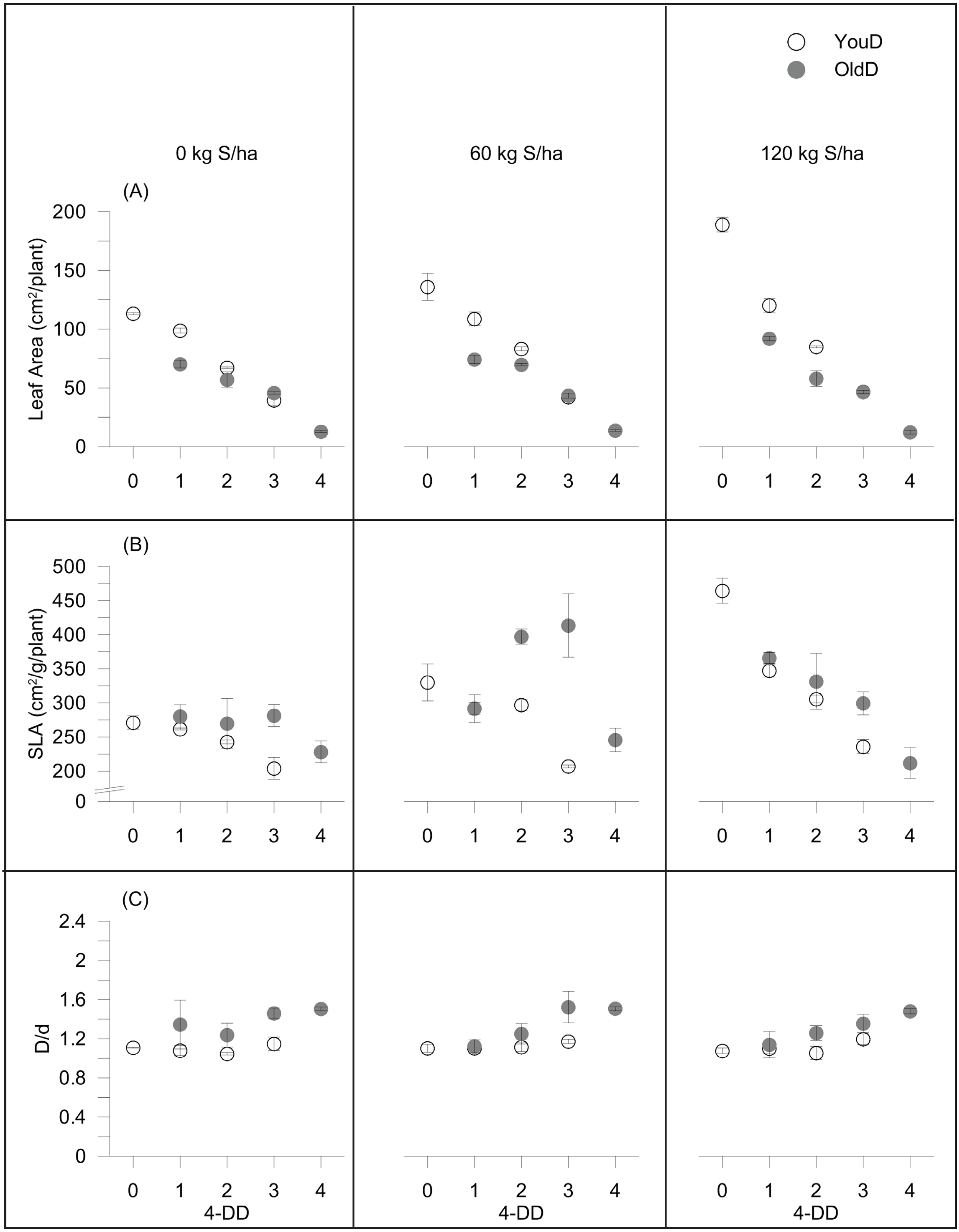

Figure 3. (A), leaf area (LA, cm^2 plant^{-1}), (B), specific leaf area (SLA, cm^2 g^{-1} plant^{-1}) and (C), longitudinally:transversely storage root diameter ratio (D/d), as recorded for radish plants subjected to 0, 1, 2, 3 and 4 periods of scalar defoliation (4-DD) (as explained in detail in Figure 1) and three S fertilization rates (0, 60 and 120 kg ha^{-1} of S). Average values ± standard errors are depicted. Empty symbols: defoliation of younger leaves (YouD treatments); fully symbols: defoliation of older leaves (OldD treatments). Teramo, University of Teramo, Italy, 2013.

Table 1. Total phenolic content (TPC, µg GAE g^{-1} FW) and antiradical activity (µmol TE g^{-1}FW) as recorded at 20 days after transplanting in storage roots of radish plants subjected to 0, 1, 2, 3 and 4 periods of scalar defoliation (4-DD) (as explained in detail in Figure 1) and three S fertilization rates (0, 60 and 120 kg ha^{-1} of S). Average values ± standard errors are reported. For each analytical determination, means labeled with the same letter did not differ significantly at P<0.05 (Tukey's HSD test). Teramo, University of Teramo, Italy, 2013.

Defoliation	Storage root total phenolic content (µg GAE g^{-1})			Storage root antiradical activity (µmol TE g^{-1})		
	S-fertilization rate (kg/ha)			S-fertilization rate (kg/ha)		
	0	60	120	0	60	120
Of Younger Leaves						
1YouD	183±1.3 ghi	217±5.7 de	327±5.6 a	3.1±0.01 hi	3.3±0.02 fg	4.1±0.06 a
2YouD	176±4.5 ghij	231±4.4 cde	251±4.2 b	3.0±0.05 i	3.7±0.01 bc	3.9±0.05 b
3YouD	180±3.0 ghi	195±0.3 fg	231±1.5 cd	3.1±0.03 ghi	3.1±0.01 ghi	3.6±0.02 cd
Of Older Leaves						
1OldD	189±3.8 gh	215±0.7 de	253±4.4 b	3.2±0.00 ghi	3.3±0.02fgh	3.8±0.00 bc
2OldD	182±1.1 ghi	212±1.4 ef	241±0.4 bc	3.0±0.01 i	3.3±0.00 fgh	3.8±0.01 b
3OldD	170±1.4 hij	193±2.4 fg	215 ±0.8 de	2.7±0.01 kl	2.9±0.01 ijk	3.3±0.02 efg
Un-defoliated control	171±0.8 hij	230±0.6 cde	215±5.6 de	3.0±0.00 ij	3.6±0.02 cde	3.5±0.12 def
Fully-defoliated control	140±0.6 k	157±0.8 jk	167±0.8 ij	2.5±0.03 m	2.7±0.02 lm	2.8±0.02 jkl
F test						
Defoliation		**			**	
S rate		**			**	
Defoliation x S rate		**			**	

**P < 0.01; *P < 0.05; n.s. = not-significant.

et al., 2014).

In conclusion, the morphological and adaptive response of radish to defoliation seems closely related to the age of the involved leaves, i.e. defoliation of younger or older leaves and to the magnitude of artificial clipping. Older leaves play a prominent role in dry matter production and partitioning to the storage organs. Besides, older leaves are more effective in the biosynthesis of phenolic compounds. Sulfur fertilization significantly enhances the polyphenol content, as well as the antiradical activity of radish storage roots, emphasizing its key role in the activation of plants' defense system. These results can be useful employed in management strategies in order to provide high nutritionally enhanced vegetables, i.e. higher phytochemicals content, slightly affecting biomass production (see the combination 1YouD treatment/120 kg ha^{-1} of S).

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