Journal Pre-proof

Plasticity of root growth and respiratory activity: root responses to above-ground senescence, fruit removal or partial root pruning in soybean

Diego Darío Fanello, Santiago Julián Kelly, Carlos Guillermo Bartoli, María Gabriela Cano, Santiago Martínez, Juan José Guiamet

PII: S0168-9452(19)30870-2

DOI: https://doi.org/10.1016/j.plantsci.2019.110296

Reference: PSL 110296

To appear in: *Plant Science*

Received Date: 24 June 2019

Revised Date: 18 September 2019

Accepted Date: 1 October 2019

Please cite this article as: Fanello DD, Kelly SJ, Bartoli CG, Cano MG, Martínez S, Guiamet JJ, Plasticity of root growth and respiratory activity: root responses to above-ground senescence, fruit removal or partial root pruning in soybean, *Plant Science* (2019), doi: https://doi.org/10.1016/j.plantsci.2019.110296

Plasticity of root growth and respiratory activity: root responses to above-ground senescence, fruit removal or partial root pruning in soybean

Diego Darío Fanello*, Santiago Julián Kelly, Carlos Guillermo Bartoli, María Gabriela Cano, Santiago Martínez and Juan José Guiamet

Instituto de Fisiología Vegetal (INFIVE), Facultades de Ciencias Agrarias y Forestales (FCAyF) y de Ciencias Naturales y Museo (FCNyM), Universidad Nacional de La Plata (UNLP), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) CCT-La Plata, cc 327, 1900, La Plata, Argentina
Tel.: (54-221) 483-8168, 453-0189 Fax 423-3698

*Corresponding author: Dr. Diego Darío Fanello, e-mail: ddfanello@gmail.com

Highlights

- Roots do not undergo a developmentally regulated senescence followed by organ death
- Fruits monopolize photoassimilates, limiting root growth
- Partially pruning roots increase root respiration and root growth
- Root show high plasticity when source-sink organs biomass are changed.

Abstract

This work focuses on the alterations in soybean root growth and activity during whole plant senescence and the contribution of roots to source-sink relations during plant development. The experiments were designed to analyze the activity of roots in relation to: a) whole plant senescence, b) total pod removal and c) root pruning (15, 25 and 50% of DW) during seed growth stages. Roots can grow until an advanced R5 stage and their specific activity decreases along the reproductive development but whole root activity declines from R6. However root respiration is maintained at a basal level until R8. Depodded plants showed a large increase of root dry matter (about 470%) and a large increase of AOX protein. Root pruning treatments showed a proportional increase of specific root respiration in 25 and 50% treatments but no differences of whole root respiration and dry matter partitioning at R7. These results indicate that roots are under the control of the requirements of above ground organs until final stages of

seed growth but, after this, roots may survive independently for some time. This suggests that roots do not suffer a senescence-like process as leaves do. Also, plants have a high capacity to buffer changes in root biomass production and specific root activity under pod removal or partial root pruning.

Key words: roots; senescence; respiration; nitrogen assimilation; depodding; root pruning.

1. Introduction

The term senescence refers to the functional and structural deterioration observed in plants during the final stages of their ontogeny [1]. It is difficult to integrate the vast literature about senescence due to a generally specific focus on cellular and molecular processes that occur in photosynthetic organs [2]. Senescence has been exhaustively studied in leaves and fruits, but scarcely in other organs such as roots. Even though recent studies show how fine roots senesce in perennial plants [3,4], the link between changes in the root system and monocarpic senescence in annuals plants is still little explored. Monocarpic refers to a life cycle with one reproductive episode followed by death [5] and monocarpic senescence involves different physiological processes occurring after flowering, including the remobilization of mineral and organic compounds from senescing leaves to growing fruits and seeds [1]. Soybean is a typical annual plant undergoing monocarpic senescence at the end of reproductive development [1].

It is not clear how the decline of root activity takes place during reproduction and the final stages of plant development. Some information indicates that both the production of new root apices and the growth of roots decline early in the reproductive phase or even before flowers appear [5]. Noodén [1] proposed that mineral absorption and transport of root-produced hormones decline during reproductive development. *Arabidopsis thaliana* plants show a decrease of nitrate uptake in the reproductive phase [6] and *Helianthus annuus* shows a progressive decrease of respiration and of the percentage of live roots during the grain filling period [7,8]. However, *Phaseolus vulgaris* does not show a correlation between root mortality and reproductive development [9] and respiration of *A. thaliana* roots increases during reproductive development, declining only when most of above ground tissues are dead [10]. Also, soybean roots still incorporate nitrogen (N) at advanced stages of the reproductive period [11,12].

Depending on the species, up to 50% of photoassimilates are consumed by roots [13]. The fraction of photoassimilates used in root respiration increases with plant age and this is partly due to the maintenance energy requirements when root growth slows down [13].

Among the macronutrients required by plants, N is consumed in the greatest abundance and often limits growth [14]. In most species, nitrate (NO_3^-) is the most abundant source of N [14]. The absorption and assimilation of NO_3^- involves an important energy cost in terms of ATP produced by root respiration (e.g., in barley approximately 23% of total root carbon catabolism is related to nitrate assimilation) [13,15]. Cells need a proton motive force to drive active NO_3^- uptake [14]. In roots, NO_3^- assimilation depends on carbon supply for the roots, and reducing power and ATP are derived from mitochondrial respiration, the pentose phosphate pathway and malate oxidation[16].

Some evidences indicate that, in general, roots tend not to reallocate their nitrogen to seeds [17–19]. However, it has been reported that during senescence about 20-30% of total root nitrogen is remobilized and, depending on the species, around 0.4-16% is remobilized to growing seeds [17].

The magnitude and timing of the decline of root activity during the reproductive period and the final phases of the development of annual species is unclear. Often, the decline in root activities is attributed to a decline in translocation of photoassimilates from photosynthetic organs [10,20], possibly due to competition by fruits. To test this idea, the responses of roots to the absence of a strong sink (i.e., when pods are removed from the plant) or in plants where root biomass is reduced (roots pruned) during the critical period of seed filling were compared. The general aim of this work was to characterize alterations in root growth and activity during the reproductive period of soybean plants, and to evaluate the effect of source-sink changes on the root system when plants are depodded or their roots are partially pruned.

2. Materials and methods

2.1 Plant material and growth conditions

The experiments were carried out with non-nodulated soybean (*Glycine max* [L.] Merr.cv. ´Williams 82´) plants. The experiments were conducted in La Plata (34°54´46´´ S; 57°55´50´´ W, Argentina). Seeds were sown in vermiculite during the second week of January and were grown until the first trifoliate leaf was expanded. Thereafter, plants were grown in a hydroponic system as described by Leggett and Frere [21] with modifications. The nutrient

solution consisted of 1.7mM $Ca(NO_3)_2$, 1.7m M KNO_3, 2.0 mM $MgSO_4$, 1.0 mM KH_2PO_4, 3.3 mM $CaCl_2$, 3.3 mM KCl, 20.0 µM FeNaEDTA, 5.0 µM H_3BO_3, 0.9 µM $MnCl_2$, 0.8 µM $ZnCl_2$, 0.3 µM $CuSO_4$ and 0.01 µM Na_2MoO_4, pH 5.5–6.5. This solution was replaced every 10-14 days and the volume of water consumed by plants was quantified and replenished every 3-5 days. Each plant was grown in a 5L pot in a climate-controlled greenhouse at an average temperature of 22±5 ºC and an irradiance ranging at midday between 500 and 900 µmol photons m^{-2} s^{-1} during January-June. Phenological stages were determined as described by Fehr and Caviness [22].

2.2 Phenological series, pod removal and root pruning treatments

For a phenological series, plants were analyzed at reproductive stages: R3 (7 days after flowering, DAF), R4 (17 DAF), R5 (23 DAF), R5ad (R5 advanced, 38 DAF), R6 (56 DAF), R7 (72 DAF) and R8 (99 DAF). Depodding and root pruning consisted of: a) complete removal of pods at R5 (23 DAF) with newly formed pods regularly removed thereafter and b) pruning of the root system (root-pruned plants) at three levels: removal of 15%, 25% or 50% of root dry weight of intact plants at R5ad (34DAF). Depodded and root-pruned plants were harvested on the date intact plants reached the R7 stage (73 DAF). Root pruning treatments were made by longitudinally cutting off the root system.

2.3 Measurement of root respiration

Respiration was measured by placing 1 g (fresh weight, FW) of roots taken from the distal part of the root system in an air-tight chamber equipped with a Clark type O_2 electrode at 25°C (Hansatech, UK) and between 10:00 and 16:00 h. Respiration of the distal part used for the measurements was representative of whole root system. This was checked by taking samples from different parts of the root system, which showed similar respiration rates (supplementary material Fig. S1). Prior to the measurements, roots were gently washed with deionized water and then blotted with paper towels to remove excess water. Total root system respiration was calculated by multiplying specific respiration by root biomass (fresh weight). Other total root system activities were calculated in the same way.

2.4Nitrate absorption

Nitrate absorption was calculated as the difference in NO_3^- concentration between fresh nutrient solution and the solution after a 3-day uptake period (before sampling, the volume of the pot was restored to 5L to account for transpired water). An aliquot of each sample was diluted to 5 mL with distilled water and mixed with 0.1 ml of 1N HCl. Nitrate was determined spectrophotometrically at 220 nm with a correction factor at 275 nm as described in APHA, AWWA and WPCF (1985) [23].

2.5 In vivo Nitrate Reductase activity (NR)

NR activity was measured as described by Deane-Drummond and Clarkson(1979) [24] with modifications. The incubation medium contained 50 mM phosphate buffer, 1% propanol (v/v), and 100 mM potassium nitrate at pH 8.0. Root samples (500 mg FW of apical root parts) were sectioned in to 1cm segments and transferred to 50 ml tubes containing cold incubation medium (20 ml/500 mg root) and then they were vacuum-infiltrated for 4 min. Two samples per plant were taken, one was incubated for 30 min and the other one for 60 min at 27 °C in the dark. The reaction was finished by boiling for 10 min. After cooling, NO_2^- was measured in 0.5 ml of incubation buffer by adding 0.5 ml sulfanilic acid (1% w/v in 3 N HCI) followed by 0.5 ml N-1-naphthylethylenediamine dihydrochloride (0.05% w/v). This mixture was vortexed for 30 s and then kept for at least 2 h in darkness. Mixtures were vortexed again for 30s and NO_2^- concentration was measured spectrophotometrically at 540 nm. NR activity was determined as NO_2^- production (i.e., the difference in NO_2^- concentration between 30 min and 60 min of incubation).

2.6 SDS-PAGE and immunobloting

Roots were harvested, frozen in liquid N_2 and kept at −80°C until analysis. Extracts (200 mg FW/mL buffer) were made in sample buffer consisting of 62.2 mM Tris-HCl pH 6.8, 3M urea, 5% v/v 2-β-mercaptoethanol, 0.005% (w/v) bromophenol blue, 4 mM cysteine, 1% w/v PVPP and protease inhibitors (0.004% w/v PMSF and 0.001% w/v Leupeptin). Homogenates were centrifuged at 1500g and 4°C for 15 min and the supernatants were collected for subsequent analysis. Soluble proteins were analyzed by SDS-PAGE according to Laemmli (1970)[25]. Samples were loaded (volume loaded onto each lane of the gels corresponded to 4 mg of root FW) on a 1 mm thick, 12% (w/v) polyacrylamide gel and electrophoresed at 20 mA gel^{-1} for 2.5 h. For western blotting, proteins were electro-transferred to a nitrocellulose

membrane at 200 mA for 1 h. Blots were blocked with 5% (w/v) non-fat dry milk dissolved in PBS (10 mM phosphate buffer, pH 7.4, and 137 mM NaCl) and probed with antibodies specific for mitochondrial proteins: cytochrome *c* oxidase (cox2; [26]) and alternative oxidase (AOX; [27]). After washing the membrane three times for 10 min each with PBS plus 0.05% (w/v) Tween 20 (PBS-T), blots were incubated with a secondary antibody (goat anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase, for polyclonal or monoclonal antibodies, respectively) for 1 h and washed with PBS-T. Blots were developed by incubation in 10 mL of chemiluminescence detection mixture: 5 ml of coumaric acid-luminol solution (0.23 mM coumaric acid and 1.2 mM luminol dissolved in DMSO) and 5 ml of hydrogen peroxyde (0.015% v/v H_2O_2), both solutions in Tris-HCl buffer 0.1 M pH 8.5. SDS-PAGE and Westerns blots were analyzed with the ImageJ software (1.46r version), Analyze Gel function. For protein quantitation, the highest value in each image (Gel or Western blot) was used as a reference (and set as 100), and then all other bands in the same gel were referred to it. Each gel contained at least two replicates of all samples to compare.

2.7 Dry biomass measurements

Dry biomass was measured by taking samples from plants at R3 (7 DAF), R4 (17 DAF), R5 (23 DAF), R5ad (38 DAF), R6 (56 DAF), R7 (72 DAF) and R8 (99 DAF); depodded plants were harvested on the date intact plants reached R7. Plants were dissected into: (i) stems and petioles, (ii) leaf blades, (iii) roots, (iv) pods and (v) seeds. All fractions were dried in a forced air oven at 60°C until reaching a constant weight.

2.8 Leaf senescence

Leaf senescence was monitored through the changes in chlorophyll concentration (average of twelve measurements per leaf, i.e. four measurements per leaflet) of leaves corresponding to nodes 1, 3, 5, 7, 9, 11 and 13 estimated with a non-destructive chlorophyll meter (SPAD 502, Minolta). Values of abscised leaves were regarded as 0. Leaves of each plant were scanned and then leaf area was calculated with the ImageJ software (version 1.46r).

2.9 Statistical analysis

Data were tested for normality by Omnibus Normality of Residuals ($P \geq 0.05$) and homogeneity of variance using the Modified-Levene Equal-Variance Test ($P \geq 0.05$). If the data were suitable for parametric testing (i.e., $P \geq 0.05$ for the normality and homogeneity of variance tests) data were analyzed using the Student's T-test and ANOVA with Tukey-Kramer Multiple-Comparison test ($P \leq 0.05$). If the data were not suitable for parametric testing a Kruskal-Wallis One-Way ANOVA with Dunn's-Bonferroni´s *post hoc* test ($P \leq 0.05$) was used. For regression analysis the residuals normal distribution was tested by Shapiro Wilk test ($P \geq 0.05$) and Constant Residual Variance by Modified-Levene Test ($P \geq 0.05$). If data were suitable for regression analysis, a significance Student's T-test ($P \leq 0.05$ of slope = zero) was used.

3. Results

3.1 Leaf senescence

Leaf area per plant (Fig. 1A) increased from 7 to 38 DAF, and then remained constant up to 56 DAF. Between 56 and 72 DAF plants showed an abrupt decline in leaf area due to the abscission of a large number of senescent leaves. In contrast, in depodded plants leaf area did not decline, at least, until 72 DAF.

Leaf 1 started to senesce between 7 and 17 DAF, therefore SPAD values decreased to almost zero between 17 and 23 DAF. Chlorophyll levels remained around 30-35 SPAD units in leaves 5, 7, 9 and 11 until 38 DAF (Fig. 1B) and these leaves showed a decrease in SPAD values between 56 and 72 DAF. The decline to 0 of SPAD values in most leaves at 72 DAF was mainly due to leaf abscission (values of abscissed leaves were regarded as 0), and finally at 99 DAF abscission of leaves was complete. At 72 DAF, depodded plants showed SPAD values similar to those of leaves of intact plants at 56 DAF, but smaller than leaves at 23 DAF (when the depodding treatment was started).

3.2 Dry matter partitioning

Dry matter of all vegetative organs (i.e. leaves, stems and roots) increased until 38 DAF (Fig. 2A, B and C), while reproductive organs (i.e. pods and seeds) achieved their maximum biomass at 72 DAF (Fig. 2D and 2E). At 72 and 99 DAF the decline of dry matter of leaves was mainly due to leaf abscission, but at 56 DAF the decline (although not significant) was due to the decrease of specific leaf weight (i.e. leaf dry mass declined without a concomitant decrease of leaf

area with respect to 38 DAF, Fig. 1B). By contrast, roots and stems maintained maximum dry matter content until 99 DAF (R8). Pod walls accumulated dry matter until 38 DAF and seeds until 72 DAF, corresponding to the final stage of seed filling. Pod number did not change from 38 DAF (65-70 pods $plant^{-1}$) and seed number was constant from 56 DAF until maturity (148-160 seeds $plant^{-1}$).

Depodded plants showed large increases of dry matter in all vegetative organs compared to intact plants. When dry matter of depodded plants at final harvest (72 DAF) is compared to biomass at 23 DAF (when treatment was started), the increments were 370%, 350% and 470% for leaves, stems and roots, respectively.

3.3 Root activities: respiration, nitrate consumption and NR activity, and plant water consumption.

Specific root respiration (i.e. µmol O_2 consumed g^{-1} FW h^{-1}) showed a gradual, but consistent decline from 7 to 99 DAF (Fig. 3A). This decline became statistically significant after 56 DAF. The rate of decline from 7 to 72 DAF in root respiration was -0.30 µmolO_2 g^{-1} FW h^{-1}DAF^{-1} (r^2 = 0.78; P < 0.05). When respiration was expressed as total root system activity (i.e. specific respiration multiplied by root biomass) respiration increased from 7 DAF to a maximum at 38 DAF and then decreased from 38 to 99 DAF (Fig. 3D). At 72 DAF, specific and whole plant root respiration rates of depodded plants were higher than those of intact, non depodded plants (Fig. 3A and 3D). Compared with plants at 23 DAF, depodded plants showed a decrease of specific root respiration and an increase of whole plant root respiration.

Nitrate consumption expressed on a root FW basis (i.e. mg NO_3^- g^{-1} FW day^{-1}) steadily decreased during the 7 DAF to 72 DAF period (Fig. 3B), at a rate of -0.097 mg NO_3^- g^{-1} FW DAF^{-1} (r^2 = 0.52; P< 0.05). Nitrate consumption of the whole root system showed relative constant values from 7 to 56 DAF, and abruptly dropped thereafter (Fig. 3E). At 72 DAF, depodded plants showed higher nitrate uptake when considering both specific and whole plant root rates compared with non depodded plants (Fig. 3B and 3E). Compared with plants at 23 DAF, depodding caused an increase of whole plant root nitrate consumption.

The specific activity of NR tended to decrease from 7 to 99 DAF (Fig. 3C). The NR activity of the whole root system (Fig. 3F) was relatively constant from 7 to 56 DAF and then it tended to decrease at 72 DAF and finally dropped significantly at 99 DAF. NR activity on a FW basis of depodded plants did not show significant differences compared with intact plants at 72 DAF, and NR activity of the whole root system of depodded plants was quite variable. Overall,

the pattern of change of root NR activity of depodded plants was similar to root respiration and root nitrate consumption (Fig. 3C and 3F).

Whole plant water consumption showed an increase from 7 DAF to 38 DAF and a drop from 38 to 99 DAF (Fig. 3G). In addition, water consumption was significantly lower from the beginning of treatment (23 DAF) up to 72 DAF in depodded plants; thereafter water consumption was higher in depodded than in intact plants due to leaf retention.

3.4 Total and mitochondrial proteins in roots

Total root protein content decreased during the reproductive period with a large decline from 17 DAF to 56 DAF (Fig 4A). The rate of total protein decrease was -0.5% d^{-1} ($r^2 = 0.75$; $P < 0.05$) from 7 to 99 DAF, with around 44% of 7 DAF root protein levels still retained at 99 DAF. The mitochondrial protein COX2 (Fig. 4B) showed a slight decline from 17 to 56 DAF (about 14%), but the rate of decrease increased thereafter and COX2 became non-detectable at 99 DAF. AOX was only detectable at 7 DAF (Fig 4C).

Depodded plants showed a small decrease (27%) of total protein between 17 and 72 DAF (Fig. 4A). Levels of COX2 did not change much, but AOX showed a large increase in depodded plants (Fig. 4A and 4B). Root respiration in the presence of KCN and SHAM was similar in depodded and control plants (supplementary material Fig. S2).

3.5 Root pruning treatments

Root pruning treatments were started at 34 DAF (R5ad) and their effects on respiration were evaluated from 48 DAF (beginning of R6, corresponding to 14 DAP, days after pruning) to 69 DAF (start of R7, corresponding to 35 DAP). Specific root respiration was increased by root pruning, with the increase being proportional to the extent of root pruning (Fig. 5A). Differences in specific root respiration were more pronounced at earlier stages after root pruning and only 25% and 50% treatments showed significant differences with respect to non-pruned plants. In the same way, only the 25% and 50% of pruning treatments resulted in significant differences in initial root DW after pruning (Fig. 5F).

There were no differences in dry matter partitioning between leaves, stem, roots, pods or seeds at the R7 stage (73 DAF, 39 DAP) (Fig. 5 B-F). At this late stage of seed growth, there were no differences in DW of pruned roots compared with those of control plants, indicating that a compensatory increase in root growth took place from the initiation of pruning treatments up to

R7, when plants were harvested. No difference was observed in the development of the plants (i.e., the days after planting to reach R5, R6 and R7 were similar).

To determine the effect of root pruning on above ground tissues activity, the net photosynthesis rate of leaves were measured. Net photosynthetic rate was not significantly affected by the root pruning treatment for leaves 7, 8 or for the most recent fully expanded leaf at any point after the start of the treatment (supplementary material Fig. S3).

4. Discussion

4.1 Root changes associated to leaf senescence during reproductive development of soybean plants

During the fruit growing period, root activity showed a decrease of specific activity (i.e. on a FW basis) in all variables measured (i.e. respiration, nitrate uptake and nitrate reductase activity). Similar results were found in *Helianthus annuus,* with plants showing a progressive decrease of root respiration during grain filling [7,8]. However, corrected for root biomass, whole root activity remained constant, or increased slightly from 7 to 38 DAF to decline later from 56 DAF to 72-99 DAF. Thus, whole root system activity depends on both the specific activity and the whole biomass until the end of seeds growth (56-72 DAF). It is remarkable that roots kept a basal respiration activity even at 99 DAF in spite of the abscission of all leaves. When most leaves had fallen down and seeds had achieved their final weight (i.e. 72-99 DAF) root contribution as a supplier of resources (e.g. nitrogen and water) dropped to almost zero, but roots still maintained a basal respiration rate and much of the biomass present at 38 DAF (R5). Similarly, after reproductive maturity in beans, a substantial portion of the roots survived [9]. Also, *Arabidopsis thaliana* roots maintained their respiratory activity until late stages of plant development even when above ground tissues were completely withered [10] and clover roots detached from the rest of the plant maintained their respiratory activity for up to 30 days [20]. This remaining activity of the root system suggests that roots do not undergo a clear-cut senescence process as leaves do, rather they may deteriorate slowly once the supply of photoassimilates ceases due to foliage abscission.

The dependence of soybean roots activity on above ground organs has been previously described in other works. In intact plants, production of new root apices and root growth may decline early during the reproductive phase or even before flowering [5]. However, our data show that root biomass increases by about 270% from 7 DAF to 38-56 DAF, an advanced stage

of development. Supporting our results, other works show that nitrogen fertilization during R5 to R7 stages delays the decline in leaf chlorophyll and nitrogen concentration, while increases grain yield [12] and seed nitrogen contents [11], which strongly suggests that roots remain quite active between R5 and R7. Furthermore, in plants where young scions are grafted near the base of old plants, nitrogen fixation recovered to its highest levels at flowering, suggesting that the decline of root activity (e.g. nitrogen fixation) is reversible and depends on the supply of assimilates from above ground organs [28]. These results indicate that roots have the capacity to maintain assimilatory activity until late stages of development if resources are available (e.g., nitrogen in the soil).

4.2 The effect of depodding on root growth and activity

The delay of whole plant senescence in depodded plants has been studied in several works [5,29-33]. In soybean, defruiting does not fully reinstate shoot elongation, although it may promote root growth and leaf and stem thickening [5]; however, no study has focused in detail on root development and activity of completely depodded plants. Our experiments show that pod removal greatly stimulated growth of soybean roots. Although other vegetative organs increased in biomass in response to fruit removal, roots showed the largest effect; root biomass increased about 470% from 23 DAF (R5, the stage at which depodding was started) to harvest at 72 DAF compared with stems and leaves (370% and 350% respectively). This indicates that roots became a strong sink in the absence of fruits, and that root growth was probably inhibited to some extent by fruit activity. Root biomass accumulation in depodded plants caused a large increase of whole root respiration and nitrate uptake with respect to 23 DAF: 3- and 2-fold respectively. Contrary to the activity of roots, photosynthesis of leaves declines in depodded plants [5].

The increased level of AOX suggests that depodding stimulates the alternative pathway in roots. Numerous environmental conditions (e.g. anoxia, nutrient deficiency, drought) and physiological changes associated with plant development (e.g. flowering, fruit development) involve modifications of AOX levels (at the mRNA or protein level), redox state or respiratory activity in the presence of CN^- [34, 35]. However, little is known about AOX in roots during fruit development and grain filling. The amount of AOX protein of soybean seedling roots increases with age but respiration in the presence of KCN declines with root age [36] and AOX protein of potato tubers is synthesized *de novo* during aging [37]. Also, in tobacco cell cultures the induction of AOX could prevent events triggering cell death [38]. Our results suggest that

maintenance of leaf area by depodding generates an excess of photoassimilates reaching root cells. A fraction of this excess might be used for roots biomass generation and respiration, but if the supply of carbon skeletons exceeds the capacity of the Cyt *c* pathway, then the AOX pathway could take over as a dissipative valve of excess reduced carbon [39]. However, we could not detect a significant increase of root respiration in presence of KCN (Fig. S2), suggesting that the capacity for cyanide-insensitive respiration was not enhanced by depodding.

4.3 Root development and source-sink relationship

Our results indicate that roots grow and maintain respiration during reproductive development, and they can even become strong sinks in the absence of pods. This implies that roots and pods are competing for resources (e.g. photoassimilates). To further test this hypothesis, we designed experiments where roots were pruned. As partial defoliation or sunlight attenuation represent a source reduction [40, 41] and fruit removal a sink reduction [4,19], root pruning may decrease the overall root demand for carbohydrates.

The results of root pruning experiments indicate that, under non-limiting conditions, an increase in the activity of remaining roots compensated for the removal of part of the root system. Remarkably, root pruning did not affect the final weight reached by each organ (i.e. leaves, stems, pods, seeds, and even roots) at 72 DAF (R7). This suggests that root-pruned plants were able to fill pods at the same time they regenerated their root system in the critical period of grain filling, without any loss of yield. Bingham and Stevenson (1993) [42] showed that root pruning of 14-16 day old wheat seedlings increased their rate of O_2 uptake and the development of lateral roots. Similarly, increases of root respiration and growth were observed after root pruning in barley seedlings [43,44]. In contrast, some plants reduce leaf and shoot growth much more than root growth after a root pruning treatment [45]. Thus, seedlings can compensate for a decrease of root biomass. On the other hand, it has been reported that grain yield increases when roots of wheat plants are pruned [46,47]. Also, at anthesis root-pruned wheat plants had a lower rate of root respiration [46], which contrasts with our results for soybean plants.

Most experiments involving root pruning show re-direction of assimilates towards the pruned root [45]. In carrots, assimilate export to roots increased by root pruning and this was at the expense of the developing leaves [48]. Similarly, pruning increased the soluble sugar content in roots of barley seedlings [43,44]. Three week old *Phaseolus vulgaris* plants show a different behaviour since removal of 50% of the roots had no effect on photoassimilate export from source leaves; however the distribution was altered and translocation of photosynthates toward the upper

plant parts was drastically reduced [49]. On the other hand, we could not detect an increase of photosynthesis in plants with roots pruned suggesting that plants can support increased root respiration, root biomass production and maintain seed weight without large photosynthetic adjustments (supplementary material Fig. S3).

4.4 Concluding remarks

The data presented here show that, under non limiting conditions, root biomass of soybean plants may increase even during early reproductive development. Roots maintain a basal respiration rate until advanced stages of development (R8) suggesting that roots do not undergo a developmentally regulated senescence process followed by death of the organ, as leaves do. While results of depodding treatments suggest that fruits monopolize photoassimilates, therefore limiting root growth, partially pruning of roots causes an increase in specific root respiration and compensatory root growth, therefore root biomass at physiological maturity is the same in intact *vs* root pruned plants. Roots accumulate biomass in the root pruning treatments without any penalty on seed growth and without any evidence of photosynthetic compensation. These data show that C allocation to different plant parts is not simply a tug-of-war between competing organs, and that other (still non-identified) controls may operate to determine plant C allocation and confer plasticity to plant growth during the reproductive period.

Declaration of interests

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

Author contributions

D. D. F. and J. J. G. formulated and designed the experiment. D. D. F., S .J. K., M. G. C. and S.

M. managed the experiments. D. D. F., J. J. G. and C. G. B analyzed the data and wrote the manuscript, and all the authors assisted in the review and editing of the final version.

Acknowledgements

DDF is a post-doctoral fellow, SJK is doctoral fellow, MGC and SM are technicians, and CGB and JJG are researchers of CONICET. This work was supported by Universidad Nacional de La Plata, Argentina (Grants 11/A258 and 11/A257). The authors are grateful to Drs. María Lorenza Costa, Corina Graciano, Virginia Luquez and Eduardo Tambussi (INFIVE) for stimulating discussions of the results, advice on biochemical and physiological techniques and materials for hydroponic cultivation. Dr. Daniel González (Universidad Nacional del Litoral, Santa Fe, Argentina) is thanked for providing the anti-COX2 antibody.

References

[1] L.D. Noodén, The Phenomena of Senescence and Aging, in: L.D. Noodén, A.C. Leopold (Eds.), Senescence and Aging in Plants, Academic Press, San Diego, 1988: pp. 1–50.

[2] L.D. Nooden, Integration of soybean pod development and monocarpic senescence, Physiol. Plant. 62 (1984) 273–284.

[3] A. Bagniewska-Zadworna, A. Stelmasik, and Jj. Minicka, From birth to death – *Populus trichocarpa* fibrous roots functional anatomy, Biol. Plantarum 58 (2014) 551-560

[4] N. Wojciechowska, K. Marzec-Schmidt, E.M. Kalemba, A. Zarzyńska-Nowak, A.M. Jagodziński, A. Bagniewska-Zadworna, Autophagy counteracts instantaneous cell death during seasonal senescence of the fine roots and leaves in *Populus trichocarpa*, BMC Plant Biol. (2018) 18:260

[5] L.D. Nooden, Whole Plant Senescence, in: L.D. Noodén, A.C. Leopold (Eds.), Senescence and Aging in Plants, Academic Press, San Diego, 1988: pp. 391–440.

[6] C. Masclaux-Daubresse, F. Daniel-Vedele, J. Dechorgnat, F. Chardon, L. Gaufichon, A. Suzuki, Nitrogen uptake, assimilation and remobilization in plants: Challenges for sustainable and productive agriculture, Ann. Bot. 105 (2010) 1141–1157.

[7] A.J. Hall, D.J. Connor, D.M. Whitfield, Root respiration during grain filling in sunflower: The effects of water stress, Plant Soil. 121 (1990) 57–66.

[8] S. Lisanti, A.J. Hall, C.A. Chimenti, Influence of water deficit and canopy senescence pattern on *Helianthus annuus* (L.) root functionality during the grain-filling phase, F. Crop. Res. 154 (2013) 1–11.

[9] M.C.T. Fisher, D.M. Eissenstat, J.P. Lynch, Lack of evidence for programmed root senescence in common bean (*Phaseolus vulgaris*) grown at different levels of phosphorus supply, New Phytol. 153 (2002) 63–71.

[10] D.D. Fanello, C.G. Bartoli, J.J. Guiamet, Qualitative and quantitative modifications of root mitochondria during senescence of above-ground parts of *Arabidopis thaliana*, Plant Sci. 258 (2017) 112–121.

[11] S. Nakasathien, D.W. Israel, R.F. Wilson, P. Kwanyuen, Regulation of seed protein concentration in soybean by supra-optimal nitrogen supply, Crop Sci. 40 (2000) 1277–1284.

[12] X. Zhao, S. Zheng, S. Arima, Influence of Nitrogen Enrichment during Reproductive Growth Stage on Leaf Nitrogen Accumulation and Seed Yield in Soybean, Plant Prod. Sci. 17 (2014) 209–217.

[13] H. Lambers, O. Atkin, F.F. Millenaar, Respiratory patterns in roots in relation to their functioning, in: Y. Waisel, A. Eshel, U. Kafkafi (Eds.), Plant Roots. Hidden Half, Third, Marcel Dekker, Inc., New York, 2002: pp. 782–838.

[14] N.M. Crawford, A.D. Glass, Molecular and physiological aspects of nitrate uptake in plants, Trends Plant Sci. 3 (1998) 389–395.

[15] A.J. Bloom, S.S. Sukrapanna, R.L. Warner, Root respiration associated with ammonium and nitrate absorption and assimilation by barley, Plant Physiol. 99 (1992) 1294–1301.

[16] N. Smirnoff, G.R. Stewart, Nitrate assimilation and translocation by higher plants: comparative physiology and ecological consequences, Physiol. Plant. 64 (1985) 133–140.

[17] M.B. Peoples, M.J. Dalling, The Interplay between Proteolysis and Amino Acid Metabolism during Senescence and Nitrogen Reallocation, in: L.D. Noodén, A.C. Leoppold (Eds.), Senescence and Aging in Plants, Academic Press, San Diego, 1988: pp. 181–217.

[18] A.J. Gordon, G.J.A. Ryle, D.F. Mitchell, C.E. Powell, The flux of ^{14}C labeled photosynthate through soybean root nodules during N_2 fixation, J. Exp. Bot. 36 (1985) 756–769.

[19] H. Nobuyasu, S. Liu, P.K. Mohapatra, K. Fujita, Variation in the export of ^{13}C and ^{15}N from soybean leaf : the effects of nitrogen application and sink removal, Plant Soil. (2003) 331–339.

[20] I.J. Bingham, R.M. Rees, Senescence and N release from clover roots following permanent excision of the shoot, Plant Soil. 303 (2008) 229–240.

[21] J.E. Leggett, M.H. Frere, Growth and nutrient uptake by soybean plants in nutrient solutions of graded concentrations, Plant Physiol. 48 (1971) 457–460.

[22] W.R. Fehr, C.E. Caviness, Stages of Soybean Development, Special Report No 80, Iowa State University Cooperative Extension Service. Ames, IA, 1977.

[23] APHA, AWWA, WPCF, Standard Methods for the Examination of Water and Wastewater, 16th ed., American Public Health Association, American Water Works Association and Water Pollution Control Federation, New York, 1985.

[24] C.E. Deane-Drummond, D.T. Clarkson, C.B. Johnson, Effect of Shoot Removal and Malate on the Activity of Nitrate Reductase Assayed in Vivo in Barley Roots (*Hordeum vulgare* cv. Midas), Plant Physiol. 64 (1979) 660–662.

[25] U.K. Laemmli, Cleavage of Structural Proteins during Assembly of Head of Bacteriophage T4, Nature 227 (1970) 680–685.

[26] C. V Attallah, E. Welchen, A.P. Martin, S. V Spinelli, G. Bonnard, J.F. Palatnik, D.H. Gonzalez, Plants contain two SCO proteins that are differentially involved in cytochrome c oxidase function and copper and redox homeostasis, J. Exp. Bot. 62 (2011) 4281–4294.

[27] T.E. Elthon, L. McIntosh, Characterization and Solubilization of the Alternative Oxidase of *Sauromatum guttatum* Mitochondria, Plant Physiol. 82 (1986) 1–6.

[28] N.S.A. Malik, Grafting experiments on the nature of the decline in N_2 fixation during fruit development in soybean, Physiol. Plant. 57 (1983) 561–564.

[29] A.C. Leopold, E. Niedergang-Kamien, J. Janick, Experimental modification of plant senescence, Plant Physiol. 34 (1959) 570–573.

[30] V.A. Wittenbach, Effect of pod removal on leaf senescence in soybeans, Plant Physiol. 70 (1982) 1544–1548.

[31] S.J. Crafts-Brandner, F.E. Below, J.E. Harper, R.H. Hageman, Effects of Pod Removal on Metabolism and Senescence of Nodulating and Nonnodulating Soybean Isolines, Plant Physiol. 75 (1984) 311–317.

[32] L.D. Noodén, J.J. Guiamét, Minireview Regulation of assimilation and senescence by the fruit in monocarpic plants, Physiol. Plant. 77 (1989) 267–274.

[33] L.D. Noodén, J.J. Guiamét, I. John, Whole Plant Senescence, in: L.D. Noodén (Ed.), Plant Cell Death Process, Academic Press, San Diego, California, 2004: pp. 227–244.

[34] I.M. Juszczuk, A.M. Rychter, Alternative oxidase in higher plants, Acta Biochim. Pol. 50 (2003) 1257–1271.

[35] C.G. Bartoli, F. Gomez, G. Gergoff, J.J. Guiamét, S. Puntarulo, Up-regulation of the mitochondrial alternative oxidase pathway enhances photosynthetic electron transport under drought conditions, J. Exp. Bot. 56 (2005) 1269–1276.

[36] D.A. Day, A.H. Millar, J.T. Wiskich, J. Whelan, Regulation of Alternative Oxidase Activity by Pyruvate in Soybean Mitochondria., Plant Physiol. 106 (1994) 1421–1427.

[37] C. Hiser, L. McIntosh, Alternative Oxidase of Potato Is an Integral Membrane Protein Synthesized de Novo during Aging of Tuber Slices, Plant Physiol. 93 (1990) 312–318.

[38] G.C. Vanlerberghe, C.A. Robson, J.Y.H. Yip, Induction of mitochondrial alternative oxidase in response to a cell signal pathway down-regulating the cytochrome pathway prevents programmed cell death, Plant Physiol. 129 (2002) 1829–1842.

[39] P.M. Finnegan, K.L. Soole, A.L. Umbach, Alternative Mitochondrial Electron Transport Proteins in Higher Plants, in: D.A. Day, A.H. Millar, J. Whelan (Eds.), Plant Mitochondria From Genome to Function, Kluwer Academic Publishers, Dordrecht, Netherlands, 2004: pp. 163–230.

[40] L. Borrás, G.A. Slafer, M.E. Otegui, Seed dry weight response to source-sink manipulations in wheat, maize and soybean: A quantitative reappraisal, F. Crop. Res. 86 (2004) 131–146.

[41] A. Quijano, E.N. Morandi, Post-flowering leaflet removals increase pod initiation in soybean canopies, F. Crop. Res. 120 (2011) 151–160.

[42] I.J. Bingham, E.A. Stevenson, Control of root growth: effects of carbohydrates on the extension, branching and rate of respiration of different fractions of wheat roots, Physiol. Plant. 88 (1993) 149–158.

[43] J.F. Farrar, C.L. Jones, Modification of Respiration and Carbohydrate Status of Barley Roots By Selective Pruning, New Phytol. 102 (1986) 513–521.

[44] I.J. Bingham, J.F. Farrar, Regulation of respiration in roots of barley, Physiol. Plant. 73 (1988) 278–285.

[45] J.B. Wilson, A review of evidence on the control of shoot:root ratio, in relation to models, Ann. Bot. 61 (1988) 433–449.

[46] S.C. Ma, F.M. Li, B.C. Xu, Z. Bin Huang, Effect of lowering the root/shoot ratio by pruning roots on water use efficiency and grain yield of winter wheat, F. Crop. Res. 115 (2010) 158–164.

[47] C. Hu, M. Ding, C. Qu, V. Sadras, X. Yang, S. Zhang, Yield and water use efficiency of wheat in the Loess Plateau: Responses to root pruning and defoliation, F. Crop. Res. 179 (2015) 6–11.

[48] L.R. Benjamin, M.J. Wren, Root Development and Source-Sink Relations in Carrot , *Daucus carota* L ., J. Exp. Bot. 29 (1978) 425–433.

[49] G.I. Ghobrial, Effects of Root Pruning on Translocation of Photosynthates in *Phaseolus vulgaris* L., J. Exp. Bot. 34 (1983) 20–26.

Figure Caption

Figure 1

Leaf area and senescence progression during reproductive development (7 to 99 days after flowering, DAF). A) Total green leaf area per plant (m^2 $plant^{-1}$). Different letters indicate significant differences (ANOVA $P \leq 0.05$). * Indicates a significant difference (Student's T-test $P \leq 0.05$) between depodded plants at 72 DAF and intact plants at 23 DAF (start of the depodding treatment), as well as between non depodded (R7) plants and depodded plants at 72 DAF. B) Chlorophyll content expressed as SPAD units. Arrows indicate the stage when SPAD values declined significantly compared to the preceding measurement (Student's T-test $P \leq 0.05$). Vertical bars represent the standard error of the mean.

Figure 2

Biomass (dry weight) of leaves (A), stems (B), roots (C), pods (D) and seeds (E) during reproductive development of intact plants (7 to 99 days after flowering, DAF, indicated by diamonds and a continuous line) and the depodding treatment (indicated by square symbols and a dashed line). Different letters indicate significant differences (Kruskal-Wallis test $P \leq 0.05$). * Indicates significant differences (Student's T-test $P \leq 0.05$) between 23 DAF (start of the depodding treatment) and 72 DAF in depodded plants, and between non depodded (R7) plants and depodded plants at 72 DAF. Vertical bars represent the standard error of the mean.

Figure 3

Root activities during reproductive development (7 days after flowering, DAF, to 99 DAF, indicated by diamonds and a continuous line) and depodded plants (indicated by square symbols and a dashed line). Specific root respiration (A), Specific root uptake of NO_3^- (B), Specific root NR activity (C), Whole plant root respiration (D), Whole plant NO_3^- uptake (E), Whole plant root NR activity (F) and Whole plant water consumption (G). Specific root activity (A-C) was expressed on the basis of FW and whole root activity (D-F) was calculated as specific activity multiplied by root FW at each stage. Different letters indicate significant differences (Kruskal-Wallis test $P \leq 0.05$). * Indicates significant differences (Student's T-test $P \leq 0.05$) between 23 DAF (start of the depodding treatment) and 72 DAF in depodded plants, and between non depodded (R7) plants and depodded plants at 72 DAF. Vertical bars represent the standard error of the mean.

Figure 4

Changes in root proteins. A) Total root protein (relative units), B) COX2 (cytochrome oxidase, a mitochondrial protein), and C) AOX (alternative oxidase, also a mitochondrial protein). Changes in intact plants, from 7 to 99 days after flowering (DAF) are represented by diamonds and a continuous line, whereas depodding is represented by grey bars. Different letters indicate significant differences (ANOVA or Kruskal-Wallis test $P \leq 0.05$).. The data shown corresponds to the amount of protein relative to the highest value (set as 100) of each Coomassie stained gel or western blot. Values were calculated using the ImageJ program. Sample volume loaded into each lane of the gels corresponded to 4 mg (FW) of roots. Vertical bars represent the standard error of the mean.

Figure 5

Responses of roots to partial pruning. Specific root respiration (A) of control plants, and plants where15%, 25% and 50% of root DW was removed at 34 DAF (t0, advanced R5 stage). Measurements were made at 14, 21, 28 and 35 days after pruning (DAP). Biomass of leaves (B), stems (C), seeds (D) and pod walls (E) at 73 DAF (R7). F) Root biomass: white bars represent root weight at 73 DAF (R7), light gray bars represent dry weight of roots removed at 34 DAF (R5ad) when root pruning treatment started and dark gray bars represent remaining root biomass immediately after root pruning. Different letters indicate significant differences (ANOVA $P \leq 0.05$) between control, 15%, 25% and 50% of root pruning treatments on each day of measurement (A) or for each plant organ (B-F). NS indicates non significant differences between treatments. * (F) Indicates significant differences (Student's T-test $P \leq 0.05$) between root biomass of intact control plants, and pruned plants at 34 DAF (t0 R5ad), when the root pruning treatment was imposed. Initial root weight of pruned plants was calculated as the difference between root weight at 34 DAF (t0 R5ad) and weight of roots removed. Vertical bars represent the standard error of the mean.

Fig 1

Fig. 1

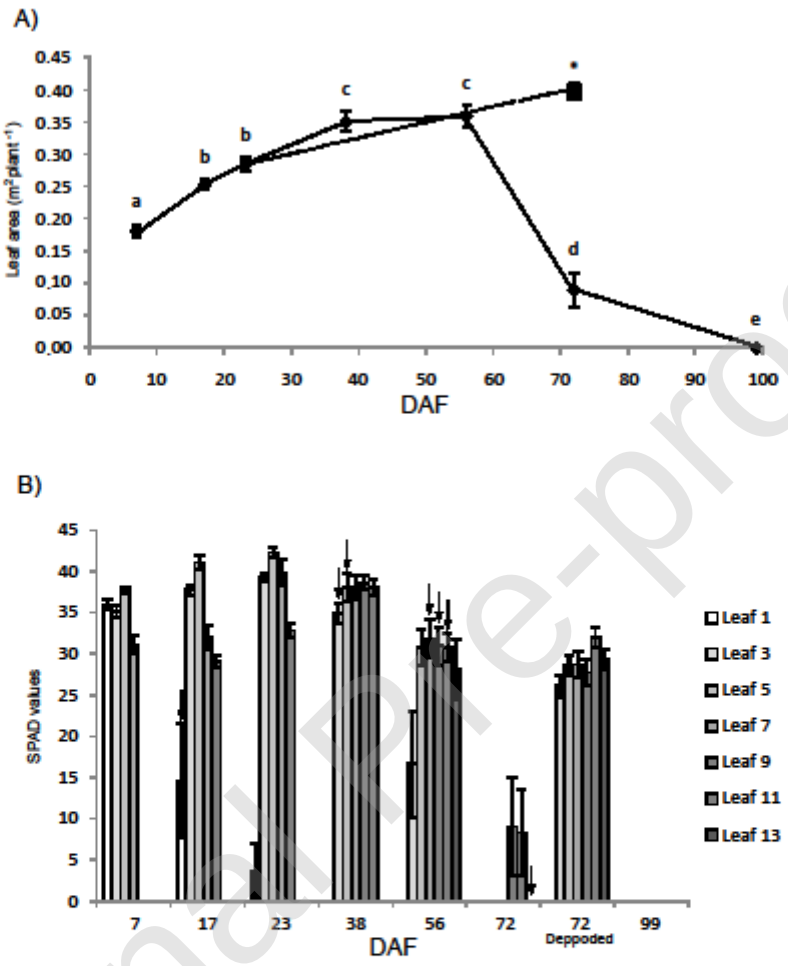

Fig 2

Fig. 2

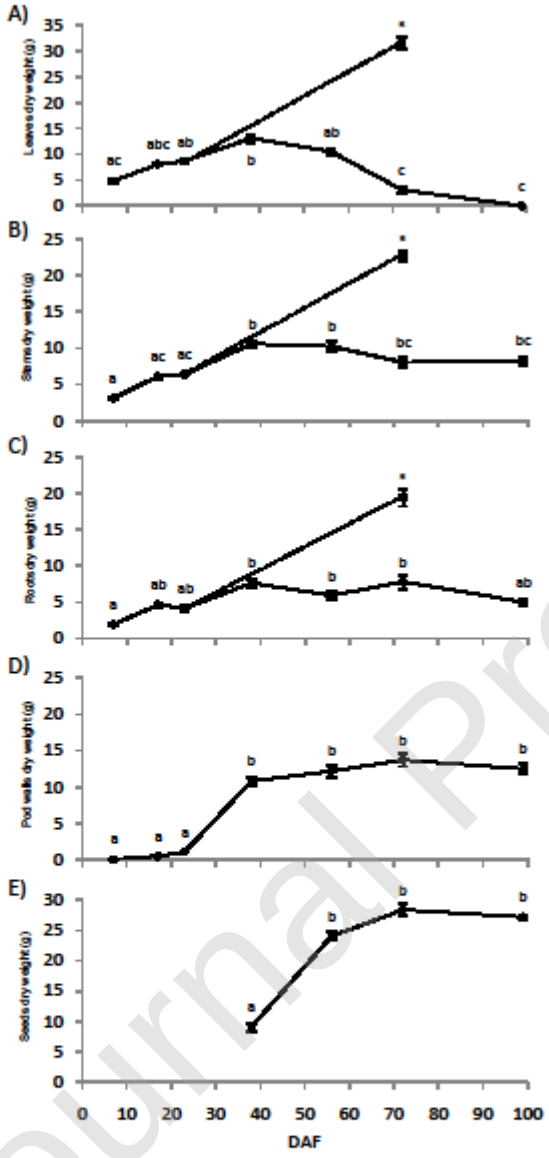

Fig 3

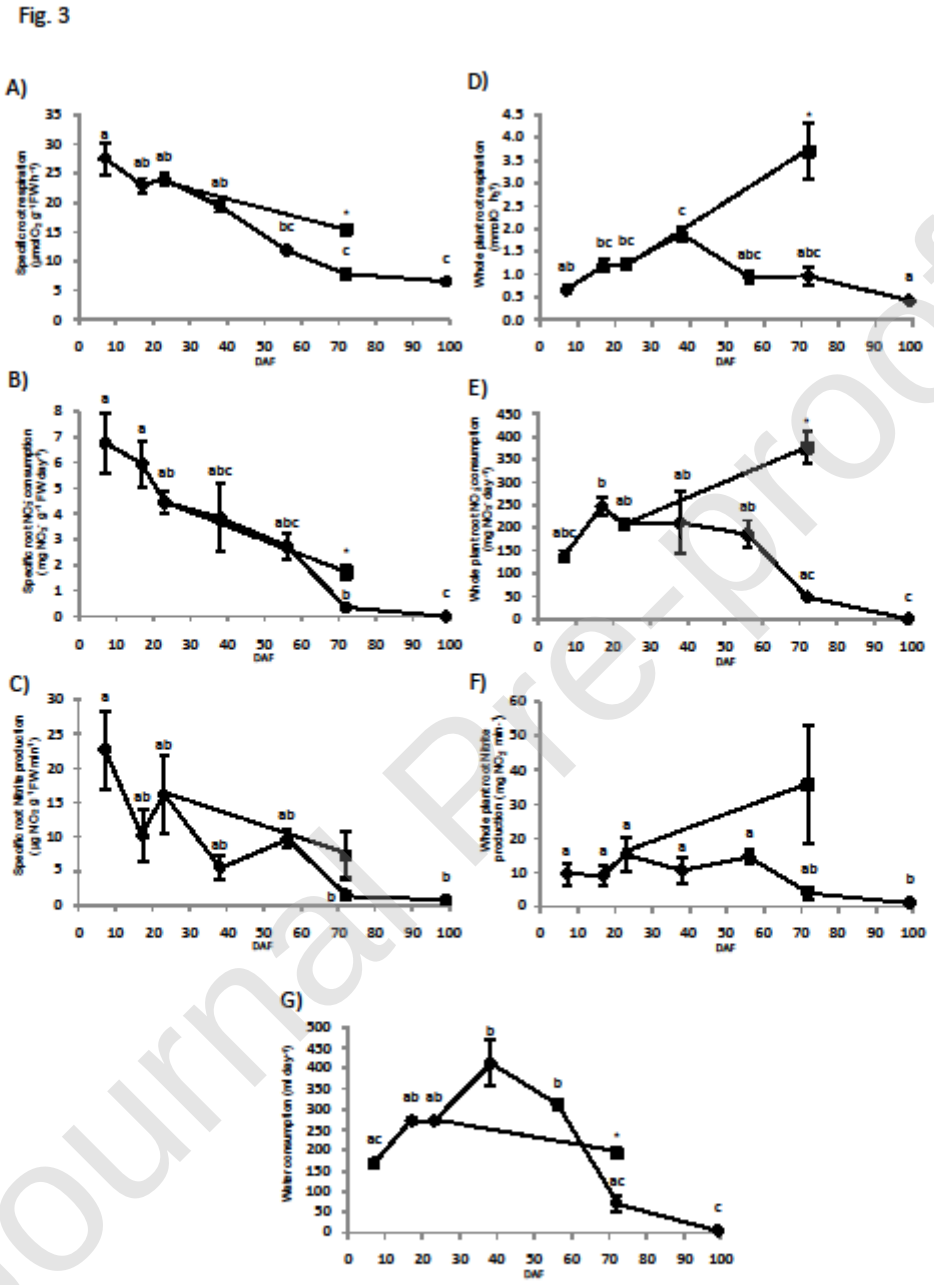

Fig 4

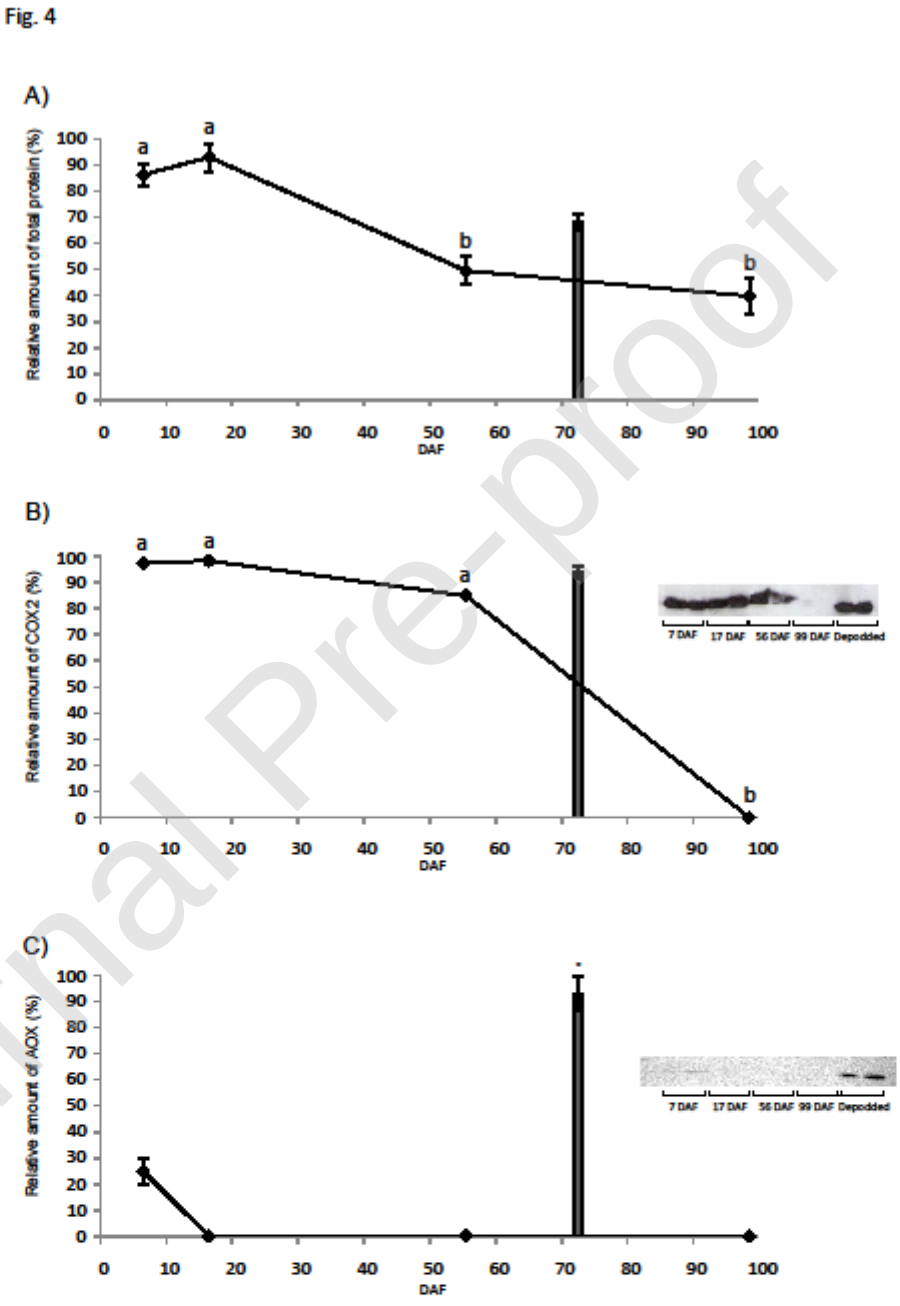

Fig 5