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Growth reduction in root-restricted tomato plants is linked to photosynthetic impairment and starch accumulation in the leaves

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Abstract: The mechanisms responsible for reduced shoot growth due to restricted root growth is still not fully understood. Therefore, this investigation was planned to determine the morphological and physiological changes induced in response to root restriction conditions and to determine the time frame within which these changes occurred. In particular, this research aims to evaluate the effect of root restriction on growth, leaf gas exchange parameters, carbohydrate production and water relations in tomato (*Solanum lycopersicum* L.). Our results show that growth reduction by root restriction is mainly linked to a photosynthetic impairment, caused by a concurrent limited stomatal conductance (probably driven by stomatal factors and hormonal substances) together with a strong accumulation of starch in the tissues, which led to a feedback inhibition of the photosynthetic process.

1. Introduction

The use of root-restricted cultivation for vegetable production has significantly grown in the last decades (Shi *et al*., 2008), as it appears an effective technique for saving resources, controlling root environment, and regulating early yield and quality (Marsh and Paul, 1988; Shi *et al*., 2008). Root restriction may occur wherever pot size or rooting volume is physically limited (Tschaplinski and Blake, 1985; Ismail and Noor, 1996; Saito *et al*., 2008; Mugnai *et al*., 2009), mostly with greenhouse-grown horticultural crops (Thomas, 1993). Root restriction leads to a denser root mass and a reduced root growth (Ismail and Noor, 1996). Besides limiting the volume of the soil available to the root system for water and nutrient uptake, it also suppresses canopy growth (Ismail and Noor, 1996; Shi *et al*., 2008) via many plant physiological and biochemical processes. The mechanisms responsible for reduced shoot growth due to restricted root growth is still not fully understood. Several hypotheses were investigated including water and nutrient stresses (Hameed *et al*., 1987), decrease in root respiration (Shi *et al*., 2007) and photosynthesis (Shi *et al*., 2008), and production of plant hormones (Liu and Latimer, 1995), but reports indicated that there are contradictory results as to which of these factors play a significant role in the response of aerial plant parts to restricted root growth and indicated differences between species. Leaf photosynthesis strongly depends on environmental conditions such as radiation, CO_2 concentration and temperature. In addition to these environmental conditions, photosynthesis is subjected to internal regulation associated with sink demand for assimilates (Marcelis, 1991). The presence of a physical restriction to root growth, a major metabolic sink for photsynthetically fixed carbon at seedling stage (Thomas and Strain, 1991) resulted in feedback inhibition mechanisms, with lower rates of carbon metabolism and photosynthesis as a result of carbohydrate accumulation (Schaffer *et al*., 1996; Shi *et al*., 2008). Therefore, this investigation was planned to determine the morphological and physiological changes induced in response to root restriction conditions and to determine the time frame within which these changes occurred. In particular, this research aims to study the effect of root restriction on growth, leaf gas exchange parameters, carbohydrate production and water relations in tomato (*Solanum lycopersicum* L.).

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2. Materials and Methods

Plant material

Experiments were carried out at the Department of Plant Biology, University of Pisa (Italy). Seeds of tomato (*Solanum lycopersicum* L.) cv. 'Cal J' were sown in seedling flats filled with vermiculite and placed in a germinating room at constant temperature (25°C) and light intensity (300 mol m^{-2} s^{-1} of PAR). After germination, seedlings with the first true leaves were selected for uniformity and single plants were transplanted into 7 ml (root restricted, RR) and 230 ml (control) speeding flats filled with vermiculite. Flats were placed in a greenhouse and suspended 15 cm above the benches to facilitate air pruning of roots and to induce root restriction treatment through out the experiment period. In each flat 24 seedlings were planted regardless of the original number of cells per flat to minimize the effect of mutual shading, to avoid light competition between plants and to allow for uniform plant density. In order to avoid any water or nutrient stress, a closed fertirrigation system controlled by a timer was established to supply water and nutrients at frequent and regular intervals. The nutrient solution was composed thus: 10 mM NO_3^-, 1 mM $H_2PO_4^-$, 8 mM K^+, 4 mM Ca^{2+}, 1.5 mM Mg^{2+}, 1 mM SO_4^{2-}, 0.04 mM Fe^{2+} and microelements (pH 6.0, EC=1.2 mS cm^{-1}). The nutrient solution was renewed every week.

Growth measurements

Five plants per treatment were sampled at weekly intervals. Roots were carefully washed, then plants were separated into leaves, stems and roots. Leaf area was measured with an area meter (Delta T-Devices Ltd., Cambridge, UK), plant height was estimated using a ruler and dry weight for each organ was obtained after oven drying (48 hr at 70°C).

Leaf gas exchange measurements

Net CO_2 assimilation (A), stomatal conductance (g) and transpiration (E) measurements were performed weekly (n=5) on the central sector of the youngest fully-expanded leaf using an open system (CMS 400, Heinz Walz, Effeltrich, Germany) connected to an assimilation chamber and equipped with a high sensitivity IRGA (BINOS, Leybold Haeraeus, Germany) under temperature (24°C) and growing light (400 mmol m^{-2} s^{-1} PAR) conditions provided by a mercury vapour lamp (OSRAM HQI-TS 250 W/NDL). Calculation of all the parameters was performed following von Cammerer and Farquhar (1981) using a specific software (Diagas 2.02, Walz, Effettrich, Germany).

Chlorophyll content

Five leaf disks (10 mm diameter) were randomly taken from the uppermost fully-expanded leaves at weekly intervals, and extracted in 2 ml of N,N-dimethylformamide for 24 hr in the dark. Absorbance was then determined for each sample using a spectrophotometer at 647 and 663 nm. Chlorophyll *a* and *b* contents, and *a*/*b* ratio were calculated according to Moran (1982).

Determination of total, osmotic and turgor potentials

Leaf water potential measurements were taken on the same leaf immediately after measuring gas exchange (n=5). Total water potential (ψ_w) was determined using a pressure chamber (Pardossi *et al*., 1991). Osmotic potential (ψ_s) of the leaf xylem sap was determined using an osmometer (Precision System, USA) by determining the freezing point depression of the sample. Leaf turgor potential (ψ_p) was calculated using the following equation (Eq. 1):

$$\psi_p = \psi_w - \psi_s \qquad \text{(Eq. 1)}$$

Measurement of sugar content

Leaf, stem, and root samples (approx. 50 mg each) were taken at weekly intervals (n=5) and directly freeze-dried in liquid nitrogen. Samples were homogenized and extracted with 1 ml hot 80% ethanol, boiled for 5 min, and centrifuged at 12000 rpm for 15 min; the supernatant was then collected. The pellet was extracted again as described above, and the supernatant was collected again. At the end of the procedure, the pellet was evaporated to remove any excess ethanol. Particulates including starch were suspended in 1 ml of KOH 20 mM, boiled and centrifuged at 8000 rpm for 15 min and the supernatant was collected. The extract from ethanol was used for sucrose, glucose and fructose determinations, and the extract from KOH was used for starch determination. For sugar determination, two 200 µl aliquots from the ethanol extract were taken, one incubated for 30 min at 37°C with 100 µl solution containing invertase (1 mg invertase ml^{-1} Na-acetate 50 mM at pH 4.6), the other with 100 µl solution containing Na-acetate 50 mM at pH 4.6, then both brought to the final volume (1 ml) with a solution containing 100 mM Tris-HCl, pH 7.6, 3 mM $MgCl_2$, 2 mM ATP, 0.6 mM NADP, 1 unit hexokinase and 1 unit glucose-6-P-dehydrogenase (incubated at 37°C for 30 min). Absorbance at 340 nm was then measured using a spectrophotometer. The concentration of glucose in each solution was determined from glucose standard curves according to Guglielminetti *et al*. (1995). The solution without invertase was used to calculate the amount of free glucose in the sample and the difference between the two gave the amount of sucrose (as glucose equivalent). For each of them 10 µl of solution containing 15 µl of phosphoglucoisomerase in 150 µl of tris-HCl 300 mM at pH 7.6 were incubated at 37°C for 15 min, then absorbance at 340 nm was determined. The difference between the one without invertase and treated with phosphoglucoisomerase and the other without invertase at the first determination gave the amount of free fructose (as glucose equivalent). For starch determination, 100 µl of extract was incubated at

37°C for 1 hr with 100 µl solution of Na-acetate 100 mM pH 5.2/10 α-amylase. This solution was incubated with 100 µl of Na-acetate 100 mM pH 4.6/10 u amyloglucosidase at 55°C for 1 hr. Finally, the solution was boiled and centrifuged to eliminate denaturated protein from α-amylase and amylogluco-sidase. 100 µl from this solution was taken and brought to 300 µl with distilled water, then starch analysis (as glucose equivalent) was carried out as mentioned above for glucose.

Statistical analysis

Data were analyzed by one-way ANOVA, and means (n=5) were separated using Duncan's Multiple Range Test (P≤0.05). Statistical analysis was performed using GraphPad Prism 4.0 (GraphPad software).

3. Results

Growth parameters were greatly affected by root restriction treatment (RR), with significant reductions in total dry weight, leaf area and plant height (Fig. 1A, B and C) starting from an early stage of seedling development. RR plants also showed a significantly higher root:shoot ratio (Fig. 1D), due to a higher allocation of biomass in the root system compared to canopy (stem and leaves).

During the first month, no significant differences were noticed in leaf gas exchange parameters. From day 29, however, stomatal conductance (g) started to significantly decrease in RR plants (Fig. 2A), leading to a significant reduction from day 36 in both net CO_2 assimilation (Fig. 2B) and transpiration (Fig. 2C) until the end of the experiment. The reduction in net CO_2 assimilation was not related to a decrease in the chloro-

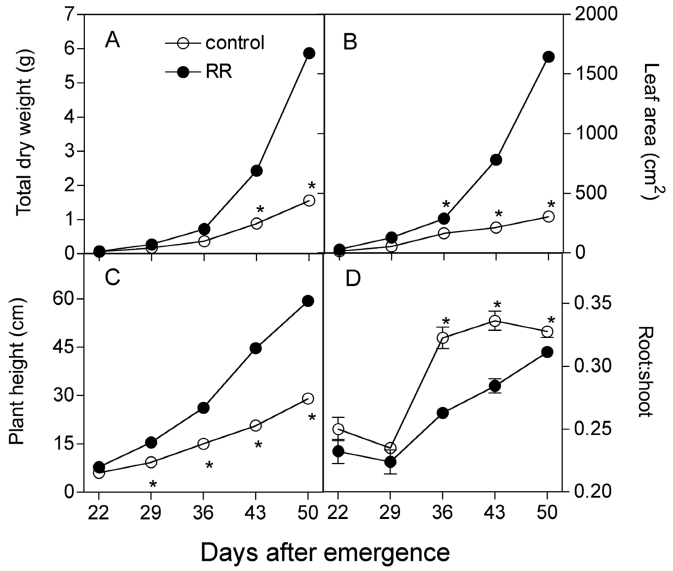

Fig. 1 - Growth parameters measured at weekly intervals from day 22 to the end of the experiment in both control and root-restricted (RR) plants: total dry weight (A), leaf area (B), plant height (C) and root:shoot ratio (D). * indicates significantly different values for P≤0.05 (n=5), when means were separated by Duncan's test.

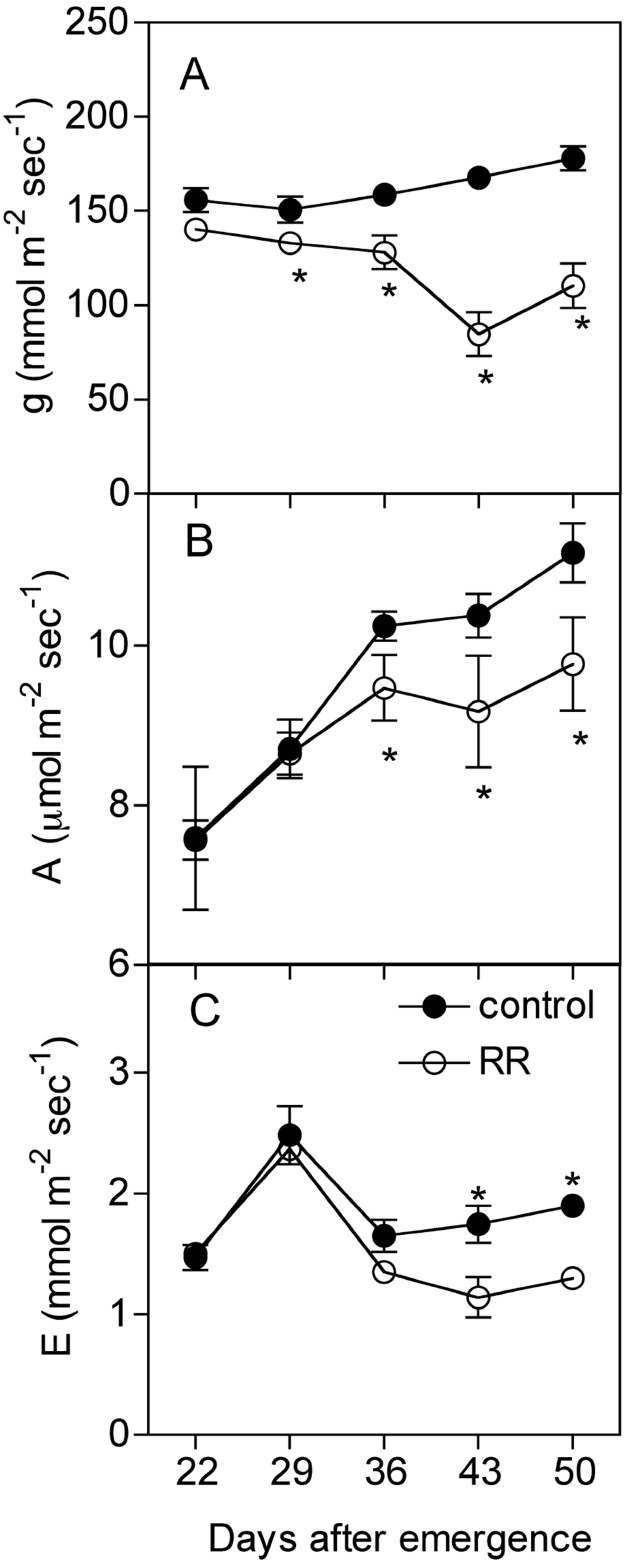

Fig. 2 - Leaf gas exchange parameters measured at weekly intervals from day 22 to the end of the experiment in both control and root-restricted (RR) plants: stomatal conductance (A), net CO_2 assimilation (B) and transpiration (C). * indicates significantly different values for P≤0.05 (n=5), when means were separated by Duncan's test.

phyll content of RR plants (Table 1), as no significant differences were noticed for chlorophyll *a*, *b,* and *a*/*b* ratio between the two treatments. Leaf water status did not affect stomatal closure, as total water potential (Fig. 3A) and turgor potential (Fig. 3C) did not show any significant difference throughout the entire experiment in both the treatments, even if a slight, but not significant, reduction in total water potential was measured on day 43 in RR plants. This behaviour also confirmed the fact that no water stress symptoms occurred during the experimental period, giving a positive feedback of our experimental system.

On the contrary, sugar content determination led to interesting results. While sucrose content trend was not uniform during the experiment, leading to contradictory results (Fig. 4A), RR treatment led to a clear increase in glucose content (Fig. 4B) and a concurrent decrease in fructose content (Fig. 4C) together with a great accumulation of starch (Fig. 4D). In particular, starch accumulation in the tissues began early in the developmental process (day 29). Starch was mainly compartimentalized in the leaves (Fig. 5A) and stems (Fig. 5B) of RR plants, whereas no significant differences were noticed in roots between control and RR plants (Fig. 5C).

4. Discussion and Conclusions

Our growth data are in line with several previous results concerning growth depression induced by root restriction in other horticultural crops (Carmi and Heuer, 1981; Tschaplinski and Blake, 1985; Thomas and Strain, 1991; Rieger and Marra, 1994; Liu and Latimer, 1995; van Iersel, 1997; Kharkina *et al*., 1999; Saito *et al*., 2008; Shi *et al*., 2008). Root restriction generally caused an increase in root:shoot ratio (Carmi *et al*., 1983; Mugnai *et al*., 2000); roots in smaller volume formed a highly branched mat, whereas plants in large volume had long tap roots and showed little branching. The increased root:shoot ratio reported by some researchers for many crop species subjected to root restriction might be attributed to an increased substrate temperature in smaller containers in conjunction with a possible temperature dependence of root elongation as suggested by Hurley *et al*. (1998).

Our results reveal that root restriction significantly reduces stomatal conductance, as previously noted by other authors for different species (Carmi *et al*., 1983; Thomas and Strain, 1991; Ismail and Noor, 1996;

Table 1 - Chlorophyll content (*a*, *b* and *a*/*b* ratio) measured at weekly intervals from day 22 to the end of the experiment in leaves collected from control and root-restricted (RR) plants

Day	Control plants			Root-restricted plants (RR)		
	Chl a (mg cm^{-2})	*Chl b* (mg cm^{-2})	*a*/*b*	*Chl a* (mg cm^{-2})	*Chl b* (mg cm^{-2})	*a*/*b*
22	8.075	3.221	0.484	8.423	3.333	0.483
29	7.403	3.274	0.703	9.261*	3.708	0.566
36	7.414	3.276	0.702	9.949*	3.500	0.216*
43	9.076	3.686	0.596	9.809	3.863	0.547
50	10.924	4.320	0.624	10.873	4.054	0.422*

* indicates significantly different values between the two treatments for the same parameters for P≤0.05 (n=5), when means were separated by Duncan's test.

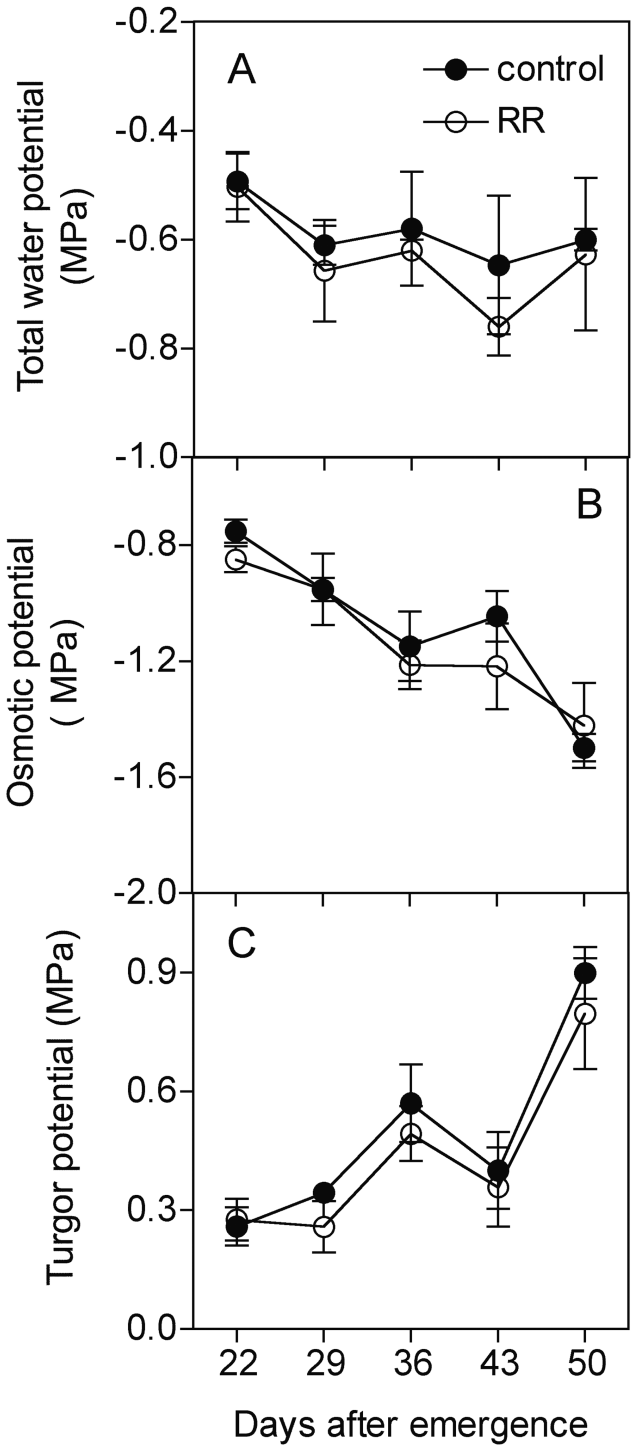

Fig. 3 - Leaf water status determined at weekly intervals from day 22 to the end of the experiment in both control and root-restricted (RR) plants: total water potential (A), osmotic potential (B) and turgor (C). * indicates significantly different values for P≤0.05 (n=5), when means were separated by Duncan's test.

Kharkina *et al*., 1999), and that stomatal conductance was the primary cause of decrease in CO_2 assimilation in root-restricted plants suggesting a stomatal factor limiting the photosynthetic rate under root-restriction conditions (Shi *et al*., 2008). The decline in stomatal conductance was not correlated to a concurrent decline in total water potential, as leaf tissues were able to maintain a high level of turgor during the whole experiment. This means that other factors are largely involved in the stomatal closure. It has been suggested that root volume restriction induces a reduction in the stomatal conductance *via* a decrease in the supply of growth substances from roots to shoots and/or an imbalance in root and shoot hormones. For example, Shi *et al*. (2008) reported that shoot growth suppression may be caused by the influence of ABA originating from the restricted roots. Carmi (1995) found that the higher level of ABA in the leaves of root-restricted plants was not a consequence of an enhanced transport from the restricted roots, concluding that root-zone restriction might promote ABA accumulation in the root and the shoot, with a possible influence of such accumulation on other processes in root-restricted plants, such as leaf gas exchange.

The decline in net CO_2 assimilation observed in root-restricted conditions was also interpreted as a feedback inhibition by carbohydrate accumulation (Pezeshki and Santos, 1998). Plant growth is strongly affected by leaf photosynthetic activity, since photosynthates are essential either as the source of carbon used for the build-up of organic compounds or as the source of energy needed for biochemical reactions involved in growth and maintenance processes. Growth rate may regulate photosynthesis either through effects on the supply of growth substances translocated into leaves or through the effect on the translocation rate of photosynthates from leaves to the growing organs (Carmi *et al*., 1983). The accumulation of photosynthates is influenced by the rate of their translocation to the sink organs (Sonnewald and Willmitzer, 1992), and sink demand for photosynthates has a marked influence on source leaf photosynthesis, which is greatly dependent on sink strength, considered as a product of sink size and sink activity (Sonnewald and Willmitzer, 1992). However, sink size is determined by different parameters. Roots are recognized as

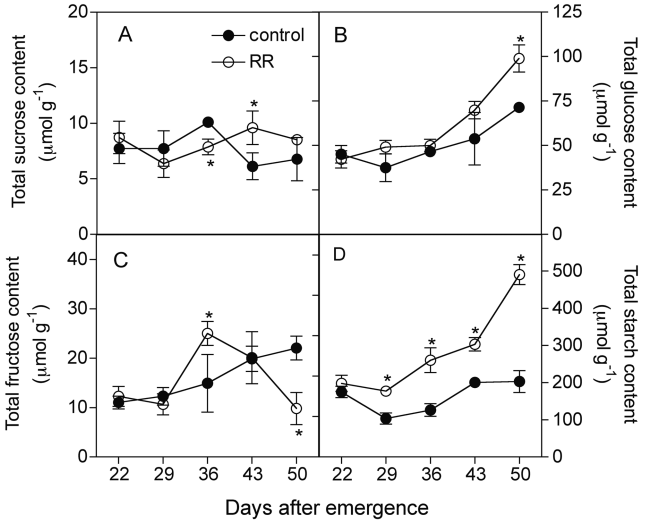

Fig. 4 - Sugar content measured at weekly intervals from day 22 to the end of the experiment in both control and root-restricted (RR) plants: total sucrose (A), total glucose (B), total fructose (C) and total starch (D). * indicates significantly different values for P≤0.05 (n=5), when means were separated by Duncan's test.

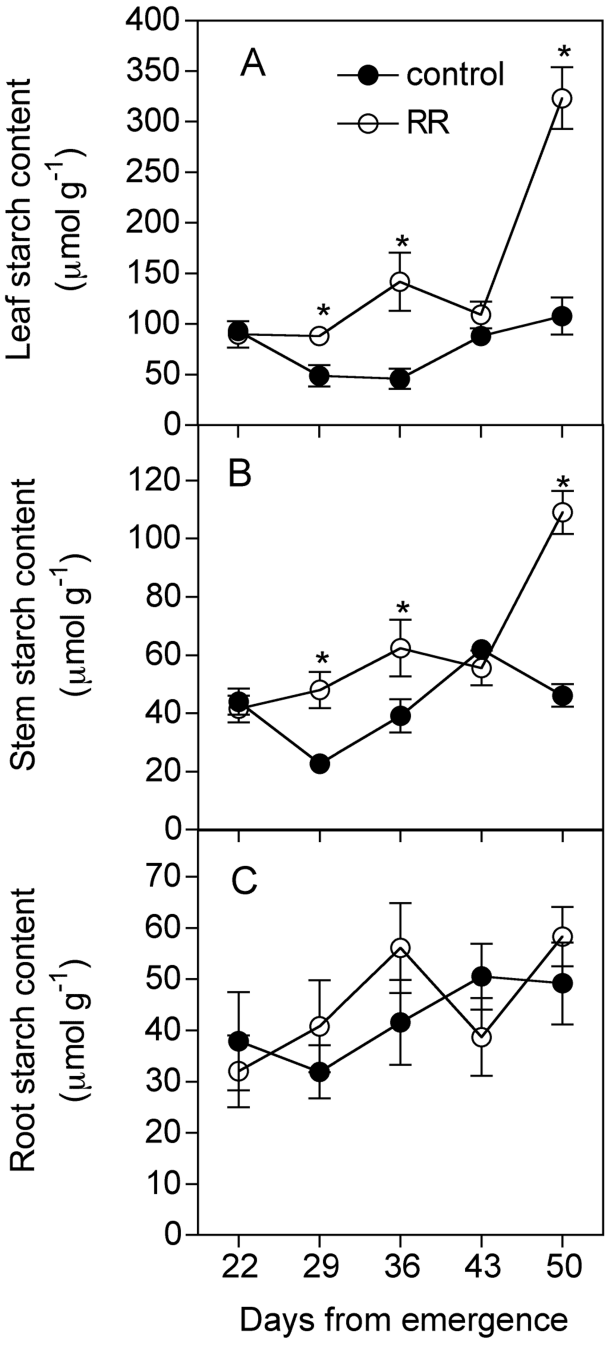

Fig. 5 - Starch content in the different plant organs measured at weekly intervals from day 22 to the end of the experiment in both control and root-restricted (RR) plants: leaf (A), stem (B) and roots (C). * indicates significantly different values for P≤0.05 (n=5), when means were separated by Duncan's test.

a metabolic sink that influences the partitioning of photosynthetically fixed carbon (Gifford and Evans, 1981; Robbins and Pharr, 1988). Sink limitation caused by root restriction can greatly reduce leaf photosynthetic rate in many crop species (Hameed *et al*., 1987; Ismail and Noor, 1996; Schaffer *et al*., 1996; Whiley *et al*., 1999; Shi *et al*., 2008), and reduced translocation of assimilates from leaves (Robbins and Pharr, 1988; Kharkina *et al*., 1999). Root volume restriction often promotes an accumulation of non-structural carbohydrates in the stem and leaves in response to the lack of the active sinks (Nishizawa and Saito, 1998), meaning that the difference in the growth rate between root-restricted and control treatments was not due to a decrease in assimilates supply to the organs whose growth was restricted (Mandre *et al*., 1995). Our results suggest that the role of the leaves and stem as sink organs may increase when root growth is extremely limited by volume restriction and a relatively larger amount of carbohydrates may accumulate in the canopy. A new shoot to root equilibrium may be established for an increased function of leaves and stem, together with a concurrent diminished function of the roots. Therefore, it can be concluded that as a result of reduced vegetative growth an excess of assimilates was produced which could not be used for growth, and thus accumulated in the form of starch, as also indicated by Carmi and Heuer (1981), Robbins and Pharr (1988) and Shi *et al*. (2008).

Accumulation of non-structural carbohydrates in the leaves in response to root restriction could provide a feedback mechanism that reduces carbon metabolism (Thomas and Strain, 1991). Starch accumulation may reduce net photosynthetic rate by avoiding intracellular CO_2 transport (Shi *et al*., 2008). However, contradictory results were obtained by Rieger and Marra (1994), who suggested that reduced CO_2 assimilation cannot always be explained by a feedback inhibition of carbohydrates. The relatively low maximum assimilation (A_{max}) rates for container-grown plants compared to field-grown plants may be attributed to containers restricting the root sink, thus causing the photoassimilate supply to exceed the capacity of demand (i.e. end-product inhibition of photosynthesis) as indicated by Arp and Drake (1991) and Whiley *et al*. (1999).

In conclusion, our results show that growth reduction by root restriction is mainly linked to a photosynthetic impairment, caused by a limited stomatal conductance (probably driven by both stomatal factors and hormonal substances) and a strong accumulation of starch in the tissues, which probably leads to a feedback inhibition of the photosynthetic process.

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