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Starch accumulation in the leaves of root-restricted pepper affects plant growth by a feedback-inhibition of the photosynthesis

H. Al-Debei*, S. Mugnai**

* *Department of Horticulture and Crop Science, University of Jordan, Amman 11942, Jordan.*

** *Dipartimento di Scienze delle Produzioni Vegetali, del Suolo e dell'Ambiente Agroforestale, Università degli studi di Firenze, Viale delle Idee, 30, 50019 Sesto Fiorentino (FI), Italy.*

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Abstract: The mechanism behind the reduced growth occurring in plants subjected to root restriction is still not fully understood. Therefore, this investigation was planned to determine the morphological and physiological changes induced in response to root volume reduction and to determine the time frame within which these changes occurred. In particular, this research focused on the effect of root restriction on growth, leaf gas exchange parameters, carbohydrate production and water relations in pepper (*Capsicum annuum* L.). Our results show that the reduced growth is mainly linked to a feedback inhibition of the photosynthesis, caused by a concurrent limited stomatal conductance (probably driven by both stomatal factors and hormonal substances) together with a strong accumulation of starch in the leaves.

1. Introduction

Root-restricted cultivation is an effective technique for saving resources, to control root environment, to anticipate yield and to regulate the quality of vegetables. For all these reasons, its use has significantly increased during the last decades in vegetable nurseries (Shi *et al*., 2008). Root restriction (RR) may occur when container size and/or rooting volume is physically constrained (Tschaplinski and Blake, 1985; Ismail and Noor 1996; Saito *et al*., 2008; Mugnai *et al.,* 2009), especially in greenhouse-grown horticultural crops (Thomas, 1993). A reduced container volume stimulates the formation of a denser root mass, with decreased root growth (Ismail and Noor, 1996). Together with a strong limitation of the soil available to the root system for water and nutrients uptake, RR also reduces canopy growth (Ismail and Noor, 1996; Shi *et al*., 2008) by affecting many plant physiological and biochemical processes. The mechanism behind the reduced shoot growth is not yet fully understood. Several hypotheses have been proposed: water and nutrient stresses (Hameed *et al*., 1987), decrease in root respiration (Shi *et al*., 2007), reduction in photosynthesis (Shi *et al.,* 2008), and synthesis and translocation of plant hormones (Liu and Latimer, 1995). However, contradictory results are reported as to which of these factors plays a significant role in the response of aerial plant parts to restricted root growth, with strong 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). In the presence of a physical restriction to root growth, a major metabolic sink for photosynthetically fixed carbon at seedling stage (Thomas and Strain, 1991) may result in feedback inhibition mechanisms (Shi *et al*., 2008). This investigation was therefore planned to determine the morphological and physiological changes induced in response to RR conditions and to determine the time frame within which these changes occurred. In particular, this research aims to study the link between leaf gas exchange parameters and carbohydrate production in regulating growth in pepper (*Capsicum annuum* L.) plants.

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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 pepper (*Capsicum annuum* L.) cv. Sienor 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} PPFD). 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 RR treatment throughout 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 as follows: 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). The nutrient solution was renewed every week.

Growth measurements

Five plants per treatment were sampled at weekly intervals. Roots were carefully washed, and 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 h 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 by 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 µmol/m*s PAR) conditions provided by a mercury vapour lamp (Osram HQI-TS 250 W/NDL). Calculation of all the parameters was performed following Von Caemmerer and Farquhar (1981) using a specific software (Diagas 2.02, Walz, Effettrich, Germany). Water use efficiency (WUE) was calculated as the ratio between A_{max} and E_{max}. For each crop species, E, A and g were also measured under different light intensities (0, 20, 50, 100, 200, 400, 600, 800 and 1000 µmol/m*s) as described above. A piece of black cloth was used to provide complete darkness, whereas different layers of wire mesh with very small holes were used to provide the required light intensity.

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 h 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):

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

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, centrifuged at 12000 rpm for 15 min and then the supernatant was collected. The pellet was extracted again as described above, and the supernatant was collected again. At the end, the pellet was evaporated to remove any excess of 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 Na-acetate 50 mM at pH 4.6), and the other one with 100 µl solution containing Na-acetate 50 mM at pH 4.6, they were 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 by 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 h with 100 µl solution of Na-acetate 100 mM pH 5.2/10 u α-amylase. This solution was then incubated with 100 µl of Na-acetate 100 mM pH 4.6/10 u amyloglucosidase at 55°C for 1 h. Finally, the solution was boiled and centrifuged to eliminate denaturated protein from α-amylase and amyloglucosidase. 100 µl of this solution was taken and brought to 300 µl with distilled water; starch analysis (as glucose equivalent) was then 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 and Discussion

Root volume reduction greatly affected growth parameters, confirming several previous results concerning growth depression induced by RR in many horticultural crops (see for example Kharkina *et al.,* 1999; Saito *et al.,* 2008; Shi *et al.,* 2008), but scarcely in pepper (Ismail and Davies, 1998). Total dry weight significantly decreased starting from day 30 after emergence with reducing container size (Fig. 1A). In detail, RR pepper plants showed a 3.85-fold lower total dry weight compared to control at the end of the experiment. Leaf area was also greatly affected by volume reduction: RR plants showed a 4.15-fold reduction (Fig. 1B) at the end of the experiment. RR plants appeared to be smaller (reduced height values) (Fig. 1C), denoting a slackened development compared to control plants, with a preferred allocation of dry matter in the root system than in the aerial system, as demonstrated by a slight increase in the root:shoot ratio (Fig. 1D). RR generally caused an increase in root:shoot ratio (Mugnai *et al.,* 2000), with roots growing in smaller volume forming a highly branched mat. The increased root:shoot ratio reported by some researchers for many crop species subjected to RR 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).

During the first month, no significant differences were noticed in leaf gas exchange parameters. Stomatal conductance (*g*) significantly decreased in RR plants (Fig. 2A)

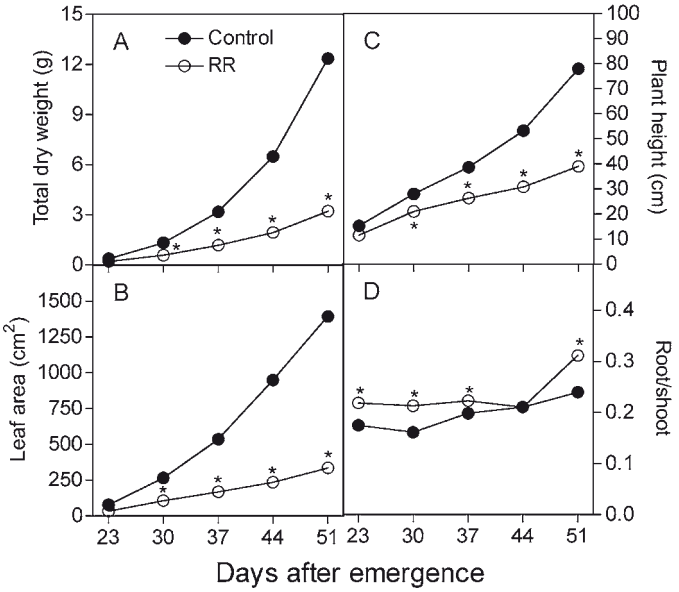

Fig. 1 - Growth parameters measured at weekly intervals from day 23 to the end of the experiment in both control and root-restricted (RR) plants:total dry weight (A), leaf area (B), plant height (C) and root:shoot ratio (D). * indicates significantly different values for P≤0.05 (n=5), when means were separated by Duncan's test.

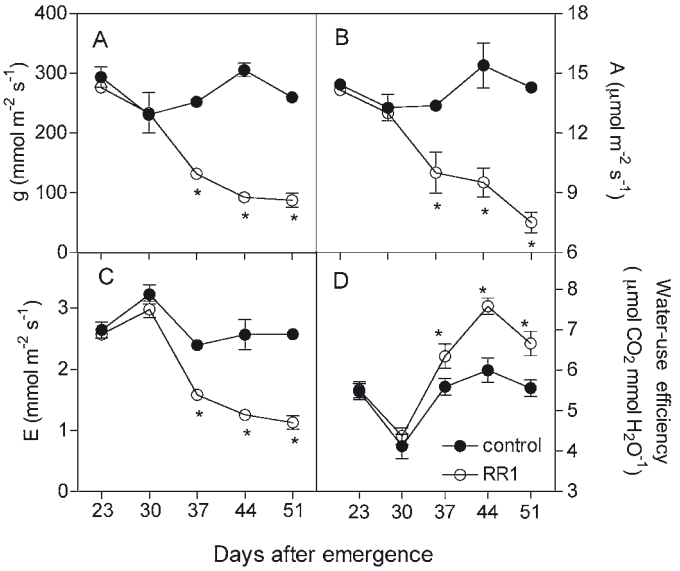

Fig. 2 - Leaf gas exchange parameters measured at weekly intervals from day 23 to the end of the experiment in both control and root-restricted (RR) plants: stomatal conductance (A), net CO_2 assimilation (B) and transpiration (C). * indicates significantly different values for P≤0.05 (n=5), when means were separated by Duncan's test.

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
23	12.469	4.485	2.780	11.453	4.170	2.746
30	13.387	4.756	2.814	13.107	4.642	2.823
37	14.671	5.639	2.601	13.845	5.259	2.632
44	16.274*	6.347*	2.564	14.058*	5.589*	2.515
51	17.784*	7.450*	2.387	11.493*	4.768*	2.410

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

from day 36, leading to a significant reduction in both net CO_2 assimilation (*A*, Fig. 2B) and transpiration (E, Fig. 2C) until the end of the experiment. The reduction in *A* was not related to a decrease in the chlorophyll content of RR plants (Table 1), as significant differences between the two treatments were noticed for chlorophyll *a*, *b,* and *a*/*b* ratio only after 43 days from the beginning of the experiment. Also, RR plants showed increased instantaneous water-use efficiency values (Wue) (Fig. 2D), as reported for several species under stress (Blum, 2009). RR treatment also affected leaf gas exchange parameters' response to light (Fig. 3). All the parameters (*g*, *A* and *E*) strongly decreased their values, leading to less pronounced response curves. In details, photosynthetic parameters, such as dark respiration, light compensation point and maximum CO_2 assimilation, started to significantly decrease after 37 days (Table 2).

Leaf water status did not seem to be the cause of the stomatal closure in RR plants, as total water potential (Fig. 4A) and turgor potential (Fig. 4C) did not show any significant difference in either of the treatments, even if slight reductions in total water potential and osmotic potential were measured at the end of the experiment in RR plants.

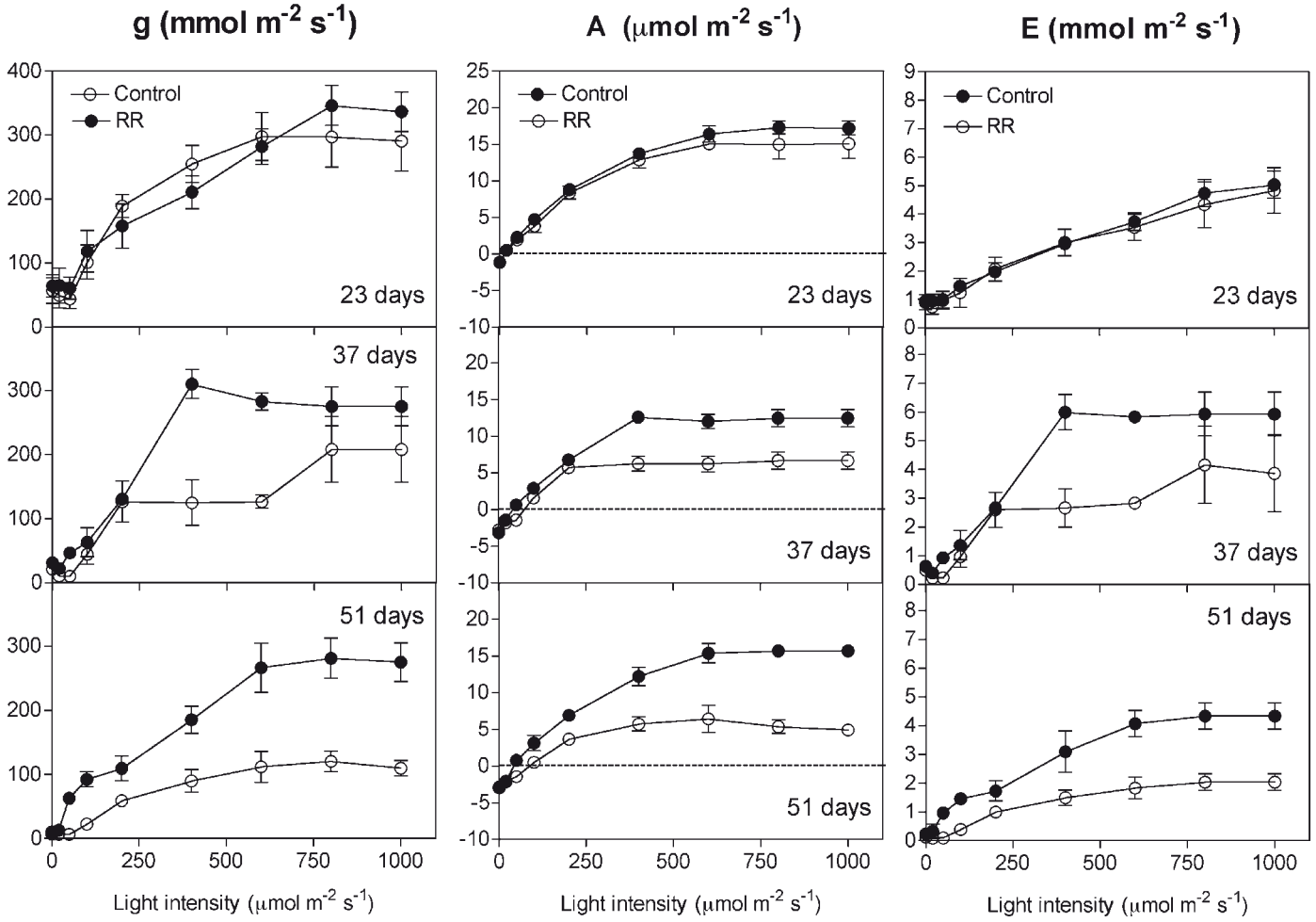

Fig. 3 - Light saturation curves for the three leaf gas exchange parameters (*g*, *A and* E) measured every two weeks from day 23 to the end of the experiment in both control and root-restricted (RR) plants.

Table 2 - Dark respiration (DR, μmol CO_2 m^{-2} s^{-1}), light compensation point (LCP, μmol m^{-2} s^{-1} PAR), maximum CO_2 assimilation (Amax, μmol CO_2 m-2 s^{-1}), water-use efficiency (WUE, μmol CO_2 mmol H_2O^{-1}) measured every two weeks 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)			
	DR	LCP	A_{max}	WUE	DR	CP	A_{max}	WUE
23	-1.07	14.06	17.31	5.47	-0.9	12.83	15.11	5.51
37	-3.31*	41.56*	24.87*	5.59*	-2.75*	71.50*	6.67*	6.34*
51	-2.93	42.17*	15.71*	5.55*	-2.87	85.56*	6.46*	6.65*

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

This result also confirmed that no symptom of water stress ever occurred during the experimental period, leading to a positive feedback about our experimental system.

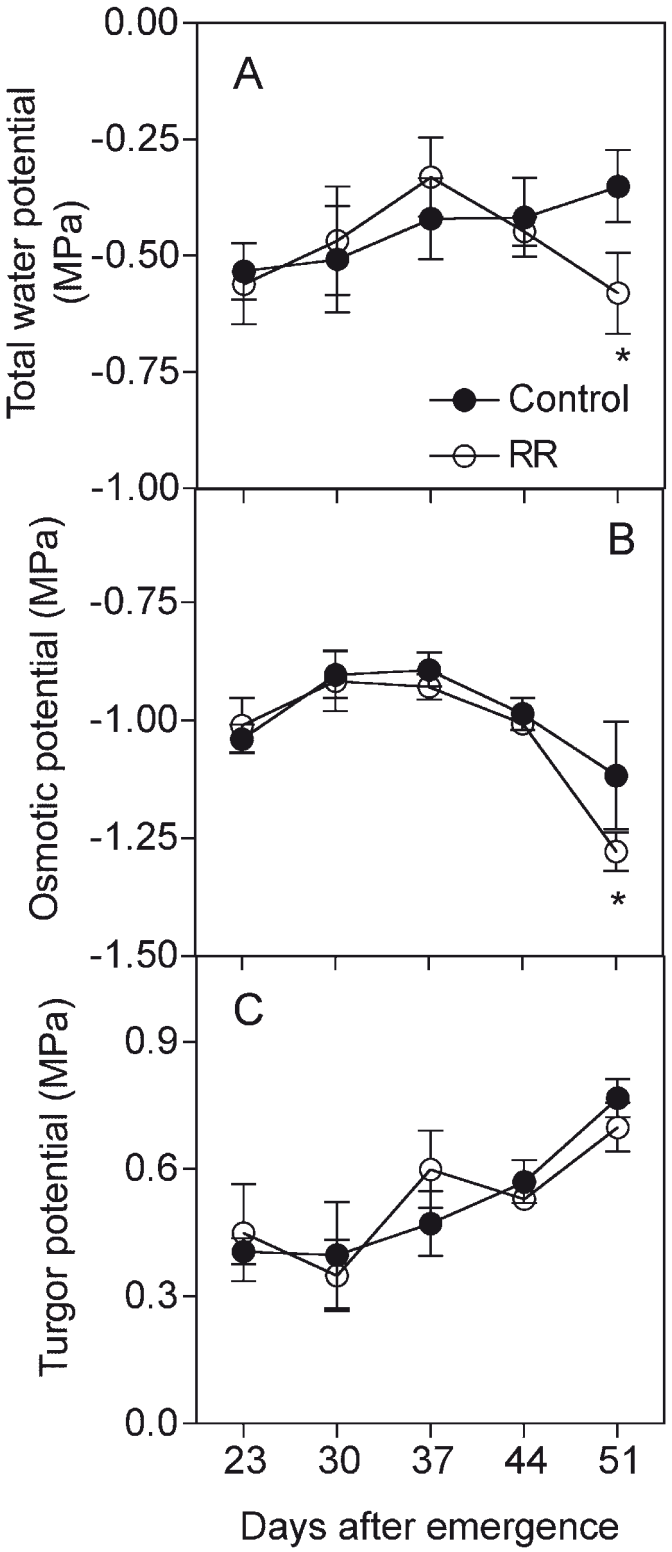

Fig. 4 - Leaf water status determined at weekly intervals from day 23 to the end of the experiment in both control and root-restricted (RR) plants:total water potential (A), osmotic potential (B) and turgor (C). * indicates significantly different values for P≤0.05 (n=5), when means were separated by Duncan's test.

Our results reveal that RR significantly reduces *g*, as previously noticed by other authors on different species (Ismail and Noor, 1996; Kharkina *et al.,* 1999), and that *g* is the primary cause of the reduction in *A* in RR plants, suggesting a stomatal factor limiting the photosynthetic rate under RR conditions (Shi *et al.,* 2008). However, the decline in *g* 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. Therefore, other factors should be involved in the stomatal closure. It has been suggested that RR induces a reduction in *g* through 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 might be caused by the influence of ABA originating from the restricted roots. Ismail and Davies (1998) found that the slight increase in xylem sap [ABA] measured in pepper plants could not account for the reduction in leaf growth and *g*. They suggested that insufficient ABA synthesis occurred to trigger the processes that cause reductions in leaf growth and *g*.

Sugar content determination led to interesting results. Sucrose content significantly increased in RR plants starting from day 37 (Fig. 5A). Also, RR treatment led to a clear increase in glucose content (Fig. 5B) and a concurrent decrease in fructose content (Fig. 5C) together with a great accumulation of starch (Fig. 5D). In particular, starch accumulation in the tissues began early in the developmental process (day 29). Starch was mainly compartmentalised in the leaves (Fig. 6A) of RR plants, whereas no significant differences were noticed both in stems (Fig. 6B) and in roots (Fig. 6C) between control and RR plants, except for day 33. The decline in *A* observed in RR conditions was often interpreted as a feedback inhibition by carbohydrate accumulation (Pezeshki and Santos, 1998). Plant

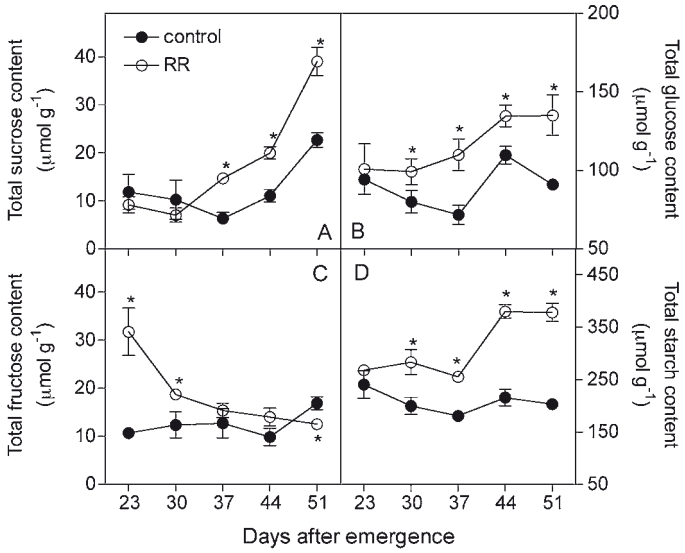

Fig. 5 - Sugar content measured at weekly intervals from day 23 to the end of the experiment in both control and root-restricted (RR) plants:total sucrose (A), total glucose (B), total fructose (C) and total starch (D). * indicates significantly different values for P≤0.05 (n=5), when means were separated by Duncan's test.

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 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 a metabolic sink that influences the partitioning of photosynthetically fixed carbon (Gifford and Evans, 1981; Robbins and Pharr, 1988). Sink limitation caused by RR can greatly reduce leaf photosynthetic rate in many crop species (Hameed *et al*., 1987; Ismail and Noor, 1996; Whiley *et al*., 1999; Shi *et al*., 2008), and reduced translocation of assimilates from leaves (Robbins and Pharr, 1988; Kharkina *et al*., 1999). RR 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 RR 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 as sink organs may increase when root growth is extremely limited by volume restriction and a relatively larger amount of carbohydrate 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 Shi *et al.* (2008). Accumulation of non-structural carbohydrates in the leaves in response to RR 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), suggesting 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 photo assimilate supply to exceed the capacity of demand (i.e. end-product inhibition of photosynthesis) as indicated by Whiley *et al.* (1999).

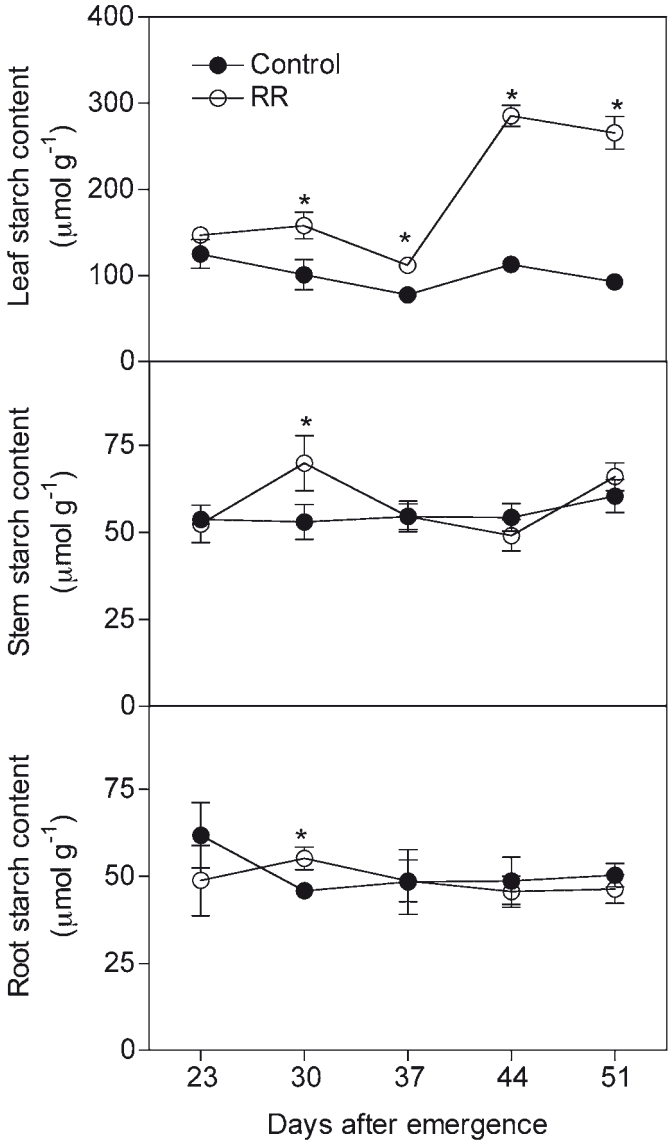

Fig. 6 - Starch content in the different plant organs measured at weekly intervals from day 23 to the end of the experiment in both control and root-restricted (RR) plants:leaf (A), stem (B) and roots (C). * indicates significantly different values for P≤0.05 (n=5), when means were separated by Duncan's test.

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

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