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Relationship between photosynthetic capacity, nitrogen assimilation and nodule metabolism in alfalfa (*Medicago sativa*) grown with sewage sludge

M. Carmen Antolín*, M. Laura Fiasconaro, Manuel Sánchez-Díaz

Dpto. Biología Vegetal, Sección Biología Vegetal Unidad Asociada al CSIC (EEAD, Zaragoza; ICVV, Logroño), Facultades de Ciencias y Farmacia, Universidad de Navarra, C/ Irunlarrea 1, 31008 Pamplona, Spain

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ABSTRACT

Sewage sludge has been used as N fertilizer because it contains some of inorganic N, principally as nitrate and ammonium ions. However, sewage sludge addition to legumes could result in impaired nodule metabolism due to the presence of inorganic N from sludge. A greenhouse experiment was conducted to examine the effects of sewage sludge on growth, photosynthesis, nitrogen assimilation and nodule metabolism in alfalfa (*Medicago sativa* L. cv. Aragón). Plants were grown in pots with a mixture of perlite and vermiculite (2:1, v/v). The experiment included three treatments: (1) plants inoculated with rhizobia and amended with sewage sludge at rate of 10% (w/w) (RS); (2) plants inoculated with rhizobia without any amendment (R); and (3) non-inoculated plants fed with ammonium nitrate (N). N_2-fixing plants had lower growth and sucrose phosphate synthase activity but higher photosynthesis than nitrate-fed plants because they compensated the carbon cost of the rhizobia. However, sewage sludge-treated plants evidenced a loss of carbon sink strength due to N_2 fixation by means of decreased photosynthetic capacity, leaf chlorophylls and N concentration in comparison to untreated plants. Sewage sludge did no affect nodulation but decreased nodule enzyme activities involved in carbon and N metabolisms that may lead to accumulation of toxic N-compounds.

1. Introduction

Municipal wastewater treatment can be considered as a continuous activity that produces increasing amounts of sewage sludge. Moreover, the progressive implementation in 2005 of the Directives 91/271/EEC and 98/15/EEC concerning urban wastewater treatment has increased the number of wastewater treatment plants operating in the EU and consequently the quantities of sewage sludge requiring disposal [1,2]. Thus, sewage sludge will remain a permanent waste problem that requires an appropriate solution [3,4]. The most common beneficial use of sewage sludge is agricultural land application because they are rich in organic matter and plant nutrients, such as phosphorus (P), some nitrogen (N) mainly organically bound, very little potassium (K) and micronutrients, and their application to soil can improve soil structure, increase soil water capacity and stimulate microbiological activity [5–7]. Land application of sewage sludge achieves a complete reuse of its nutrients and organic carbon at a relatively low cost since reduces the amount of organic waste disposed in landfills [8]. However, with the sludge, heavy metals, pathogenic bacteria and different organic contaminants can be added to agricultural fields [9–12].

Leguminous plants acquire N by assimilation of nitrate and ammonium from the soil solution, or from atmospheric N (N_2) fixation through a symbiotic association with N_2-fixing bacteria. Thus, nitrogen-fixing legumes provide the major N input into the rizosphere as a result of their ability to convert N_2 to a form that can be assimilated by the plant [13]. Although fixation of N_2 is not restricted to this group of bacteria, only the rhizobia induce the formation of nodules on the roots of their legume hosts. Plant provides an environment conducive to sustain bacterial metabolism by reducing the internal free O_2 level and providing a source of energy, usually in the form of succinate and malate. Sucrose, formed by photosynthesis in the leaves, is translocated to the root system where it is converted to malate and succinate, while the ammonium is assimilated within the infected nodule tissue to form amides or ureides that are translocated to the shoot.

Sewage sludge has been used as N fertilizer in different crops [8,14,15] because it contains some of inorganic N, principally as nitrate and ammonium ions [16,17]. In addition, it was shown that sludge application can improve growth and yield of nodulated legumes [18–21] but also, this treatment can induce a certain degree of oxidative stress in nodules due to accumulation of heavy metals in rizhosphere [20,22]. On the other hand, it was well established that combined N (especially nitrate) inhibits both nodulation

* Corresponding author. Tel.: +34 948425600; fax: +34 948425649.
E-mail address: cantolin@unav.es (M.C. Antolín).

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and activity of nodules, which legumes will utilize as N source in preference to forming the N_2-fixing symbiosis [23]. Despite apparent benefits of sludge application for growth of free-living rhizobia [24], its influence on nodule metabolism is still not well understood. Thus, we hypothesize that sewage sludge addition to legume crop could result in impaired nodule metabolism due to the presence of inorganic N from sludge. Therefore, the aim of this study was to compare the effects of sewage sludge and mineral fertilizer on growth, photosynthesis and N assimilation of alfalfa plants. Specifically we sought to determine the impact of sewage sludge addition on nodule activity.

2. Materials and methods

2.1. Sludge

The sewage sludge was collected at the wastewater plant of Tudela (Navarra, Spain) which processes domestic wastewater amounting to 38,969 person equivalents per year. The sludge had been obtained from autothermal termophilic aerobic digestion. The most significant characteristics of the sludge were: dry mass 29.6%, volatile solids 52.3%, pH 7.8, electric conductivity 7.39 mS cm^{-1}, total organic carbon 26.2%, N Kjeldhal 2.5%, total P 1.4%, total K 0.5%, C:N ratio 10.5, ammonia 190 mg l^{-1}, Fe 1.4%, Cd 1 mg kg^{-1}, Cr 74 mg kg^{-1}, Cu 243 mg kg^{-1}, Mn 190 mg kg^{-1}, Ni 32 mg kg^{-1}, Pb 56 mg kg^{-1}, Zn 755 mg kg^{-1}. Heavy metal content in the sludge was not a significant issue, since concentrations in pots were below the limits established by European legislation for soil sludge addition [25].

2.2. Experimental design

Plants were cultivated in an inert medium to appropriate assessment of nitrogenase activity of a selected strain of *Sinorhizobium meliloti* [20,21]. Two hundred grams of a mixture of perlite and vermiculite (2:1, v/v) was packed into 15 cm × 12 cm pots (1.0 dm^3 volume). The experiment included three treatments: (1) plants inoculated with rhizobia and amended with the provided sewage sludge (RS) at rate of 10% (w/w), which was equivalent to approximately 30 t dry matter (DM) ha^{-1}; (2) plants inoculated with rhizobia without any amendment (R); and (3) non-inoculated plants fed with ammonium nitrate (N) as a control for comparison. Five replications per treatment were prepared. The sludge was added to the substrate 30 days before planting, in order to allow that the processes of chemical degradation, biodegradation and volatilization of toxic compound in sludges reach equilibrium in substrate and thus reduce the risk of phytotoxicity, as recommended by Epstein [26]. All plants were watered twice a week with Evans N-free nutrient solution [27] alternating with deionized water to avoid salt accumulation in pots. Plants fed with ammonium nitrate (N) were watered throughout all experimental period with a Evans' solution supplemented with ammonium nitrate at the same rate of N as contained in the sewage sludge. Similarly, the amount of P and K were adjusted at the same rate as in the sewage sludge in all treatments.

Seeds from alfalfa (*Medicago sativa* cv. Aragón) were surface disinfected in a 0.1% (w/v) $HgCl_2$ solution for 10 min, washed five times with sterile water to remove any trace of chemical that could interfere in seed germination and placed in Petri dishes to germinate. Petri dishes were watered daily till seed germination using sterile distilled water. One seedling of alfalfa was transplanted into each recipient. During the first month, plants were inoculated four times with *Sinorhizobium melioti* strain 102F34 maintained on yeast extract mannitol agar. Plants were grown in a glasshouse at 25 °C/15 °C and 50%/70% RH (day/night). The photoperiod was 14 h under natural daylight, supplemented with high pressure sodium lamps (SON-T Agro Phillips, Eindhoven, The Netherlands), which provided a minimum photosynthetic photon flux (PPF) of about 400 μmol m^{-2} s^{-1} at the upper level of canopy. One week prior to measurements, the plants were transferred to a controlled environment chamber with a day/night regime of 25/15 °C and 80/90% relative humidity. A PPF of 400 μmol m^{-2} s^{-1} at the canopy level was provided by fluorescent lamps (Sylvania F 48T12 CW-WHO, München, Germany) for a 14 h photoperiod. Plants were harvested when green pods were evident, corresponding to growth stage 7 (early seed pod) [28].

2.3. Soil and plant elemental analysis

The pH of substrate was measured in an aqueous solution (1:10, w/v) and electrical conductivity (EC) was measured in 1:10 dilution. Nitrogen content was determined in dried samples by using the Kjeldahl method. Phosphorus (P) was extracted with $NaHCO_3$ [29]. Potassium (K) was extracted with ammonium acetate and analyzed by flame spectrometry. Plant heavy metal concentrations were determined following nitric–perchloric acid digestion. The "plant available" metal concentrations in substrate were determined after extraction with 0.005 M DTPA [30]. All material digests were analyzed for Cd, Cr, Cu, Fe, Mn, Ni, Pb and Zn using inductively coupled plasma mass spectrometry (ICP-MS). Quality control was assured by the use of certified reference materials SRM 1575a (pine needles) and BCR 100 (beech leaves) for plants and CMI 7003 (silty clay loam soil) for soils, procedural blanks and duplicates of the analysis.

2.4. Plant determinations

Net photosynthetic rates were measured 3 h after the onset of the photoperiod at ambient CO_2 (350 μmol mol^{-1}), PPF of 1000 μmol m^{-2} s^{-1}, 80% relative humidity and 25 °C following procedures described by Antolín and Sánchez-Díaz [31] with a portable photosynthesis system (GFS-3000, Walz, Effeltrich, Germany). CO_2–response curves were measured at ambient O_2 (21%) and saturating PPF (1000 μmol m^{-2} s^{-1}), starting at 0 and increasing to 1800 μmol mol^{-1} of external CO_2 concentration (11 points with 3 min of adaptation). All measurements were made in young fully expanded leaves. Mechanistic analyses of CO_2–response curves provide estimations of the maximum rate of carboxylation by RuBPCO (V_{cmax}), the PPFD-saturated rate of electron transport (J_{max}) and the rate of triose phosphate utilization (TPU). These parameters were estimated with the formulas utilized in 'Photosynthesis Assistant' version 1.1 software (Dundee Scientific, Dundee, UK). After measurements of leaf exchange, plants were harvested and leaf samples were stored at −80 °C until analysis.

Leaf chlorophylls were extracted in 95% (v/v) ethanol and its concentration were quantified spectrophotometrically. Calculations were made using the equations of Lichtenthaler [32]. Total soluble proteins (TSP) in leaves and roots were analyzed by the protein dye-binding method using bovine serum albumin as a standard [33]. Leaf appearance rates were recorded weekly and expressed as the number of leaves produced per day. Leaf area was measured with a portable leaf area meter (Model LI-3000, LiCor, Lincoln, NE). Plant dry matter (DM) was determined by drying samples at 85 °C to constant mass.

Sucrose phosphate synthase (SPS) (EC 2.4.1.14) was extracted in a medium containing 50 mM Mops–NaOH (pH 7.4), 12 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM benzamidine, 1 mM aminocaproic, 1 mM DTT, 1 mM PMSF, 0.1 mM Triton X-100 and 2% PVPP. Maximum SPS activity was assayed with saturating substrates as described in Hekneby et al. [34]. Reaction mixtures contained 1 mM Mops (pH 7.4), 50 mM $MgCl_2$, 10 mM EDTA, 65 mM UDP-glucose, 35 mM fructose 6-phosphate, 53 mM glucose 6-phosphate

Table 1
Main properties of the mixture of perlite and vermiculite (2:1, v/v) in substrates fed with mineral-fertilizer (N), amended with sewage-sludge (RS) and in untreated soils (R) measured at the end of experimental period.

Measurement	N	R	RS
pH	6.43 ± 0.08b	7.38 ± 0.11a	6.66 ± 0.07b
EC ($mS\,cm^{-1}$)	1.29 ± 0.15ab	0.81 ± 0.07b	1.40 ± 0.17a
$N_{Kjeldhal}$ ($g\,100\,g^{-1}$)	0.16 ± 0.02a	0.01 ± 0.00b	0.184 ± 0.01a
P_{Olsen} ($mg\,kg^{-1}$)	45.80 ± 5.43a	4.86 ± 0.49b	46.58 ± 5.17a
$K_{available}$ ($mg\,g^{-1}$)	542.74 ± 69.19a	506.66 ± 49.24a	244.71 ± 12.96b
$Fe_{available}$ ($mg\,kg^{-1}$)	14.94 ± 1.74b	10.65 ± 1.08b	35.38 ± 3.01a
$Cd_{available}$ ($mg\,kg^{-1}$)	0.02 ± 0.00b	0.03 ± 0.00b	0.05 ± 0.00a
$Cr_{available}$ ($mg\,kg^{-1}$)	0.65 ± 0.01a	0.61 ± 0.01a	0.64 ± 0.02a
$Cu_{available}$ ($mg\,kg^{-1}$)	4.56 ± 0.47b	2.34 ± 0.19c	6.85 ± 0.74a
$Mn_{available}$ ($mg\,kg^{-1}$)	4.96 ± 0.50b	6.74 ± 0.79ab	7.22 ± 0.42a
$Mo_{available}$ ($mg\,kg^{-1}$)	0.07 ± 0.00b	0.07 ± 0.00b	0.39 ± 0.03a
$Ni_{available}$ ($mg\,kg^{-1}$)	0.73 ± 0.08a	0.55 ± 0.04a	0.64 ± 0.04a
$Pb_{available}$ ($mg\,kg^{-1}$)	0.15 ± 0.02b	0.09 ± 0.00b	1.13 ± 0.11a
$Zn_{available}$ ($mg\,kg^{-1}$)	1.06 ± 0.15b	0.83 ± 0.05b	14.81 ± 2.56a

EC: electric conductivity. Within each line, means followed by different letter are significantly different ($P < 0.05$) according to a Tukey's test. Values are means ($n = 5$) ± standard error (S.E.).

and 70 μl extract. Reaction was run for 10 min at 25 °C and was terminated by addition of 70 μl of 30% KOH (w/v), followed by a 10 min incubation in a boiling water bath. After the material was cooled, 1 ml of 0.15% anthrone (w/v) in 13.8 M H_2SO_4 was added, and the tubes were incubated at 40 °C for 20 min before absorbance was measured at 620 nm.

Total soluble sugars (TSS) in leaves and roots were analyzed by reacting 0.25 ml of the extracts with 3 ml of freshly prepared anthrone and placing in boiling water for 10 min. After cooling, the absorbance at 620 nm was determined in a spectrophotometer [35].

2.5. Nodule determinations

Nitrogenase activity was measured as H_2 evolution on the intact plants with root systems sealed in the growth pots and housed inside a chamber in an open flow-through system under N_2:O_2 (79:21%) according to Witty and Minchin [36], using an electrochemical H_2 sensor (Qubit System Inc., Canada) as described previously [20]. After measurement of nitrogenase activity, roots were carefully washed and nodules were detached, counted, weighed and stored at −80 °C until analysis.

Five hundred mg of frozen nodules were crushed in 10 ml of 50 mM K-phosphate buffer (pH 7.8), with 0.2% (v/v), 2-mercaptoethanol, 0.1 mM Na_2–EDTA and 10% (w/w) polyvinilpolypirrolidone (PVPP) in a cold mortar following protocol described by Irigoyen et al. [37]. Phosphoenolpyruvate carboxylase (PEPC, E.C. 4.1.1.31), malate dehydrogenase (MDH, EC 1.1.1.37) and glutamate–oxaloacetate transaminase (GOT, EC 2.6.1.1) activities in the nodule plant fraction were assayed spectrophotometrically by NADH oxidation at 340 nm. PEPC reaction was performed as described by Deroche et al. [38]. The reaction medium and assay conditions for MDH and GOT were based on those of Vance and Stade [39].

Extraction of nodule nitrate reductase (NR, EC 1.7.99.4), nitrite reductase (NiR, EC 1.7.7.1) and glutamate synthase (GOGAT, EC 1.4.1.14) activities was made with one gram of frozen nodules, which were crushed in 5 ml of 50 mM K-phosphate buffer pH 7.5, containing 2 mM EDTA, 1.5% (w/v) soluble casein, 2 mM DTT and 1% (w/v) insoluble PVPP at 0 °C as described by Sánchez et al. [40]. The NR assay followed the methodology of Kaiser and Lewis [41]. Each assay mixture tube contained 0.1 ml potassium phosphate buffer pH 7.5, 0.1 ml NADH ($1\,mg\,l^{-1}$), 0.2 ml 0.1 M KNO_3 and 0.5 ml extract made up to a final volume of 2 ml with distilled water. After 15 min incubation at 28 °C, the reaction was stopped by the addition of 1 ml of 1% (w/v) sulphanilamide in 1.5 M HCl and 1 ml of 0.02% (w/v) N-(1-naphthyl)-ethylenediamine dihydrochloride solution. All samples were centrifuged at 500 × *g* for 5 min to remove suspended matter. Nitrite production was determined by measuring absorbance at 540 nm. The NiR activity was determined on its basis of the drop in nitrite concentration in the reaction medium using methyl viologen as the reductant [42]. The assay contained in a total volume of 1 ml, 4 mM $NaNO_2$, 50 mM potassium phosphate buffer pH 7.0 and 0.1 ml extract. Freshly prepared $Na_2S_2O_4$ (0.2 ml $17\,mg\,ml^{-1}$ in 0.29 M $NaHCO_3$) was added to start the reaction. After incubation at 27 °C for 15 min, 50 ml of distilled water was added, and air was led into the mixture to ensure that oxidation of methyl viologen was complete. The nitrite content was determined as described elsewhere. The GOGAT activity was measured spectrophotometrically at 30 °C by monitoring the oxidation of NADH at 340 nm, essentially according to Singh and Srivastava [43]. The assay mixture contained 0.4 ml 20 mM L-glutamine, 0.4 ml 5 mM 2-oxoglutarate, 1 mM EDTA (added in assay buffer), 0.1 ml 100 mM KCl, 0.6 ml 1 mM NADH and 0.5 ml of the extract in a final volume of 3.0 ml completed with 25 mM sodium phosphate (pH 7.5). The reaction was started by adding L-glutamine immediately following the enzyme preparation. The drop in absorbance (linear at least for 10 min) was recorded for 5 min.

Extracts for assay of glutamine synthetase (GS, 6.3.1.2) were prepared as described by Aráujo et al. [18] and GS activity was determined in the supernatant by the hydroxamate biosynthesis method [44]. The reaction mixture consisted of 600 μl of 250 mM Tris–HCl buffer, pH 7.0, 200 μl of 30 mM glutamate, 200 μl of 30 mM ATP, 200 μl of 500 mM $MgSO_4$, 500 μl of enzyme extract and 200 μl of 1 M hydroxylamine. Hydroxylamine was omitted for the control. The reaction was stopped after 20 min at 30 °C by addition of $FeCl_3$ reagent. γ-Glutamyl hydroxamate formed from hydroxylamine and glutamate was determined spectrophotometrically at 540 nm after complexation with $FeCl_3$.

Leghemoglobin was extracted three times, each with 2 ml of Drabkin's solution [45]. Nodule samples were centrifuged at 30,000 × *g* at 4 °C for 15 min and the supernatant was collected for leghemoglobin determinations. Leghemoglobin was measured by reading the absorbance at 540 nm [46]. Nodule extracts from measurement of PEPC, MDH and GOT were also utilized for determination of total soluble sugars (TSS), which were analyzed as described elsewhere.

2.6. Statistical analysis

Data were submitted to an analysis of variance (ANOVA). Means ± standard errors (S.E.) were calculated and, when *F* ratio was significant, least significant differences were evaluated by a

Table 2
Heavy metals of nitrate-fed (N) and nitrogen-fixing alfalfa plants grown in soils amended with sewage sludge (RS) or in untreated soils (R).

Treatment	Cd (mg kg^{-1})	Cr (mg kg^{-1})	Cu (mg kg^{-1})	Fe (mg kg^{-1})	Mn (mg kg^{-1})	Ni (mg kg^{-1})	Pb (mg kg^{-1})	Zn (mg kg^{-1})
Leaves								
N	0.01 ± 0.00c	0.60 ± 0.07b	23.77 ± 3.71a	231.91 ± 12.08b	140.52 ± 17.09a	1.88 ± 0.25a	0.44 ± 0.06b	37.36 ± 1.46b
R	0.17 ± 0.03b	0.51 ± 0.04b	7.04 ± 0.45c	193.14 ± 23.92b	151.64 ± 8.70a	0.61 ± 0.03b	0.14 ± 0.02c	20.59 ± 0.83c
RS	0.03 ± 0.00c	0.51 ± 0.04b	8.84 ± 1.32c	179.37 ± 10.09bc	116.40 ± 8.74ab	0.68 ± 0.03b	0.24 ± 0.03bc	48.71 ± 4.55a
Roots								
N	0.03 ± 0.00c	1.75 ± 0.27a	12.94 ± 1.10b	638.73 ± 102.59a	93.42 ± 12.38b	2.30 ± 0.36a	0.42 ± 0.05b	33.15 ± 4.69b
R	0.30 ± 0.04a	0.54 ± 0.04b	4.99 ± 0.51d	150.72 ± 19.49c	35.51 ± 6.25c	0.72 ± 0.10b	0.19 ± 0.02bc	11.61 ± 0.60c
RS	0.07 ± 0.01c	1.42 ± 0.29a	8.38 ± 0.88c	605.84 ± 81.73a	36.94 ± 5.01c	1.81 ± 0.31a	0.68 ± 0.11a	43.86 ± 4.63ab

Within each element, means followed by different letters are significantly different ($P < 0.05$) according to a Tukey's test. Values are means ± S.E. ($n = 5$).

Tukey's *t*-test, as found in the Statistical Package for the Social Sciences (SPSS) (SPSS Inc., Chicago, USA) version 15.0 programs for Windows XP.

3. Results and discussion

It has been frequently reported that sewage sludge application to soil produced modifications of soil properties as decreases of pH and increases of EC [47–49], which have also been evident in the present study (Table 1). Sewage sludge addition also improves soil fertility due to increased availability of some nutrients (N and P), and addition of mineral fertilizer (N) produced the same fertilizer effect as the sludge because our experimental design was performed by equalizing the amounts of main nutrients added (Table 1). Sewage sludge application also led to increases in soil DTPA-extractable heavy metals (Cd, Cu, Fe, Mn, Mo, Pb and Zn) as it has been observed previously [20,21,50–52]. Besides, the addition of organic (RS) or inorganic (N) amendments to substrate produced similar amount of heavy metals in plant tissues being significant from some elements (Cr, Cu, Fe, Ni and Zn) with respect to untreated (R) alfalfa (Table 2). Nevertheless, plants do not develop heavy metal-related symptoms during the whole experiment because tissue concentrations were always below the limits established for crops [53].

Increased soil fertility after sludge application often result in improved plant growth of legumes [18,21,54]. However, this growth stimulation effect was not evident in our study because sewage sludge-treated (RS) and unamended (R) plants exhibited similar plant dry matter (DM), leaf area and nodule DM (Table 3). It is well understood that positive response of crops to sludge application is not a general phenomenon, and could depend, at least in part, on the soil type, the sludge applied and/or the technology used for processing raw material [20]. Although other authors reported that application of organic waste products improved growth and yield of legumes compared to those amended with inorganic fertilizer [19], our data showed that nitrate-fed (N) plants exhibited higher growth and leaf area than N_2-fixing plants (R and RS) (Table 3). These results are in agreement with the idea that N from organic fertilizers often shows little effect on crop growth in the year of application, because of the slow-release characteristics of organically bound N [55].

Nodulation in RS plants was similar than in R plants suggesting that the available forms of N were not enough for supplying alfalfa needs (Table 3). Although some authors have observed that sludge application can diminish plant nodulation ability [19], it was also observed that sludge addition caused no negative effects on the formation of nodules [18,56] contrary to the common view that nodulation is suppressed in soil high in N. Currie et al. [57] hypothesize that sludge N do not repress N_2 fixation for two reasons: firstly, sludge N may not be evenly distributed within the rooting zone of plants, and secondly, sludge N is made available to the plant over the entire growing period, not just the early period when nodules are being formed. Also, it has been shown that a small dose of N can stimulate seedling growth and early nodulation such that N_2 fixation and yield are enhanced [58].

Acquisition of N_2 from symbiotic fixation requires energy and photosynthetic carbon for rhizobia metabolism and legumes are able to compensate the carbon cost of their microbial symbionts by increased photosynthesis [59,60]. Our findings agree with this affirmation because it has been shown that nitrogen-fixing alfalfa plants (R and RS) have higher net photosynthetic rates than N-fed plants (Fig. 1). Assuming that carbon cost of N_2 fixation is higher than those of nitrate uptake [61], our data support the idea that higher carbon sink strength of N_2 fixation increases the rates of photosynthesis [60]. When compared R and RS treatments it can be observed that R plants had higher photosynthetic capacity than sludge-treated plants, as indicated by higher PPFD-saturated rate of electron transport (J_{max}) and maximum rate of carboxylation by RuBPCO (V_{cmax}) (Fig. 1), and higher chlorophyll concentration (Table 3). Our data

Table 3
Growth and main plant characteristics of nitrate-fed (N) and nitrogen-fixing alfalfa plants grown in soils amended with sewage sludge (RS) or in untreated soils (R).

Measurement	N	R	RS
Plant growth			
Plant DM (g plant^{-1})	5.44 ± 0.18a	3.10 ± 0.41b	3.55 ± 0.37b
Leaf area (cm^2 plant^{-1})	600.70 ± 50.54a	253.63 ± 20.47b	260.04 ± 31.25b
Nodulation (g plant^{-1})	0 ± 0b	0.17 ± 0.03a	0.15 ± 0.02a
Plant characteristics			
Total chlorophylls (mg m^{-2})	348.76 ± 59.14b	586.97 ± 41.42a	379.96 ± 24.76b
Leaf TSP (mg g^{-1} DM)	33.88 ± 1.91a	21.57 ± 1.68b	16.51 ± 1.79b
Root TSP (mg g^{-1} DM)	16.76 ± 2.20a	7.20 ± 0.83b	10.18 ± 0.89b
Leaf nutrients			
N (mg g^{-1} DM)	55.28 ± 1.88a	48.52 ± 4.14a	35.80 ± 4.38b
P (mg g^{-1} DM)	19.10 ± 1.54a	9.61 ± 0.65b	2.23 ± 0.17c
K (mg g^{-1} DM)	28.06 ± 2.44a	21.66 ± 1.82a	20.46 ± 1.93a
Mg (mg g^{-1} DM)	7.72 ± 0.50a	6.22 ± 0.55ab	4.60 ± 0.35b

DM: dry matter; TSP: total soluble proteins. Within each file, means followed by different letters are significantly different ($p \leq 0.05$) according to a Tukey's test. Values are means ± S.E. ($n = 5$).

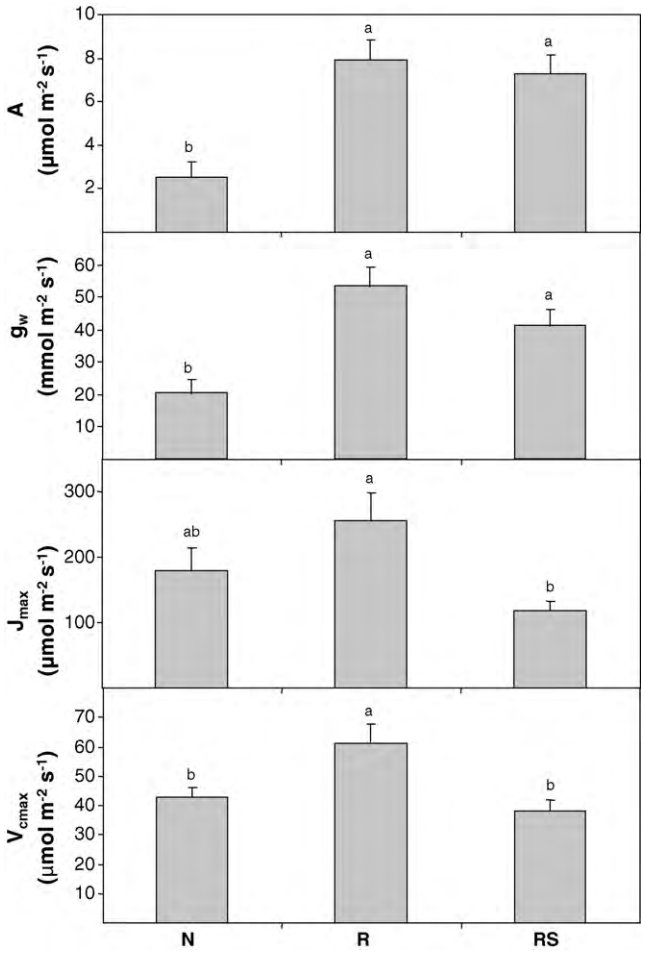

Fig. 1. Net photosynthetic rate (A), leaf conductance to water vapour (g_w), intercellular concentration of CO_2 and some photosynthetic parameters estimated from CO_2-response curves (J_{max}: the PPFD-saturated rate of electron transport; V_{cmax}: maximum rate of carboxylation by RuBPCO; TPU: rate of triose phosphate utilization) in nitrate-fed (N) and nitrogen-fixing alfalfa plants grown in soils amended with sewage sludge (RS) or in untreated soils (R). Values represent means ($n = 5$); bars indicate standard error (S.E.) of the mean. Different letters indicate significant differences ($P \leq 0.05$) treatments according to a Tukey's test.

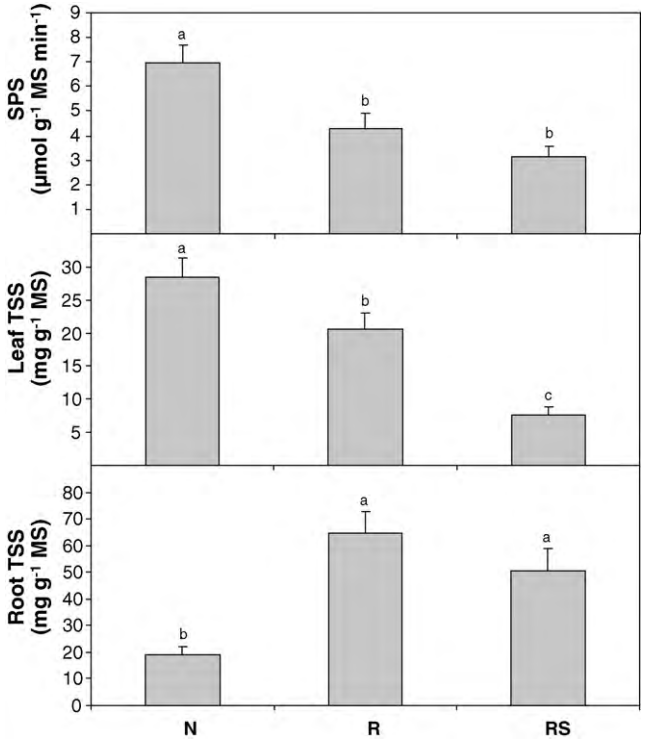

Fig. 2. Sucrose phosphate synthase (SPS) activity and leaf and root concentrations of total soluble sugars (TSS) in nitrate-fed (N) and nitrogen-fixing alfalfa plants grown in soils amended with sewage sludge (RS) or in untreated soils (R). Otherwise as in Fig. 1.

suggest that R plants achieved higher photosynthetic capacity because they had a larger demand for photoassimilates from more active nodules. Another important evidence for increased carbon sink strength due to N_2 fixation was that N-fertilized plants had increased sucrose phosphate synthase (SPS) activity that led to accumulation of more carbohydrates in leaves, despite lower rates of photosynthesis (Fig. 2). Our data agree with those from other authors that have demonstrated that increased photoassimilate demand prevents accumulation of carbohydrates in leaves [62]. In roots, R and RS plants accumulated more carbohydrates suggesting that photoassimilates were mainly translocated to nodules (Fig. 2).

Sewage sludge-treated plants did no depend exclusively on N_2 fixation because they can utilize combined N of soil as a N source, which was evidenced by stimulation of nodule NR activity (Fig. 3). For a long time it has been well known that presence of nitrate inhibits nitrogenase activity in nodules because it may cause changes in the resistance of O_2 diffusion [23]. However, this seemed not be our case because no significant alterations of nitrogenase activity and leghemoglobin content were found in RS nodules receiving nitrate from sludge (Table 4). The fact that RS plants had lower concentration of N in leaves than in R plants (Table 3) suggests a decrease in the efficiency of N_2 fixation of RS nodules that could result from the competition for reductive power between nitrogenase and NR [23] which, in alfalfa, is mostly localized in bacteroids [63,64]. Nevertheless, our data suggest other

Table 4
Main nodule characteristics and nitrogen fixation-related activity in nitrogen-fixing alfalfa plants grown in soils amended with sewage sludge (RS) or in untreated soils (R).

Measurement	R	RS
Nodule characteristics		
TSS (mg g^{-1} NDM)	22.18 ± 3.72a	18.99 ± 1.25a
TSP (mg g^{-1} NDM)	21.91 ± 2.75b	30.15 ± 2.46a
Leghemoglobin (mg g^{-1} NDM)	31.48 ± 5.00a	45.72 ± 9.41a
Nodule activity		
PEPC (μmol mg^{-1} TSP min^{-1})	7.98 ± 1.42a	1.82 ± 0.31b
MDH (mmol mg^{-1} TSP min^{-1})	0.073 ± 0.022a	0.021 ± 0.004b
Nitrogenase (μmol mg^{-1} TSP min^{-1})	0.023 ± 0.004a	0.032 ± 0.009a
GOT (μmol mg^{-1} TSP min^{-1})	6.98 ± 1.07a	0.58 ± 0.13b

NDM: nodule dry matter; TSS: total soluble sugars; TSP: total soluble proteins. Within each file, means followed by different letters are significantly different ($p \leq 0.05$) according to a Tukey's test. Values are means ± S.E. ($n = 5$).

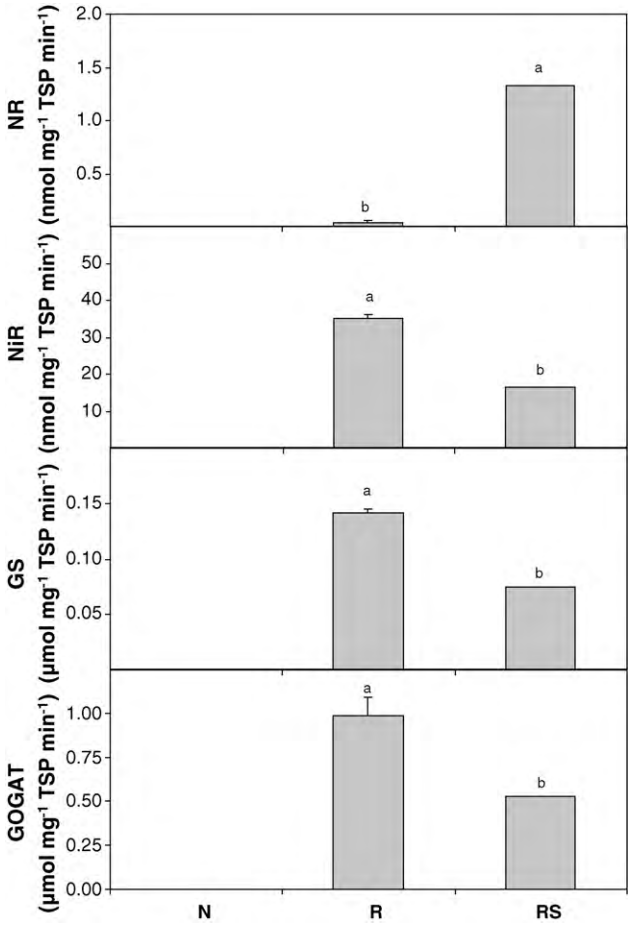

Fig. 3. N assimilation enzyme activities in nodules of nitrogen-fixing alfalfa plants grown in soils amended with sewage sludge (RS) or in untreated soils (R). Otherwise as in Fig. 1.

possibilities to explain the decrease in the efficiency of N_2 fixation in RS plants. Firstly, sewage sludge application led to the inhibition of enzymes involved in carbon (i.e. MDH and PEPC) and N (i.e. GOT) metabolisms of nodules (Table 4), indicating a lower flux of carbon and nitrogen in the nodule. Secondly, the increase in NR activity in RS nodules was not accompanied by any increase in NiR as it decreased (Fig. 3). Moreover, the ammonium released as a result of NiR or nitrogenase activities should be incorporated into glutamine and glutamate primarily by GOT and the GS/GOGAT system, which also was significantly inhibited by sludge application (Fig. 3). The inhibition of N assimilation in sludge-treated nodules could be due to oxidative stress induced by the accumulation of heavy metals in roots (Table 2), as shown previously [20]. Other authors have shown that the presence of heavy metals in the rhizosphere can reduce N assimilation due to inhibition of NR [65] and the GS/GOGAT system in nodules [66] leading to nitrite and ammonium accumulation respectively. In the same way, our data suggests that the inhibition of nodule NiR, GOT and GS/GOGAT activities in sludge-treated nodules could have induced nitrite and ammonium accumulation which, in turn, could have contributed to decreased enzyme activities of nodules [67].

4. Conclusions

This study shows that sewage sludge application to nodulated alfalfa plants affected photosynthetic capacity and N metabolism in nodules because sewage sludge-treated plants did no depend exclusively on N_2 fixation and can utilize combined N of soil. N_2-fixing plants had lower growth and sucrose phosphate synthase activity but higher photosynthesis than nitrate-fed plants because they compensated carbon cost of the rhizobia. However, sewage sludge-treated plants evidenced a loss of carbon sink strength due to N_2 fixation by means of decreased photosynthetic capacity, leaf chlorophylls and N concentration in comparison to untreated plants. In our experimental conditions, sewage sludge application did no affect nodulation ability but data provide evidence for inhibition of efficiency of N_2 fixation, which is characterized by decreased nodule enzyme activities involved in carbon and N metabolisms that may lead to accumulation of toxic N-compounds. To our knowledge, this is the first study reporting relationship between photosynthetic capacity, N assimilation and nodule metabolism in sewage sludge-treated plants.

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