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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