Effects of nitrogen source and water availability on stem carbohydrates and cellulosic bioethanol traits of alfalfa plants

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Abstract

Symbiotic association of legumes with rhizobia frequently results in higher photosynthesis and soluble carbohydrates in comparison with nitrate-fed plants, which might improve its potential for biomass conversion into bioethanol. A greenhouse experiment was conducted to examine the effects of nitrogen source and water availability on stem characteristics and on relationships between carbohydrates, phenolic metabolism activity and cell wall composition in alfalfa (*Medicago sativa* L. cv. Aragón). The experiment included three treatments: (1) plants fed with ammonium nitrate (AN); (2) plants inoculated with rhizobia (R); and (3) plants inoculated with rhizobia and amended with sewage sludge (RS). Two levels of irrigation were imposed: (1) well-watered and (2) drought stress. Under well-watered conditions, nitrogen-fixing plants have increased photosynthesis and stem fermentable carbohydrate concentrations, which result in higher potential for biomass conversion to bioethanol than in AN plants. The latter had higher lignin due to enhanced activities of phenolic metabolism-related enzymes. Under drought conditions, the potential for bioethanol conversion decreased to a similar level in all treatments. Drought-stressed nitrogen-fixing plants have high concentrations of fermentable carbohydrates and cell wall cellulose, but ammonium nitrate-fed plants produced higher plant and stem biomass, which might compensate the decreasing stem carbohydrates and cellulose concentrations.

Keywords: Cell wall; drought; *Medicago sativa* L.; nitrogen fixation; soluble carbohydrates

1. Introduction

Leguminous plants as alfalfa acquire nitrogen by assimilation of nitrate and ammonium from the soil solution, or from atmospheric nitrogen fixation through a symbiotic association with nitrogen-fixing bacteria by developing nodules in its roots. The symbiotic fixation of nitrogen constitutes a free and renewable resource, which either alone or in combination with fertilizers or organic amendments may prove to be a better solution to supply nitrogen to the cropping system [1, 2]. The plant provides sucrose to nodule host cells, where it is oxidized to dicarboxylic acids and used as energy source by the bacteroids to fix atmospheric nitrogen, which is converted into ammonium and assimilated as amides or ureides. The association rhizobia-legume results in an extra sink of additional carbon for exchange with the bacterial symbiont [3]. In fact, it has been reported that nodulated root can require up to 60% of photoassimilates produced during photosynthesis [4]. This increase in the sink: source ratio due to higher carbon costs of nitrogen fixation compared with nitrate uptake increases the rate of photosynthesis in nodulated plants because they are able to compensate demand of carbohydrates for nitrogen fixation [5, 6].

Lignocellulosic biomass is an abundant renewable resource that can be used for the production of alternative transportation fuels [7-9]. Lignocellulosic biofuel production involves collection of biomass, deconstruction of cell wall polymers into component sugars, and conversion of the carbohydrates to biofuels (fermentation) [10]. The lignocellulose originating from forage crops represents a second generation of biomass feedstock for conversion into bioethanol [11]. Alfalfa has a great potential as a bioenergy crop because of its high biomass production, perennial nature, and ability to provide its own nitrogen fertilizer due its ability to establish symbiotic relations with nitrogen-fixing soil bacteria. Thus, different studies considered alfalfa (especially stems) as a good sustainable crop for second-generation bioethanol production [12-14]. Alfalfa stems constitutes $\geq$ 50% of the total alfalfa herbage and contain greater concentrations of lignin and cellulose and less crude protein than leaves. When alfalfa is utilized as a feed for livestock, it is well understood that forage (leaves and stems) quality is higher in water-deficit-stressed plants than in nonstressed plants

and it was related to delay on plant maturity, increase of leaf to stem weight ratio and reduction of fiber concentrations [15, 16]. Thus, digestibility and crude protein increased in drought stressed plants, and cellulose concentration decreased whereas hemicellulose increased in both leaves and stems. However, when alfalfa is utilized as a feedstock for biofuel production, stem cellulose and lignin concentrations are major determining factors [17] because the key step for using stem carbohydrates for bioethanol production is the degradation of cell walls into its monomers. The recalcitrance of cell walls to hydrolysis is a major limitation for conversion of lignocellulosic biomass to bioethanol and is due to the presence of lignin [18, 19].

Bearing in mind nodulated alfalfa plants improved net photosynthesis resulting in higher soluble carbohydrates in roots in comparison with plants receiving ammonium nitrate [6] we hypothesize that symbiotic association of alfalfa with rhizobia could result in higher carbohydrates in stems, which might improve alfalfa potential for its biomass conversion into bioethanol. To our knowledge no studies have been conducted to explore this possibility under drought conditions. Therefore, the aims of the present study were to: (1) determine if the establishment of rhizobia symbiosis can modify stem traits suitable for its utilization as a feedstock for bioethanol production; (2) analyze the relationships between stem carbohydrates, phenolic metabolism activity and cell wall composition in nitrogen-fixing and nitrate-fed alfalfa subjected to drought conditions. In addition, the combined effects of nitrogen source and water availability were evaluated.

2. Materials and methods

2.1. Plant material and growth conditions

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 chemicals and placed in the Petri dishes to germinate. Four seedlings were transplanted into each pot. Plants were cultivated in an inert medium to assure nodulation of roots with a selected strain of *Sinorhizobium meliloti* as described previously [6] in a factorial design with two factors and five replicates. Briefly, 200 grams of a mixture of perlite and vermiculite (2:1, v/v) was packed into 25 x 10 cm pots (2.0 dm^3). Factors applied were type of nitrogen source and level of water. For the nitrogen source, the treatments were: (1) plants fed with ammonium nitrate (AN); 2) plants inoculated with rhizobia (R); and (3) plants inoculated with rhizobia and amended with the sewage sludge (RS) at rate of 10% (w/w), which was equivalent to approximately 30 t dry matter (DM) ha^{-1}. During the first month, seedlings of nitrogen-fixing treatments were inoculated four times (one inoculation each week) with *Sinorhizobium melioti* strain 102F34 growing on yeast extract mannitol agar. *S. meliloti* cultures in the mid-to-late exponential phase of growth were resuspended in 2% (w/v) sucrose (cell density: 10^9 cells ml^{-1}). The inoculum consisted in 3 ml of this suspension applied around roots. The sludge was added to the substrate 30 days before planting to reach equilibrium in the substrate, as recommended by Epstein [20]. Plants of nodulated treatments (R and RS) were watered twice a week with a N-free nutrient solution [21] alternating with deionized water to avoid salt accumulation in pots. Non-inoculated plants were watered throughout the experimental period with an Evans' solution supplemented with ammonium nitrate (8 mM). Thus, total amount of nitrogen added with Evans 'solution was similar to that contained in the sewage sludge.

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 the canopy. When the late vegetative stage

corresponding to growth stage 2 (stem length ≥ 31 cm) [22] was reached, half of the plants were subjected to drought conditions by withholding irrigation in a cyclic way [23]. In the well watered treatments, pots were maintained at 80% of pot capacity, which was previously assessed by determining water retained after free draining water had been allowed to pass from the holes in the bottom of the pot. The surface of the plant containers was covered with quartz stones during the experiments to avoid water loss due to evaporation. The stress level was defined as the degree of moisture stress that occurs when the plants show visible signs of wilting in the afternoon and recover turgor during the night. Plants were rewatered individually as they began to show visible signs of wilting. Rewatering was performed with nutrient solution or deionized water taking precautions to supply the different water treatments with the same amount of nutrients during drought. The drought treatment lasted approximately 25 days (4-5 cycles).

Prior to the last cycle of stress, plants were transferred to a controlled environment chamber with a day/night regime of 25°C/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. All plant measurements were taken at the end of the last cycle of drought. Then, plant organs were carefully separated, weighed and stored at -80°C until analysis. Plants were harvested at the green pod stage (70 days after sowing), corresponding to growth stage 7 (early seed pod) because it was proposed that delaying harvest until green pod would maximize both leaf and stem yield for an alfalfa biomass energy production system [24].

2.2. Sludge and substrate analyses

The sewage sludge was collected at the wastewater plant of Tudela, Navarra (northern of Spain) (latitude: 42°03'55'' N; longitude: 1°36'24'' W), which processes domestic wastewater amounting to 38,969 person equivalents per year. The most significant characteristics of the sludge were: dry mass 28.9%, volatile solids 49.8%, pH 7.4, electric conductivity 7.14 mS cm^{-1}, total organic carbon 24.5%, N Kjeldhal 2.5%, total P 1.6%, total K 0.5%, C:N ratio 9.8, Fe 1.2%, Cd 1 mg kg^{-1}, Cr 72 mg kg^{-1}, Cu 263

mg kg^{-1}, Mn 223 mg kg^{-1}, Ni 34 mg kg^{-1}, Pb 55 mg kg^{-1}, Zn 780 mg kg^{-1}. Heavy metals were below the limits permitted by EU Directive 86/278/EEC [25].

The pH of the substrate was measured in an aqueous solution (1:10 w/v) and electrical conductivity (EC) was measured at a 1:10 dilution. N content was determined in dried samples by using the Kjeldahl method. P was extracted with $NaHCO_3$ and measured by Olsen'method [26]. K was extracted with ammonium acetate and analyzed by flame spectrometry. The "plant available" metal concentrations in the substrate were determined after extraction with 0.005 M diethylenetriaminepentaacetic acid [27]. Extracts were digests and analysed for Cd, Cr, Cu, Fe, Mn, Ni, Pb and Zn using inductively coupled plasma mass spectrometry (Agilent 7500, Agilent Technologies, Spain). 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.

2.3. Plant measurements

Leaves were collected, weighed, and rehydrated for 24 h at 4°C in darkness and subsequently oven-dried at 85°C until constant mass. Leaf relative water content (RWC) was calculated as 100*(FM-DM)/(TM-DM), where FM is the fresh matter, TM is the turgid matter after tissue rehydration, and DM is dry matter. These measurements were made at predawn. Three hours after the onset of the photoperiod, net photosynthetic rate (A) and leaf conductance to water vapour (g_w) were measured at ambient CO_2 (350 µmol mol^{-1}), PPF of 1000 µmol m^{-2} s^{-1}, 80% relative humidity and 25°C with a portable photosynthesis system (GFS-3000, Walz, Effeltrich, Germany). Starch analyses were carried out with 0.1 g of fresh stems that were ground in an ice-cold mortar and pestle containing potassium phosphate buffer (50 mM, pH 7.0). The homogenates were filtered through four layers of cheesecloth and centrifuged at 3,500 * g at 4°C for 15 min. The pellet was used to determine starch that was estimated after iodine reaction [28]. Samples were dissolved in 5 ml 1M KOH and 5 ml distilled water. Next 1 ml of this solution was neutralized with 5 ml 0.1 M HCl, 0.5 ml of

iodine reagent was added and the volume made up to 50 ml with distilled water. After 15 min at room temperature, absorbance was measured at 630 nm.

After harvest, the main morphological traits as height plant, number and diameter of stems were recorded. Plant and nodule DM were determined after drying samples at 85°C to constant mass.

2.4. Determination of soluble carbohydrates by HPLC

Samples (0.1 g DM of stems) for soluble carbohydrate analyses were freeze crushed and polar compounds were extracted into 1 ml aqueous 80% ethanol at 80°C, in three steps, each lasting 20 min as described by Jiménez et al. [29]. The mixture of each step was centrifuged for 5 min at 4,800 * g and slurries were pooled. Ethanol was evaporated under vacuum in a speed vac system (Thermo Fisher Scientific Inc., Waltham, MA, USA) and dry extracts were solubilized in 500 µl double-distilled water. The soluble carbohydrates of the samples were purified using about 3.5 g g^{-1} plant material ion exchange resins (Bio-Rad AG 50 W-X8 Resin 200-400 mesh hydrogen form, Bio-Rad AG 1-X4 Resin 200-400 chloride form). The samples were concentrated to 200 µl, filtered through a 0.22 µm filter and 20 µl was injected and analyzed by high-performance liquid chromatography (HPLC), using Ca-column (Aminex HPX-87C 300 mm x 7,8 mm column Bio-Rad) flushed with 0,6 ml min^{-1} double distilled water at 85°C with a refractive index detector (Waters 2410). Concentrations of the main carbohydrates, raffinose, sucrose, galactinol, glucose, xylose, fructose and sorbitol were calculated for each sample using manitol as internal standard since it was not present in alfalfa samples. Carbohydrate quantification was performed with the Empower Login software, Waters (Milford, Mass, USA) using standards of analytical grade from Panreac Quimica S.A. (Barcelona, Spain) and Sigma-Aldrich (Schnelldorf, Germany). Concentrations of carbohydrates were expressed as mg kg^{-1} DM.

2.5. Stem fiber analysis

Fresh stems were oven dried at 60°C for 48 h and ground in a Fritsch pulverisette mill (Fritsch GmbH, Idar-Oberstein, Germany) through a 1 mm screen. Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) content were determined according to Van Soest et al. [30] using the Ankrom Filter Bag method. NDF was used as an estimate of cell wall (CW) concentration. Cellulose was calculated as ADF minus ADL and hemicellulose as the difference between NDF and ADF values after removing ash. Lignin, hemicellulose, and cellulose were expressed as a proportion of CW [15].

2.6. Phenolic metabolism-related enzymes

Measurements of phenolic metabolism-related enzymes were performed following procedures described by Kováčik et al. [31]. For determination of shikimate dehydrogenase (SKDH, EC 1.1.1.25) and cinnamyl alcohol dehydrogenase (CAD, EC 1.1.1.195) activities, stem samples were ground in an ice-cold mortar and pestle containing potassium phosphate buffer 50 mM (pH 7.0). The homogenates were filtered through four layers of cheesecloth and centrifuged at 15,000 * g for 15 min and the supernatant was used as enzymatic extract. The SKDH activity was assayed in 0.1 M Tris-HCl buffer (pH 9) and the reaction mixture consisted of 1.45 ml of 2 mM shikimic acid, 1.45 ml of 0.5 mM NADP and 0.1 ml of enzyme extract. SKDH activity was assayed spectrophotometrically by NADP reduction at 340 nm and activity was calculated using molar absorptivity 6.22 mM^{-1} cm^{-1}. The CAD activity was assayed in 0.1 M Tris-HCl buffer (pH 8.8) and the reaction mixture consisted of 1.45 ml of 1 mM coniferyl alcohol, 1.45 ml of 1 mM NADP and 0.1 ml of enzyme extract. Measurements and calculations were done as for SKDH. Blanks for enzyme assays were carried out exactly as the samples but without NADP.

2.7. Statistical analysis

Data from substrate analyses were subjected to an analysis of variance (ANOVA). Data from plant determinations were subjected to a two-factor analysis of variance (ANOVA). Variance was

related to the main treatments (nitrogen and water treatment) and to the interaction between them. Means ± standard errors (S.E.) were calculated and, when the F ratio was significant, least significant differences were evaluated by a Tukey's t-test, as available in the Statistical Package for the Social Sciences (SPSS) (SPSS Inc., Chicago, USA) version 15.0 program for Windows XP. All values shown in the figures are means $\pm$ S.E. The significance of regression equations was also evaluated using this program.

3. Results

3.1. Substrate properties and heavy metals

The main properties of substrate are shown in Table 1, and it revealed that the addition of sludge to the substrate increased pH and EC when compared with untreated soils (R). Although the sludge meets the standards of heavy metal contents, application of this significantly increased substrate availability of most of the heavy metals analyzed (Cu, Fe, Mn, Pb and Zn). In plants, drought leads to significant accumulation of Cu, Pb and Zn in roots of sewage sludge-treated plants (38 mg kg^{-1} DM of Cu, 2.3 mg kg^{-1} DM of Pb and 120 mg kg^{-1} DM of Zn). No significant differences between treatments were detected for the rest of heavy metals analyzed (Cd, Cr, Fe, Mn and Ni) (data not shown).

3.2. Plant growth and gas exchange rates

The main plant growth characteristics are shown in Table 2. Plant dry matter (DM) was higher in AN than in nitrogen-fixing alfalfa grown in substrate amended with sewage sludge (RS) or untreated soil (R) both, in well-watered and in drought stressed conditions. Water deficit had a significant impact in plant DM of all treatments. On the other hand, nodulation was better in R than in RS plants under well-watered conditions but nodule growth was decreased to similar values in plants subjected to water deficit. However, this decrease was less pronounced in plants of RS treatment reaching 45% of values quantified in well- watered plants, whereas it decreased to ca. 35% in R plants. Under well-watered conditions, stem production and its height, diameter and number were significantly improved by AN treatment. However, water restriction reduced significantly stem DM and stem height, diameter and number in all treatments. Two-way ANOVA analysis showed significant interaction between nitrogen and water level in for stem height (Table 2).

Drought treatment reduced leaf RWC similarly in all treatments assayed (Table 3). In well-watered conditions, nitrate-fed plants (AN) had lower rates of net photosynthesis (A) and leaf conductance to water vapour (g_w) than nitrogen-fixing plants. Under drought conditions, A and g_w

was significantly reduced in R and RS plants, whereas less changes were found in AN treatment. This differential pattern was emphasized by two-way ANOVA because there was significant interaction between nitrogen source and water treatment for photosynthetic assimilation rate.

3.3. Soluble carbohydrates and estimated bioethanol yields

The nitrogen source affected significantly concentrations of total soluble carbohydrates in alfalfa stems, which was higher in nodulated (R and RS) than in AN plants (Table 3). The main soluble carbohydrates identified in alfalfa were sucrose, raffinose, galactinol, glucose, xylose, fructose and sorbitol (Table 4). Under well-watered conditions, nitrogen-fixing plants contained higher concentrations of sucrose, galactinol and glucose than AN plants. Although water stress had not significant effect on concentrations of total carbohydrates, individual carbohydrate composition differed according to water level and nitrogen regime applied. The type of nitrogen source resulted in significant changes in the concentration of raffinose, sucrose, galactinol, glucose, fructose and sorbitol. Water level affected carbohydrate composition of different treatments. Thus, in the AN treatment raffinose, galactinol and fructose decreased in drought-stressed plants, but sucrose, xylose and sorbitol concentrations increased; in the R treatment drought reduced concentrations of sucrose, glucose and sorbitol, but increased concentration of xylose. Finally, in the RS treatment raffinose and sucrose was declined under water deficit, but xylose and fructose concentrations increased. These differential patterns were shown in two-factorial ANOVA that showed significant interactions between both factors (nitrogen and water) for sucrose, fructose and sorbitol (Table 4). On the other hand, imposition of water stress markedly decreased stem concentration of starch in all treatments. The estimated bioethanol yields from non-structural carbohydrates calculated with the formulas reported by Vogel et al. [32] are shown in Figure 1. Under well watered conditions, bioethanol yield per plant was higher in nodulated alfalfa (R and RS) than in AN plants, but drought conditions results in reduced bioethanol yield to similar levels in all treatments. Thus, nitrogen

source, water availability and the combination of both factors significantly affected the estimated bioethanol yields.

3.4. Cell wall components and phenolic metabolism-related activities

The cell wall components of alfalfa stems are shown in Table 5. Under well-watered conditions concentration of cell wall (CW) was significantly higher in nitrogen-fixing grown in untreated soils (R) than in ammonium nitrate-fed (AN) plants. The stems of water-stressed AN plants exhibited lower CW, cellulose and lignin concentrations, and higher hemicellulose in comparison to unstressed condition. By contrast, no changes in CW components were detected in R and in RS plants. The differential pattern of CW components was reinforced by two-way ANOVA showing significant interactions between nitrogen source and water treatment for hemicellulose and lignin. Combining all measurements data revealed that improved bioethanol yield from alfalfa stems was significantly correlated with higher concentrations of CW ($r = 0.64$, $P < 0.001$), which also had increased amount of cellulose ($r = 0.65$, $P < 0.001$) (Figure 2).

Some phenolic metabolism-related enzyme activities in alfalfa stems are shown in Figure 3. Under well-watered conditions, the highest SKDH and CAD activities were detected in AN plants, but upon drought conditions, both phenolic enzyme activities strongly decreased. The R plants exhibited lower CAD and SKDH activities than AN, but drought induced a significant decrease only for SKDH. Under water deficit, sewage sludge-treated (RS) plants showed a marked reduction in CAD but not in SKDH activity, which was maintained as in unstressed situation. Data showed that stem lignin content was significantly and positively correlated with SKDH activity ($r = 0.51$, $P < 0.01$) and negatively related to concentrations of soluble carbohydrates ($r = -0.77$, $P < 0.001$) (Figure 4).

4. Discussion

The present study raises the influence of nitrogen nutrition and water supply on the carbohydrate and cell wall composition of alfalfa stems to estimate its impact on a feedstock suitable for bioethanol production. Several approaches have aimed to increase the efficiency of biomass conversion to bioethanol: (1) to reduce the lignin content of plants by developing crop varieties with less lignin [18, 33-35]; (2) to produce crops that self-produce cellulose enzymes for cellulose degradation and ligninase enzymes for lignin degradation [7]; (3) to obtain plants that have increased cellulose [11]; and (4) to develop crops that have overall biomass yield and/or increased fermentable carbohydrates [36-37]. The last approach was addressed in our study, and it was obtained that nitrogen source affected the plant DM in favor of ammonium nitrate-fed (AN) plants whatever the water treatment (Table 2). However, nitrogen-fixing plants have higher concentrations of soluble carbohydrates than nitrate-fed plants (Table 3). Having into account that soluble carbohydrate amounts depend primarily on photosynthesis, results can be explained by the fact that nitrogen-fixing plants had higher photosynthesis than AN plants (Table 3). Acquisition of nitrogen from symbiotic fixation requires energy and photosynthetic carbon for rhizobia metabolism and legumes can compensate this carbon cost by increased photosynthesis [6, 38]. When calculated the total amounts of soluble sugars in stems, the net yields for AN treatment became similar to R (approximately 77 mg $plant^{-1}$) but was lower than in RS plants (107 mg $plant^{-1}$), indicating that increased biomass could only partially compensate lower carbohydrate concentrations. Therefore, nitrogen-fixing plants have high potential for bioethanol production (Figure 1). Although, drought provoked similar stem biomass in all treatments, the larger concentration of monosaccharides (glucose and fructose) in R and RS plants might be advantageous for a more efficient bioethanol production because glucose and xylose can be converted at higher yields to ethanol than most other carbohydrates [14, 39]. Regarding carbohydrate composition, our results indicated that plants of the three treatments performed differently upon drought conditions (Table 4). Thus, in drought-stressed AN plants, amounts of glucose and fructose accounted 10% of total carbohydrates whereas in

nodulated plants, the contribution of glucose and fructose to total carbohydrates increased to 25% and 27% in R and RS, respectively.

Some studies have reported that plants with the highest carbohydrate levels had the least lignin, reflecting compensation of the reduction in lignin level on a mass balance basis [19]. In our case, the significant relationship found between carbohydrates and lignin concentrations ($r = -0.77$, $P < 0.001$) supported this idea (Figure 4). Well-watered ammonium nitrate-fed (AN) plants had higher lignin than R and RS plants (Table 5) and high lignin coincided with enhanced activities of phenolic metabolism-related enzymes as SKDH and CAD (Figure 3). The SKDH, a member of shikimate pathway, is one of enzymes converting simple carbohydrates into aromatic amino acids as phenylalanine, whereas enhanced CAD activity is considered as a basic indicator of increased lignin biosynthesis [40]. In our case lignin content was significantly correlated with SKDH activity ($r = 0.51$, $P < 0.01$) (Figure 4) but no with CAD activity (data not shown). Relationship between lignification and CAD activity was not always evident as showed in other studies where CAD down-regulation did not result in a decrease in the amount of lignin in alfalfa [41]. More recently it has been shown that reduction of the CAD gene expression in lignifying tissues did not show any reduction in lignin [9].

Cell wall (CW) components have a major effect on the ability to convert biomass to bioethanol via enzymatic hydrolysis and fermentation [42]. The present study showed that AN plants have lower CW concentration in stems than nitrogen-fixing plants (Table 5) suggesting that much of the carbon fixed is used to support levels of sugars rather than for CW development [15]. Drought imposition decreased CW concentration in AN and R treatments, which was often associated with reduced plant size under water stress that may diminish the need for structural components for support [15, 16]. It is well understood that stem cellulose and lignin concentrations are major determining factors of alfalfa forage quality and environmental factors such as drought could produce relevant changes on both traits, especially in stem cellulose [17, 43]. In our study, the response of CW traits to drought varied depending of nutritional treatment applied. Thus, AN plants showed decreased lignin and cellulose and increased hemicelluloses, but in R plants, the main effect of water deficit was the

reduction in CW concentration (Table 5). Drought-stressed nodulated plants (R and RS) had higher cellulose than AN plants, suggesting that biomass from nitrogen-fixing plants might be converted more efficiently into bioethanol (Figure 1). However, in drought-stressed AN plants, lower efficiency in biomass conversion into bioethanol could be compensated by high plant biomass (Table 2).

Organic amendments as sewage sludge have been utilized as an effective fertilizer to crops that also improves soil physical and nutritional properties [44, 45]. However, it contains various toxic compounds especially heavy metals which cause harm to soil-plant system and further might pose a serious risk to animal and human health [46]. As expected, application of sewage sludge led to increase of available heavy metals in substrate (Table 1), which were accumulated in roots of drought-stressed plants (data not shown), although plants do not develop heavy metal-related symptoms during the whole experiment. The present study also demonstrated that, under drought conditions, sewage sludge addition to nitrogen-fixing alfalfa can be a good option for residue reutilization because the estimation of bioethanol yields showed that both nodulated treatments (R and RS) performed in a similar manner, having higher potential for biomass conversion to bioethanol than ammonium nitrate-fed plants (Figure 1). The potential use of sludge sewage to fertilize bioenergy crops provide a mean of recycling the organic waste at low costs without harming the crop and avoiding potential risks to animal and human health.

5. Conclusions

This study shows that in well-watered conditions, nitrogen-fixing alfalfa increased photosynthesis providing additional carbon for sustain bacterial symbiont that could result in higher potential for biomass conversion to bioethanol than mineral fertilized plants. Under drought conditions, estimated bioethanol production was similar in both types of plants. Water-stressed nodulated plants have high concentrations of fermentable carbohydrates and cell wall cellulose, but ammonium nitrate-fertilized plants produced higher plant biomass, which might compensate the decreasing stem carbohydrates and cellulose concentrations. This study provides new data supporting the influence of nitrogen source and water availability on biomass and stem traits of interest for increasing efficiency of biomass conversion to bioethanol in alfalfa.

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Table 1.

Some substrate properties in the mixture of perlite and vermiculite (2:1, v/v) in substrates fed with mineral-fertilizer (AN), amended with sewage-sludge (RS) and in untreated soils (R).

Measurement	AN	R	RS
pH	6.70 c	7.36 b	7.72 a
EC (mS cm^{-1})	1.92 a	0.84 b	1.35 a
$N_{Kjeldhal}$ (g 100 g^{-1})	0.16 a	0.01 b	0.18 a
P_{Olsen} (mg kg^{-1})	177.50 a	179.85 a	136.67 a
$K_{available}$ (mg g^{-1})	91.24 a	106.75 a	74.23 a
$Fe_{available}$ (mg kg^{-1})	3.56 b	2.55 b	14.87 a
$Cd_{available}$ (mg kg^{-1})	ND	ND	ND
$Cr_{available}$ (mg kg^{-1})	ND	ND	ND
$Cu_{available}$ (mg kg^{-1})	0.39 b	0.33 b	2.44 a
$Mn_{available}$ (mg kg^{-1})	3.41ab	2.37 b	4.13 a
$Ni_{available}$ (mg kg^{-1})	0.36 a	0.04 c	0.20 b
$Pb_{available}$ (mg kg^{-1})	ND	0.12 b	0.44 a
$Zn_{available}$ (mg kg^{-1})	1.16 b	1.71 b	7.51 a

EC: electric conductivity. Within each line, the means followed by different letter are significantly different ($P<0.05$) according to a Tukey's test. Values are means (n=5). N.D. not detected.

Table 2.

Plant growth and main stem characteristics of ammonium nitrate-fed (AN) and nitrogen-fixing alfalfa plants harvested 70 days after sowing grown in substrates amended with sewage sludge (RS) or in untreated soils (R) under well-watered (WW) or soil water deficit (D) conditions.

Treatment	Water level	Plant DM (g plant^{-1})	Nodule DM (g plant^{-1})	Stem DM (g plant^{-1})	Stem height (cm)	Stem diameter (mm)	Stem number
AN	WW	6.21 a	---	2.49 a	68.70 a	2.01 a	5.23 a
	D	4.38 b	---	1.09 bc	51.35 c	1.67 b	3.53 bc
R	WW	3.71 bc	0.099 a	1.42 b	47.38 d	1.54 b	4.18 b
	D	2.71 c	0.036 c	0.55 c	43.20 e	1.23 c	2.73 cd
RS	WW	3.86 bc	0.060 b	1.56 b	55.00 b	1.62 b	4.25 b
	D	2.92 c	0.026 c	0.59 c	44.80 de	1.44 bc	2.48 d
Nitrogen source (N)		***	***	***	***	***	***
Water level (W)		***	***	***	***	***	***
NxW		ns†	*	ns	***	ns	ns

DM: dry matter. Two-way ANOVA analysis was performed for linear model on raw data. Comparison means by Tukey's test ($P \leq 0.05$) were shown for the significant interaction between nitrogen source (N) and water treatment (W). Within each column data followed by the same letter are not significantly different. Values are means (n=5.

† Not significant.

* Significance at 0.05 probability level.

*** Significance at 0.001 probability level.

Table 3

Leaf water status and gas exchange parameters, and stem carbohydrates of ammonium nitrate-fed (AN) and nitrogen-fixing alfalfa plants harvested 70 days after sowing grown in substrates amended with sewage sludge (RS) or in untreated soils (R) under well-watered (WW) or soil water deficit (D) conditions.

Treatment	Water level	Leaf RWC (%)	A ($\mu mol\ m^{-2}\ s^{-1}$)	g_w ($mmol\ m^{-2}\ s^{-1}$)	Total soluble sugars ($g\ kg^{-1}$ DM)	Starch ($g\ kg^{-1}$ DM)
AN	WW	88.16 a	4.96 b	96.26 b	34.12 b	3.69 a
	D	60.36 b	3.29 bc	38.55 c	43.78 b	0.66 b
R	WW	88.81 a	8.18 a	140.93 a	55.24 a	3.43 a
	D	60.70 b	2.13 c	80.52 b	58.73 a	1.06 b
RS	WW	91.13 a	8.48 a	131.20 a	59.59 a	2.96 a
	D	64.50 b	3.95 bc	31.83 c	61.18 a	1.72 b
Nitrogen source (N)		ns†	**	**	***	ns
Water level (W)		***	***	***	*	***
NxW		ns	**	ns	ns	*

RWC: relative water content. Two-way ANOVA analysis was performed for linear model on raw data. Comparison means by Tukey's test ($P \leq 0.05$) were shown for the significant interaction between nitrogen source (N) and water treatment (W). Within each column data followed by the same letter are not significantly different. Values are means (n=5).

* Significance at 0.05 probability level.

** Significance at 0.01 probability level.

*** Significance at 0.001 probability level.

† Not significant.

Table 4.

Soluble carbohydrate characterization of the stems of ammonium nitrate-fed (AN) and nitrogen-fixing alfalfa plants harvested 70 days after sowing grown in substrates amended with sewage sludge (RS) or in untreated soils (R) under well-watered (WW) or soil water deficit (D) conditions.

Treatment	Water level	Sucrose (g kg^{-1} DM)	Raffinose (g kg^{-1} DM)	Galactinol (g kg^{-1} DM)	Glucose (g kg^{-1} DM)	Xylose (g kg^{-1} DM)	Fructose (g kg^{-1} DM)	Sorbitol (g kg^{-1} DM)
AN	WW	6.80 d	0.93 b	2.17 b	2.75 c	15.80 b	6.61 c	0.05 bc
	D	13.08 c	0.43 c	1.37 c	1.83 c	23.95 a	2.65 d	0.10 a
R	WW	21.27 ab	1.45 ab	3.46 a	5.51 a	15.24 b	8.04 bc	0.08 ab
	D	14.39 c	0.99 b	2.71 ab	4.05 b	25.70 a	10.85 ab	0.04 c
RS	WW	23.38 a	1.81 a	3.23 a	5.17 ab	17.73 b	8.41 bc	0.05 bc
	D	15.66 b	1.01 b	2.65 ab	4.59 ab	24.87 a	12.37 a	0.04 c
Nitrogen source (N)		***	***	***	***	ns	***	**
Water level (W)		*	***	***	**	***	ns	ns
NxW		***	ns†	ns	ns	ns	***	***

DM: dry matter. Two-way ANOVA analysis was performed for linear model on raw data. Comparison means by Tukey's test ($P \leq 0.05$) were shown for the significant interaction between nitrogen source (N) and water treatment (W). Within each column data followed by the same letter are not significantly different. Values are means (n=5).

* Significance at 0.05 probability level.

** Significance at 0.01 probability level.

*** Significance at 0.001 probability level.

† Not significant.

Table 5.

Cell wall components of stems of ammonium nitrate-fed (AN) and nitrogen-fixing alfalfa plants harvested 70 days after sowing grown in substrates amended with sewage sludge (RS) or in untreated soils (R) under well-watered (WW) or soil water deficit (D) conditions.

Treatment	Water level	CW (g kg^{-1} DM)	Cellulose (g kg^{-1} CW DM)	Hemicelulose (g kg^{-1} CW DM)	Lignin (g kg^{-1} CW DM)
AN	WW	565.26 b	542.71 a	276.92 c	180.37 a
	D	511.91 c	511.57 b	343.44 a	144.99 b
R	WW	610.61 a	553.56 a	301.55 bc	144.89 b
	D	556.32 b	537.52 a	314.89 ab	147.58 b
RS	WW	586.15 ab	553.43 a	305.70 bc	140.87 b
	D	559.25 b	540.13 a	314.34 ab	145.53 b
Nitrogen source (N)		***	**	ns	**
Water level (W)		***	***	***	ns
NxW		ns†	ns	***	**

CW: cell wall; DM: dry matter. Two-way ANOVA analysis was performed for linear model on raw data. Comparison means by Tukey's test ($P \leq 0.05$) were shown for the significant interaction between nitrogen source (N) and water treatment (W). Within each column data followed by the same letter are not significantly different. Values are means (n=5).

** Significance at 0.01 probability level.

*** Significance at 0.001 probability level.

† Not significant.

FIGURE LEGENDS

Figure 1. Estimated bioethanol yield from non-structural carbohydrates in stems of ammonium nitrate-fed (AN) and nitrogen-fixing alfalfa plants grown in substrate amended with sewage sludge (RS) or in untreated substrate (R) under well-watered or soil water deficit (Drought) conditions. Values represent means (n=5) and bars indicate standard error (S.E.) of the mean. Different letters indicate significant differences ($P \leq 0.05$) between treatments according to a Tukey's test. Two-way ANOVA analysis to evaluate the nitrogen source (N), water treatment (W) and interaction (NxW) effects was performed. Significance: *** $P \leq 0.001$.

Figure 2. Relationships between estimated bioethanol yield and cell wall (A), and cellulose (B) concentrations in stems of ammonium nitrate-fed (AN) and nitrogen-fixing alfalfa plants grown in substrate amended with sewage sludge (RS) or in untreated substrate (R) subjected to different water treatments. Straight lines correspond to the regression lines fitted for the joint data of all determinations. The corresponding equations were: (A) $y = 0.0004x^2 – 0.21x + 25.68$ ($r = 0.64$, $P < 0.001$); (B) $y = -0.0028x^2 – 2.66x + 648.6$ ($r = 0.65$, $P < 0.001$).

Figure 3. Phenolic metabolism-related enzyme activities (shikimate dehydrogenase, SKDH and cinnamyl alcohol dehydrogenase, CAD) measured in stems of ammonium nitrate-fed (AN) and nitrogen-fixing alfalfa plants grown in substrate amended with sewage sludge (RS) or in untreated substrate (R) under well-watered or soil water deficit (Drought) conditions. Values represent means (n=5) and bars indicate standard error (S.E.) of the mean. Different letters indicate significant differences ($P \leq 0.05$) between treatments according to a Tukey's test. Two-way ANOVA analysis to evaluate the nitrogen source (N), water treatment (W) and interaction (NxW) effects was performed. Significance: *** $P \leq 0.001$; ** $P \leq 0.01$.

Figure 4. Relationships between soluble carbohydrate and lignin concentrations (A), and between lignin and shikimate dehydrogenase (SKDH) activity (B) in stems of ammonium nitrate-fed (AN) and nitrogen-fixing alfalfa plants grown in substrate amended with sewage sludge (RS) or in untreated substrate (R) subjected to different water treatments. Straight lines correspond to the regression lines fitted for the joint data of all determinations. The corresponding equations were: (A) $y = 606979x^{-1,87}$ ($r = -0.77$, $P < 0.001$); (B) $y = 6*10^{-6}x^2 + 0.017x + 138,03$ ($r = 0.51$, $P < 0.01$).

Figure 1.

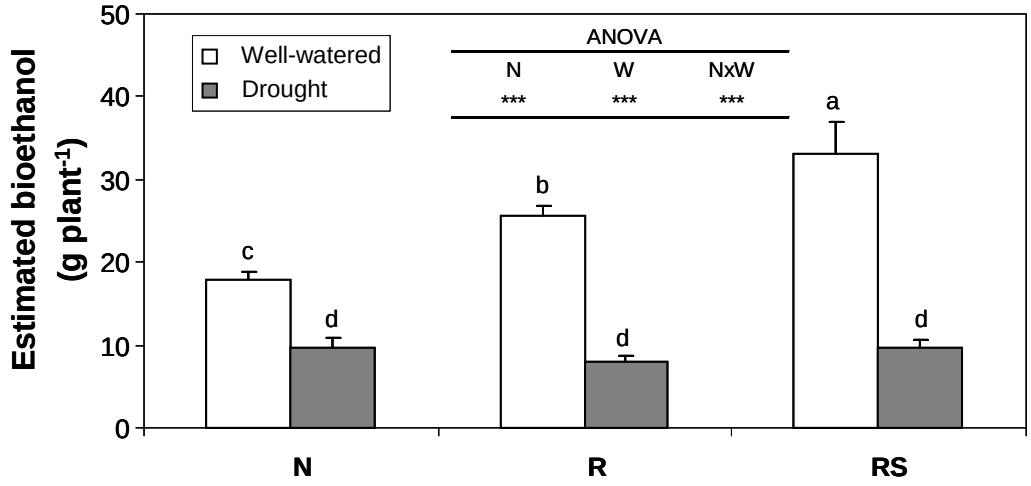

Estimated bioethanol (g plant-1)
50
40
30
20
10
0
Well-watered
Drought
ANOVA
N
W
NxW
***
***
***
c
d
b
d
a
d
N
R
RS

Figure 2

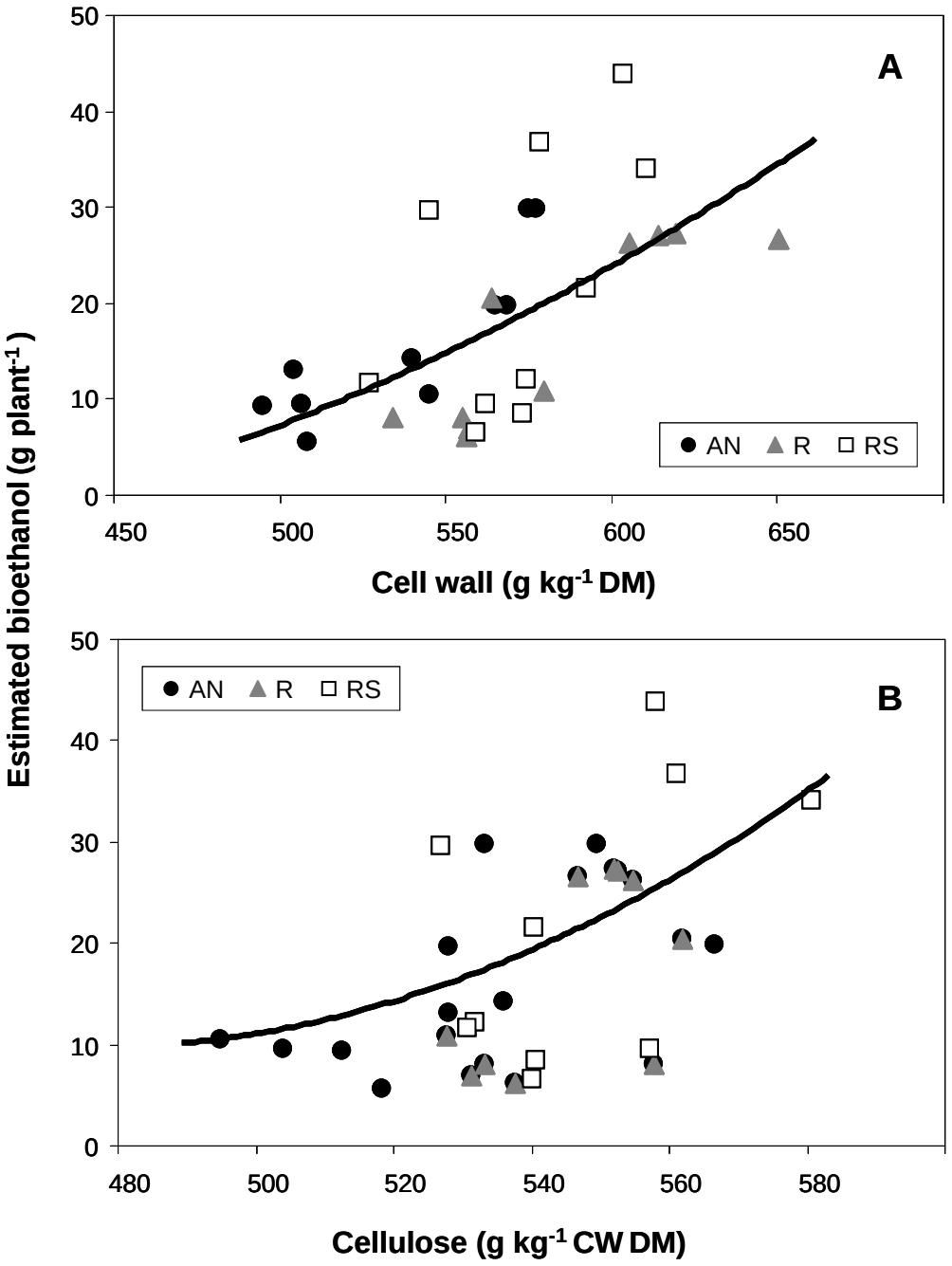

A
B
AN
R
RS
Estimated bioethanol (g plant-1)
Cell wall (g kg-1 DM)
Cellulose (g kg-1 CW DM)
0
10
20
30
40
50
450
500
550
600
650
480
500
520
540
560
580

Figure 3

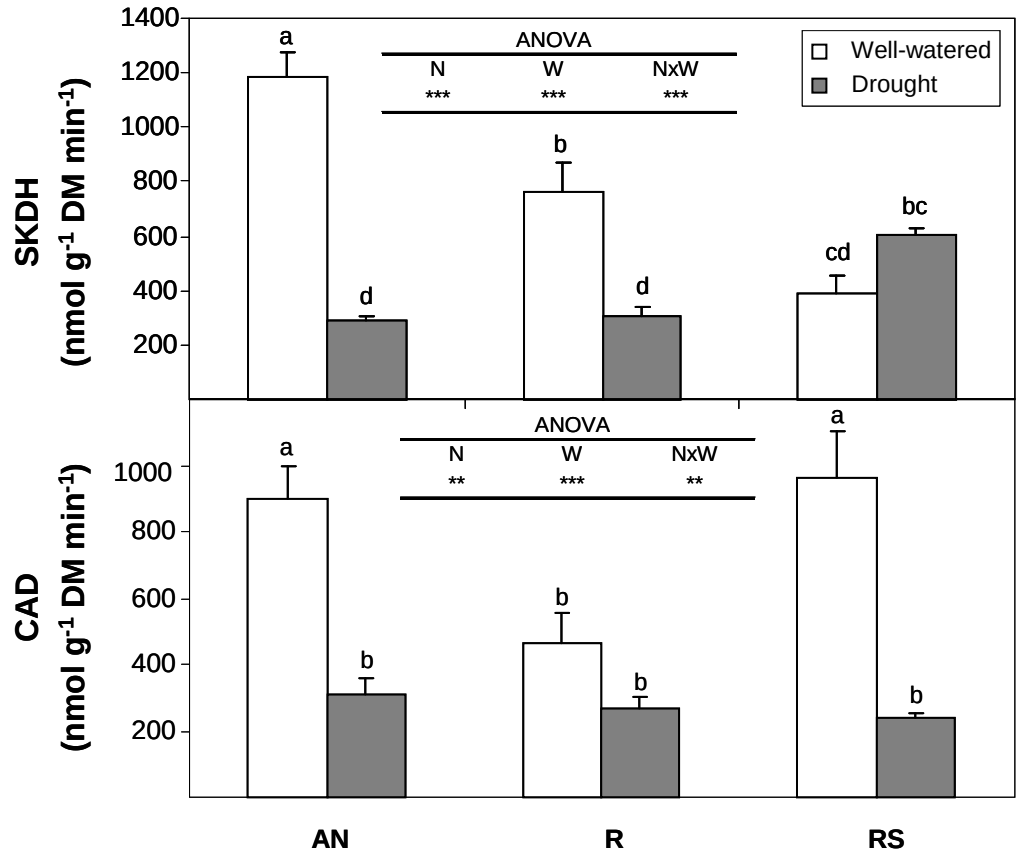
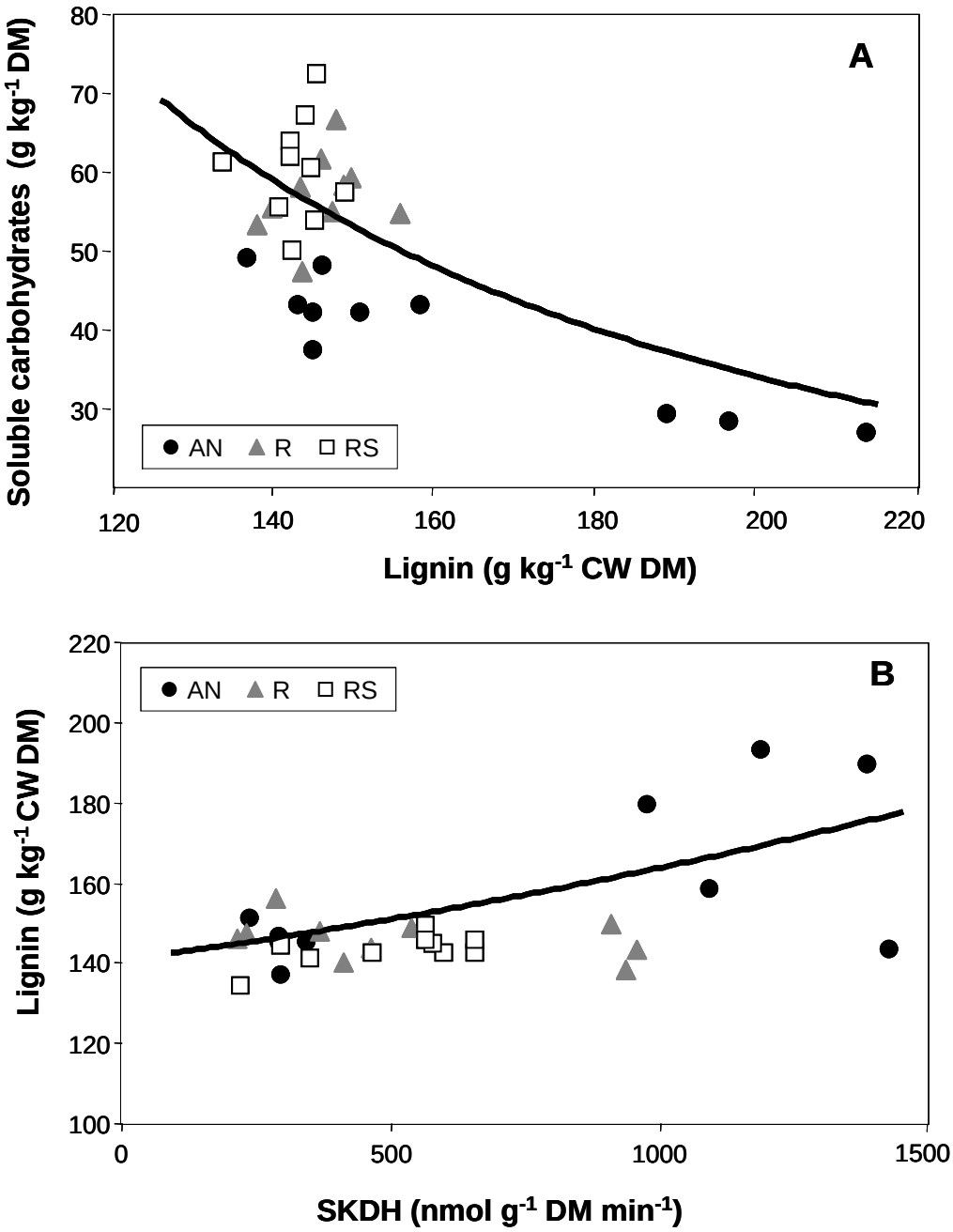

SKDH
(nmol g-1 DM min-1)
1400
1200
1000
800
600
400
200
a
d
b
d
cd
bc
ANOVA
N
W
NxW
***
***
***
Well-watered
Drought
CAD
(nmol g-1 DM min-1)
1000
800
600
400
200
a
b
b
b
a
b
ANOVA
N
W
NxW
**
***
**
AN
R
RS

Figure 4


A
Soluble carbohydrates (g kg-1 DM)
Lignin (g kg-1 CW DM)
AN
R
RS
80
70
60
50
40
30
120
140
160
180
200
220
B
Lignin (g kg-1 CW DM)
SKDH (nmol g-1 DM min-1)
AN
R
RS
220
200
180
160
140
120
100
0
500
1000
1500