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BRIEF COMMUNICATION

Responses of the fructose-1,6-bisphosphatase and glutamate dehydrogenase activities of alfalfa to boron, gypsum, and limestone amendments of soil

G.O. OSUJI[*,+], V.A. HABY[**], D.J. CHESSMAN[***], and A.T. LEONARD[**]

Cooperative Agricultural Research Center, P.O. Box 4079, Prairie View, TX 77446, USA[*]
Texas Agricultural Experiment Station, P.O. Box 200, Overton, TX 75684, USA[**]
Soil Testing Laboratory, Box 13000 SFA Station, Nacogdoches, TX 75962, USA[***]

Abstract

Plants cultivated on acid soils that contain toxic levels of Al^{3+} usually produce low yields. A multi-factorial treatment of gypsum (G), boron (B), and limestone (Lm) was applied to such soil in order to determine the biochemical basis of the best management plan for ameliorating the soil acidity for sustainable growth of alfalfa. The alfalfa shoots were subjected to analysis for hexose, protein, nucleotide, and chlorophyll (Chl) contents, fructose 1,6-bisphosphatase (FBPase) activity, and the RNA synthetic activity of glutamate dehydrogenase (GDH). Hexose and protein contents of control alfalfa without B and G, but with Lm (672 g m^{-2}) amendment were 0.87 and 38.30 g, respectively, per kg shoot. Increasing the G doses at fixed moderate doses of 0.15 and 0.30 g m^{-2} B decreased the FBPase activity by ~53 and ~31 %, respectively. However, increasing the B doses at higher fixed G (1 kg m^{-2} = $G_{1.0}$) increased the FBPase activity by ~91 % thus indicating that G_1 optimized the saccharide metabolism by neutralizing the soil acidity. In the absence of B, increasing the G doses also maximized the hexose and Chl contents, but minimized the nucleotide amount. In the absence of G, increasing the B doses maximized the RNA synthetic activity of GDH, but lowered the hexose and Chl contents as well as the FBPase activity without affecting the protein contents, thereby permitting the selection of B (0.45 g m^{-2}) with Lm as the best amendment for the sustainable growth of alfalfa. Treatment with 0.45 g B and 0.5 kg G (= $G_{0.5}$) induced the strongest B–Ca antagonism by maximizing the hexose and Chl contents but severely suppressing the FBPase activity and the RNA synthetic activity of GDH. Therefore, the coordinate optimization of saccharide metabolism through the G-dependent neutralization of soil acidity, and of RNA metabolism through the B-dependent detoxification of Al^{3+} are the biochemical options for the mitigation of the adverse effects of soil acidity for the optimization of sustainable alfalfa production.

Additional key words: Al toxicity; Ca–B antagonism; Calvin cycle; chlorophyll; free nucleotides; hexose; *Medicago sativa*; nucleotides; proteins.

Only a few studies have been conducted on the responses of photosynthesis to the combination of gypsum (G), boron (B), and/or limestone (Lm) amendments of soil (Brown and Hu 1997, Mahboobi and Yucel 2000, Dixit *et al*. 2002); the lack of interest being due in part to the complexity of the physiological antagonism between Al toxicity and B deficiency (Yang and Zhang 1998, Camacho-Cristobal and Gonzalez-Fontes 1999, Matsumoto 2000, Yau 2000). Toxic Al levels in the soil are neutralized by treatment with Lm and/or G (Sumner *et al*. 1986, Liu and Hue 1996, Zaifnejad *et al.* 1996). By neutralizing the soil acidity, Lm ties up available soil B (Haby *et al.* 1998a). B deficiency, like Al toxicity, causes cessation of root growth (Bohnsack and Albert 1977, Heyes *et al*. 1991, Ciamporová 2002). Boron is important in cell wall structure, saccharide, phenol, and RNA metabolism (Brown and Hu 1997, Marschner 1998). On the other hand, Al^{3+} retards senescence and stabilizes plant protein and chlorophyll (Chl) contents (Subhan and Murthy 2000). Acid soils on the USA Coastal Plain that are limed to pH 7 in the surface for alfalfa production usually have phytotoxic levels of Al in the subsoil

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[+]Author for correspondence; fax: 936-857-2325, e-mail: Godson_Osuji@PVAMU.edu
Abbreviations: Chl – chlorophyll; FBPase – fructose-1,6-bisphosphatase; G – gypsum; GDH – glutamate dehydrogenase; Lm – limestone; ND – not determined; NDP – nucleoside diphosphate; NMP – nucleoside monophosphate; NTP – nucleoside triphosphates; P_i – inorganic phosphate.

(Bouton *et al.* 1991, Beedy *et al*. 1995). Soils that contain toxic concentrations of Al^{3+} (>10 μM) usually have negative impact on crop yields (Kochian 1995). Boron application at ~0.42 g m^{-2} along with limestone to pH 7 mitigated the adverse effects of Al toxicity and gave excellent yields of alfalfa (Clary and Haby 1998, Haby *et al*. 1998a,b). However, the excellent yield was not explained on the basis of Ca–Al antagonism. Therefore, there was the need to investigate the biochemical mechanism by which Ca, Al, and B coordinately regulate the alfalfa metabolism to permit the achievement of excellent crop yield.

In order to elucidate the basis of the antagonistic response, we analyzed the fructose-1,6-bisphosphatase (FBPase) and the glutamate dehydrogenase (GDH) activities. FBPase catalyzes the irreversible hydrolysis of fructose-1,6-bisphosphate to fructose-6-phosphate and P_i in the synthesis of sucrose, and in doing so it transduces photon energy in the Calvin cycle at the interface between electron transport and product synthesis (Buchanan 1980). Also the sensitivity of FBPase to Ca^{2+} (Kreimer *et al.* 1988) further supported its suitability as a target for assessing the response of carbon metabolism to B and Ca amendments of soil. GDH synthesizes some RNA (Osuji *et al.* 2003a), thereby regulating biomass enhancement (Osuji and Braithwaite 1999, Ameziane *et al*. 2000, Osuji *et al*. 2003/4) and tissue differentiation (Osuji and Madu 1997). Because B forms complexes with ribose, it interferes with nucleotide, NADH, and nucleic acid metabolism (Johnson and Smith 1976). Nucleoside triphosphates are the substrates of GHD besides being the strongest inducers of the enzyme's isomerization (Osuji *et al.* 2003b). Therefore, the effect of B on nucleotide metabolism might illuminate the antagonistic response of saccharide metabolism to B and Ca amendments of soil. In this paper we report that B treatments of the soil coordinately optimized the RNA metabolism, whereas G treatments coordinately optimized the saccharide metabolism.

Soil containing toxic levels of exchangeable Al was dug by depth intervals from a Kirvin soil (fine, mixed, semiactive, thermic Typic Hapludult) on the Texas Agricultural Experiment Station near Overton, Texas, USA (32°20'51"N, 94° 58'13"W – Kilgore SW guad). The soil was replaced by depth and packed into 10×127-cm PVC pots. The top 15-cm of the soil was treated with Lm (672 g m^{-2}) to increase pH to ~7, and fertilized with P, K, Mg, and S for alfalfa. Four replications of G equivalent to 0, 0.5, and 1.0 kg m^{-2} (further G_0, $G_{0.5}$, $G_{1.0}$) and B equivalent to 0, 0.15, 0.30, and 0.45 g m^{-2} (further B_0, $B_{0.15}$, $B_{0.30}$, $B_{0.45}$) were applied into the top 2.5-cm of the soil. De-ionized water was applied to leach the G into the subsoil. Inoculated alfalfa (*Medicago sativa* L.) was planted and water was applied to continue the leaching. At maturity, the alfalfa shoots were harvested, ground to powder with dry ice, and stored at –80 °C.

FBPase (EC 3.1.3.11) was extracted by homogenizing 3 g of powdered alfalfa leaves with 10 cm^3 of 0.1 M Tris-HCl buffer (pH 8.0) containing 2 mM EDTA. The supernatant after centrifugation (20 000×*g* for 20 min at 4 °C) was kept on ice and treated with solid $(NH_4)_2SO_4$. The protein that precipitated between 40 and 70 % $(NH_4)_2SO_4$ saturation was pelleted (20 000×*g* for 20 min at 4 °C), dissolved in 5 cm^3 extraction buffer, and dialyzed exhaustively at 4 °C against 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA. The dialyzed extract was used for the assay of FBPase activity. The P_i liberated from the substrate fructose-1,6-bisphosphate was measured by the malachite green method of Leegood (1990) against a standard curve of P_i.

GDH (EC 1.4.1.2) oxidative and reductive activities were assayed photometrically (Osuji and Madu 1995). The RNA synthetic activity of GDH was determined by the method of Osuji *et al.* (2003a) using ~7 μg of the cryoelectrophoretically purified enzyme per assay that contained the 4 NTPs, NH_4Cl, α-ketoglutarate, $CaCl_2$, NADH, rifampicin, and DNase 1. The RNA product was quantitated by photometry at 260 nm. The control assay contained GDH but no substrates and modulators.

Total Chl content was determined with 3 g of the powdered alfalfa using the method of Isaac (1990). Total hexose content was determined with 3 g of the alfalfa using the o-toluidine method of Feteris (1965). Total protein content was determined with 1 g of the alfalfa using the biuret method (Gornal *et al.* 1949). All assays including enzymes were done in triplicate with a fresh sample of powdered alfalfa; the average data were then analyzed (SAS 1994).

Free nucleotides were extracted by homogenizing 9 g of the powdered alfalfa leaves with 30 cm^3 of ice-cold 70 % (v/v) ethanol in a blendor at maximum speed for 3 min. The homogenate was centrifuged (10 000×*g*, 15 min, 4 °C) to remove precipitated proteins and nucleic acids. The supernatant was completely recovered and an aliquot corresponding to the extract from 3 g of the alfalfa was vacuum-concentrated to ~1 cm^3, and then chromatographed through a 5-cm^3 cartridge of *Econo-Pac Q* (*Bio-Rad*, USA) strongly basic anion exchanger. After loading the extract into the cartridge, the automated *Econo System* (*Bio-Rad*) was programmed at a flow rate of 5 cm^3 per min, and a multi-step of discontinuously increased NaCl concentrations, using distilled water as the initial eluant. The chart recorder speed, and the *Econo* UV monitor wavelength were set at 12 cm per h and 254 nm, respectively. Elution peaks were collected by time windows. The absorbance at 260 nm of each peak was measured. Nucleotides present in each peak were identified by chromatography of authentic samples. Concentrations were calculated based on the volume of each elution peak, and the molar absorbance at 260 nm (Perbal 1988). Nucleotide extractions were repeated three times and used for the triplicate chromatography of the free nucleotides for each treated alfalfa. The free nucleotide concentrations were then averaged.

In the absence of B, G treatments progressively decreased the alfalfa total protein but increased the hexose content. This showed that the increasing G concentration neutralized the soil acidity and detoxified the Al. Plant protein content is stabilized by Al^{3+} (Subhan and Murthy 2000). In the absence of G, $B_{0.15}$ increased the hexose content, but higher B doses decreased the hexose contents without affecting the protein contents (Table 1). $B_{0.15}$ decreased the total protein with or without G thereby demonstrating the independent ability of B to detoxify the soil Al. $B_{0.45}$ together with $G_{0.5}$ did not affect the alfalfa protein content, but with or without $G_{1.0}$ it induced a 14 % decrease in the protein content thereby suggesting that B and Ca did not synergistically detoxify the Al. G without B and Lm decreased the alfalfa protein content by ~25 % thus confirming its independent ability to detoxify the soil Al. Therefore, B and G exerted independent effects on the alfalfa protein contents but antagonistic effects on the hexose contents.

Increases in G doses at fixed B generally increased the Chl contents. $B_{0.45}$ and $G_{0.5}$ gave the highest increase (~30 %) in Chl content as compared to the control. This is in agreement with the ameliorative effects of B and Lm on Al toxicity (Haby *et al*. 1998a). Apart from some exceptions, the changes that were induced in the Chl content by B, and G treatments followed closely the general trend of the hexose (Table 1) because the $B_{0.45}$ with $G_{0.5}$ induced the highest increase of hexose content (28.7 %) by inducing the highest increase (~30 %) in Chl content. But in the absence of G, $B_{0.15}$ suppressed (52 %) the Chl content whereas there was increase (~15 %) in the hexose content. These signs of antagonism, however, showed that the neutralization of soil acidity by G also optimized the saccharide metabolism of alfalfa.

Increasing the B doses in the absence of G and also at $G_{0.5}$ decreased the FBPase activity by ~37 and ~69 %, respectively (Table 1). Similarly, increasing the G doses at fixed $B_{0.15}$ and $B_{0.30}$ decreased the FBPase activity by ~53 and ~31 %, respectively. However, increasing the B dose at $G_{1.0}$ alleviated the Ca–B antagonism because of the increased FBPase activity (~91 %). Also, the FBPase activity increased when $G_{1.0}$ was applied in the absence of both B and Lm. Therefore unlike the responses of the protein and hexose contents, the response of FBPase to the spectrum of G, B, and Lm amendments was biphasic, the point of inflection ($G_{1.0}$) being indicative of the endpoint of the neutralization of the soil's acidity. Although FBPase is sensitive to Ca^{2+} (Kreimer *et al*. 1988), it had hitherto not been applied for assessing the response of photosynthesis to the neutralization of soil acidity.

Table 1. Responses of alfalfa metabolism to Ca–B antagonism: Responses of hexose, protein, and chlorophyll contents [g kg^{-1}(FM)], FBPase activity [mg(P_i) kg^{-1}(Chl) s^{-1}], oxidoreductase ratio of GDH, RNA synthetic activity of GDH [mg kg^{-1} s^{-1}], and the free nucleotides [mmol kg^{-1}(FM)] of alfalfa to boron [g m^{-2}], gypsum [kg m^{-2}], and limestone [672 g m^{-2}, or none in the last two variants] amendments of soil. Means ± SD under each effect were significant at >5 %. ND = not determined.

Treatment		Hexose	Protein	Chl (*a*+*b*)	FBPase	GDH ratio	RNA synth. by GDH	Nucleotides
Boron	Gypsum							
0	0	0.87±0.03	38.3±1.3	14.1±1.1	11.95±1.11	0.67	28.9±2.6	22.75±1.91
	0.5	0.75±0.04	37.1±1.3	13.1±1.2	9.19±0.58	0.60	36.3±3.1	9.60±0.27
	1.0	1.13±0.03	24.3±1.2	17.6±1.4	8.27±0.50	1.71	18.3±1.4	4.23±0.23
0.15	0	1.00±0.06	32.6±1.6	6.7±0.5	23.63±0.61	0.29	30.0±2.8	13.70±1.05
	0.5	0.75±0.05	38.5±1.1	11.3±1.0	11.83±0.31	0.33	35.1±2.2	5.29±0.54
	1.0	0.77±0.07	25.0±0.8	14.0±1.2	10.95±0.53	0.38	20.4±0.9	8.69±0.78
0.30	0	0.83±0.08	31.6±1.2	8.1±0.4	18.06±0.50	0.47	29.4±2.3	9.37±0.69
	0.5	0.82±0.06	35.5±1.3	11.8±1.0	11.30±1.00	0.86	21.9±1.3	10.36±1.09
	1.0	0.87±0.07	39.9±0.7	12.8±0.9	12.37±0.78	0.81	17.2±1.0	15.70±1.30
0.45	0	0.79±0.05	38.6±0.9	10.1±0.8	14.71±1.36	0.35	35.1±1.8	10.46±0.85
	0.5	1.12±0.04	36.4±1.1	18.5±1.7	7.24±0.58	0.58	15.0±1.1	8.20±0.88
	1.0	0.91±0.08	32.8±1.5	9.3±0.6	15.83±1.06	0.81	13.0±0.9	12.25±1.06
0	1.0	0.87±0.05	28.9±1.1	12.3±1.2	15.33±1.33	0.71	24.2±1.4	ND
0.45	1.0	0.83±0.06	32.6±0.6	15.8±0.7	10.77±1.08	ND	ND	12.97±1.39

The GDH oxidation:reduction ratios ($GDH_{ox:red}$) were generally less than 1 except in $G_{1.0}$ without B (Table 1). The alfalfa with the high (1.71) $GDH_{ox:red}$ (Table 1) also had ~25 % higher Chl content (17.6 g kg^{-1}) and correspondingly ~20 % higher hexose content (1.13 g kg^{-1}), but ~36 % lower protein content (24.3 g kg^{-1}) than the control alfalfa. The optimization of the GDH redox activity showed that its oxidative and reductive components were functionally interdependent, in agreement with the roles ascribed to its oxidative activity in carbon metabolism (Srivastava and Singh 1987, Robinson *et al*. 1991, Aubert *et al*. 2001). But low $GDH_{ox:red}$ did not always induce high protein contents because in the $B_{0.15}$ with and without G, the alfalfa protein contents decreased by up to 34 % despite very low (0.29 to 0.38) $GDH_{ox:red}$. Therefore, like the hexose, Chl, and protein contents, the

amination and de-amination activities of alfalfa GDH attained optimum, outside which they deviated from the trends of the G and B concentrations.

$G_{0.5}$ in the absence of B increased the RNA synthetic activity of GDH by ~25 %. Also in the absence of G, increasing the B doses to $B_{0.45}$ optimized the RNA synthetic activity of GDH (Table 1). But increase from $G_{0.5}$ to $G_{1.0}$ decreased the RNA synthetic activity of GDH by ~48 and ~55 %, respectively. Also, increase from $B_{0.30}$ to $B_{0.45}$ decreased the RNA synthetic activity of GDH by ~41 and ~63 %, respectively. In the absence of B and Lm, $G_{1.0}$ decreased the RNA synthetic activity of GDH by ~16 %, but Lm and $G_{1.0}$ decreased the activity by ~36 %. Therefore, detoxification of Al by B optimized the RNA synthetic activity of GDH whereas neutralization of soil acidity by G suppressed the enzyme activity.

$B_{0.15}$ in the absence of G, or $G_{0.5}$ in the absence of B decreased the free nucleotide content by ~40 and ~57 %, respectively (Table 1). Also, increasing the doses of B in the presence of $G_{0.5}$ decreased the content of free nucleotides. But, increasing the doses of B at $G_{1.0}$ increased the content of the free nucleotides. Therefore the free nucleotides and the FBPase activity responded similarly to the soil amendments thereby confirming that the point of inflection at $G_{1.0}$ marked the endpoint of the neutralization of the soil acidity. The most vivid effect of the soil amendments was that they induced more than 90 % decrease in the NMP contents (Fig. 1) with concomitant increases in the RNA synthetic activity of GDH and FBPase activity by ~25 and ~97 %, respectively, with or without changes in the hexose, protein, and Chl contents. The contents of free nucleotides are regulated by reciprocating feedback mechanisms (Wasternack 1978, Osuji and Ory 1986). Therefore, the decrease in NMP content suggested that the Ca and B coordinately optimized the alfalfa carbon and nitrogen assimilation pathways (Coruzzi and Bush 2001).

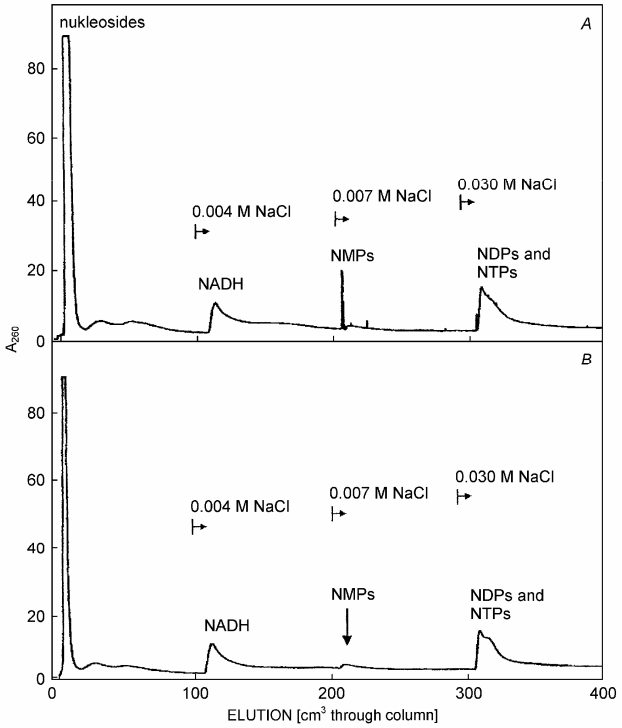

Fig. 1. Free nucleotides of alfalfa. Alfalfa shoots were extracted with 70 % ethanol; the extract was concentrated by freeze-drying, chromatographed on *Econo-Pac Q* strongly basic anion exchanger using increased concentrations of NaCl as the eluant, and the UV absorbance of eluate was measured with the automated *Econo* System. *A*: Control (limed, but without boron and gypsum). *B*: Limed with B (0.15 g m^2) without gypsum.

Consideration of the effects of each treatment on alfalfa metabolism shows that increasing the doses of G in the absence of B maximized the hexose and Chl contents but decreased the RNA synthetic activity of GDH, FBPase activity, and nucleotide concentration (Table 1). In particular, $G_{1.0}$ without B gave the highest yields of hexose and Chl. Therefore neutralization of soil acidity by G coordinately regulated metabolism by optimizing saccharide metabolism. Conversely, increasing the doses of B in the absence of G progressively raised the RNA synthetic activity of GDH while progressively depressing the FBPase activity and the hexose, nucleotide, and Chl contents until at $B_{0.45}$ when optimal coordination was attained. Therefore, detoxification of Al^{3+} by B coordinately regulated metabolism by optimizing RNA metabolism. The optimization of saccharide metabolism by G, and of RNA metabolism by B were the key mechanisms by which alfalfa achieved optimal sustainable growth on the acid soils. The Ca–B antagonism is part of the role expected of G, but it did not adequately explain the sustainable growth of alfalfa. Simultaneous neutralization of acidity and detoxification of Al^{3+} by combined application of $G_{0.5}$ and increasing doses of B switched the coordinate regulation from the optimized RNA to the optimized saccharide metabolism (Table 1). Combined application of $G_{1.0}$ and increasing B doses progressively depressed the hexose and Chl contents until at the combined $B_{0.45}$ and $G_{1.0}$ there was still optimal FBPase activity but the Chl and hexose contents and the RNA synthetic activity of GDH became extremely low, with neither the saccharide nor the RNA metabolism being coordinately optimized. Therefore, the switch-over in the coordinate regulation of alfalfa metabolism from the B-dependent optimization of RNA metabolism to the Ca-dependent optimization of saccharide metabolism and *vice versa* are the parameters that determine the best management plan for mitigating the adverse effects of soil acidity for enhanced sustainable alfalfa production. Hence the neutralization of soil acidity with B (0.42 g m^{-2}) and Lm enhanced the sustainability of alfalfa production (Clary and Haby 1998, Haby *et al.* 1998a,b) through the coordinate optimization of the RNA metabolism of the crop.

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