Symbiosis, 27 (1999) 147–156
Balaban, Philadelphia/Rehovot

Inoculation with *Acetobacter diazotrophicus* Increases Glucose and Fructose Content in Shoots of *Sorghum bicolor* (L.) Moench

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Received July 21, 1999; Accepted October 19, 1999

Abstract

This work analyzes the effect of inoculation with *Acetobacter diazotrophicus* and of applications of GA_3 and IAA on total carbohydrates, sucrose, glucose and fructose in shoots of *Sorghum bicolor*. Sorghum seedlings grown in plastic pots with sand-vermiculite were inoculated with pure cultures of *A. diazotrophicus* strain PAL 5 or sterile water. Two days later, GA_3, IAA and a combination of the two hormones were applied to the first leaf of un-inoculated seedlings. After 30 days, shoot samples were extracted with 1% TCA, carbohydrates assessed by phenol reaction, and individual sugars (sucrose, glucose and fructose) measured by capillary gas chromatography-flame ionization detection (GC-FID). *A. diazotrophicus*, as well as GA_3 and IAA, promoted total carbohydrate accumulation. GA_3 was the most effective treatment at 30 pmol.plant^{-1}. However, neither the inoculation with the bacterium nor the different concentrations of hormones produced sucrose increase as assessed by GC-FID. Notwithstanding fructose and glucose levels were significantly augmented by *A. diazotrophicus* as compared to control and GA_3 and IAA treatments.

Keywords: *Acetobacter diazotrophicus*, *Sorghum bicolor*, GA_3, IAA, carbohydrates

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0334-5114/99/$05.50

1. Introduction

Acetobacter diazotrophicus is a diazotrophic bacterium which has been isolated from leaves, shoots and roots of plants with a high sucrose content, mainly sugar cane and other Gramineae representatives like Cameroon-grass, all of them of vegetative propagation (Döbereiner et al., 1988). It is an obligatory endophyticum which lives in the apoplast (Dong et al., 1997) and is not found as a free-living bacterium in the soil solution, or in the rhizosphere of infected plants (Baldani et al., 1997). *A. diazotrophicus* tolerates high sucrose concentrations (above 10%) and grows and fixes N_2 under low pH conditions (5.0 or less).

Sorghum bicolor (L.) Moench accumulates significant amounts of sugars, mainly sucrose, especially in the parenchymatous shoot tissues. Depending on the carbohydrate content and its composition, this type of sorghum has been identified as a source for considerable quantities of biomass useful in fermentation for methanol or ethanol (Lipinsky and Kresovich, 1982). However, it can be also used in more traditional ways for fresh forage or silage, situations in which increased levels of sugars are desirable. In this plant, it has been shown that sucrose accumulation is not necessarily associated with the beginning of the reproductive phase during the plant ontogenesis, as it has been assumed in the first investigations regarding sorghum maturation. It possibly follows the pattern of sugar cane, in which the sugar content increases rapidly right after the internode elongation ceases (Hoffmann-Thoma et al., 1996). Moreover, in sugar cane great differences can be observed in sucrose content related to the age of the plant and the environment in which this plant grows. The sucrose content is lower during periods of fast growth, but higher when the plant grows slowly because of unfavorable environmental factors, like low temperatures or shortage of water and minerals. The cultivars accumulating great quantities of sucrose usually contain a low amount of fibers and have a high fresh weight (Zhu et al., 1997).

Since two decades the relationship between the increase of the endogenous levels of sucrose and the applications of phytohormones, like gibberellins (GAs) and IAA, has been studied (Nickell, 1982; Daie, 1987). In fact, the application of GA_3 to sugar cane significantly increases sucrose production without affecting quality (Nickell, 1988). Although a clear correlation was found, especially with GAs, no explanation regarding the type of action and the possible interactions with other factors of stress could be established. There have been discrepancies among authors because of the different results found depending on the experimental conditions. Martínez Cortina et al. (1994) postulated that GAs might regulate the increase in sucrose accumulation throughout a direct effect on the membranes and, indirectly by modulating

ATPase activity. Xu et al. (1995) proposed that the GA effect is just indirect, through the activation of specific amylases for starch hydrolysis.

Treatments with GA_3 increase the total dry weight in sorghum (Pao et al., 1986). Rood et al. (1992) demonstrated that some hybrids present high levels of GAs, mainly GA_1, and corroborated that in slow growing lines, growth can be promoted by application of GA_3 (Rood, 1995). Inoculation of sorghum with *A. diazotrophicus* was previously studied in association with mycorrhizae (VAM) (Isopi et al., 1995), and increases in N_2 fixation and root length have been found.

We previously characterized by capillary gas chromatography-mass spectrometry the GAs GA_1 and GA_3 and the auxin IAA produced by chemically-defined cultures of *A. diazotrophicus* (Bastián et al., 1998). These antecedents lead to the hypothesis that *A. diazotrophicus* might increase sucrose production in *Sorghum bicolor* throughout the production of IAA and GA_3. However, rather than to measure sucrose content at the end of the plant life cycle, the interest was to search for levels of carbohydrates in early stages of active growth, when sucrose accumulation might not have started yet. This situation was expected according to the antecedents reported for sugar cane (Hoffmann-Thoma et al., 1996). Thus, the objective of this work was to analyze the effect of inoculation with the bacterium and of applications of GA_3 and IAA on growth, total carbohydrates and sugar production in plants of *Sorghum bicolor* in early ontogeny and grown under controlled conditions.

2. Materials and Methods

Plant material and bacterial cultures

Seeds of *Sorghum bicolor* hybrid Pioner 8118 were disinfected with ethanol 70% 20 sec, and finally washed thoroughly with sterile distilled water 18 to 20 times. The seeds were imbibed all night, and then allowed to germinate in Petri dishes, on two wetted layers of filter paper, 24 to 48 h in a Conviron G-30 germination chamber at 30°C and ca. 90% RH. The seedlings were then transferred to plastic pots filled with sand-vermiculite (1:1 v/v), previously sterilized for 10 h at 180°C and inoculated with *A. diazotrophicus* or sterile water (controls). The seedlings were grown under a 24-h cycle of 16 h with cool-white fluorescent light of 33.7 $\mu mol.m^{-2}.s^{-1}$ at 32°C followed by 8 h of darkness at 17°C, in a growth chamber with ca. 90% RH. Then *A. diazotrophicus* was inoculated with 1 ml of PBS buffer solution containing 10^3 to 10^5 $CFU.ml^{-1}$. The inoculum was prepared with washed cells (in PBS) from pure cultures of *A. diazotrophicus* strain PAL 5 (ATCC 49037, kindly provided by Dr.

J. Döbereiner, EMBRAPA, Itajai, Brasil) grown in liquid medium LB (Luria Broth, Sigma). Two days later, 1 µl of 95% ethanol solutions of GA_3 (Sigma Chem. Co., 90% purity) or IAA (a gift of J.D. Cohen, ARS Beltsville, USDA, USA), and a combination of the two, were spread to the first leaf with a microsyringe. Thus, the variants were control water, GA_3 3, 30, 300 pmol.plant^{-1}, IAA 6, 60, 600 pmol.plant^{-1}, GA_3 300 + IAA 60 pmol.plant^{-1}, *A. diazotrophicus* 10^3 and 10^5 CFU.ml^{-1}. Every two days the plants were watered with sterile distilled water, and once a week with sterile Hoagland solution. After 30 days under such conditions the plants were collected, and fresh and dry weight assessed from aliquots prior to total carbohydrates and sugars determinations. The experiment was done by triplicate.

Sampling for quantification of total carbohydrates and sugars

The shoots were washed thoroughly with running tap water and then with sterile distilled water. Six samples were processed for each treatment and experiment, thus the total sampling was of 18 per treatment. One g of shoot per treatment was lyophilized and homogenized in a mortar with one ml of 1% TCA. The homogenate was loaded in Eppendorf tubes, centrifuged twice 7 min at 7500 rpm, neutralized with 2% $OHNH_4$, and freeze-dried.

Carbohydrates quantification by phenol reaction

Aliquots of 20 µl were taken from 9 samples of the above, added to 500 µl of phenol reactive (phenol 0.5% in water), followed by 2.5 ml of sulfuric acid. A serial dilution of glucose from 0 to 200 mg.ml^{-1} was used to determine the calibration curve. After 10 min at room temperature, the mixture was recorded by spectrophotometry at 488 nm, and the results of the samples compared with those of the glucose calibration curve (modified from Daniels et al., 1994).

Sugar quantification by capillary gas chromatography-flame ionization detection (GC-FID)

For the evaluation of the soluble carbohydrates the lyophilized samples neutralized in the Eppendorf tubes mentioned above were used for analysis of fructose, glucose and sucrose. The equivalent of 0.1 g of fresh weight, plus 2.5 mg of phenyl-glucopyranoside as internal standard, was extracted overnight at room temperature with 7 ml of 80% ethanol and 2 ml of imidazole buffer 0.1 M, pH 7, in order to avoid acid hydrolysis of sucrose. The extract was then centrifuged at 4000 rpm for 10 min. The supernatant was withdrawn and made up to a volume of 10 ml with 80% ethanol. Four ml of this solution were dried by

air stream at room temperature and dissolved in 200 µl of anhydrous pyridine-hexamethyldisilazane-trimethylchlorosilane mixture (4:2:1) for 2 hours at 60°C. The cooled samples were stored at 4°C and 3 µl were used for the injection into capillary GC. The GC used was a Chrompack CP 9000 (Chrompack, Middelbung, The Netherlands) equipped with a flame ionization detector (FID) and a capillary fused-silica column (25 m length, 0.25 mm I.D.), coated with CP-Sil-5 CB, DF 0.12. Injector and detector temperatures were 280°C and 320°C, respectively. The temperature program was: 120°C for 1 min, followed from 120°C to 152°C at 8°C.min^{-1}; from 152°C to 176°C at 12°C.min^{-1}; from 176 to 198°C at 16°C.min^{-1}; from 198°C to 238°C at 20°C.min^{-1}; from 238°C to 300°C at 24°C.min^{-1} and held at 300°C for 5 min. Flow rates of He, H_2, air and N_2 (used as a make-up gas) were 2, 30, 250 and 30 ml.min^{-1}, respectively, with a split ratio of 80:1. The quantification of each compound was performed using the internal standard calculation method.

3. Results

According to the results shown in Table 1, the inoculation with *A. diazotrophicus* PAL 5 was effective in promoting total carbohydrate accumulation in shoots of young plants of *S. bicolor*, as measured by the phenol reagent method. This promoting effect was especially noticeable with the smaller inoculum size of 10^3 CFU.ml^{-1}, which was significantly different from both, the control and the highest bacterial concentration (10^5 CFU.ml^{-1}).

Table 1. Quantification of total carbohydrates in *Sorghum bicolor* by phenol reaction (n = 6), expressed as g of glucose equivalents

Treatments	µg.g^{-1} d wt
Control	2993.00 a
A. diazotrophicus 10^3 CFU.ml^{-1}	5842.25 b
A. diazotrophicus 10^5 CFU.ml^{-1}	3806.25 c
GA3 3 pmol.plant^{-1}	3802.88 ac
GA3 30 pmol.plant^{-1}	7987.50 d
GA3 pmol.plant^{-1}	6501.33 bd
IAA 6 pmol.plant^{-1}	3005.50 a
IAA 60 pmol.plant^{-1}	3299.50 ac
IAA 600 pmol.plant^{-1}	4853.88 e

Same letter after values means no significance with $p>0.95$.

The application of both hormones in different concentrations, GA_3 at 30 and 300 pmol.plant^{-1} and IAA at 600 pmol.plant^{-1} also promoted total carbohydrate accumulation showing significant differences to the control. This effect was more remarkable for GA_3 at 30 pmol.plant^{-1}, which multiplied the control values by a factor of 2.7.

However, when different sugars were measured individually by GC-FID (Fig. 1) there was a different picture. The levels of sucrose were very similar in the control, and the inoculated and hormone-treated plants, but the accumulation of fructose and glucose was quite different among treatments. With the smaller inoculation with *A. diazotrophicus* with 10^3 CFU.ml^{-1}, there was not a significant increase in accumulation of fructose and glucose compared to the control. However, by inoculation with 10^5 CFU.ml^{-1} there were 2 to 3 times more fructose and glucose than with 10^3 CFU.ml^{-1}. Obviously, the total accumulation of sugars individually measured is higher than in the control and in most cases with regulator treatments. However, especially noticeable was also the increase in glucose and fructose accumulation with the lowest GA_3 concentration. The question of why are differences between the results obtained with the two techniques of measuring in the inoculated and hormone-treated samples? These differences between the measurements can be explained by the fact that in the phenol method individual sugars are not identified but the total amount of carbohydrates was quantified. While in the GC-FID technique single sugars were measured, so it is likely that other carbohydrates (mainly starch) may account for the final result.

Fig. 2 shows the effect of the different treatments on fresh weight of the sorghum plants (as there were no significant differences between fresh and dry weight determinations, only data for fresh weight are shown). The growth of the aerial portion did not present significant differences between the two inoculation treatments, but they were substantially increased in relation with the hormone-treated and the controls. Although the hormonal treatments did not affect overall growth, they increased shoot growth.

4. Discussion

When the results of fresh weight measurement of the aerial plant part (Fig. 2) are compared with those of total carbohydrates (Table 1) and sugars (Fig. 2), some correlation can be established among growth promotion, the increase in total carbohydrates and the sugars measured. Inoculation with the bacterium increased overall growth and, depending on the inoculum size, it has a moderate effect increasing total carbohydrates and more notably on levels of glucose and fructose. On the contrary, hormonal treatments had a moderate effect on glucose and fructose levels, a null one on overall growth promotion, but

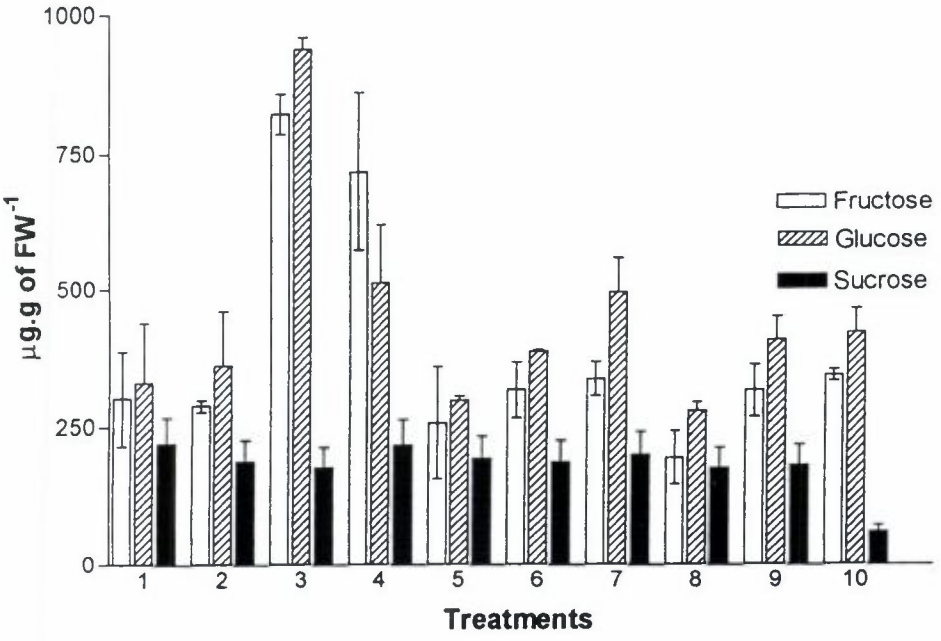

Figure 1. Sugar content (glucose, fructose and sucrose) in *Sorghum bicolor* measured by GC-FID. Treatments: 1, control; 2, *Acetobacter diazotrophicus* 10^3 UFC.ml^{-1}; 3, *Acetobacter diazotrophicus* 10^5 UFC.ml^{-1}; 4, GA_3 3 pmol.plant^{-1}; 5, GA_3 30 pmol.plant^{-1}; 6, GA_3 300 pmol.plant^{-1}; 7, IAA 6 pmol.plant^{-1}; 8, IAA 60 pmol.plant^{-1}; 9, IAA 600 pmol.plant^{-1}; 10, GA_3 300 pmol.plant^{-1} + IAA 6 pmol.plant^{-1}.

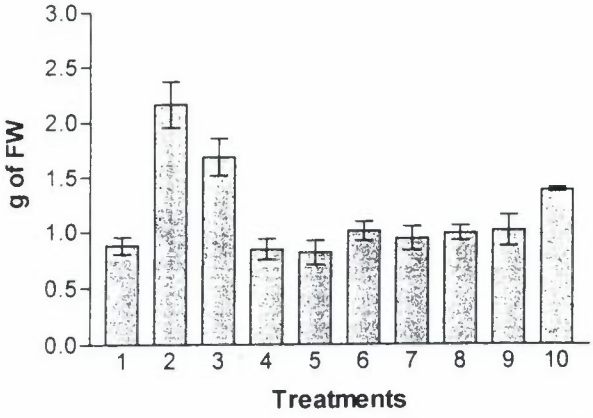

Figure 2. Fresh weight of *Sorghum bicolor* stems and leaves of one month. Treatments: 1, control; 2, *Acetobacter diazotrophicus* 10^3 UFC.ml^{-1}; 3, *Acetobacter diazotrophicus* 10^5 UFC.ml^{-1}; 4, GA_3 3 pmol.plant^{-1}; 5, GA_3 30 pmol.plant^{-1}; 6, GA_3 300 pmol.plant^{-1}; 7, IAA 6 pmol.plant^{-1}; 8, IAA 60 pmol.plant^{-1}; 9, IAA 600 pmol.plant^{-1}; 10, GA_3 300 pmol.plant^{-1} + IAA 6 pmol.plant^{-1}.

they promoted shoot growth and (especially GA_3) remarkably increased total carbohydrate accumulation.

These results suggest that the bacterium may act in part by producing hormones, but it may also have another effect. It has been shown that *A. diazotrophicus* has the ability to fix N_2 and, although the amount of N_2 fixed is too small compared with the plant requirements, an extra input of the element may account for a surplus of growth in the different plant parts. Such a situation has been reported for *Azospirillum* spp. inoculated maize in field experiments (Fulchieri and Frioni, 1994).

Also, the inoculated plants showed higher amounts of fructose and glucose than the non-inoculated (both controls and hormone-treated). This suggests that *A. diazotrophicus* might stimulate either invertase activity or another enzymatic system related to mono-sugar synthesis, an effect also found with the lower GA_3 concentration. This activity may account for better growth and increasing in carbohydrate accumulation. However, these effects are not exclusive, and the total response might be a sum of them.

Regarding the effect of hormones (mainly GA_3) on sucrose accumulation, it has been claimed that the mechanism involved can be indirect. When different enzymes purportedly involved in sucrose accumulation were measured in different systems and species, the results have been rather confusing and erratic (Martínez Cortina et al., 1994; Xu et al., 1995). Our results obtained either, with bacterial inoculation or hormone application, reinforce the hypothesis that their effects on carbohydrates accumulation may be the consequence of stimulated growth (overall or localized). Although there was no sucrose accumulation, the increased levels of glucose and fructose may account for higher amounts of carbohydrates stored (perhaps, sucrose levels may increase later on in the plant ontogeny). This means that by stimulating growth, for example GAs in the internode-growing zone, this plant part increases its sink strength, and thus accumulates more sugars. The bacterium may have a more general effect on the whole plant, by providing hormones and reducing N_2, so increasing the overall growth (and carbohydrate accumulation).

These results generate a great expectation in field application of both, GA_3 and the bacterium, for *S. bicolor* crops used for alcohol production, fresh forage or silage. They also confirm the importance of studying the relationship between plants and microorganisms in the managing of agricultural and biological systems.

Acknowledgements

This work has been supported by a scholarship to Fabiola Bastián and grant N°3933 to Rubén Bottini provided by Consejo de Investigaciones Científicas y

Tecnológicas de la Provincia de Córdoba (CONICOR). Also funding support from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) research grant N°4939, and Secretaría de Ciencia y Técnica of the Universidad Nacional de Río Cuarto (Secyt UNRC) grant N°93[a] to R. Bottini is recognized.

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