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Nitrogen compounds in the apoplastic sap of sugarcane stem: Some implications in the association with endophytes

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Summary

Several nitrogen compounds were identified and quantified in the apoplastic and symplastic sap of sugarcane stems. The sap of stems was composed mainly of soluble sugars, which constituted 95% of the total organic compounds detected. Sap also contained nitrogen compounds, with amino acids (50–70% of N) and proteins (20–30% of N), being the main nitrogenous substances, as well as inorganic forms as ammonium, nitrite and nitrate, in low concentrations (<20% of N). Serine, proline, alanine and aspartic acid together represented around 60% of the amino acids of the sap of both field grown and high nitrogen fertilized plants, and non-nitrogen fertilized plants inoculated with *Gluconacetobacter diazotrophicus*. The total amino acid content of apoplastic sap was six to nine times lower in non-nitrogen fertilized plants than in fertilized ones. The possible roles of these substances to regulate endophytic associations with sugarcane are also discussed.

Introduction

Sugarcane is one of the major productive plant species in the world. Sugarcane can potentially produce approximately 45 tons of dry weight $ha^{-1}\,year^{-1}$, and 22 tons of sugar $ha^{-1}\,year^{-1}$. The stem tissues of this plant, consisting of intercellular spaces (apoplast) and vacuolar spaces (symplast), have been the subject of study over many years, since the maturation of sugarcane is characterized by the accumulation of sucrose in developing internodes (Glasziou and Gaylor, 1972; Moore, 1995). Most interest of sugarcane stem knowledge has risen from the finding of Döbereiner's group in Brazil (Baldani

Abbreviations: AA, amino acids; APS, apoplastic sap; Prot, proteins; SPS, symplastic sap

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et al., 1997) regarding the presence of nitrogen fixing endophytes in sugarcane stems.

Endophytes in the apoplast may support plant growth in many instances by increasing resistance to biotic or abiotic stress factors, as well as by contributing directly to plant mineral nutrition (Sattelmacher, 2001). *Gluconacetobacter diazotrophicus* is an obligatory aerobe with the ability to fix atmospheric nitrogen (Stephan et al., 1991; Alvarez and Martínez-Drets, 1995), well adapted to extreme conditions such as high sugar concentrations and low pH (James and Olivares, 1997; Chanway, 1998), but also shows a high tolerance to abiotic factors such as heat treatments and salt concentrations in the culture media (Tejera et al., 2003). Results of Dong et al. (1994) demonstrated the presence of the N_2-fixing endophyte (*G. diazotrophicus*) living in the apoplastic fluid of sugarcane plants, and Fuentes Ramirez et al. (1999) confirmed the presence of this bacterium within the xylem and intercellular apoplast. McCully (2001) also demonstrated that the intercellular space apoplast (the space within the plant outside the symplast) is the most suitable niche for bacterial endophytes.

Various organic and inorganic nitrogen compounds are also present in both apoplast and symplast sap of many higher plants. In sugarcane stems, Tejera et al. (2004) described the existence of amino acids and ammonium in the apoplastic and symplastic saps. In the xylem sap of squash roots, Satoh et al. (1992) detected proteins and carbohydrates. In addition, Pate (1973) reported that in higher plants, N is transported from roots to shoots, primarily as nitrate, amino acids, amides and ureides. On the other hand, Bollard (1957) demonstrated that aspartic acid, asparagine and glutamine were the most abundant N compounds in the xylem sap of apple trees, whereas in coffee seedlings, Mazzafera and Goncalves (1999) found that NO_3^- was the most abundant N compound. However, in nitrogen fixing plants, ureides (allantoin and allantoic acid) constitute an important group of compound in which N_2 is incorporated (Schubert, 1986).

The aim of this study was to investigate the composition of sugarcane stems, as well as to relate the presence of amino acids in the apoplastic sap with the endophytic association *G. diazotrophicus*-sugarcane.

Materials and methods

Plant material and growth conditions

Saccharum officinarum L. (varieties NCo310, RD 75–11 and PR 60–170) aged 6 months old (ratton sugarcane), grown in a field located in Motril, near Granada (Spain) were sampled. The field has been in monoculture for more than 10 years and sugarcane plants, fertilized with 400–500 kg $N\,ha^{-1}\,year^{-1}$, grew in a typical Mediterranean environment. Mature, non-flowering stalks were randomly selected from separate plants. Samples were taken from the top 10 internodes of plants with approximately 25 aboveground internodes. In addition, sugarcane plants were sampled (var. NCo310, 6 month old) from cultures maintained in a controlled environmental chamber without N application, and some were inoculated with *G. diazotrophicus* PAL-5 strain, while others were not inoculated. Culture conditions were: 16–8 h light–dark cycle, 28–20 °C day-night temperature, relative humidity 55–75% and photosynthetic photon flux density (400–700 nm) of 450 $\mu mol\,m^{-2}\,s^{-1}$, supplied by combined fluorescent and incandescent lamps.

Sap collection

The extraction of the apoplastic fluid of the sugarcane stem was carried out according to Dong et al. (1994). Pieces of stem internodes were cut transversely into lengths of approximately 3–4 cm. The apoplastic sap was extracted by centrifugation at 3000 × *g* for 20 min for the stem pieces that were previously dipped in ethanol and surface flamed. After centrifugation, stems were squeezed and the released fluid, containing vacuolar cell juice (symplastic sap), was collected. The sap was kept on ice during collection and stored at −80 °C until analysis.

Analyses

Total soluble sugars in the sap were analyzed following the colorimetric method of Irigoyen et al. (1992). Free amino acids were determined using ninhydrin reagent (Yemm and Cocking, 1955) and calculated with a standard curve prepared with L–glutamine. Protein concentration was measured at 660 nm by the method of Lowry et al. (1951) using the Folin–Ciocalteau reagent with bovine-serum albumin as standard. Ammonium content in the sap was quantified by the colorimetric method of Solórzano (1969), while mean nitrate and nitrite levels were determined at 210 and 540 nm, respectively, according to Linblad and Guerrero (1993). Pure standards of ammonium, nitrate and nitrite were used to calculate their concentrations in the sugarcane sap. A total of 17 amino acids were separated by high-performance liquid chromatography (HPLC) and quantified by the fluorimetric