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Fructan Variation in Plants of *Lolium multiflorum* ssp. *italicum* 'Lema' (Poaceae) Exposed to an Urban Environment Contaminated by High Ozone Concentrations

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ABSTRACT

Fructans are the main reserve carbohydrates in vegetative tissues of Poaceae from temperate regions. The synthesis and degradation of fructans vary according to physiological and ecological alterations, including high concentrations of air pollutants. *Lolium multiflorum* (Italian ryegrass) is a temperate grass species, cultivated as forage in the South of Brazil and has also been used for biomonitoring purposes since the plants accumulate heavy metals in the leaves, in addition to high concentrations of fructans. In this study, six week-old *L. multiflorum* plants were exposed during 28 consecutive days as well as for 24, 48 and 72 h in a polluted site affected by high levels of ozone (Ibirapuera Park) and in a glasshouse with filtered air (reference site) in the City of São Paulo (Brazil). After each exposure period, the content and composition of leaf carbohydrates were analysed. Plants from the polluted site contained higher concentrations of fructans, especially those with an intermediate degree of polymerization, when compared with plants maintained in the reference site. The pattern of fructan accumulation seemed to follow the diurnal pattern of ozone concentrations in the air, i.e., higher contents of fructose were found in the afternoon, when the levels of that pollutant were also high. The data obtained confirmed that fructan metabolism was affected by high concentrations of ozone, being a potential indicator of the stress imposed by tropical urban environments to plants of Italian ryegrass.

Keywords: air pollution, carbohydrate, fructans, grasses, ozone, stress

INTRODUCTION

Carbohydrates derived from photosynthesis may be allocated to current growth and metabolism or accumulated as reserve of carbon and energy. Fructan (polyfructosylsucrose) is an important storage carbohydrate in many plant families, including grasses. Italian ryegrass accumulates large amounts of high molecular weight fructans in the leaf bases (Pollock and Eagles 1988), which add quality and nutritive value to ruminants (Shewmaker *et al*. 2006).

In addition to their role as sources of carbon and energy, fructans have versatile regulatory functions at both cellular and whole organism levels, controlling cellular metabolism, growth and development, and stress resistance of plants (Nishizawa *et al*. 2008; Valluru and Van den Ende 2008). These compounds and the enzymes associated with their metabolism might interact indirectly with reactive oxygen species (ROS) signalling pathways. Furthermore, fructans and other carbohydrates themselves might act as signals in pathways associated with stress tolerance (Van den Ende *et al*. 2004), like that observed in plants of the bioindicator species *Lolium multiflorum* submitted to air pollution (Sandrin *et al.* 2008).

In urban environments, ozone is considered to be one of the most phytotoxic air pollutants, causing severe stress effects in plants (Paoletti *et al*. 2007). The effects of ozone in plants are generally consequences of the enhanced production of ROS, which firstly result from reactions between ozone and water in the leaf apoplast. These oxygen compounds may promote an oxidative burst in leaf cells and cause injury to biomacromolecules, such as lipids and proteins, if the equilibrium of the oxidation/reduction state is not preserved (Baier *et al*. 2005; Foyer and Noctor 2005; Iriti and Faoro 2008). So, the lipid peroxidation in cellular membranes is one of the most precocious effects of ozone in plants (Iriti and Faoro 2008). These toxic by-products of ozone also affect photosynthesis, carbon partitioning and plant development (Sild *et al*. 2002). The ozone-induced changes in different plant processes can increase (Meyer *et al*. 1997) or reduce (Balaguer *et al*. 1995; Sild *et al*. 2002) the concentrations of carbohydrates.

Considering that fructans interact with ROS and are accumulated in plants exposed to air pollution, we may raise the hypothesis that the enhanced production of ROS in plants growing under high levels of ozone would interfere on their non-structural carbohydrate metabolism, being potential indicators of the stress imposed by the urban environment. Therefore, we investigated the composition and concentrations of these compounds in *L. multiflorum* plants growing in a highly contaminated site by ozone in São Paulo city in comparison to *L. multiflorum* plants growing in a reference site without air pollution.

MATERIALS AND METHODS

Seeds (0.8 g per pot) of *Lolium multiflorum* Lam. ssp. *italicum* Beck cv. 'Lema' were germinated and cultivated in plastic pots inside a glasshouse with filtered air (reference site) at the Institute of Botany (South Region of São Paulo city; 23° 38′ S and 46° 37′ W; 805 m above sea level) using a commercial substrate (Eucatex Plantmax HT^{TM}) mixed with fine vermiculite (3:1, v/v). During cultivation, the plants were weekly excised to a height of 4 cm from the substrate and fertilized with nutrient solution (40 cm^3 per pot) containing only macronutrients (5.8 g KH_2PO_4, 8.5 g KNO_3 and 5.3 g NH_4NO_3 per litre deionised water) according to VDI (2003).

After six weeks under controlled conditions, two experiments were performed at the Ibirapuera Park of São Paulo, which is cha-

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racterized by high concentrations of ozone and low concentrations of PM_{10}, CO, SO_2 and NO_2 (CETESB 2008). Ozone concentrations during the experiments were obtained from a continuous monitoring station settled at the Ibirapuera Park.

The first experiment was conducted in October, November and December 2005. In each month, six pots of *L. multiflorum* plants (average of 100 plants per pot) were maintained in the field, under standardized conditions for 28 days. The second experiment was performed in March 2006 and consisted of exposing a group of 12 pots of plants for 24, 48 and 72 h. The plants of six pots were collected in the morning and the other six pots in the afternoon of each exposure time. During both experiments, a similar number of pots were maintained in parallel at the mentioned glasshouse with filtered air, representing the reference situation. The pots remained equipped with nylon wicks over deionised water basins for providing a continuous water supply. *Lolium* cultures were produced and exposed following the draft of the VDI guideline 3957/2 (VDI 2003).

Stubbles of *L. multiflorum* were harvested in the morning after each 28-days period of exposure in the first experiment, and in the morning (7 and 10 h) and in the afternoon (13 and 16 h) after 24, 48 and 72 h of exposure in the second experiment. Non-structural carbohydrates (monosaccharides, sucrose and fructans) were extracted from the stubble according to the method described previously (Sandrin *et al*. 2008). The concentrated extracts were quantified for free and combined fructose (Jermyn 1956). Aliquots of the carbohydrate extracts were purified in columns containing cation exchange resin (Amberlite, IR-120) and anion exchange resin (Amberlite, IR-400) and analyzed by ascending thin-layer chromatography (TLC) on silicagel plates (Silicagel 60, layer thickness 0.2 mm, Merck or F1500, Schleicher and Schuell) and developed in butan-1-ol/propan-2-ol/water (3:12:4, v:v:v) as solvent system (Kanaya *et al*. 1978). Fructose-containing sugars were visualized after spraying with the ketose-specific urea-phosphoric acid reagent (Wise *et al*. 1955). Extracts of tubers of *Helianthus tuberosus* and *Allium cepa*, obtained in the market, were used as reference material for different fructan series (Pollock 1982).

Component sugars were separated by high-performance anion exchange chromatography and pulsed amperometric detection (HPAEC-PAD DX-300, Dionex, USA) on an analytical Carbo-Pac PA1 column (4 × 250 mm) using a sodium hydroxide (150 mM) and sodium acetate gradient (500 mM in 150 mM NaOH). The elution programme followed Itaya *et al*. (1997). Relative molecular mass of fructans was determined by gel-permeation chromatography (GPC) using polyacrylamide gel (Bio-Gel P-10), as described by Carvalho and Dietrich (1993). The degree of polymerization (DP) was calculated dividing the relative molecular mass of fructans by the relative molecular mass of fructose free of water.

Statistics

Differences in the contents of total fructose in plants from both sites and among the periods of exposure were identified by two-way and three-way ANOVA in the first and second experiments, respectively. In the last case, site, time of exposure and sampling time in each 24 h experiment were adopted as factors. Data from the second experiment of total fructose were $\log_{10}$ transformed to reach normal distribution and equal variances. After testing interactions between factors, differences among treatments were identified by the multiple comparison method of Student-Newman-Keuls.

RESULTS AND DISCUSSION

The mean levels of ozone in the polluted site were high throughout the first experimental period (October, November and December 2005), showing peaks between 13 and 17 h when solar irradiation was also high. The maximum hourly concentration per day in the period varied from 33 to 194 $\mu g.m^{-3}$. The daily pattern was similar in the second experiment, but the maximum hourly was lower (110 $\mu g.m^{-3}$; first two days) compared to the maximum concentration observed in the first experimental period (**Fig. 1**).

The contents of total fructose in plants after all periods of 28 days of exposure at Ibirapuera Park were significantly

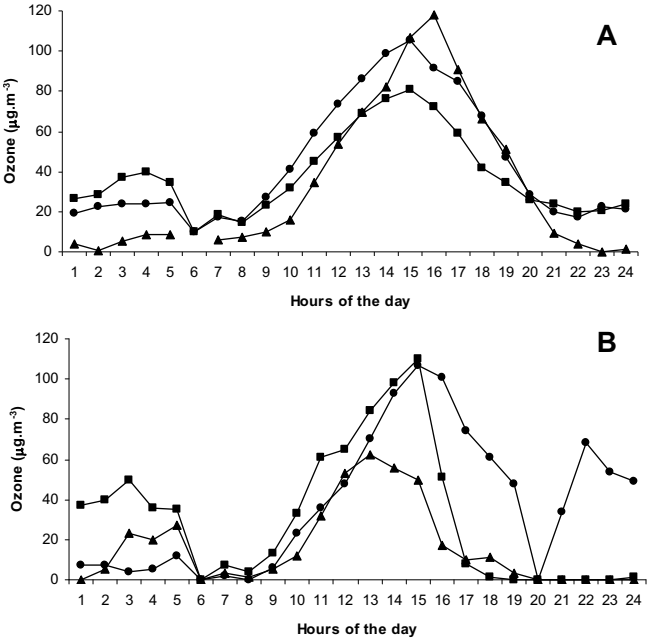

Fig. 1 Hourly variations of ozone concentrations during one day period in both exposure experiments performed with *L. multiflorum* plants at the Ibirapuera Park. (**A**) = Average values in (●) October, (■) November and (▲) December 2005. (**B**) = Values during the three consecutive days of March 2006 when the second experiment was performed (● = 24 h, ■ = 48 h, ▲= 72 h for the first to third day, respectively).

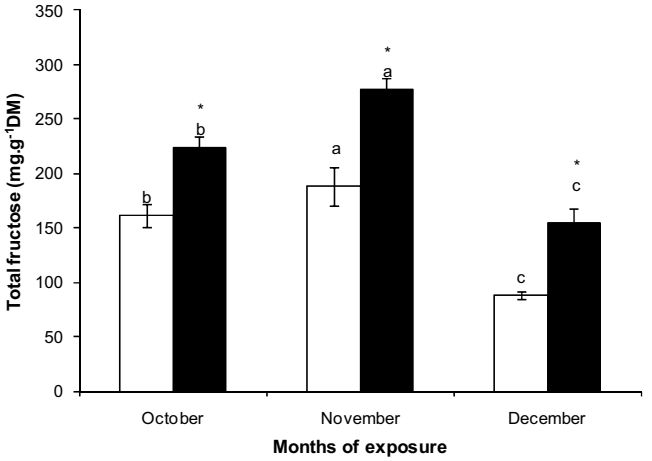

Fig. 2 Total fructose content in *Lolium multiflorum* plants exposed for twenty eight days at the (□) reference site (glasshouse) and at the (■) polluted site (Ibirapuera). Distinct letters indicate significant differences among monthly values obtained in each site and (*) the site where the leaf accumulation of fructose was higher. $p < 0.05$. Bars indicate the standard error.).

higher than those determined in plants maintained under filtered air inside the glasshouse (**Fig. 2**). Similar results were found in plants of the same species exposed to particulate matter and other pollutants in São Paulo city (Sandrin *et al*. 2008). These authors also discussed the co-influence of climatic conditions, especially temperature, on the carbohydrate levels. Although these climatic data were not recorded in the present work, their influence on the levels of fructose could not be ignored, since variations among months were found, which may not be explained only by the levels of ozone in the atmosphere. Significant higher concentrations of fructose were found in November, for example, a month that was characterized by lower mean levels of ozone in the air, while lower contents of fructose were found under higher atmospheric concentrations of ozone in December. Thereby, some climatic factors, such as high temperature and relative humidity, together with high ozone levels could

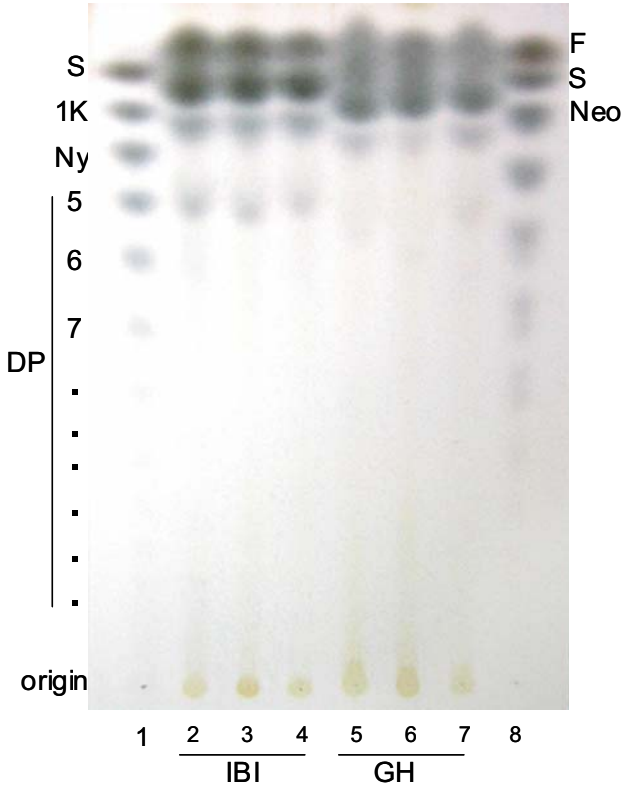

Fig. 3 Thin Layer Chromatography of fructoligosaccharides in *Lolium multiflorum* plants exposed for twenty eight days at the glasshouse (GH) and at the polluted site (IBI) in October (2 and 5), November (3 and 6) and December (4 and 7). *Helianthus tuberosus* (1) and *Allium cepa* (8). S = sucrose, F = fructose, 1K = 1-kestose; N = nystose; Neo = neo-kestose. DP = degree of polymerization.

have increased the fructose accumulation during the experimental period. In fact, reductions in the photosynthetic rate and in carbohydrate metabolism of plants exposed to ozone due the production of ROS have been reported (Gelang *et al*. 2001; Moraes *et al*. 2006; Bulbovas *et al*. 2007).

Sandrin *et al*. (2008), among other works (see Valluru and Van den Ende 2008, and refs therein), showed accumulation of fructans under stressing conditions, possibly playing a role in osmoregulation (Wiemken *et al*. 1995) and in cell membrane protection (Hincha *et al*. 2000, 2003). Recently, the putative roles of fructans localized in the vacuole (Kawakami *et al*. 2008) and in the apoplast (Van den Ende *et al*. 2005; Valluru *et al*. 2008) were established. The role in oxidative stress defence has also been proposed (Parvanova *et al*. 2004). These studies suggest that fructans act directly as ROS scavengers or indirectly by stimulating other specific antioxidative defence mechanisms. In the present study, the higher concentrations of fructose polymers could promote the survival of plants in the stressing urban environment of São Paulo.

The chromatograms (**Figs. 3, 4**) showed the accumulation of mainly low- and medium-DP fructans in plants growing under high levels of ozone, suggesting that polysaccharides were hydrolysed to accelerate the mobilization of fructans within the plant. These results were also confirmed by molecular mass analyzes that showed fructans with higher degree of polymerization in plants exposed to the reference site (mean value of 9.8 KDa) than in plants exposed to the polluted site (mean value of 7.65 KDa). This mechanism could be interpreted as a strategy for short-term storage, especially during a stress situation (Guerrand *et al*. 1996). In contrast, high-DP fructans preferentially accumulated by plants at the reference site could be related with their role as long-term reserve carbohydrate. In fact, the preferential accumulation of low- or high-DP fructans by plants under stress conditions is very variable. Some varieties of *Dactylis* and *Lolium*, for example, are able to survive severe drought in the Mediterranean regions, due to their ability to accumulate high DP fructans (Volaire *et al*. 1998). But, it is suggested that partial degradation of longer DP fructans increases the total number of molecules (fructose, sucrose, and lower DP fructans), increasing scaveging ROS capacities and dealing with the increased oxidative stress (Van den Ende and Valluru 2009). According to these and other authors, lower DP fructans as soluble polyhydroxy compounds might be still stronger antioxidants, being more efficient in radical quenching than higher DP fructans due their high total number of hydroxyl groups, as well as sucrose (Smirnoff and Cumbes 1989; Morelli *et al*. 2003; Van den Ende and Valluru 2009).

The significantly higher accumulation of fructans in plants exposed at Ibirapuera Park than in plants from the glasshouse was also observed in the second experiment. However, this experiment proved that the level of accumulation was time dependent. The longer the exposure time in both sites the higher the accumulation of fructose (**Fig. 5**). Additionally, this aspect seems to be reinforced by the fact that the concentrations of total fructose were comparatively higher in plants grown for 28 days in the same sites.

Lower levels of fructose were found in the morning in comparison to the results found in the afternoon, in all consecutive sampling days and in both exposure sites (**Fig. 5**). Studies concerning the diurnal variations of carbohydrates in grasses showed great changes in sucrose and starch contents (Ciavarella *et al*. 2000; Shewmaker *et al*. 2006), whilst no (Souza *et al*. 2005) or irregular variation was observed in the concentrations of fructose and fructans (Waite and Boyd 1953; Lechtenberg *et al*. 1972). According to Pollock and Cairns (1991), the storage and mobilization of sucrose and starch largely regulate the diurnal production, export, and consumption of photosynthates in the leaves, while fructans are preferably subjected to seasonal changes throughout plant development and respond quickly to weather changes. In the present study, the pattern of fructan accumulation seemed to follow the diurnal pattern of ozone concentrations in the air. Lower contents of fructose were found during the hours of the day when lower levels of ozone were registered, while higher contents of fructose coincided with higher atmospheric contamination by this pollutant. However, we may not also ignore the probable influence of meteorological variations, such as of air temperature and irradiance, on the fluctuation of fructose in plants in one day, since they straightly follow the hourly curve of ozone concentrations. Therefore, the real influence of ozone on hourly levels of fructose must be confirmed under laboratory conditions.

Analysing the results obtained by HPAEC/PAD, we observed that the composition of sugars in *L. multiflorum* plants did not change during the increasing period of exposure (data not shown). The oligosaccharides, mainly monosaccharides, tended to be in lower proportions in the afternoon, in plants from both sites, but fructans seemed not to proportionally change during the day (**Fig. 6**). These results indicate that short-term changes in ozone concentrations and/or on climatic factors during one day might influence more the diurnal variation in the concentration of fructose as a whole than its polymerization degree.

In the present work, the results suggest that the increase in fructan concentrations of plants growing in a contaminated environment by ozone, mainly those of lower degree of polymerization, could be a strategy for plant defence and also used as indicator of stress conditions.

CONCLUSION

Results presented here confirmed the hypothesis that stressing conditions as found in the studied urban area highly contaminated by ozone may alter qualitative and quantitatively the fructan pool in plants of *Lolium multiflorum* ssp. *italicum* cv. 'Lema', and is a potential indicator of the stress imposed by urban environment.

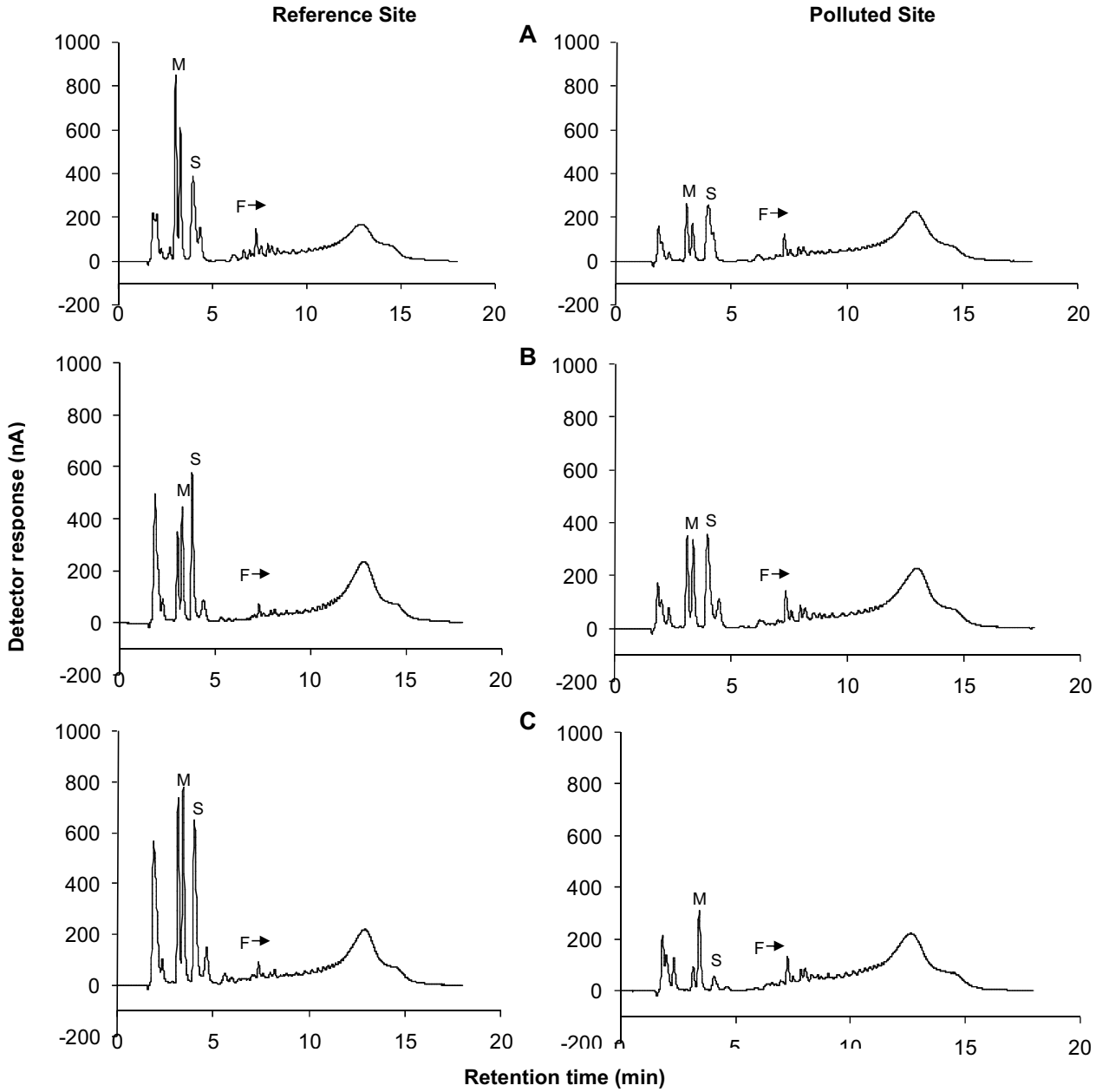

Fig. 4 HPAEC/PAD analysis on CarboPack PA-1 column of fructans in *Lolium multiflorum* plants exposed for twenty eight days at the reference site (glasshouse) and at the polluted site (Ibirapuera). (**A**) = October, (**B**) = November, (**C**) = December, M = monosaccharides, S = sucrose, F = fructans.

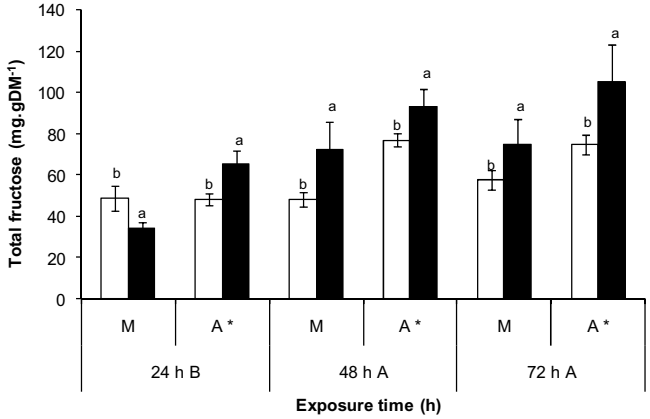

Fig. 5 Average contents of total fructose in *Lolium multiflorum* plants exposed for three consecutive days at the (□) reference site (glasshouse) and at the (■) polluted site (Ibirapuera). M = morning, A = afternoon. Small letters compare sites in each morning and afternoon sampling period. Capital letters compare the increasing times of exposure. Distinct letters indicate significant differences. * Significantly higher than the average values obtained in the morning in each time of exposure. Bars indicate the standard error.

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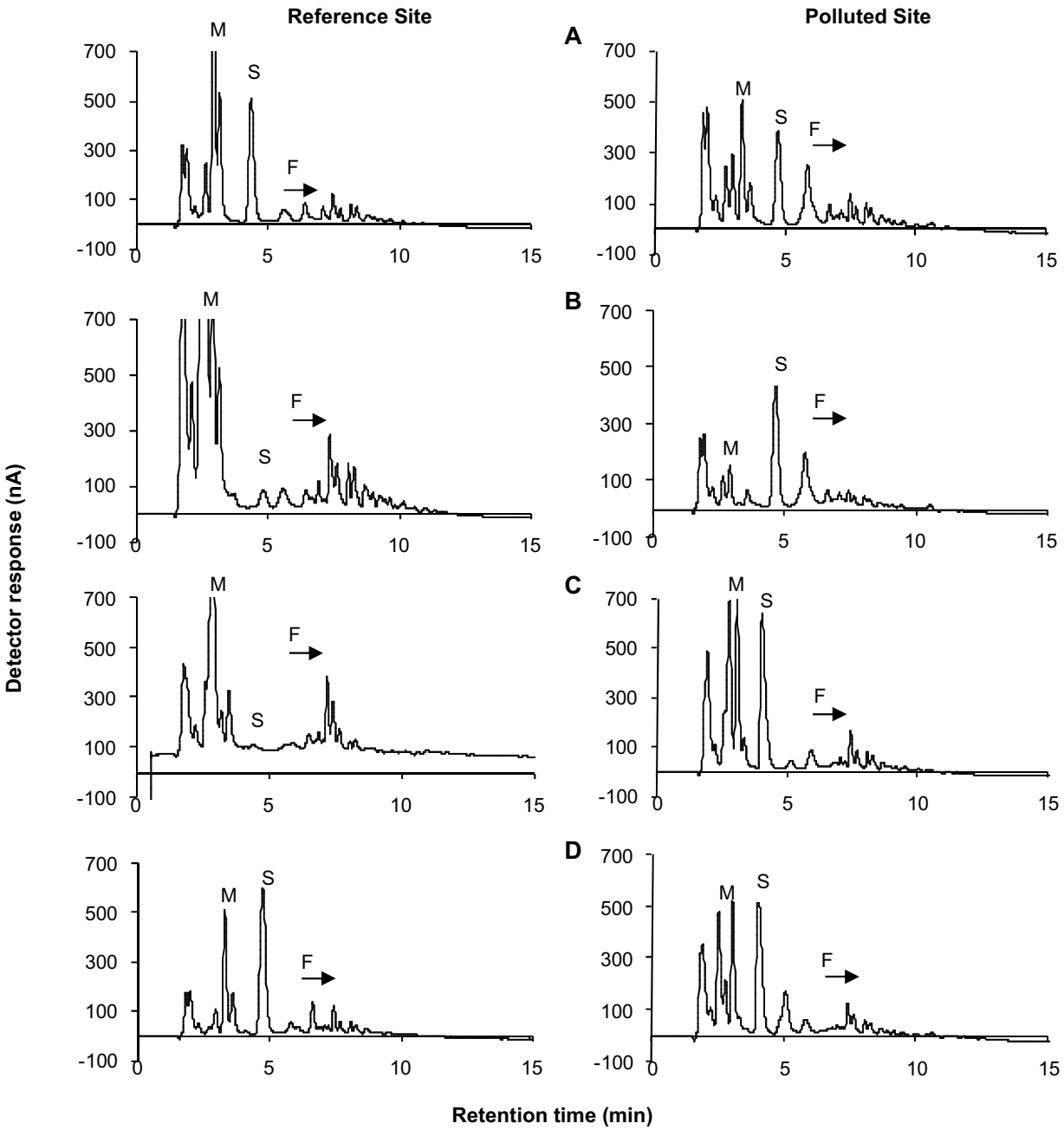

Fig. 6 HPAEC/PAD analysis on CarboPack PA-1 column of fructans in *Lolium multiflorum* plants exposed for three consecutive days at the reference site (glasshouse) and at the polluted site (Ibirapuera). **(A)** = 7 h, **(B)** = 10 h, **(C)** = 13 h, **(D)** = 16 h, M = monosaccharides, S = sucrose, F = fructans.

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