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Fructan variation in the rhizophores of *Vernonia herbacea* (Vell.) Rusby, as influenced by temperature

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ABSTRACT - (Fructan variation in the rhizophores of *Vernonia herbacea* (Vell.) Rusby, as influenced by temperature). The influence of climatic variations on fructan content in tropical regions is not well known. The present study deals with the effects of temperature on fructan contents in rhizophores of plants of *Vernonia herbacea*, a native species from the Brazilian cerrado vegetation. Intact plants and fragmented rhizophores were subjected to different temperatures under natural and controlled environmental conditions. Rhizophores of plants in pre-dormant stage (aerial parts showing some yellowish leaves) presented higher fructan content at 5ºC than those kept at 25ºC, whereas in dormant plants (aerial parts absent) temperature treatments did not affect fructan contents. Fragmented rhizophores obtained from dormant plants presented higher levels of fructo-polysaccharides at the end of the experiment than at the beginning of the treatment, regardless of the temperature they were stored, whereas fragments obtained from vegetative plants showed a decrease in fructan content under the same treatments. It was concluded that variations observed in fructan contents are related to the phenological state of the plants prior to the treatment rather than to extraneous temperatures they are subjected to during this stage.

RESUMO - (Variações nos frutanos de rizóforos de *Vernonia herbacea* (Vell.) Rusby, em função da temperatura). As relações entre variação nos conteúdos de frutanos e a variação nas condições climáticas e edáficas em regiões tropicais são pouco conhecidas. O objetivo deste trabalho foi investigar o efeito da temperatura no conteúdo e composição dos frutanos presentes em rizóforos de *Vernonia herbacea*, uma planta nativa do cerrado brasileiro. Plantas intactas e fragmentos de rizóforos foram submetidos a diferentes temperaturas. Ao final do tratamento de frio (5ºC) plantas intactas em início de dormência (parte aérea presente) apresentaram conteúdo de frutanos maior que o das plantas mantidas a 25ºC. Plantas completamente dormentes (sem a parte aérea) apresentaram poucas diferenças no conteúdo de frutanos quando mantidas a 5 ou 25ºC. Independentemente da temperatura utilizada, fragmentos de rizóforos, isolados de plantas em fase de dormência, apresentaram ao final do experimento um aumento no conteúdo de polifrutanos em relação ao início do tratamento; no entanto, fragmentos isolados de plantas em fase de brotação apresentaram diminuição no conteúdo de polifrutanos. Concluiu-se que as variações observadas estão mais relacionadas com o estádio fenológico da planta do que com o tratamento de temperatura.

Key words - Asteraceae, fructans, cerrado, low temperatures

Introduction

Fructans have been regarded as second to starch in importance as storage carbohydrates (Meier & Reid 1982, Soja et al. 1989), being present in approximately 15% of the contemporary Angiosperm (Hendry & Wallace 1993). The species that contain fructans are distributed within a diverse range of families including Poaceae and Asteraceae, and several families within the Liliales and Campanulales. This diversity of distribution together with the occurrence of fructans among highly evolved families indicate that the genes for fructan metabolism in angioperms may have arisen in response to one or a few selective pressures in the relatively recent past (Hendry 1987).

Variation in fructan contents and composition have been associated not only with the plant growth but also with seasonal changes in temperature and other environmental factors (Pollock 1986, Pontis 1989, Housley & Pollock 1993, and references there in). Since fructo-oligosaccharide accumulation is frequently correlated with plants exposed to low temperatures, it has been suggested that fructans may act as cryoprotectants (Eagles 1967, Wiemken et al. 1986, Pollock & Chatterton 1988). Corroborating this hypothesis is the fact that grasses including cereals (wheat, oats and barley) accumulate fructans during winter (Meier & Reid 1982, Pontis & Del Campillo 1985, Pollock 1986, Chatertton et al. 1989, Prud'home et al. 1993). Also, several species of Asteraceae, as exemplified by chicory (*Cichorium intybus* L.), accumulate fructans of low degree of polymerization (DP) when exposed to chilling

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temperatures (Van den Ende et al. 1996). However, Tognetti et al. (1990) pointed out that the role of fructan in cold acclimation remains an open question since its accumulation seems to be the result of sucrose accumulation (Wagner et al. 1983, Cairns & Pollock 1988, Pollock & Cairns 1991) rather than the low temperature *per se*.

Vernonia herbacea (Asteraceae) is a perennial herb that grows spontaneously in the Brazilian cerrrado and shows well defined seasonal developmental patterns (Figueiredo-Ribeiro et al. 1986, Carvalho & Dietrich 1993). The plants bear an underground reserve organ known as rhizophore which accumulates fructan of the inulin type as the main non-structural carbohydrate. The amounts and degree of polymerization of fructan in this species display very distinctive seasonal variation (Carvalho & Dietrich 1993). Due to these observations and since most of the reported studies on fructan variation have been conducted with plants native or adapted to temperate climate, the aim of the present study was to determine the effects of low temperature on the contents and the composition of fructan in intact and excised rhizophores of *Vernonia herbacea*.

Material and methods

Plant material - Two year old plants of *Vernonia herbacea* (Vell.) Rusby (Asteraceae), obtained through vegetative propagation, according to Carvalho et al. (1997) and cultivated in individual pots under natural environmental conditions, were used in the experiments.

At the start of the first experiment, the plants were at early dormancy (winter). Plants were separated into five groups of five plants each. One group was harvested immediately before the beginning of the treatments (day zero - D0) and the others were maintained under different temperature treatments for seven days; control (C) was maintained under natural environmental conditions, i.e., minimum air temperature of 8°C and maximum of 21°C and 10 h photoperiod. The three other groups were kept in growth chambers under 8 h photoperiod, 15 $mE.m^{-2}.s^{-1}$ and the following temperature regimes: 5°C, 25°C and 25°C/5°C (day/night). At the end of the treatments the rhizophores from each plant were collected for carbohydrate analyses.

On a second experiment, a lot of 24 plants at the dormant stage (without aerial organs) was separated into two groups and maintained in complete darkness for five days in growth chambers in two different conditions: 5°C and 25°C. Samples of rhizophores were harvested daily for carbohydrate analyses.

A third experiment was designed to detect a possible relationship between the phenological phase of the plants and the effect of temperature on fructan metabolism. Eighty fragments of rhizophores of about 3 cm in length were excised from plants at early dormancy and at the sprouting phase. The fragments were stored in black plastic bags for four weeks at 5°C and 20°C and 20 fragments were collected from each treatment every week for carbohydrate analyses. The temperature of 20°C was used instead of 25°C due to the fact that fragmented rhizophores and longer experimentation periods were used in these cases; 20°C was considerable more suitable to avoid tissue contamination and deterioration.

Extraction and analyses of soluble carbohydrates - Water soluble carbohydrates, including free-fructose, sucrose, fructo-oligosaccharides and fructo-polysaccharides were extracted from rhizophores according to Pollock & Jones (1979). Three samples (3 g each) of rhizophores were sliced into thin sections, killed by boiling in 80 % aqueous ethanol for 5 min and homogenized in a blender. The suspension was filtered under vacuum and the residue re-extracted with hot 80% ethanol for 5 min. After filtration, the residue was extracted twice (60 min each time) with hot water (60°C). The ethanolic and aqueous supernatants were pooled and concentrated under vacuum at 37°C, frozen, thawed and centrifuged at 13000 *g* for 10 min at 5°C. The supernatant was concentrated under vacuum and precipitated with three volumes of absolute ethanol. This mixture was maintained overnight at 5°C and then centrifuged at 9000 *g*, for 10 min at 5°C. The precipitates obtained were combined, thus constituting the fructo-polysaccharide fraction, whereas the remaining supernatant contained the fructo-oligosaccharides.

Free and combined fructose were quantified by a ketose-specific modification of the anthrone reaction (Jermyn 1956) using fructose (Sigma Co.) and inulin from *Helianthus tuberosus* L. as standards.

For qualitative analysis the oligosaccharides were desalted using a 5x1 cm column consisting of half anionic resin (Amberlite IR 400) and half cationic resin (Amberlite IR 120) as described by Pollock & Jones (1979). After elution with 10 volumes distilled water, the eluate was concentrated under vacuum at 37°C and the neutral sugars analyzed by ascending thin-layer chromatography (TLC) as described by Kanaya et al. (1978). Carbohydrates were visualized by the ketose-specific urea-phosphoric acid reagent (Wise et al. 1955). Components were identified by comparison with oligosaccharides of the inulin series from tubers of *H. tuberosus* chromatographed simultaneously.

The purified fructo-oligosaccharides were also analyzed on a 4x250 mm CarboPac PA-1 anion-exchange column using a Dionex DX 300 gradient chromatography system with pulsed amperometric detection (HPAEC/PAD). The gradient was established according to Shiomi (1993) with the following modifications: eluent A (150 mM NaOH) and eluent B (500 mM sodium acetate in 150 mM NaOH). The gradient utilized was 0-1 min, 25 mM; 1-2 min, 25-50 mM ; 2-14 min, 50 mM to 500 mM; 14-22 min, 500 mM; 22-30 min, 25 mM. The applied PAD potentials were for $E_1 = 0.10$ (540 ms); $E_2 = 0.60$ (120 ms); $E_3 = -0.60$ (60 ms).

The polysaccharides were analyzed by gel-permeation chromatography (GPC). After dialysis overnight against water, samples containing 5 $mg.ml^{-1}$ in fructose equivalent were applied into a column of polyacrylamide gel (Bio-Gel P-10) calibrated with a series of linear dextrans (Sigma Chemical Co.). Fractions (2.3 ml) were collected by elution with 10 $mol.l^{-3}$ ammonium bicarbonate buffer containing 0.002 % sodium azide, at a rate of 0.3 $ml.min^{-1}$. Free and combined fructose were measured by the anthrone reaction (Jermyn 1956). Apparent molecular mass and degree of polymerization (DP) were estimated according to Andrews (1965).

Statistical significance of the data at 5% level was determined by the Tukey-test (Snedecor 1962).

Results and Discussion

When plants at early dormancy (aerial organs with signs of senescence) were kept for seven days at 5ºC, fructan contents in the rhizophores increased as compared to plants maintained at 25ºC. Intermediate values were found for alternate temperature treatment - 25ºC/5ºC (day/night) (figure 1). Senescence of the aerial parts was also higher at 5ºC than in the two other treatments. The DP of the fructo-polysaccharide fraction was slightly higher at 5ºC (DP=36) and lower at 25ºC (DP=30) relative to the remaining treatments (DP=33). Plants at dormancy, lacking aerial parts and maintained at 5ºC and 25ºC, did not differ significantly from each other in fructan content at the end of the treatment (figure 2), although fluctuations not statistically significant occurred throughout the experiment.

It is known that fructan content is influenced by sink-source manipulations, low temperature promoting accumulation of fructans in roots and tubers (Kuhbauch & Thomé 1989). This is due to an excess of photoassimilates since photosynthesis is less sensitive to low temperature than growth processes (Pollock 1986, Chatterton et al. 1989, Prud'home et al. 1993). Furthermore, the enzymes of fructan metabolism are not affected by low temperature (Pollock & Lloyd 1987, Farrar 1988, Tognetti et al.1989, Bancal & Triboi 1993).

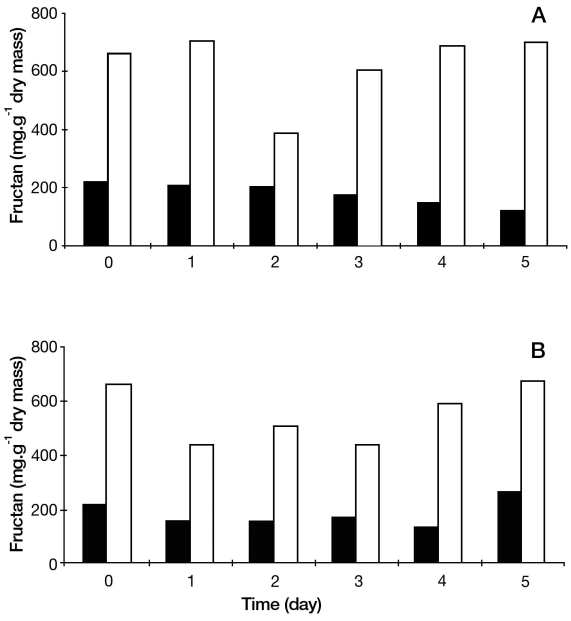

Figure 2. Contents of fructo-oligosaccharides (■) and fructo-polysaccharides (□) in rhizophores of intact plants of *V. herbacea* at dormancy, subjected to 5ºC (A) and 25ºC (B) for five days under darkness. Statistical differences between treatments and time of sampling were not detected by analysis of variance.

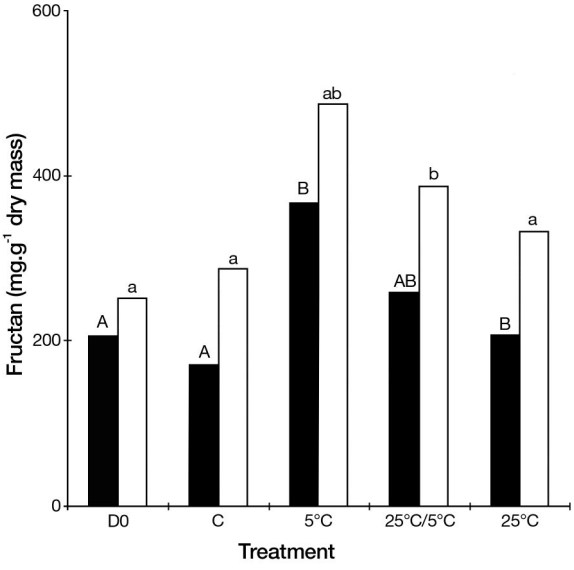

Figure 1. Contents of fructo-oligosaccharides (■) and fructo-polysaccharides (□) in rhizophores of intact plants of *V. herbacea* at early dormancy subjected to different temperature treatments for seven days. D0 - day zero, C- control (after seven days under natural environmental conditions), 5ºC and 25ºC constant temperatures and 25ºC/5ºC (day/night). Capital letters compare oligosaccharide contents and small letters compare polysaccharide contents among different treatments (LSD 5% Tukey).

The increase in fructan contents in senescing plants of *V. herbacea* at low temperature observed in figure 1 could be the result of more intense translocation of remaining photoassimilates from the decaying aerial organs to the rhizophores. The relatively constant levels of fructans in plants treated during dormancy (figure 2), when the source of photoassimilates is absent is consistent with this hypothesis.

Fragments of rhizophores obtained from plants at early dormancy and maintained for four weeks under 5ºC in darkness presented an overall increase in the fructo-polysaccharide fraction from the first week of the experiment (figure 3A). Variation in this

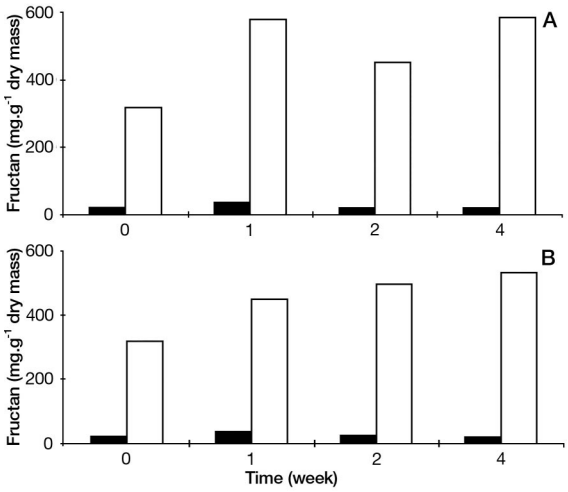

Figure 3. Contents of fructo-oligosaccharides (■) and fructo-polysaccharides (□) in fragmented rhizophores from plants of *V. herbacea* at early dormancy phase subjected to 5ºC (A) and 20ºC (B) for four weeks under darkness. Statistical differences between treatments and time of sampling were not detected by analysis of variance.

fraction was also shown by fragments maintained at 20ºC, leading to a final increase in polysaccharides (figure 3B). In contradistinction, fragments from plants in the sprouting phase had the fructan content decreased during the same period regardless of the temperature (figure 4). These results, in addition to those obtained by Carvalho & Dietrich (1993) on the increase of fructan during dormancy and decrease during sprouting, suggest that the changes in fructan contents are more related to the phenological phase of the plant than to the temperature *per se*.

In fact, the endogenous status of the rhizophores at the moment they are excised from the plant, i.e. its phenological state, seems to be a key aspect when dealing with other physiological process, like sprouting. Carvalho & Dietrich (1993) reported that although fragmentation of rhizophores induces break of dormancy regardless of the phenological state of the plant from which they originate, the velocity of the response is lower in rhizophores from plants fully vegetative and in dormancy than in sprouting and flowering stages.

Analysis of the relative contents of individual sugars from the rhizophores of intact plants (figures 5 and 6) showed that the proportion of fructo-oligosaccharides to free fructose is higher

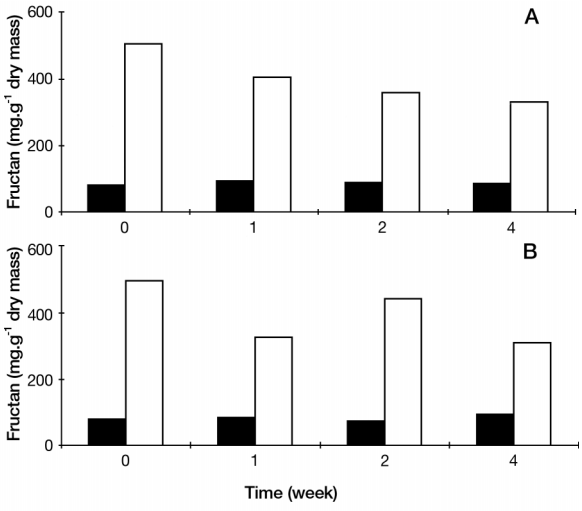

Figure 4. Contents of fructo-oligosaccharides (■) and fructo-polysaccharides (□) in fragmented rhizophores from plants of *V. herbacea* at sprouting phase subjected to 5ºC (A) and 20ºC (B) for four weeks under darkness. Statistical differences between treatment and time of sampling were not detected by analysis of variance.

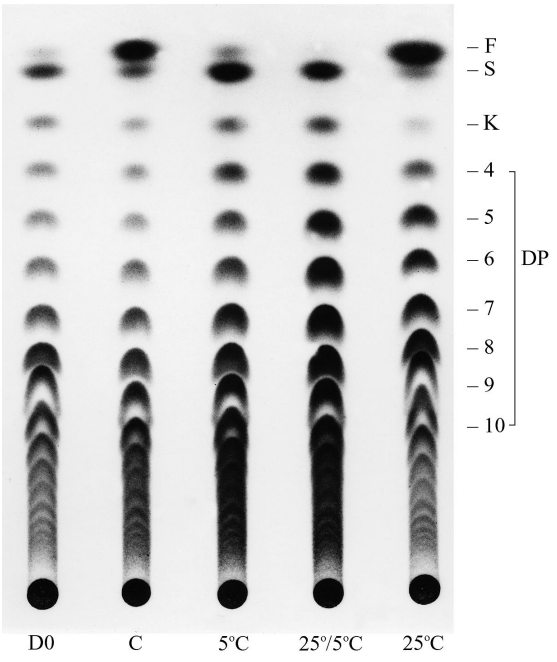

Figure 5. Thin layer chromatography of fructo-oligosaccharides present in rhizophores of intact plants of *V. herbacea* at early dormancy subjected to different temperature treatments for seven days. Legends for treatments as in figure 1. F - fructose, S - sucrose, K - 1-kestose, DP - degree of polymerization.

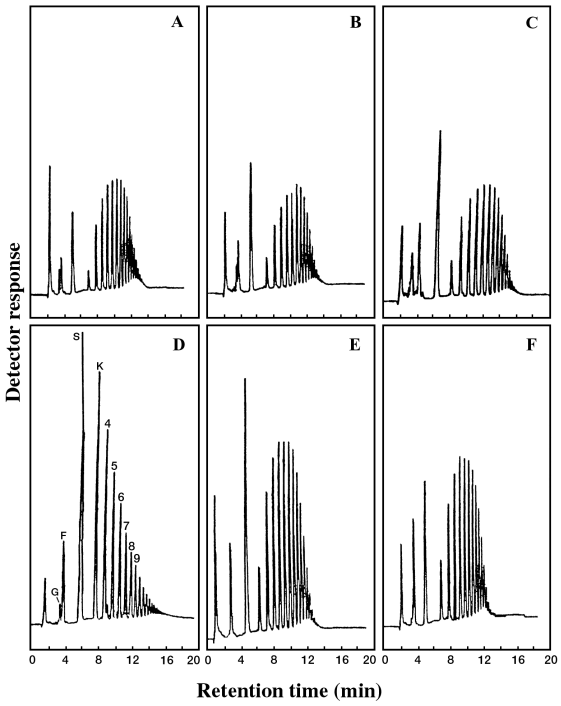

Figure 6. HPAEC/PAD profiles of fructosaccharides from rhizophores of *V. herbacea* at dormancy, subjected to 5ºC and 25ºC for five days. A - beginning of experiment; B - three days at 5ºC; C - five days at 5ºC; D - fructo-oligosaccharides from tubers of *Helianthus tuberosus*; E - three days at 25ºC; F- five days at 25ºC. G - glucose, F - fructose, S - sucrose, K - 1-kestose. Numerals 4-9 identify peaks of inulin oligosaccharides of DP 4-9.

than in fragmented rhizophores (figures 7 and 8). This indicates that the depolymerization of the fructan chain occurs more markedly in rhizophores which are fragmented than in intact rhizophores still attached to the aerial parts, regardless of the storage temperature. The depolymerization in fragmented rhizophores is evidenced by the marked increase in free fructose when the chromatographic profile at the beginning of the experiment (figures 7 A and 8 A) is compared to the following sampling periods.

Rhizophores from intact plants and fragmented rhizophores excised from dormant plants subjected to low temperatures present, at the end of the treatment, sucrose:fructose ratios higher than those kept at 25ºC or 20ºC (figures 5, 6 and 7). This seems to be a consequence of the increase of sucrose possibly due to the enhancement of the activity of sucrose synthesizing enzymes at low temperature. Indeed, in wheat it was observed that sucrose phosphate synthase and sucrose synthase increase in plants stored at low temperatures (Calderon & Pontis 1985, Salerno et al. 1989, Tognetti et al.1989, 1990).

Levels of glucose and fructose in fragments of rhizophores excised from sprouting plants were high, regardless of the temperature they were submitted to (figure 8). Since the metabolism of plants is more active towards depolymerization during the sprouting phase (Carvalho & Dietrich 1993), increase in free fructose in fragments from sprouting plants suggests that fructan hydrolysis was stimulated. It is known that fragmentation represents an injury to the tissues promoting several changes which include fructan hydrolysis in reserve organs of plants (Rosenstock & Kahl 1978).

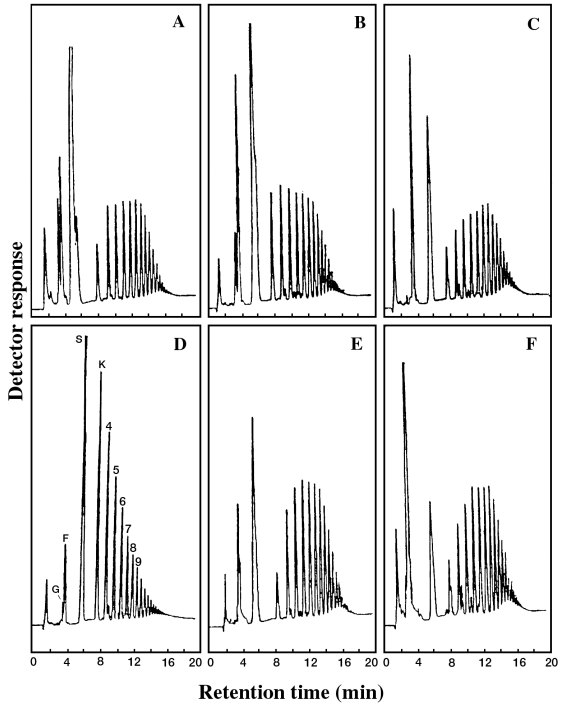

Figure 7. HPAEC/PAD profiles of fructosaccharides from fragmented rhizophores of *V. herbacea* at early dormancy, subjected to 5ºC and 20ºC for one and four weeks; A - beginning of experiment; B - one week at 5ºC; C - four weeks at 5ºC; D - fructo-oligosaccharides from tubers of *Helianthus tuberosus*; E - one week at 20ºC; F - four weeks at 20ºC. G - glucose, F - fructose, S - sucrose, K - 1-kestose. Numerals 4-9 identify peaks of inulin oligosaccharides of DP 4-9.

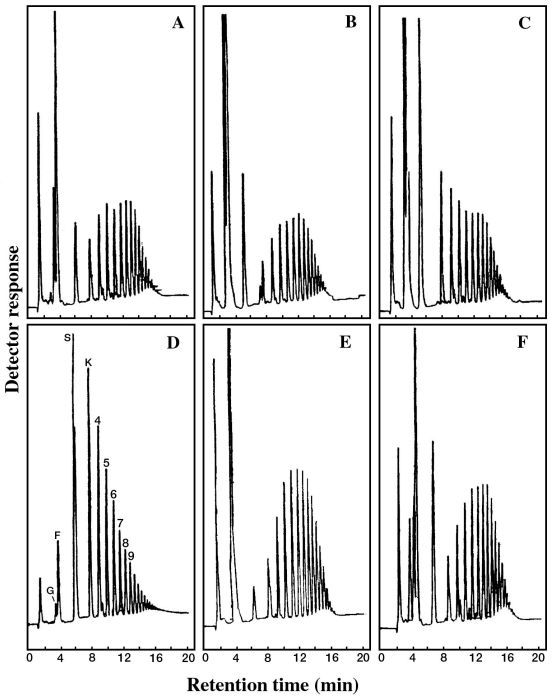

Figure 8. HPAEC/PAD profiles of fructosaccharides from fragmented rhizophores of *Vernonia herbacea* at sprouting, subjected to 5ºC and 20ºC for one and four weeks; A - beginning of experiment; B - one week at 5ºC; C - four weeks at 5ºC; D - fructo-oligosaccharides from tubers of *Helianthus tuberosus*; E - one week at 20ºC; F - four weeks at 20ºC. G-glucose, F - fructose, S - sucrose, K - 1-kestose. Numerals 4-9 identify peaks of inulin oligosaccharides of DP 4-9.

From the results obtained so far with *V. herbacea* it can be concluded that low temperature *per se* does not alter the direction of fructan metabolism from synthesis to hydrolysis or vice versa but seems to change the rate of the changes which is characteristic of each phenological stage of the plant.

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