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A thin layer chromatography study of the effect of low temperature on the molecular size distribution of fructans in Osteospermum Sinuatum (DC.) Norlindh (Aste aceae).

by Jaana-Maria Ball

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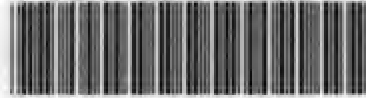

A THIN LAYER CHROMATOGRAPHY STUDY OF THE EFFECT OF LOW TEMPERATURE ON THE MOLECULAR SIZE DISTRIBUTION OF FRUCTANS IN OSTEOSPERMUM SINUATUM (DC.) NORLINDH (ASTERACEAE).

by

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ABBREVIATIONS AND SYMBOLS

^{14}C or $^{14}CO_2$	A radioactive form of carbon
C3 PLANTS	Plants with PGA as the primary product of photosynthetic CO_2 assimilation
C4 PLANTS	Plants with OAA or a related 4C-acid as the first identifiable product of the photosynthetic CO_2 assimilation
DP	Degree of polymerization
FFT	Fructan-fructan fructosyl transferase
GPC	Gel permeation chromatography
HPLC	High performance liquid chromatography
MW	Molecular weight
SST	Sucrose-sucrose fructosyl transferase
TLC	Thin layer chromatography
WSC	Water soluble carbohydrates

ABSTRACT

Plants of a palatable dwarf deciduous Karoo shrub *Osteospermum sinuatum* (DC.) Norlindh were grown in contolled environment chambers at 12/5°C, 25/18°C and 35/28°C day/night temperatures with a 12 h day/12 h night cycle. Shock treated plants were grown at 35/28°C day/night temperatures and transferred to 12/5°C day/night temperatures for either 12, 24 or 48 hours. Different molecular weight fructans were separated by TLC and identified using oligofructans extracted from *Allium cepa* L. (onion) and a fructose and sucrose mixture (1 g/100 ml) as reference compounds. *Osteospermum sinuatum* contained fructose, sucrose and fructans of the inulin series. Growth at low temperature caused the breakdown of high-DP (>DP 8) fructans and the accumulation of low- (DP 1 and 2) and medium-DP (DP 3-7) fructans. Slight differences in accumulation were observed in the above and below ground material. Growth at high temperatures resulted in the synthesis of high-DP (>DP 8) fructans. Transfer of plants from high to low temperature caused the breakdown of high-DP fructans between 12 and 24 hours after transfer.

A review of the recent literature pertaining to fructans, especially their potential role in low temperature tolerance of plants is also presented.

1. INTRODUCTION

Cold is a stress factor of widespred occurrence. Various mechanisms are involved which help the plant to reduce, prevent or escape excessive low temperature stress. Degrees of coldhardiness or freezing resistence of plants vary among species, and depend upon developmental stages and environmental conditions (Sakai and Larcher, 1987).

Plants are able to store carbon fixed during photosynthesis to serve as an energy substrate during periods when carbon fixation cannot take place. The strategy of storing the nonstructural carbohydrate, fructan, as an extension of the sucrose pool in vacuolated cells, is employed by many vascular plants (Pollock, 1986).

The relationship between fructans and cold stress response in higher plants, especially in economically important genera such as temperate cereals and grasses has received considerable attention recently (Suzuki, 1989). Several reviews on the occurrence, structure and metabolism of fructans have been published (Pontis, 1989; Pontis, 1990; Pollock, 1986; Pollock and Chatterton, 1988; Pollock and Cairns, 1991). A function of fructan in acclimation is considered to be osmoregulation, as molecular size of fructan changes in response to varying environments. There is however continued debate as to whether fructan is directly involved in the mechanism of cryoprotection (Pollock, 1986). The potential role of sugar and polyhydric alcohol accumulation in cold tolerant plants have been well

studied (Sakai and Larcher, 1987), but few studies have included fructan. Furthermore, there have been no studies to date on the importance of fructans in cold tolerance in the flora of Southern Africa, although fructans have been reported to occur in the Karoo shrubs *Pteronia pallens*, *Ruschia spinosa* and *Osteospermum sinuatum* (van der Heyden and Stock, unpublished). The purpose of this study was to examine and compare variation in molecular size of fructans of above and below ground tissues of a palatable dwarf deciduous Karoo shrub, *Osteospermum sinuatum* (DC.) Norlindh (Asteraceae) grown in different day/night temperatures regimes. The effects of low temperature on the molecular size of fructans of above ground tissues during the acclimation process was also studied by shock treating the plants for various periods of time. *O. sinuatum* is found in the Little and Great Karoo (Bond and Goldblatt, 1984) which have a high occurence of frost days a year. From the period June 1990 to May 1991 (12 months) 17 severe frost days and 40 frost days were recorded at the weather station at Beaufort West. In the same period, lowest minimum temperatures less than $0^{o}C$ and less than $5^{o}C$ were recorded for three months and eight months of the year, respectively (Weather Bureau, 1990 and 1991).

2. LITERATURE REVIEW

2.1. HISTORICAL BACKGROUND

Fructans have been recognised as a distinct class of carbohydrates since 1905 (Rose, 1904 as cited by Pollock and Cairns, 1991), and their chemistry, biochemistry, and physiology have been studied periodically ever since. Recent work has concentrated on the biochemistry, enzymology, and cell biology of these compounds (Pollock and Cairns, 1991; Wagner *et al.*, 1987; Housley *et al.*, 1989).

2.2. FRUCTAN STRUCTURE

Fructans, previously called fructosans are small oligmers and polymers of fructose carrying a D-glucosyl residue at the end of the chain attached via a β -(2→1)-linkage (Nelson and Spollen, 1987). Most fructans are distributed in an unbroken series between a fructosyl-sucrose and the polymer of the highest molecular weight found in that plant, which may be a mixture of neighbouring homologues (Pontis, 1990). Fructans differ in molecular structure and in molecular weight. The degree of polymerization varies with plant species and with life cycle (Pollock and Cairns, 1991; Pontis, 1990), but all fructans have a low degree of polymerization compared with starch. The number of fructose units range from 5-10 in the fructans of *Avena sativa* (Pollock, 1986) to as high as 260 in *Phleum pratense* (Pontis, 1990).

Fructans are classified into three major types: (a) the inulin group or those with β -(2→1)-D-fructofuranosyl units, (b) the phlein group or those with β -(6→2)-D-fructofuranosyl units, and (c) the branched group or those with both kinds of glycosidic linkages (Meir and Reid, 1982; McDonald, 1946).
As implied in many reviews (Pollock, 1986; Nelson and Spollen, 1987; Pollock and Chatterton, 1988; French, 1989; Nelson and Smith, 1968) the structure of fructans is neither simple nor well understood. There is a need for detailed structural analyses using modern techniques on a wide range of species. Future studies must include both low and high molecular weight fructans in species where a range of polymer size exists, and mixtures of single linkage chains must be distinguished from true mixed linkage polymers. The distribution of branched forms of fructans, in particular needs examination. It is the view of Pollock (1986) that only when a better understanding of the range of fructan structures present in fructans of vascular plants is obtained, will researchers be able to test hypotheses concerning synthetic and degradative mechanisms. There is also a growing need for the availability of documented standards of fructans.

2.3. OCCURRENCE

Fructans are widely distributed in the plant kingdom and assumed to be found in approximately 36,000 plant species making up over 12% of the angiospermous flora (Hendry, 1987; Pontis, 1990). They are present in monocotyledons, dicotyledons, green algae and blue-green algae (Hendry, 1987). The majority of species are members of 10 families within 5 orders (Table 1) (Pollock and

Chatterton, 1988), with Asteraceae being the dominant family (24 000 fructan-containing species) (Pontis, 1990). Fructan is the principal reserve carbohydrate in four of the most evolutionary advanced families, the Asteraceae, Campanulaceae, Boraginaceae and Graminaee (sub family Pooideae). Fractionation by TLC of fructans from a range of Gramineae indicated some similarities in the distribution in the generic, sub-tribal, and tribal levels (Smouter and Simpson, 1989 as cited by Pollock and Cairns, 1991). It has been proposed by Pollock and Cairns (1991) that molecular weight distribution patterns of fructans may be used as a sensitive chemotaxanomic aid within the Gramineae and the data obtained compared with other taxonomic observations. Chatterton *et al.* (1989) found variations in fructan levels within species suggesting significant genetic differences for carbohydrate partitioning within species. Of the 5 orders in which fructan occurs, Liliales is the only order not highly evolved, therefore fructan chemistry may have developed recently and should not be considered a minor pathway of declining evolutionary significance (Pollock and Chatterton, 1988). Hendry (1987) believes that fructan metabolism has arisen in response to a few selective pressures in the recent past. Studies on fructan metabolism have concentrated on the Asteraceae, Graminae, Liliaceae and Agavaceae families (Pollock and Chatterton, 1988). The inulin group is found in most dicotyledonous plants and the phlein and branched groups in most monocotyledonous plants (Pontis, 1990). However, the first member of the inulin series, isoketose is found in all Graminea (Pontis, 1990).

Hendry (1987) studied 20 fructan-containing species from the British flora and from this study and generalized evidence from the world flora, concluded that despite the confinement of fructan-containing species to a small number of families, the species themselves tend to have evolved a wide range of life histories and exploit a diverse selection of habitats and climates. Chatterton *et al.* (1989) examined 185 grass accessions and reported that no plant with C-4 type photosynthesis accumulates significant amounts of fructans and that C-3 type plants generally are fructan accumulators.

It is generally assumed that the localization of fructan is in the vacuole, although until recently there was no direct evidence for this hypothesis. The absence of any other obvious storage compartment, together with the large quantities of fructan accumulated in the storage organs of both dicots and monocots strengthened this argument. Precise fractionation of cells from the leaves of *Hordeum vulgare* and tubers of *Helianthus tuberosus* in a study by Wagner *et al.* (1983 as cited by Pollock, 1986) confirmed these suggestions. This location may be significant if the function of fructan is the regulation of osmotic potential and low temperature tolerance (Hendry, 1987). Hendry (1987) while studying 20 British fructan-containing species noted that fructan concentration was correlated to large cell size and high 2C DNA values.

Although various authors have reported distribution of fructan throughout a particular plant, unequal fructan concentration in

the different parts of the same plant is a common occurence (Pontis, 1990). In general, leaves tend to have small concentrations of fructans, whereas roots, bulbs, tubers, rhizomes (Brocklebank and Hendry, 1989) and immature fruits (Meir and Reid, 1982) tend to have high concentrations. The lower grass stem internodes generally contain large amounts of fructans (Pollock and Cairns, 1991). Fructan values exceeding 50% dry weight of tissue have been reported for members of the Asteraceae, Liliaceae and Gramineae (Pontis, 1990; Brocklebank and Hendry, 1989). The amount of fructan in a given tissue depends on the stage of development and fructan contents vary throughout a plants life cycle (Soja *et al.*, 1989; Suzuki, 1989). Changes are also associated with seasonal temperature variations (Pontis, 1989). Pontis (1989) reports that in all cases high molecular weight (DP >25) fructans were found in late summer and autumn, whereas in winter and early spring, low molecular weight (DP >3-6) fructans predominated.

In a study of 185 accessions of Gramineae it was found that tissues which function as a reservoir of nonstructural carbohydrates stored a greater proportion of high molecular weight fructans than leaf blades (Chatterton *et al.*, 1989). In field grown forage grasses (Pollock and Jones, 1979) extracts of stems contained higher molecular weight fructans than those of leaves. In a study by Housley and Volenec (1988) the mean molecular weight of leaf blade fructan was smaller than that of the leaf sheath in *Festuca arundinaceae*.

2.4. GEOGRAPHICAL DISTRIBUTION

From the literature studied there seems no strong relationship between fructan accumulating plant species and geographical distribution. Fructan accumulators frequently share their habitats with species which do not accumulate fructan; thus, their metabolism should be viewed as one adaptive mechanism out of many which would increase fitness in any given environment (Pollock, 1986). Even in cool climates the majority of species do not have fructan metabolism (Pollock and Chatterton, 1990). For this reason Hendry (1987) suggests that the examination of geographical distributions of fructan containing species will not provide evidence for the selective advantages of fructan metabolism. Chatterton *et al.* (1989) however found no fructans in intact leaves in genera of cool-season grasses of Gondwanic origin, although all other cool-season grass studied contain significant levels of fructan. The authors also found no warm-season grass species accumulating fructan.

2.5. EXTRACTION, PURIFICATION AND MEASUREMENT OF FRUCTAN

This section will attempt to present the current status of experimental techniques and methods used for studying the different aspects of fructan biochemistry. There is to date no simple, reliable and sensitive test for the presence of fructans in plant tissues. For this reason and the fact that many methods used have limitations, it is difficult to accurately assess the distributions of fructans among plants (Pollock and Chatterton, 1990). Precise analytical methods are required to discriminate among sucrose, low-DP fructo-oligosaccharides, and other soluble oligosaccharides (Pollock and Chatterton, 1988). Errors in separation are greatest when soluble carbohydrate contents are low and when modern chromatographic techniques are not used (Pollock and Cairns, 1991).

Initially separation methods were based upon the differential solubility of fructans of different sizes in ethanol, and it proved difficult to obtain pure preparations which were free from sucrose and yet representative of the size distribution of the native polymer (Pollock, 1984a as cited by Pollock, 1986; Livingston, 1990).

Resolution of individual fructo-oligosaccharides by the use of paper chromatography (Bacon, 1959 as cited by Pollock, 1986), thin-layer chromatography (TLC) (Collins and Chandokar, 1971; Schaffler and Morel Du Boil, 1972), gas-liquid chromatography (Pollock *et al.*, 1979), or gel-permeation chromatography (GPC) (Darbyshire and Hendry, 1978) has helped to further the

understanding of the structural characteristics of fructans with different DPs. High-performance liquid chromatography (HPLC) using amino-bonded silica, calcium based ion-exchange resins and high-performance gel permeation columns give improved resolution and shorter separation times. The general resolution limits are restricted to oligosaccharides with degree of polymerization (DP) of 10-12. The use of organic modifiers with silica-based columns, allow resolution of fructans up to DP 30. Estimates of mean molecular weight for higher DP fructan can be made on incompletely separated fractions by conventional physiological methods (Pollock and Chatterton, 1988). To date there exists a limited range of plant species for which detailed information on the structural relationships of fructans is available and research has tended to to concentrate on the simplest and most easily characterized forms (Pollock, 1986).

2.6. CONDITIONS FAVOURING FRUCTAN ACCUMULATION

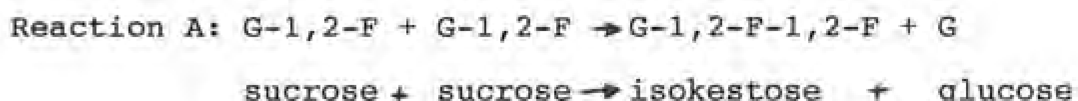
Many reviews have been written examining the effects of environmental variables upon fructan accumulation (Meir and Reid, 1982; Pollock, 1986). Conditions favouring fructan accumulation are those which stimulate carbon fixation or reduce plant growth (e.g. occasinal cutting or grazing, long photoperiods, low levels of nitrogen fertilization, low temperatures and biotic stresses such as fungal infection or removal of fruit). Fructan accumulation is minimized when carbon fixation is low or growth stimulated (e.g. frequent defoliation, short photoperiods, high levels of fertilization and high temperatures) (Byeong-Ryong and Housley, 1990). Frossard *et al.* (1989) found that fructan

accumulated in ryegrass as heavy metal content increased. The accumulation occured in the absence of yield reduction and visible symptoms of phytotoxicity in the case of Cd, Zn and Ni. Fructan content of the shoots increased by 25% with 0.4mg Cd kg^{-1} soil, but decreased at higher doses. Fructan content increased by 25% with 100mg Ni kg^{-1} soil, by 42% with 100mg Cu kg^{-1} soil and by 118% with 360mg Zn kg^{-1} soil.

2.7. THE ENZYMES OF FRUCTAN SYNTHESIS

2.7.1. Sucrose utilization

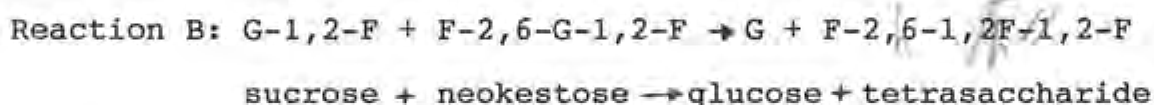
Evidence suggests that fructans are not synthesised from sugar nucleotide intermediates, but are formed from sucrose by the action of a number of glycosyl transferases (Meir and Reid, 1982; Pollock, 1982a). Enzymes from many plant species can catalyse the synthesis of fructan with sucrose as the only substrate. A wide range of glycosyl transferases are involved in fructan accumulation in vascular plants (Pollock 1986). All cell-free systems which have been characterized in detail and shown to synthesize fructans appear to catylyse the production of isokestose from two molecules of sucrose. The reaction (reaction A) is catalysed by the enzyme sucrose-sucrose fructosyl transferase (SST, EC 2.4.1.99) (Scott, 1968 as cited by Pollock, 1986). Evidence available at present suggests that this reaction represents the major route of carbon from sucrose to fructan, although confirmation will require studies on a wider range of species.


Reaction A: G-1,2-F + G-1,2-F → G-1,2-F-1,2-F + G
sucrose + sucrose → isokestose + glucose

SST activity has been detected in species whose fructans have structures not based upon isokestose. An example being *Lolium temulentum* seedlings which accumulate fructans of the levan type (Pollock, 1984).

Studies on the physical properties of the enzyme SST indicate a specific degree of homology in size (65-70 kD molecular weight) and an optimum pH range of 5.0-5.7 (Pollock, 1986). Reported substrate affinities vary greatly. A study by Shiomi *et al.* (1985, as cited by Pollock 1986) on purified SST from *Allium cepa* reported high K_m values (0.1 M) for sucrose, which suggests that sucrose concentration might control fructan synthesis *in vivo* via a direct effect on SST activity. A study by Labhart *et al.* (1983, as cited by Pollock, 1986) report that in *Festuca arundinaceae* a specific concentration of sucrose is required prior to the onset of fructan synthesis.
SST from the tubers of *H. tuberosus* fails to catalyse the reverse of reaction A, however SST from the roots of *Asparagus officinalis* have been shown to catalyse the reverse reaction at a reduced rate and only when reactants were present at high concentrations (Pollock, 1986). SST, although inactive with isokestose as an acceptor, will catalyse fructosyl transfer from sucrose to neokestose or its higher homologues, generating

oligosaccharides with glucose residue included in the chain (reaction B).



Reaction B: G-1,2-F + F-2,6-G-1,2-F → G + F-2,6-1,2F-1,2-F

sucrose + neokestose → glucose + tetrasaccharide

2.7.2. <u>Chain elongation</u>

Examination of SST from different sources might suggests how some of the stuctural diversity arises. Further complexities in the structure of fructans are introduced when the enzymes which catalyse chain elongation are studied. A number of enzyme systems have been partially characterized, in which higher fructans are produced from *in vitro* by the action of cell-free systems upon oligofructosides. Tubers of *H. tuberosus* contain an enzyme designated fructan-fructan fructosyl transferase (FFT) (EC 2.4.1.100) which catalyses reaction C. FFT catalyses the transfer of fructose from the trisaccharide to the other oligomers and between oligomers. However little is known about this enzyme.

Reaction C: $G\text{-}F\text{-}(F)_n + G\text{-}F\text{-}(F)_m = G\text{-}F(F)_{n+1} + G\text{-}F\text{-}(F)_{m-1}$

Studies have shown that whereas sucrose can act as a fructosyl acceptor but not as a donor, isokestose and its higher homologues are effective both as donors and acceptors (Kanabus *et al.*, 1991). The relative rates of transfer were affected greatly by the size of the oligosaccharides involved. Sucrose and high DP fructan were the most avid acceptors, thus net fructan synthesis would need a continuous supply of isokestose, produced by the

action of SST and would be enhanced by the re-utilization of any sucrose liberated after transfer of fructose residues from isokestose. Transfer of fructosyl residues by the action of FFT could also occur in the absence of net fructan accumulation (Edelman and Jefford, 1968 as cited by Pollock, 1986). The distribution of FFT enzymes in species such as *A. officinalis*, with more complex fructans have been studied in detail (Shiomi, 1981 as cited by Pollock, 1986). Extracts from some species produce fructans from sucrose alone, although the specific enzymes involved have not been characterized.

2.7.3. Fructan breakdown

It is generally accepted that the utilization of fructan proceeds by the sequential removal of terminal fructose residues by specific -fructofuranosidase (fructan hydrolase); the activity of which has been detected in a number of species (Pollock, 1986). Fructan hydrolases have been characterized in barley (*Hordeum vulgare*) (Henson, 1989 as cited by Byeong-Ryong and Housley, 1990) and *Dactylis glomerata* (Pollock, 1982b) as fructan exohydrolases. Two forms of this enzyme have been isolated from tubers of *H. tuberosus* (Edelman and Jefford, 1964) and the tap roots of *Taraxacum officinale* (Rutherford and Deacon, 1972 as cited by Pollock, 1986). Both are inactive against sucrose and liberate only fructose *in vitro*; the enzyme from *H. tuberosus* being inhibited by sucrose.

2.7.4. Fructosyl transfer from sucrose *in vivo*

Evidence from studies on the accumulation of fructan in tissues supplied with radioactive precursors ($^{14}CO_2$ or ^{14}C sucrose) is consistent with the present understanding of the operation of enzyme systems described above. Chandorkar and Collins (1972,1974; as cited by Pollock, 1986) observed the sequential movement of radioactivity from sucrose, via a trisaccharide into higher oligosaccharides during *de novo* synthesis of fructan in leaf discs of various compositae floated on sugar solutions. Pollock (1982a) reports similar observations in the leaves of *L. temulentum* following exposure to $^{14}CO_2$. A study by Pollock (1979) in the leaves of *Dactylis glomerata* exposed to ^{14}C suggests

direct transfer of fructose residues from sucrose to high molecular weight fructan, with no accumulation of radioactivity in oligosaccharides. Rocher (1967 as cited by Pollock, 1986) suggested that observed patterns of uptake of radioactivity into leaf sections of *L. italicum* incubated on ^{14}C sucrose indicate that although oligosaccharides are formed secondarily from high molecular weight fructan, the data are also consistent with the existence of two separate synthetic systems, one producing oligosaccharides and the other larger fructans, both utilizing trisaccharide probably generated by SST. This could explain the presence of multiple linkages in oligosaccharides and their apparent absence in high molecular weight fructan (Pollock, 1986). The existence of such discrete pathways, however, remains speculative at present, as does the selective advantage of the accumulation of fructan of different sizes in different species.

Synthesis of fructans from sucrose rarely leads to a noticable increase in the amounts of free glucose within the tissue. This suggests that following phosphorylation, the glucose, a product of SST action, is re-synthesized into sucrose (Edelman and Jefford, 1968 as cited by Pollock, 1986). The activities of a number of enzymes needed for such re-utilization are indeed sufficient to metabolize the amount of glucose produced by the rates of fructan accumulation observed in the leaf tissue of *L. temulentum* (Pollock, 1986). Some studies however show that the fructosyl residues of exogenously supplied sucrose were utilized preferentially during fructan biosynthesis, suggesting that re-synthesis of sucrose at the site of accumulation was insufficient

to utilize all the released glucose in the stems of *Triticum aestivum* (Hendrix, 1985 as cited by Pollock, 1986).

2.7.5. Enzyme localization

Wagner *et al.* (1983 as cited by Pollock, 1986) found that all the glucose, fructose and oligofructan (DP 3) were localized in the *H. vulgare* vacuoles together with all the SST activity, wheras only 65% of the sucrose was vacuolar. Vacuoles from *H. tuberosus* contained all the fructan together with all the SST, FFT and fructan hydrolase activity but only a small proportion of the sucrose and monosaccharide content of the tissue. In a study by Wagner and Wiemken (1986 as cited by Byeong-Ryong and Housley, 1990) on *H. vulgare*, both SST and fructan hydrolase were found to be present in the vacuole. Byeong-Ryong and Housley (1990) hypothesized that fructan, SST and fructan hydrolase are located exclusively in the vacuole. Their findings imply that fructan synthesis and degradation takes place simultaneously. Further research is needed to determine how the direction of fructan metabolism is regulated. The results of Byeong-Ryong and Housley (1990) indicate that changing leaf sheath temperature can be utilized to control the direction of fructan metabolism and thus provide a system in which the synthesis or degradation of fructan can be examined. These and other (Bancal and Gaudillere, 1989) results strongly suggest that all the major events of fructan metabolism occur within the vacuole, rather than in the cytoplasm and vacuole as proposed by Edelman and Jefford (1968 as cited by Pollock, 1986). The location of sucrose outside the vacuole is consistent with the distribution of enzymes involved in its

synthesis and with the kinetic evidence for the existence of multiple pools with different turnover rates (Pollock, 1986). At present the regulation of carbon movement across the tonoplast is not well understood.

It was noted by Pollock (1986) that it can only be speculated, that differentiation of enzyme types is associated with the greater precision of regulation of fructan metabolism required for intracellular storage of fructan in vascular plants. It is essential that individual enzymes from a range of fructan accumulating species are purified, if the mechanisms controlling accumulation and breakdown are to be fully understood (Pollock, 1986). Further studies are needed to clarify the effects of compartmentation of fructans and fructan enzymes on freezing resistance of plants.

2.8. THE FUNCTIONS OF FRUCTANS IN VASCULAR PLANTS

The role of fructans has not been clarified completely, but there is growing evidence that fructans may function in 1) short-term carbohydrate storage, 2) regulation of sucrose pool size in photosynthetic tissue, 3) regulation of sucrose metabolism during phloem unloading, 4) osmoregulation of cellular water potential, 5) adaptation to low-temperature photosynthesis, and 6) lowering of the freezing point of tissue water upon depolymerization to fructose; none of these would be mutually exclusive.

The role of fructans in carbohydrate storage and the possible role of fructans as cryoprotectants and osmotically active solutes is reviewed in the following section.

There seems no uncertainty regarding the role fructan plays as a reserve carbohydrate. Carbon storage is divided into short-term storage, mainly restricted to the leaves of temperate Gramineae and long-term storage which occurs throughout the range of fructan accumulators (Pollock, 1986).

2.8.1. Long-term storage in vegetative tissues

As fructan undergoes no rapid turnover it forms an important long-term storage carbohydrate (Hendry, 1987). It is the major storage carbohydrate during vegetative growth in four of the most evolutionary advanced families, namely Asteraceae, Campanulaceae, Boraginaceae and Gramineae (subfamily Pooideae) (Brocklebank and Hendry, 1989).

Most fructan accumulators are able to store fructan for long periods in specialized organs, with accumulation being maximal toward the end of the growing season. Storage of this type is frequently associated with perennation and examples exist where it is only observed in perennial members of a particular genus. Long-term storage in temperate grasses and cereals also occurs during reproductive development (Pollock and Jones, 1979). Jefford and Edelman (1963, as cited by Pollock, 1986) observed that maintenance of *Helianthus tuberosus* tubers around $5^{o}C$ results in a decline in the mean chain length of the fructans with no significant loss of carbohydrate. This implies an

increase in the number of fructan chains and synthesis of new short chain acceptors, presumably sucrose. It is presumed that the glycosyl residues for such acceptor synthesis are generated by the action of fructan hydrolases (Edelman and Jefford, 1968 as cited by Pollock, 1986). It has been shown using measurements of enzyme activity that both SST and FFT are active during net fructan accumulation and there is a subsequent SST decline and FFT increase. In spring when the tubers sprout the products of fructan hydrolase activity (sucrose) are utilized in growth and overall carbohydrate content of the tuber falls.

Pollock and Jones (1979) however report that the maintenance of temperate Gramineae at low temperatures does not lead to a decline in mean molecular size. Net fructan breakdown occurs before the start of rapid spring growth, with a transient rise in sucrose and monosaccharide levels. Mobilization of fructan is associated with developmental changes in the stem apex which lead to increased rates of leaf extension. The major site of fructan accumulation, associated with reproductive development, in temperate grasses and cereals is the elongated stem internodes. Stoy (1979 as cited by Pollock, 1986) found that part of this material can be remobilized into the developing grains and in perennial species it may be used for early tiller growth (Pollock and Jones, 1979).

2.8.2. Long-term storage in repoductive tissues

Little data exists regarding the storage of fructan in seeds. It is however known that mature seeds of species within the Gramineae, as well as *Allium cepa* contain small amounts of

fructan (1-2% dry weight) (Pollock, 1986). Observations by Housley *et al.* (1985 as cited by Pollock, 1986) suggest that a period of active fructan metabolism occurs early in grain development and it has been speculated that accumulation of fructans during rapid endospermic cell division may serve to establish a gradient of sucrose necessary for phloem unloading (Pollock, 1986). The same author suggests that future studies on carbohydrate metabolism in grain development should consider in greater detail the contribution of fructan synthesis to total carbon flux in the period immediately following anthesis.

2.8.3. Short term storage of fructans

The carbohydrate storage pool in the actively photosynthesizing leaves show very different kinetic characteristics and regulatory mechanisms to other organs. The most important difference being the daily rather than monthly alternation of synthesis and mobilization. Starch is the most significant short-term storage pool in vascular plants, although several species accumulate fructans in the leaves (Incoll *et al.*, 1989). Fructan accumulation in the leaves of such species are usually in small amounts, always associated with low starch/sucrose ratios (Brockelbank and Hendry,1989) (see Pollock and Cairns, 1991 for a conflicting view point). However, in some species including the temperate Gramineae substantial amounts of fructan are accumulated in the leaves. Of the many species studied by Pollock (1986) only *H. tuberosus* accumulated fructans in storage organs, although high starch/sucrose ratios were observed. Some species

utilized sucrose as the major leaf carbohydrate, although they lacked fructan metabolism.

In a study by Pollock (1984) it was observed that in the leaves of temperate Gramineae, decline in need for sucrose leads to rapid increases in the amounts of soluble carbohydrates and the beginning of fructan synthesis. Farrar and Farrar (1985a,b as cited by Pollock, 1986) showed in an experiment on the leaves of *Hordeum distichum* that rates of fructan turnover were very high despite low demand-led changes in the export of carbon, indicating that the significance of vacuolar storage of sucrose and fructan is considerable. In field conditions where demand-led changes in leaf sucrose would be great as the temperature of the leaf meristem changes; vacuolar storage would facilitate balancing of supply and demand, while buffering chloroplast metabolism from changes in metabolic status caused by fluctuating rates of sucrose export (Pollock, 1986).

2.8.4. Fructans as cryoprotectants and osmotically active solutes

In many temperate species, the accumulation of carbohydrates in storage tissues in late fall is assumed to be associated with an increase in resistance to frost (Suzuki and Nass, 1988; Yelenosky and Guy, 1977; Green and Ratzlaff, 1975). Soluble sugars in the cytoplasm lower the freezing point by simple colligative and/or noncolligative effects (Suzuki and Nass, 1988). The observation that storage of fructan is associated with perennation of temperate plants has led to many suggestions for the role of

fructans as cryoprotectants (Pollock, 1986; Gonzalez *et al.*, 1990).

Although there is still continued debate regarding the role fructan plays in providing the plant with a distinct and direct mechanism for freeze tolerance, evidence is rapidly growing for the support of the hypothesis (Pontis, 1990). According to Pollock (1986) suggestions that fructans act as cryoprotectants have been made on correlative evidence only, and the relevant literature is characterized by conflicting observations, qualifications and exceptions. Brockelbank and Hendry (1989) believe that fructan has no unique or specified role in low temperature tolerance as similar attributes are seen in non-fructan accumulating species. Hendry (1987) notes a number of reservations, including the fact that fructan occurs in some species in the same storage organ as starch and that growth in some species without fructans occurs at low temperatures. It was also noted that the accumulation of fructans occurs as a result of growth stopage at low temperatures.

Advantages of accumulation of fructan over starch accumulation under low temperature conditions include the high solubility in water, the resistance to crystallization at subzero temperatures as crystals produce extensive membrane damage (Santarius and Bauer, 1983 as cited by Suzuki and Nass, 1988) and the insensitivity of the biosynthetic pathways of fructan to low temperatures (Pollock, 1986).

The increase in low molecular weight oligosaccharides accompanied by an increase in starch in late fall and winter is a well-documented phenomenon in frost-resistant plants and is generally assumed that there must be a casusal significance to this correlation. Although sucrose is the most common oligosaccharide to be accumulated during cold acclimation, raffinose and stachose are often dominating (for example in gymnosperms) (Suzuki and Nass, 1988).

Fructan metabolism is however especially evident in species which are active at low temperatures, including monocots of the Gramineae and Liliaceae.

Fructan accumulation in vacuoles allows photosynthesis to continue providing a carbohydrate sink at cool temperatures when the rate of growth is reduced and other carbohydrate storage pools are saturated (Chatterton *et al.*, 1989). Fructans are associated with plants with early season or low temperature growth (Brockelbank and Hendry, 1989).
Cool-season grass, studied by Chatterton *et al.* (1989) that did not synthesise fructans begun growing later in spring than their fructan accumulating counterparts. The presence of fructans in *Osteospermum sinuatum* may allow early season growth of the species.

Low temperatures have effects upon isolated enzymes and whole metabolic processes associated with carbohydrate metabolism (Pollock *et al.*, 1989).

Some authors believe that fructan accumulation is not caused by cold temperature *per se*, but by accumulation of sucrose. Sucrose is thought to provide the free energy for fructan synthesis and the fructosyl residues required. Fructans synthesis is initiated by sucrose:sucrose fructosyl transferase which transfers fructose from one sucrose molecule to another, producing a trisaccharide (Housley *et al.*, 1989). Sucrose is also closely associated with regulation of enzyme activity (Pollock *et al.*, 1989). Tognetti *et al.* (1989) supplied plants with exogenous sugars and fructan levels rose. Pollock *et al.* (1989) claim that a homeostatic compensatory mechanism serving to maintain a steady state of sucrose synthesis after environmental disturbances comes into operation following specialised cytoplasmic changes. Chatterton *et al.* (1989) however found low correlation between leaf fructan and sucrose. The author concluded that the hypothesis that fructan accumulating grasses have high levels of sucrose cannot be universally applied across all cool-season grasses.

Studies also indicated that a threshold value of 15% total nonstructural carbohydrates (TNC) must be present before fructan accumulation can occur and that fructan accumulation calculated as a percentage of TNC is highly correlated with TNC in cool-season grasses at 10°C and 25°C. High TNC levels did not occur unless fructan levels were at least 40% of TNC. This suggests that fructan is required for accumulation of high TNC contents (Chatterton *et al.*, 1989).

Pressman *et al.* (1989) found that withholding water during cold treatment of asparagus caused further increase to the initial sucrose increase observed in the shoot and also in the fructan content of the roots. After 50 days of drought sucrose levels dropped, but fructan levels were unaffected. Root sugar levels also increased compared to the cold treatment alone. Drought at normal to high temperatures caused loss of storage fructans. The results indicate that low temperature is the overriding factor, causing fructan accumulation despite the water stress factor.

It is thought that fructans act as osmotic regulators (Pontis, 1990), by helping maintain turgor and control water movement (Pontis, 1989). Fructans are nearly ideal osmotically active solutes, because they are present in tissues as a series of soluble homologous oligosaccharides, each containing one more fructose residue than the previous member of the series (Pontis, 1989).

It is unlikely that all forms of fructans have the same effect on freezing resistance (Suzuki, 1989). Certain types may also be more closely associated with coldhardiness than others.

The vacuolar location of fructans would appear to prevent direct effects on the cytoplasmic or organelle functions, although high vacuolar concentrations of solute generated by hydrolysis of fructans would appear to increase the cell water potential and reduce the level of freezing-induced dehydration. In temperate Gramineae there is no good correlation between the extent of

accumulation of low molecular weight, osmotically active fructan and the freezing resistance of different species. Considerable levels of frost-tolerance can be observed in species which accumulate short or medium-length fructans (Johansson, 1970 as cited by Pollock) or those which accumulate long-chain fructans (Suzuki, 1968 as cited by Pollock, 1986)

Polymerization or break down of fructan will effect vacuolar osmotic potential, and thus may alter turgor pressure. Fructan depolymerization has been correlated with cell expansion in developing onion bulbs and it has been suggested that this is essential to maintain osmotic balance (Darbyshire and Hendry, 1978). It has been suggested that the hydrolysis of fructan is a mechanism for regulating turgor in guard cells of *Allium cepa* where staech is absent (Darbyshire and Allaway, 1981 as cited by Pollock, 1986). It has been shown by Flood *et al.* (1967, as cited by Pollock, 1986) that increased water uptake by discs of storage tissue from *H. tuberosus* is associated with an increase in fructan hydrolase activity. Pollock (1984) reports that exposure to low temperature ($5^{o}C$) induced the accumulation of large quantities of fructan in the leaf extension zone in the intercalary meristems of the temperate grass *Lolium temulentum*. The author suggests that metabolism of fructans may be involved in turgor regulation during leaf growth. In the same study, it was shown that where the growth of *L. temulentum* is limited by chilling, subsequent accumulation of fructan in the extension zone has no effect upon the rate of leaf extension.

In a study by Pollock and Jones (1979) fructan concentration and the mean molecular weight of fructans from the leaf and stem tissues of meadow fescue (*Festuca pratensis*) was shown to vary with season. The major period of fructan synthesis was late autumn and early winter when plant growth was limited. Fructan concentration decreased in spring during regrowth. In an experiment by Chatterton *et al.* (1988; 1990) fructan concentrations increased 3- to 10-fold in all parts of crested wheatgrass (a fertile hybrid of *Agropyron desertorum* and *A. cristatum*) and redtop (*Agrostis alba* L.) when plants were transferred from warm (20°C) to cold temperatures (5°C), although starch became the most dominant carbohydrate. Cold stress (5°C at night) induced fructan accumulation in the leaf blades of barley (*Hordeum vulgare*) and wheat seedlings (Wagner *et al.* 1983 as cited by Byeong-Ryong and Housley, 1990). In other studies, fructans have been shown to be synthesized by continuous illumination of excised primary blades of barley and degraded by transferring leaves to darkness (Wagner *et al.*, 1986, 1987).

Pollock and Jones (1979) in a study on the seasonal patterns of fructan metabolism in forage grasses identified three phases of fructan metabolism. The first, where growth was most rapid had little or no detectable fructan, and low levels of low molecular weight sugars. This is followed by the accumulation phase during the later stages of reproductive growth and autumn and winter growth of vegetative tillers. In this phase growth is slow and levels of fructan reach their highest. The breakdown phase is

characterized by a decline in mean molecular weight and a tempory rise in low molecular weight sugars.

It has been suggested by Pollock (1986) that temperature relationships of the accumulation of sucrose, starch and fructan favour vacuolar accumulation in tissues where metabolism continues in the range $0^{o}C$ to $10^{o}C$. It has been shown by Wagner *et al.* (1983 as cited by Pollock, 1986) that SST from the leaves of *Hordeum vulgare* were very insensitive to temperature and that starch synthesis is much more sensitive to temperatures below $5^{o}C$ than sucrose. To date the enzymatic basis for these differences in sensitivity is not known but the data provide futher indication of the advantages of sucrose and fructan storage in metabolically active tissues at low temperatures.

In *Helianthus tuberosus* a partial depolymerization of inulin occurs during cold storage in winter, whereas complete depolymerization to sucrose and fructose occurs during sprouting (Meir and Reid, 1982). Bacon and Loxely (1952 as cited by Meir and Reid, 1982) studied the seasonal changes in fructans in tubers of *Helianthus tuberosus*. Extracts from late summer and autumn had large amounts of high molecular weight inulin, whereas extracts from winter and spring had large amounts of fructan oligosaccharides of low molecular weight. In winter part of the fructose released by the hydrolases may be transformed to glucose from which more sucrose may be produced, thus allowing the FFT-enzyme to catalyze a redistribution of fructose residues and increase the amounts of fructans of smaller molecular size at the

expense of larger ones. Frost resistance should be improved by this process.

It is well recognized that excess starch accumulation in chloroplasts, especially at low temperatures, can lead to loss of photosynthetic activity. Fructan accumulating species, however, can accumulate large quantities of fructans in the leaf, while still retaining a low starch content by shunting photosynthetically fixed carbon into fructans more effectively than to starch. This could explain why fructan-accumulating species active photosynthesis at low temperatures, even near $0^{o}C$, allowing plants to be adapted for photosynthesis and growth at low temperatures.

In a study by Pollock (1984) plants of *Lolium temulentum* grown at $20^{o}C$ and transferred to $5^{o}C$, showed a marked increase in sucrose accumulation, which was detectable an hour after transfer. Exposure to low temperature for seven days resulted in the initial synthesis of oligosaccharides and the eventual synthesis of higher molecular weight polymers. Sucrose accumulation was most marked in the blades of mature leaves, wheras the accumulation of fructan was maximal in the meristematic regions of developing leaves. Chilling also led to a parallel increase in the activity of SST, the first enzyme specifically involved in the biosynthsis of fructans. Activity of the enzyme was also higher in the leaf meristems.

The results of a study by Suzuki and Nass (1988) indicate that the accumulation of low DP fructans, sucrose, and fructose in winter cereals did not possibly relate to freezing resistance when the concentration was expressed as a percentage of total fructans. High DP fructans appeared to be more closely associated with freezing resistance. Effects of fructans and other soluble carbohydrates on freezing resistance depend on their molecular size, linkage type, and other molecular properties. An important factor influencing effectiveness of cryoprotectants may be their locations in cells. It is believed that unless fructans are situated on or near frost-sensitive cell membranes, they may not exhibit cryoprotective effects (Suzuki and Nass, 1988). If they are confined to the vacuoles (Wagner *et al.*, 1986), their participation in cryoprotection would be indirect, through maintenance of osmotic balance and supply of energy. *Phleum pratenense*, which attains a higher freezing resistance than winter cereals (LT_{50} of -22°C) contains a high concentration of phlein with a DP of 100 or higher. This led to two assumptions, phlein rather than inulin and higher rather than lower DP fructans are more closely associated with freezing resistance. Observations that the concentration of phlein in hardy wheat and hardy rye was low, suggest that phlein-type fructans are unlikely to have important functions in cold hardiness of winter cereals. Suzuki and Nass (1988) believe the second assumption to be partially true, as the hardier cultivars tended to contain higher concentrations of high DP fructans. The authors suggest that determination of the high DP fructan could be used for separating

a hardy from a less hardy cultivar, although it may be of little value in selection of hardier lines.

It is concluded that the complexity and variety of fructan structures and of the associated enzyme systems has resulted in an incomplete understanding of their physiology and biochemistry, but their significance as an alternative storage polysaccharide in storage organs and leaves should not be underestimated.

3. MATERIALS AND METHODS

3.1. PLANT MATERIAL AND EXPERIMENTAL CONDITIONS

Osteospermum sinuatum (DC.) Norlindh (Asteraceae), a palatable dwarf deciduous Karoo shrub was used in this study. Seeds were sown in plastic containers and thinned to four plants per pot after germination. Plants were watered with deionized water. At 90 days of age, the plants were transferred to controlled environment chambers set at three different day/night air temperature regimes, ie. $12^{o}C/5^{o}C$, $25^{o}C/18^{o}C$ and $35^{o}C/28^{o}C$. Twelve pots were used for each temperature treatment. An additional 12 pots were placed in the $35^{o}C/28^{o}C$ chamber to determine the effect of transfer of plants from a high temperature to a low temperature ($12^{o}C/5^{o}C$) on the fructan degree of polymerization. The relative humidity was kept constant at 75 ± 5 % and the photoperiod set to provide 12 hours light and 12 hours dark cycles. Photosynthetically active radiation (500 μmol $m^{-2}s^{-1}$ at plant height) was supplied by fluorescent lamps. No developing inflorescences were observed on the plants during the study period.

Plant material was harvested after 30 days of growth in the controlled environments. The assumption was made that a 30 day controlled temperature treatment was sufficient to establish 'equilibrium' in the carbohydrate contents. At 12, 24 and 48 hour periods before harvesting four pots were removed from the $35^{o}C/28^{o}C$ chamber and transferred to the $12^{o}C/5^{o}C$ chamber for the

shock treatment experiment. The tissues were sampled 6-8 hours into the daily light cycle. Plants were divided into above ground (leaves, twigs and stems) and below ground plant material (root crown and roots), except for the shock treated plants, for which only above ground plant material (leaves, twigs and stems) was used. Six replicate plants from the three different temperature regimes and four replicate plants from the three different shock treatments were used. The potting soil was carefully extracted and the soil removed to ensure that no below ground plant material was lost during harvest.

3.2. THIN LAYER CHROMATOGRAPHY (TLC)

The following fructan extraction procedure was also undertaken on onion bulb (*Allium cepa* L.) purchased locally, because the range of oligosaccharides present in this species have previously been identified (Darbyshire and Hendry, 1981; Suzuki and Cutcliffe, 1989). Fructose, sucrose, inulin and the oligofructans separated from onion were used as reference compounds. Purified neokestose, isokestose, nystose and inulin pentasaccharide are used as chromatographic standards in many other TLC studies (Cairns and Pollock, 1988a and b). This allows determination as to whether the separated sample oligosaccharides co-chromatographed with the standards. These standards were unobtainable and costly.

The above and/or below ground *Osteospermum* plant material was washed with distilled water, dried, diced and weighed using an electronic balance. The below ground and above ground plant material was homogenized, in 100 ml and 200 ml distilled water,

respectively in a Waring blender. The homogenate was boiled for 5 min, filtered through a muslin cloth and then through Whatman No. I filter paper. The filtrate was cooled and centrifuged at 1000 x *g* for 15 min. A 25 ml aliquot of the supernatant was passed through a mixed-bed ion-exchange resin column (Dowex H^+ and Cl^- forms). Sample purification procedures included decoloring with charcoal and filtration through a millipore filter (0.45 µ). These procedures removed proteins and pigments, but no significant improvement in the separation of oligofructans was observed. The eluate was then evaporated to dryness. The dessicated sample was sealed using parafilm and stored at -18°C until further analysis. The extract was thawed and dissolved in 15 ml 50 % ethanol to give a final concentration of 2ug fructose equivalents per ul as determined by the reducing power titration technique (Smith, 1981).

Kieselgur chromatoplates coated with a fluorescent indicator (20 x 20 x 0.25 mm) (DC-Fertigplatter, Germany) were used to separate fructo-oligosaccharides. Seven µl of onion extract, fructose and sucrose mixture (1 g/100 ml) and the individual fructo-oligosaccharide mixtures were spotted along a line 2.5 cm from the lower edge of each plate. The plates were developed in solvents containing I-propanol, ethyl acetate, and water (solvent ratio 40:45:15) for 90 minutes. Various ratios of the solvent (Collins and Chandorkar, 1971) were tested, and only the above mentioned ratio resulted in good separation of oligofructans of DP up to 10-11. The plates were then removed and air-dried, and the carbohydrates located by means of urea-phosphoric acid spray reagent described by Wise *et al.* (1955). After spraying the

plates were placed in an oven at 110°C for 15 minutes and viewed under an UV light. Chromatograms were run once and if the results were not conclusive, the procedure was repeated. Tracings were made of the separated low-DP fructans (DP of 3-10) using high quality tracing paper.

4. RESULTS

Results from the thin layer chromatograms (Figs. 1, 2 and 3) show a number of different patterns, which are summarized in Table 1.

TLC separation was found to be an effective way of resolving oligosaccharides ranging from DP 1 to DP 8 in *Osteospermum sinuatum* and in the reference material (*Allium cepa*). The amount of oligofructans was not quantified. The TLC procedures could not facilitate the separation of multiple isomers (DP 3).

It was confirmed that onion bulb extracts contain a series of oligofructans from DP 1-8 of the inulin series, as previously reported by Darbyshire and Hendry (1981) and Suzuki and Cutcliffe (1989) using HPLC methods. The fructose and sucrose mixture (1 g/100 ml) clearly resolved into fructose (DP 1) and sucrose (DP 2) fractions. Consistent with the findings of van der Heyden and Stock (unpublished), *O. sinuatum* contained fructan as part of the total nonstructural carbohydrate pool.

Above and below ground tissue extracts of *O. sinuatum* grown at 12/5°C (Fig. 1 and 2) showed faint spots of medium- and low-DP fructans (DP 1-7) and a very dark spots of high-DP fructans (>DP 8) on the baseline. Above and below ground tissue extracts differed in that the below ground tissue extract contained DP 1-8 and DP >8, whereas the above ground tissue extract showed only DP 1-7 and DP >8.

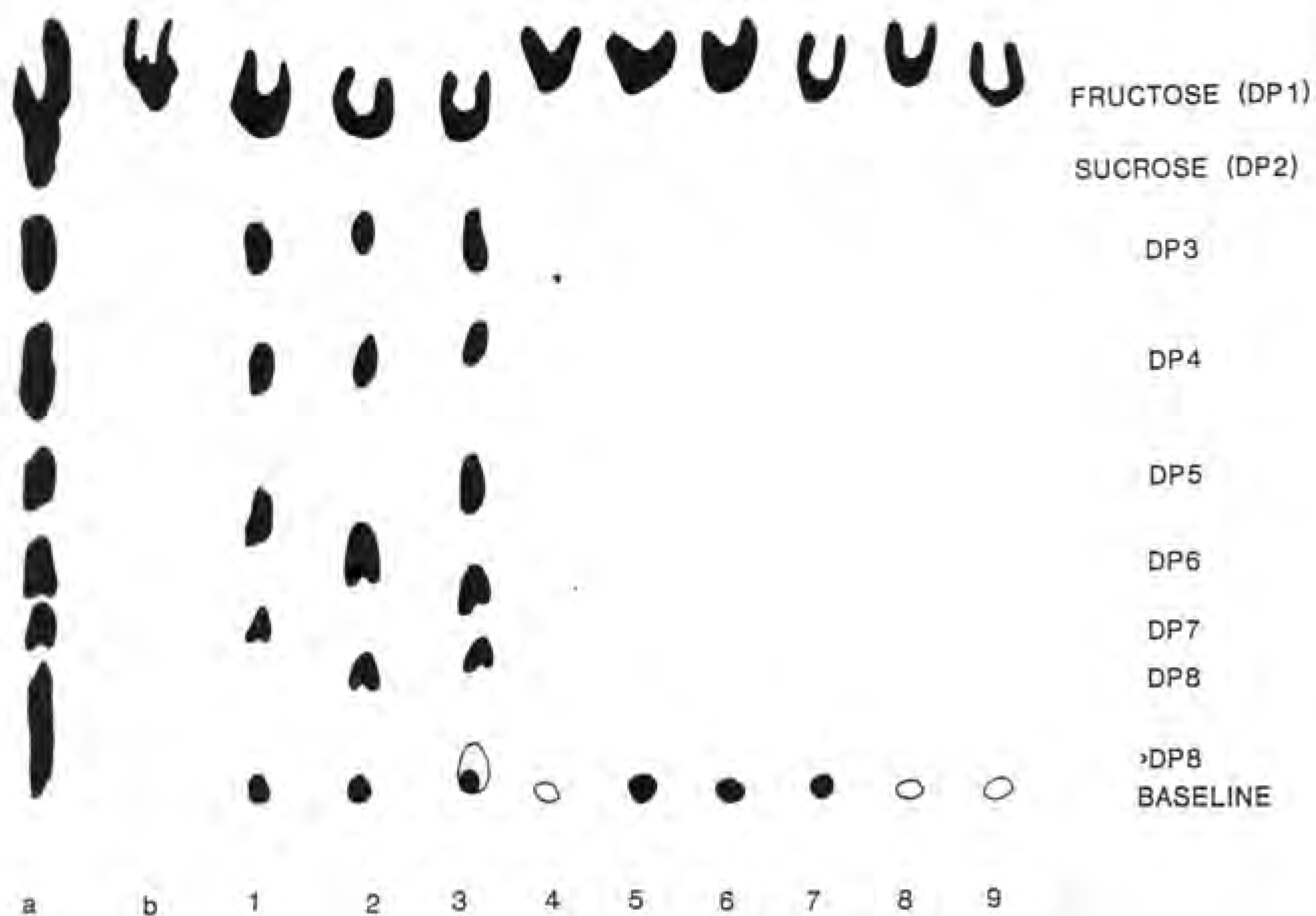
Above and below ground tissue extracts of plants grown at $25/18^{\circ}C$ (Fig. 1 and 2) showed faint spots of low-DP fructans (DP 1 and 2) and a slightly darker spot of high-DP fructans (DP >8) on the baseline. The characterization of combined fructose units at the point of spot application confirmed the presence of longer chain polymers of fructose in these tissues. Spots on the baseline (DP >8) for the above ground tissue extract were lighter than those of the below ground tissue extract. The incompleteness of the colour forming reaction of the spot at the point of application suggests that the fructans of the above ground tissue extract were present at lower levels than in the below ground tissue extract.

Above ground tissue extract of the $35/28^{\circ}C$ treatment (Fig. 1) showed a dark spot of low-DP fructan (DP 1 and 2) and high-DP fructan (>DP 8) on the baseline. Below ground tissue extract at the same day/night temperature regime (Fig. 2) showed a light spot of high-DP fructan (>DP 8) and no medium- or low-DP fructans.

<u>Shock treatment</u>

The following results refer to TLC profiles of *O. sinuatum* above ground tissue extract only (Fig. 3). Plants were transferred from high to low temperature regimes for various periods of time. Extracts from plants transferred for 12 hours showed a dark spot of low-DP fructans (DP 1 and 2) and light spot of high-DP fructans (>DP 8). Extracts from plants transferred for 24 hours

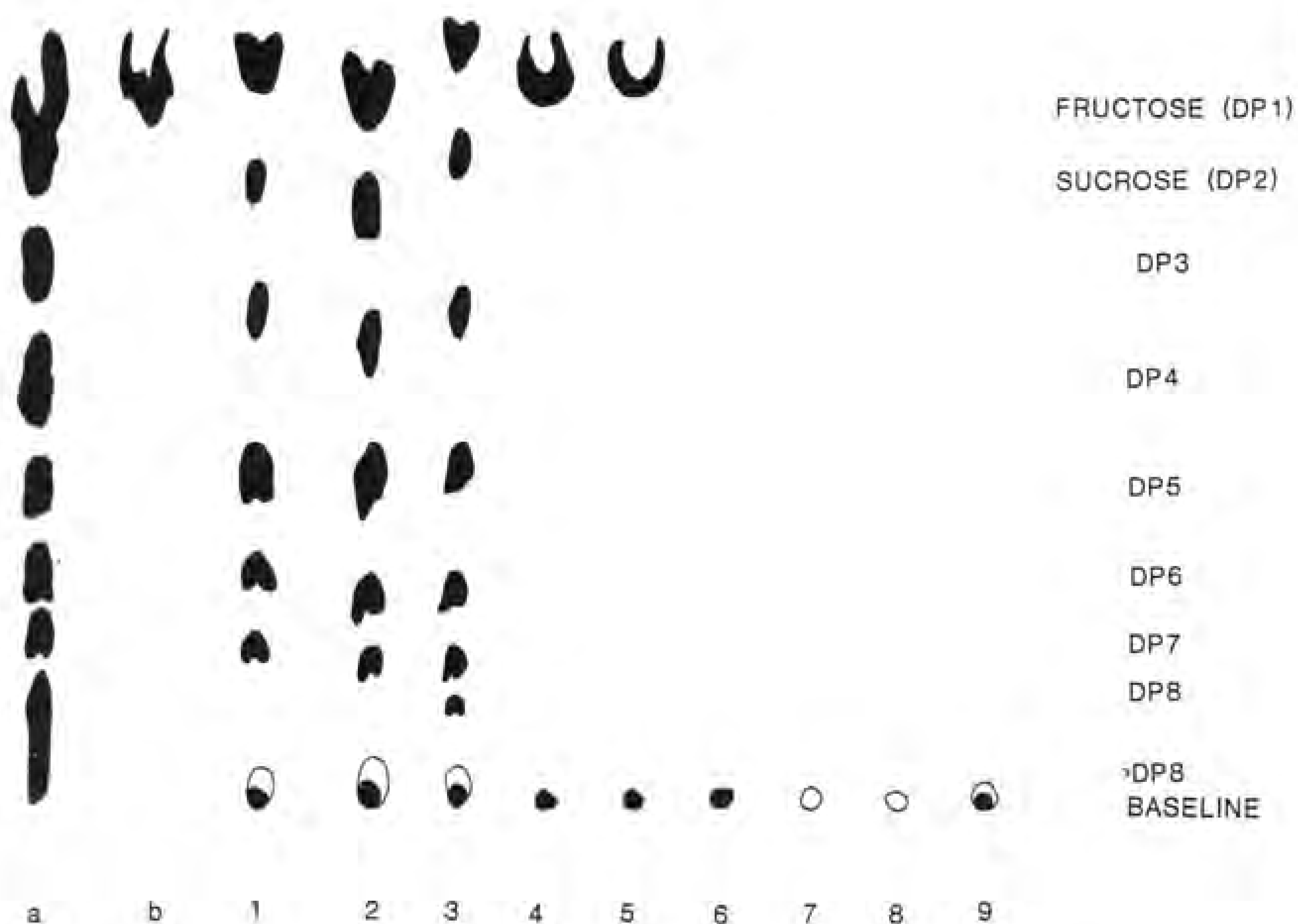
showed a dark spot of low-DP fructan (DP 1 and 2) and DP 3, and no high-DP fructans on the baseline. Extracts from plants transferred for 48 hours showed a dark spot of low-DP fructans and no high-DP fructans on the baseline. This indicates that on transfer of plants from high temperature to low temperature the high DP fructans are hydrolysed to oligofructans of DP 1 and 2.



Key: Faint fructan spot

Dark fructan spot

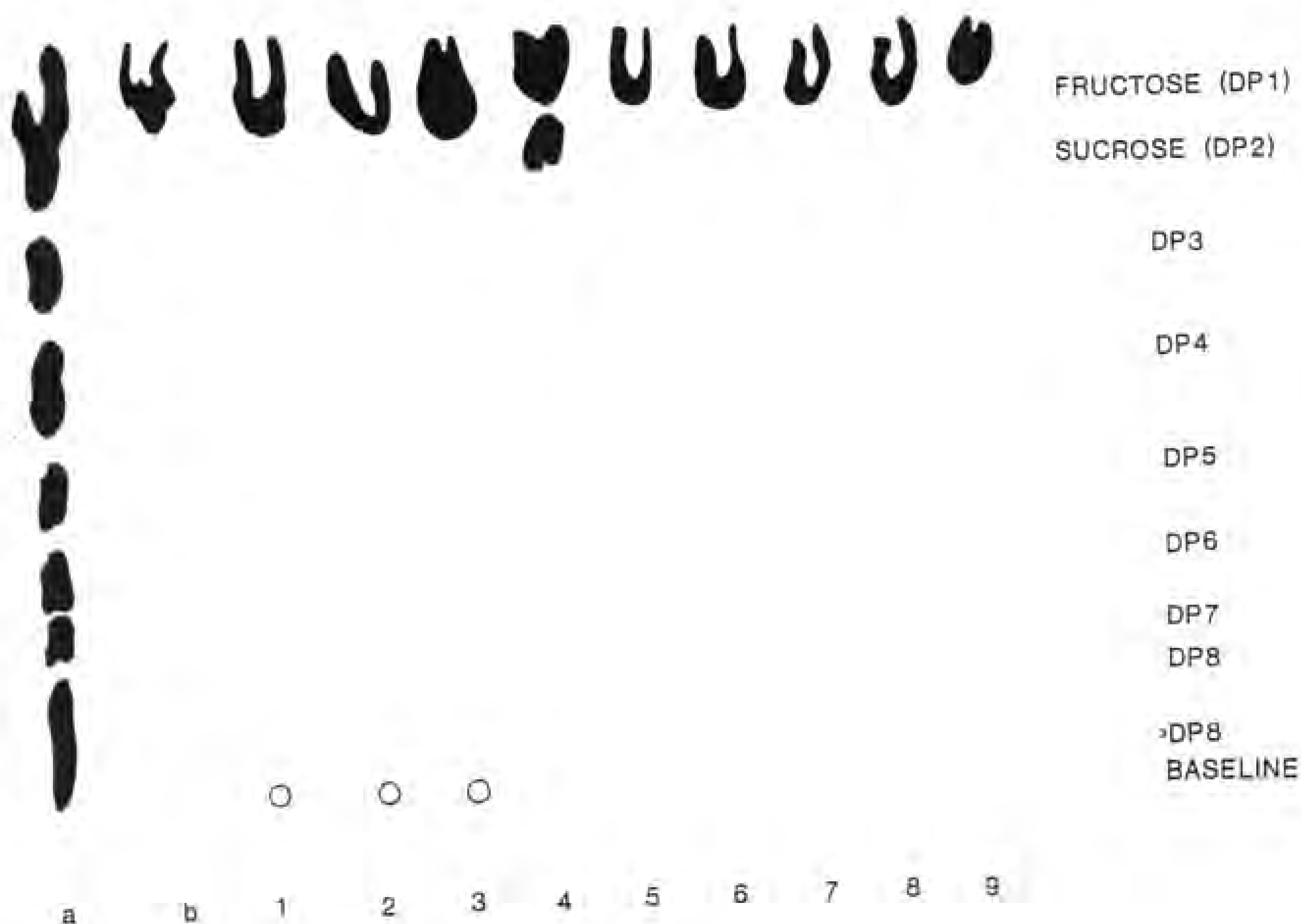
Fig.1: Separation of water-soluble carbohydrates in extracts from above ground *Osteospermum sinuatum* tissues (leaves, twigs and stems) and *Allium cepa* bulb tissue by thin-layer chromatography. a = *Allium cepa*; b = fructose and sucrose mixture (1 g/100 ml); 1 = 12/5°C treatment; 2 = 12/5°C; 3 = 12/5°C; 4 = 25/18°C; 5 = 25/18°C; 6 = 25/18°C; 7 = 35/28°C; 8 = 35/28°C and 9 = 35/28°C.



Key: Faint fructan spot

Dark fructan spot

Fig.2: Separation of water-soluble carbohydrates in extracts from below ground *Osteospermum sinuatum* tissues (root crown and roots) and *Allium cepa* bulb tissue by thin-layer chromatography. a = *Allium cepa*; b = fructose and sucrose mixture (1 g/100 ml); 1 = 12/5°C treatment; 2 = 12/5°C; 3 = 12/5°C; 4 = 25/18°C; 5 = 25/18°C; 6 = 25/18°C; 7 = 35/28°C; 8 = 35/28°C and 9 = 35/28°C.



Key: Faint fructan spot

Dark fructan spot

Fig.3: Separation of water-soluble carbohydrates in extracts from shock treated above ground *Osteospermum sinuatum* tissues (leaves, twigs and stems) and *Allium cepa* bulb tissue by thin-layer chromatography. a = *Allium cepa*; b = fructose and sucrose mixture (1 g/100 ml); 1 = 12/5°C for 12 hours; 2. 12/5°C for 12 hours; 3 = 12/5°C for 12 hours; 4 = 12/5°C for 24 hours; 5 = 12/5°C for 24 hours; 6 = 12/5°C for 24 hours; 7 = 12/5°C for 48 hours; 8 = 12/5°C for 48 hours and 9 = 12/5°C for 48 hours.

Table 1: Occurence of TLC-separated oligofructans in above ground (AG) and below ground (BG) tissues of *Osteospermum sinuatum* grown under various day/night temperature regimes. Shock treated plants were grown at 35/28°C day/night temperatures and transferred to 12/5°C day/night temperatures for various periods of time. Oligofructans present in *Allium cepa* (bulb) are also shown.

		Fructans								
Treatment		Fr	Su	*DP3	DP4	DP5	DP6	DP7	DP8	>DP8**
12/5°C	AG	++	++	++	++	++	++	++		++
	BG	++	++	++	++	++	++	++	++	++
25/18°C	AG	++	++							+
	BG	++	++							++
35/28°C	AG	++	++							++
	BG									++
Shock treatment:										
12 hours	AG	++	++	++						+
24 hours	AG	++	++							
48 hours	AG	++	++							
Allium cepa		++	++	++	++	++	++	++	++	++

* DP=Degree of polymerization.

** Fructans above this degree of polymerization were not separable by TLC methods.

Fr - Fructose (DP 1).

Su - Sucrose (DP 2).

++ indicates presence in large quantities.

+ indicates presence in small quantities.

5. DISCUSSION AND CONCLUSIONS

Results of the present study indicate that plants of *Osteospermum sinuatum* contain fructose and sucrose and high-DP fructans at intermediate temperatures (25/18°C). Asteraceae are well-known for containing fructans of the inulin series only (Bacon and Edelman, 1951 as cited by Pollock, 1986). These results were consistent with the findings of van der Heyden and Stock (unpublished), using thin layer chromatographic procedures, who report the presence of fructose and sucrose, and no oligofructans with a DP of less than 7 in *O. sinuatum* grown at similar temperatures. Results of their study also indicate similar fructose, sucrose and range of oligofructans for below and above ground tissue extracts in *O. sinuatum* grown at intermediate temperatures. Growth at higher temperatures results in fructan synthesis and chain elongation, resulting in a decrease in fructose and sucrose (DP 1 and 2). In the below ground material this results in no free fructose and sucrose, providing evidence for the synthesis of fructans from sucrose by the action of a number of glycosyl transferases. These findings are consistent with the current understanding of fructan synthesis (see Meir and reid, 1982). Growth at low temperatures caused a depolymerization of high-DP fructans, resulting in the accumulation of low-DP fructans, sucrose, and fructose. The accumulation of free fructose is a result of the sequential removal of terminal fructose residues by fructan hydrolases, as fructan is utilized. The below ground tissues contained fructan from DP 1 to DP 8,

whereas the above ground material contained DP 1 to DP 7. This slight difference in the DP values between above and below ground plant tissue extracts is not sufficient evidence to suggest distribution of fructans of different DP values in different plant tissues, as suggested by other studies (Pontis, 1990).

The results of the shock treatment experiment indicate that fructan breakdown, following transfer from high to low temperatures, takes place between 12 to 24 hours of transfer. This was indicated by the presence of fructose, sucrose and high-DP fructans in the plant tissues transferred for 12 hours to cold temperature. The TLC profile was similar to that of the above ground tissue extracts of plants grown at high temperatures, indicating that oligofructan fraction of the nonstructural carbohydrate pool of the plant had not undergone any detectable metabolic changes in the 12 hours following cold shocking. Extracts from plants cold shock treated for 24 hours contained only fructose, sucrose and low-DP fructans (DP 3), indicating that all the high-DP fructans had been hydrolysed. The results suggest that a period of between 12 and 24 hours is needed for complete breakdown of high DP fructans into low DP fructans following cold shocking. Extracts from plants transferred for 48 hours contained only fructose and sucrose, indicating that transferral for 48 hours results in breakdown of long chain fructans only and no other metabolic activity. Resynthesis of fructans after a period of time in cold temperatures has been reported in a number of studies (Byeong-Ryeong and Housley, 1990), but this did not occur within 48 hours in this study.

Accumulation of low-DP fructans thus resulted from transferral to cold temperatures. The results from this study indicate that fructan breakdown does occur in plants subjected to low temperatures and provides futher indication of the advantages of sucrose and fructan storage in metabolically active tissues at low temperatures.

It is appealing to suggest that the occurrence of fructans in karoo shrubs is an essential or contributing factor to their adaption to extremely cold winters and particularly dry summers. A study on the effect of low temperature and drought on the molecular size distribution of fructans would indicate which of the factors has an overriding effect on fructan metabolism. It may also be an adaption for growth during the cold season, as reported for other species growing in habitats with a cold growing season (Brocklebank and Hendry, 1989). Van der Heyden and Stock (unpublished) suggest that degree of fructan accumulation could be related to the rate of plant growth following defoliation (selective advantage) since predominantly fructan accumulators (*Osteospermum sinuatum* and *Pteronia pallens*) are relatively fast growers, compared to *Ruschia spinosa*, the partial fructan accumulator.

Free fructose in the shock treatment extracts and the extract from the plants grown at low temperatures suggests that the hydrolases were active. This study did not investigate the enzymology envolved in fructan metabolism and it can only be speculated that fructan breakdown in the study material occurs by

the sequential removal of the terminal fructose residues by fructan hydrolases, as discussed in the literature review. Analysis of hydrolysis products viz. free glucose/free fructose ratios of individual oligosaccarides would also provide further evidence as to whether the plants were actively synthesizing and storing fructan or hydrolyzing and mobilizing them.

The exact role of fructans in low temperature tolerance of plant species is still not clearly understood. Futhermore no studies have been undertaken in Southern Africa on fructan metabolism. This study presents evidence that in the species studied low temperatures causes the breakdown of high-DP fructans and the accumulation of low-DP fructans, within 24 hours of transferral to low temperatures. It is thought that this accumulation of fructans allows the plants to photosynthesize at low temperatures, a common occurence in the habitat in which it grows. It is hoped that the present study on the effect of low temperature on fructan metabolism of a palatable dwarf deciduous Karoo shrub, *Osteospernum sinuatum* (DC.) Norlindh will be first of many studies undertaken to further understanding of fructan function in plants of southern Africa.

Future research is needed to determine the contribution of fructans to total nonstructural carbohydrates of economically important crops, rangeland plants and fruit trees because of the close relationship between the production and utilization of carbohydrates and plant growth. Studies of the localities, the quantities and forms of carbohydrate accumulation therefore have

definate management implications in terms of the assessment of plant growth potentials and in the understanding of plant adaptive features (van der Heyden and Stock, unpublished). It would also be particularly useful to determine the nonstructural carbohydrate content of *O. sinuatum* plants of differing ages, to determine whether age affects forms of carbohydrate accumulation. Another interesting investigation could involve a survey of the carbohydrate content of various Karoo shrubs, and the results related to habitat, distribution, life history and taxanomic group, to determine correlation of fructan accumulation with any of these factors.

More studies are needed on the kinetic properties and temperature responses of enzymes associated with fructan metabolism. Enzymological reasons for fructan accumulation at low temperatures is at present not understood.

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