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Pinitol is a major carbohydrate in leaves of some coastal plants indigenous to New Zealand

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Abstract D-pinitol (an O-methyl inositol) is identified as a major soluble carbohydrate in the leaves and other parts of the parapara, *Heimerliodendron brunonianum*. Leaves of two other members of the Nyctaginaceae, *Mirabilis jalapa* and *Bougainvillea glabra*, show the same feature; a high pinitol content may be a family characteristic. Four other native coastal species from three other plant families, *Tetragonia trigyna*, *Disphyma australe*, *Carmichaelia aligera*, and *Spergularia marginata*, also show pinitol as their major soluble carbohydrate in the leaves, but *Euphorbia glauca* does not. The possibility that presence of pinitol contributes to the ecological behaviour of these native species, in conferring resistance to salinity stress, is discussed.

Keywords polyol; sugars; D-pinitol; salinity stress; *Heimerliodendron*; Nyctaginaceae; *Tetragonia*; *Disphyma*; *Spergularia*; *Carmichaelia*; *Euphorbia*

INTRODUCTION

In the course of a broad study on the role of carbohydrates in plant stress and senescence responses, leaf material from the parapara (*Heimerliodendron brunonianum*) was included on the grounds that the plant is typically found in the coastal hedge of islands like Little Barrier, and therefore is presumably reasonably resistant to salt stress. The soluble carbohydrate fraction was found to be dominated by a single compound having the characteristics of a polyol, rather than by the usual leaf carbohydrates of sucrose, glucose, and fructose. This note identifies the compound, and records its occurrence in four other native coastal species.

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MATERIALS AND METHODS

Materials

One collection of plant material from *Heimerliodendron brunonianum* (Endl.) Skottsb., *Euphorbia glauca* Forst., *Bougainvillea glabra* Choisy, and *Disphyma australe* (Sol.) J. M. Black was made between 8.30 and 9.30 a.m. from plants growing in the grounds of the Horticulture Institute, and processed into liquid nitrogen within 10 min of collection. A second collection of material of *Heimerliodendron*, *Bougainvillaea*, and *Euphorbia* plus one of *Mirabilis jalapa* Linn. was taken between 8.30 and 9.00 a.m. from plants growing in my garden at Manukau City and was processed 35–45 min after collection. *Tetragonia trigyna* Banks et Sol ex Hook. and *Spergularia marginata* (D.C.) Kittel were collected twice between 9.00 and 10.30 a.m., at different sites, and *Disphyma* was collected once, from natural plant communities on coastal cliffs south of Karekare Beach in the Waitakere Ranges near Auckland. One collection of *Carmichaelia aligera* Simpson was taken from coastal scrub adjacent to the mouth of the Pararaha Stream in the same area, and a second collection of *Mirabilis* was made at Kawakawa Bay. All material from remote sites was transported in a plastic bag, placed in darkness at 4°C within 4 h of collection, and processed into liquid nitrogen within 24 h. The duplicate collections, which were separately analysed, in each case gave substantially the same values for total soluble carbohydrate and proportion of pinitol.

Preparation of samples and extraction of tissue

The following method has been developed for

studying sugar composition of numerous small leaf samples. By not homogenising and minimising transfer steps, it reduces handling losses, and gives >97% recovery of tissue sugars while minimising hydrolysis of sucrose through post-mortem invertase action (a problem with some hot extraction methods). It relies on the finding that mechanical damage to leaf tissue while it is frozen in liquid nitrogen makes a heavy cuticle more easily penetrated by the extraction solvent (Bieleski et al. 1992). Leaf tissue was cut into 1.0–1.5 × 10–15 mm slices, which were mixed to provide uniform subsamples as necessary, then 1.00 g fresh weight was folded into an aluminium foil packet which was dropped into liquid nitrogen. The frozen packet was briefly crushed between two blocks of wood to fragment the tissue it contained, returned to –196°C, then taken and unfolded so as to slip the frozen meal into a vial containing 20 ml methanol-chloroform-water (12–5–3 v/v)(MCW) at –20°C. The sample was held at –20°C overnight to let the solvent penetrate the tissue then warmed to room temperature, and the solvent was transferred by pasteur pipette to a large centrifuge tube. The tissue residue was extracted with a further 10 ml MCW with occasional mixing for 10 min, then the solvent was added to that in the centrifuge tube and the residue discarded. Chloroform (7 ml) then water (10 ml) were added, the contents of the tube were stirred, and the tubes were centrifuged at low speed to separate the two phases that formed. The lower chloroform phase containing pigments and lipids was removed by pasteur pipette and discarded. The remaining aqueous phase was filtered through Miracloth (Calbiochem) to remove tissue fragments and a small curd that sometimes formed, and dried in a 150 ml rotary film evaporator flask at 35°C. The extract was redissolved in 5 ml water and deionised by passing it through two Econopack columns (Biorad) containing Sephadex SP25-H^+ (2 ml) followed by Sephadex QAE25-formate (2 ml) (Redgwell 1980). The eluate which was the sugar fraction was lyophilised, dissolved in 1.00 ml 10% propan–2-ol, and stored at –25°C till required. Smaller amounts of tissue (down to 0.20 g fresh weight) were handled in the same way, dissolving the lyophilised sugar in sufficient 10% propan–2-ol to still give 10 mg tissue ≡ 10 µl extract. Additional extracts were made from other tissues of the parapara plant, using the same extraction methods.

Separation of sugars

The sugar fraction was studied by standard methods in use in the laboratory for quantifying and identifying normal and unusual sugars and polyols: TLC, GLC, capillary GC, and mass spectrometry (Redgwell 1980; Redgwell et al. 1990; Bieleski et al. 1992). Most separations were carried out by GLC of trisilyl derivatives using OV101 silicone in a stainless steel column (Redgwell et al. 1990). Additional information was provided by capillary GC on a DB–1701 column (J & W Scientific). These methods showed that the sugar fraction from parapara contained a polyol in addition to the usual sugars.

Preparation of bulk polyol

To conclusively identify the polyol and to provide a standard for quantitative measurements, a bulk preparation was made. Parapara leaf, 100 g fresh weight, was extracted and deionised by a scaled-up version of the method given above, yielding 0.95 g of dried sugar fraction. The sugars were separated, 0.5 g at a time, by column chromatography on a 2.5 × 40 cm (200 ml) column of Dowex 50-WX4, 200–400 mesh (Biorad) in the Ca^{++} form with 10% isopropanol as a solvent. The method is based on that described by Angyal et al. (1979), the polyol exiting in a peak between 140 and 160 ml eluent, compared with blue dextran 65–75 ml, sucrose 110–125 ml, glucose 125–145 ml, and fructose 190–210 ml. The combined polyol fraction (0.53 g) was dissolved at 1.8% (w/v) in 90% acetone at 50°C and allowed to crystallise at 0°C (0.29 g). Standard quebrachitol (Calbiochem), a commercially available pinitol isomer, was recrystallised in the same way to provide a known reference sample.

Mass spectrometry and optical rotation

Samples of the polyol and of recrystallised quebrachitol were examined by electron impact mass spectrometry at 70 ev, and by FAB-MS (in glycerol), on a VG 70-SE mass spectrometer. Other samples were examined in water solution at 20°C by conventional optical rotation methods in a Perkin-Elmer 241 polarimeter using a 10 cm cell.

RESULTS AND DISCUSSION

Standard pinitol was not available from commercial sources, but comparisons were made with extracts prepared from *Trifolium repens* and *Ginkgo biloba*, reported to contain it (Plouvier 1963). The following findings identified the polyol in parapara as D-pinitol:

(1) It was first noted during GLC of trisilated sugar derivatives from various species, in which the retention time of the main peak from parapara was identical with that of polygalatol and pinitol from *Protea eximia* and *Trifolium repens*, respectively (Plouvier 1963; Bieleski et al. 1992), close to but distinct from one of the fructose peaks, and clearly distinguishable from other compounds under study (standard sugars, sorbitol, mannitol, bornesitol, inositol, quebrachitol, adonitol).

(2) Capillary GC gave a major peak having the same retention time as pinitol, but clearly distinct from those of fructose and polygalatol.

(3) TLC on silica gel gave a spot travelling with the same Rf and having the same reagent colour reactions as standard quebrachitol and pinitol in *Trifolium* extracts.

(4) The purified polyol, when submitted to EI-MS and FAB-MS, gave the mass spectrum expected of an O-methyl-substituted inositol, with a pseudomolecular ion at $m/z = 195$, $(M+1)^+$, and a fragmentation pattern virtually identical to that obtained from the pinitol isomer, quebrachitol.

(5) The specific optical rotation of the purified unknown was $[\alpha]D$ +64.0° compared with +64° quoted for pinitol and clearly distinct from that of other O-methyl inositols (Plouvier 1963). It was concluded that the polyol in parapara leaves was D-pinitol.

Pinitol was the dominant soluble carbohydrate in the leaves and bud, was of lesser importance in the young wood and pith, and was virtually absent from the young roots (Table 1). All samples were taken early in the day, and it is probable that the leaf concentration of sugar would be higher and the proportion of pinitol would be rather lower in the evening (cf. Bieleski et al. 1992). A horticultural cultivar of parapara has areas of leaf totally lacking chlorophyll (and so lacking photosynthetic capacity). This material was separately harvested and studied, and consistently found to have a rather lower concentration of sugars, but the proportion of pinitol in those sugars was essentially the same as in the green leaf (Table 1). It is clear that pinitol plays an important part in the carbohydrate economy of the parapara.

The taxonomic relationships of parapara are of some interest. With polyols in plants, there is typically a strong taxonomic pattern in the occurrence of a particular polyol in various species—thus, sorbitol is characteristic of the woody Rosaceae and mannitol of the Oleaceae and Umbelliferae (Bieleski 1982). Parapara is placed within the Nyctaginaceae. Four other members of the Nyctaginaceae have been reported to contain pinitol (*Mirabilis jalapa*, *M.*

Table 1 Composition of the soluble carbohydrate fraction in early to mid photoperiod from different tissues of parapara. "Opening bud" material was from buds that had broken and which were initiating extension growth; "green leaf" leaf material was from the fully green areas of mature leaves from the current season's growth; "white leaf" leaf material was from portions of the variegated leaf containing no chlorophyll; "bark" samples were split from young wood at the cambium layer. The young wood was split into quarters and the pith excised from the inside to give the "pith" and "wood" fractions. The "root" material was of young white terminal rootlets. A single unknown accounted for up to 1.9% of the total sugar content (peak 7 in Fig. 1). No other sugars were identified in the extracts at levels above 0.2% of the total.

Tissue	Soluble sugars, mg/g.fw.	Percent of total sugar in:				
		Pinitol	Sucrose	Glucose	Fructose	Inositol
Opening bud	9.31	57.8	31.8	5.9	1.2	1.5
Green leaf	11.07	59.8	36.2	1.4	0.3	1.3
White leaf	8.84	49.0	47.2	1.8	0.2	0.6
Young bark	6.69	20.3	64.7	10.5	3.6	0.4
Young wood	16.96	9.0	86.3	2.9	1.2	0.4
Young pith	15.78	9.6	84.3	5.1	0.8	<0.2
White roots	3.78	<1.0	80.1	7.9	6.1	1.3

longiflora, *Bougainvillea glabra*, and *Oxybaphus viscosus*) (Plouvier 1963); and its presence in parapara might have been predicted. However, Plouvier (1963, 1990) was interested primarily in the presence of such compounds, and did not report their concentrations relative to the other soluble carbohydrates. Two of the species he studied, *Bougainvillea glabra* and *Mirabilis jalapa* (Marvel of Peru), were available to me; and the present results extend our knowledge by showing that pinitol is not merely present, but that, as in parapara, it can be the dominant soluble carbohydrate present in the leaves (Table 2, Fig. 1).

Considering the relatively widespread occurrence of pinitol (Plouvier 1963, 1990), there has until recently been surprisingly little attention paid to its physiological function, or indeed that of most plant polyols. One of the most plausible suggestions to date is that the polyols are in some way able to protect cell structures against various types of desiccation stress (Bieleski 1982; Redgwell et al. 1990), and recent studies are beginning to provide some direct evidence of that. At least seven species from widely separated families have been shown to respond to increased drought or salinity stress by increasing their pinitol content in the leaves: *Aster tripolium* (Compositae), *Honkenya* (=*Arenaria*) *peploides* (Caryophyllaceae) (Gorham et al. 1981), *Macroptilium atropurpureum* and *Sesbania aculeata* (tropical Leguminosae; Ford & Wilson 1981; Gorham et al. 1988), *Medicago sativa* (temperate Leguminosae; Fougère et al. 1991), *Pinus pinaster* (Pinacae; Nguyen & Lamant 1988), and *Mesembryanthemum crystallinum* (Aizoaceae; Paul & Cockburn 1989). Recently there has been an exciting advance by the discovery (Vernon & Bohnert 1992) that salt stress in *Mesembryanthemum crystallinum* rapidly induced the formation of a specific mRNA (Imt1), which in turn was shown to code for a specific protein having the structural characteristics and activity of a myo-inositol O-methyl transferase, the enzyme known to catalyse the first step in biosynthesis of pinitol from inositol. Far more than any other study, this work establishes the importance of pinitol in conferring tolerance to osmotic stress in this species.

The likelihood that pinitol might act in this way with parapara prompted a brief study of some other native coastal species related to those already shown to contain pinitol (Plouvier 1963, 1990). When the New Zealand relatives were tested, *Disphyma australe* (Aizoaceae), *Carmichaelia aligera* (Leguminosae), *Tetragonia trigyna* (Aizoaceae), and *Spergularia marginata* (Caryophyllaceae) were all found to contain pinitol as the major soluble carbohydrate in their leafy tissue (Table 2). Though a *Euphorbia* species was shown to contain pinitol (Plouvier 1990), *E. glauca* did not, despite (or perhaps because of) a high general sugar concentration. Pinitol prepared from parapara is currently being studied for potential membrane-stabilising properties in an in vitro system (L. Crowe pers. comm.).

Clearly, the occurrence of pinitol as a major carbohydrate in parapara has a taxonomic basis, and is likely to turn out to be a general characteristic of the Nyctaginaceae. It is too early to say whether pinitol contributes to the salt and desiccation

Table 2 Composition of the soluble carbohydrate fraction from young but fully expanded leaves or leafy tissues of two exotic members of the Nyctaginaceae and five endemic New Zealand dicotyledons in early to mid photoperiod. Values for both the opening bud and the fully expanded leaf of *Mirabilis* are given. True leaves are not present in mature plants of *Carmichaelia*, and the tissue studied was the leaf equivalent, the flattened branchlets. "Other" sugars are dominated by a single compound in the position shown as 7 in Fig. 1.

Tissue	Soluble sugars, mg/g.fw.	Percent of total sugar in:					
		Pinitol	Sucrose	Glucose	Fructose	Inositol	Other
Bougainvillea leaf	35.76	71.1	23.2	2.0	2.0	1.3	0.5
Mirabilis shoot	7.68	28.0	15.8	21.9	15.2	0.7	18.5
Mirabilis leaf	4.24	39.2	38.9	0.7	0.5	4.7	13.4
Carmichaelia stem	5.84	66.8	27.6	2.7	0.7	0.9	1.4
Euphorbia leaf	36.87	<0.5	87.2	3.9	3.0	5.0	0.8
Spergularia leaf	5.37	82.1	14.2	0.6	0.4	1.3	1.5
Tetragonia leaf	3.06	63.1	24.8	3.6	2.0	3.3	3.3
Disphyma leaf	2.35	61.7	17.4	11.9	3.4	2.6	3.0

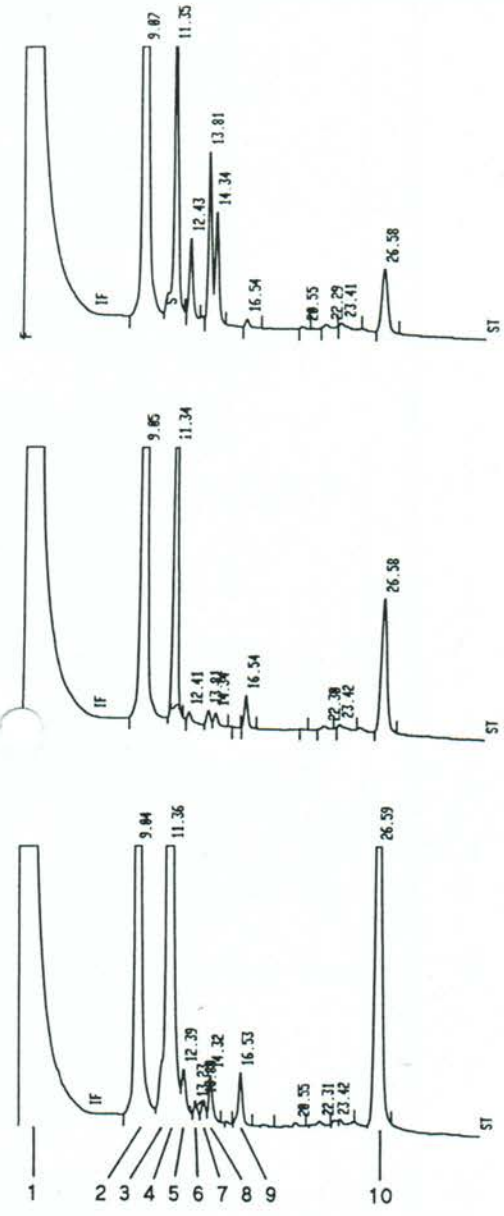

Fig. 1 Comparison of gas-chromatograph traces of the trisilyl derivatives from the soluble carbohydrate fraction of (bottom) mature *Bougainvillea* leaves; (middle) young full[illegible]panded parapara leaves; (top) young shoot of *Mirabilis*. Peaks are identified as follows: 1, solvent peak; 2, adonitol (internal standard); 3, a shoulder on the left side of the pinitol peak, is fructose A (two anomers of fructose that are in equilibrium in plant extracts give separate peaks and the same is so for glucose); 4, pinitol; 5, glucose A; 6, fructose B; 7, an unknown with the mobility of sorbitol; 8, glucose B; 9, inositol; 10, sucrose. Small peaks to the left of sucrose are reagent impurities.

tolerance of parapara or of some of our other coastal species. However, it seems more than a coincidence that at least four characteristically coastal species of the Auckland region contain pinitol, not just as a metabolite, but as the major soluble carbohydrate in their leaves. There is clearly room here to extend the work on *Mesembryanthemum crystallinum* (Paul & Cockburn 1989), and carry out physiological experiments relating the degree of exposure to drought and salt stress in parapara (or another of these coastal members of New Zealand's flora) to its pinitol content. *Tetragonia trigyna* might be particularly well suited to such a study.

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