The structures of the honeydew oligosaccharides synthesized by *Claviceps africana*

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ABSTRACT

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The structures of the principal oligosaccharides in the honeydew exudate of the sorghum ergot pathogen *Claviceps africana*, which has become epidemic in the Americas, have been characterized through linkage analysis using FAB-MS and GC-MS techniques, as 1,6-di-b-D-fructofuranosyl-D-mannitol and 1,5-di-b-D-fructofuranosyl-D-arabitol trisaccharides, 1-b-D-fructofuranosyl-D-mannitol and 5-b-D-fructofuranosyl-D-arabitol disaccharides and other minor disaccharides and trisaccharides. Their structural diversity is explained according to perceived biosynthetic interrelationships in pathways that appear to be unique amongst ergot fungi, particularly concerning intra-molecular reduction of fructose. The oligosaccharide, 1,6-di-b-D-fructofuranosyl-D-mannitol, which inhibits *C. africana* macrospore germination at a concentration in water of 1 g/mL or more, forms together with other slightly less bioactive oligosaccharides, the basis of a novel strategy to limit ergot disease losses in hybrid sorghum seed production.

Additional key words: ergot, linkage analysis, FAB-MS, GC-MS.

RESUMO

Bogo, A.; Mantle, P.; Casa, R. T.; Guidolin A. F. Análise estrutural dos oligossacarídeos presentes no exsudato da doença açucarada do sorgo, sintetizados por *Claviceps africana***.** ***Summa Phytopathologica***, v.32, p. 16-20, 2006.

As estruturas dos principais oligossacarídeos presentes no exsudato da doença açucarada do sorgo, produzidos pelo patógeno *Claviceps africana*, foram caracterizadas através de espectroscopia de bombardeamento rápido de átomos-espectrometria de massa (FAB-MS) e cromatografia gasosa-espectrometria de massa (GC-MS). Dois grupos de oligossacarídeos foram elucidados, sendo o principal formado pelos trissacarídeos 1,6-di-b-D-frutofuranosil-D-manitol e 1,5-di-b-D-frutofuranosil-D-arabitol e os dissacarídeos 1-b-D-frutofuranosil-D-manitol e 5-b-D-frutofuranosil-D-arabitol e outros grupos menos importantes de dissacarídeos e trissacarídeos. As diversidades estruturais foram elucidadas de acordo com a inter-relação biossintética de sua rota metabólica, a qual parece ser exclusiva entre os fungos produtores da doença "ergot", particularmente com relação a redução intramolecular da frutose. O oligossacarídeo 1,6-di-b-D-frutofuranosil-D- manitol, o qual inibe a germinação dos macrosporos de *C. africana* a uma concentração de 1 g/mL ou mais, forma juntamente com outros oligossacarídeos menos bioativos a base de uma nova estratégia para limitar as perdas na produção de sementes de híbridos de sorgo.

Palavras-chave adicionais: ergot, análise de ligação, doença açucarada do sorgo.

The sugar composition of the honeydew exudate from ergot fungi parasitising host grasses and cereals has, until recently, been largely of academic interest, concerning only the presumed mixture of oligosaccharides arising from the pathogen's preferred use of the glucose component of the host's sucrose (14). However, a prominent feature of the sorghum ergot pathogen *Claviceps africana* Frederickson, Mantle & De Milliano (8), indigenous to parts of Africa, is the development of a white crust of secondary spores on the surface of exuded honeydew (7), from which the name "sugary disease" arises. Unlike other important ergot pathogens, the asexually produced secondary spores of *C. africana* are potentially windborne (9), which makes them an important epide-

miological factor.

Following the discovery of this disease in the Americas in 1995, secondary sporulation facilitated the dramatic spread of the pathogen in sorghum within South America in 1996 and prompted the first unequivocal recognition of the pathogen's identity (15). The disease spread through Central America (16) into the USA in 1997 (11), where the fungus now is a serious economic threat to commercial hybrid sorghum seed production. *C. africana* occurs in Australia, Japan and Thailand and has also recently been recognised in India (2). Recognition of the potential global epidemiological significance of the secondary sporulation phenomenon (12) of this pathogen has focused attention to the sugar composition of the honeydew exudate in allowing or facilitating production of airborne inoculum (10, 3). The present saccharides studies have particularly addressed the report on the occurrence of oligosaccharides in the honeydew of *C. purpurea* (Fr.) Tul. (5). *C. africana* has alditol components and these were quite different from the honeydew sugars of *C. purpurea* which the biosynthesis is different from those ergot fungi pathogens (3).

The present paper reports on a fresh evaluation of *C. africana* oligosaccharides, in the context of the new global significance of sorghum ergot disease, primarily applying methodology (FAB-MS and GC-MS in electron impact and chemical ionisation modes) to define new oligosaccharide components. It is also reported that the main oligosaccharides are inhibitors to *C. africana* macrospore germination at quite low concentration, thereby offering a novel strategy to limit epidemics of ergot disease in hybrid sorghum seed production through the use of female lines expressing a sweet character.

MATERIAL AND METHODS

Ergot parasitism. Male-sterile sorghum (*Sorghum bicolor* L.)(IS 2219 A) was grown in a horticultural tunnel at the Chelsea Physic Garden, London, and flowered in August and September. Florets were inoculated with *C. africana* at floret gaping by spraying diluted natural honeydew with spore concentration around $1x10^4$ conidia/mL. New parasitized tissues exuded honeydew 1-2 weeks after floret inoculation.

Oligosaccharides isolation. Descending paper chromatography [Whatman 3MM No. 1 paper; solvent, propan-1-ol:ethyl acetate:water (7:1:2)] for 48-55h resolved standards (D-fructose, D-glucose, sucrose and raffinose) and analytically and/or preparatively separated sugars in 100 ml of honeydew. To localise the sugars, lateral strips of the chromatograms were stained in aniline hydrogen phthalate reagent (3) and heated at 120°C for 20 min. The oligosaccharides separated in the chromatograms were eluted though a 2.5 x 40 cm column of Bio-Gel P-2 (Bio-Rad) equilibrated in degassed water at 0.4 mL/min. The eluate was monitored with a Waters RI-detector R-403 and the fractions (5 mL) were collected with an LKB fraction collector, using an Hitachi 561 Recorder. The fractions collected were freeze-dried prior to saccharide analysis and bioassay.

Linkage analysis. Oligosaccharide composition was determined by a combination of FAB-MS and GC-MS analysis. Oligosaccharides were reduced on a DuPont Model 21-491 instrument with dry DMSO plus 5 pellets of NaOH, reduced with 200 mL of borohydride and permethylated with 0.5 mL methyl iodide, as described by Dell et al. (6), before FAB-MS analyses. GC-MS analysis after the standard procedure of hydrolysis in 1M trifluroacetic acid (TFA) at 25°C for 30 min, followed by reduction with sodium borodeuteride and acetylation with acetic anhydride had been applied to the permethylated saccharides, as described by Carpita & Shea (4). GC-MS was carried out on a Finnigan/MAT 9610 gas chromatograph equipped with a 30 m x 0.2 mm DB-5 capillary column in a temperature gradient 90-190°C (20°C min^{-1}), 190-210°C (1°C min^{-1}), 210-300°C (25°C min^{-1}). In A a Fisons 8000 quadrupole mass spectrometer system in electron impact mode, and B employed a VG autospek Q system in chemical ionisation with negative ion mode to show molecular ions of derivatised monosaccharides.

Macrospore germination. Potato dextrose agar (Difco) was diluted to 1/8 normal concentration and supplemented with agar (2%). Purified honeydew oligosaccharides, or raffinose, mannitol or arabitol as controls, were incorporated into the medium at concentrations up to 5% and a drop spread as a 1 cm diameter film on a microspore slide. The surface was inoculated by sedimenting in the air an aerosol of a suspension of parasitically-produced conidia in water. Slides were incubated in a water-saturated atmosphere in Petri dishes at 18°C. Without added oligosaccharides, macrospores germinated by an iterative process (8), giving a secondary spore on a sterigma projecting above the medium surface within 1-2 days. Spore germination was assessed microscopically.

RESULTS

Apparently, FAB-MS spectra of *C. africana* honeydew indicated the presence of two groups of oligosaccharides, one with ions around *m/z* 450-500 and the other around *m/z* 650-700 (Figure 1). The first one seems to be a disaccharide group with ions *m/z* 449, 477 and 493, which are the respective pseudomolecular ion fragments of the permethylated sodiated disaccharide $[M + Na]^+$. The most prominent FAB-MS disaccharides, eluted from paper chromatograms, are the ions *m/z* 449 and 493 (Figure 1, A and C). Ion *m/z* 449 is consistent with an oligosaccharide composed of one permethylated hexose plus one permethylated pentose ($C_{19}H_{38}O_{10}$), an A-type fragment ion corresponding to the mass of the free reducing end terminal group and the mass of the cationising ion (Na= 23), *i.e.* (1 x 204) + (1 x 191) + 31 + 23. The ion *m/z* 477 (Figure 1, B) equate to di-hexose and can apply to several disaccharides including inulin, levan, sucrose and/or others which are composed of two permethylated hexoses, an A-type fragment ion corresponding to the mass of the non-reducing and free reducing end terminal groups and the mass of the cationising ion, *i.e.* (2 x 204) + 15 + 31 + 23. The data from GC-MS linkage analysis, of the ion m/z 477, showed small amounts of fructoses linked in positions 1 or 6 (Table 1) which, when each is associated with 2-fructose from this dominant component, reveals the presence of both levanbiose and inulobiose. The ion *m/z* 493 (Figure 1, C) by analogy shows an oligosaccharide composed of two permethylated hexoses, one of which was reduced, an A-type fragment ion of which corresponds to the mass of the non-reducing end terminal group, an additional increment of the reduced-reducing end and the mass of the cationising ion, *i.e.* (2 x 204) + 15 + 47 + 23. GC-EIMS and GC-CIMS data of the electron impact mode and chemical ionisation of the 1-b-D-fructofuranosyl-D-mannitol (Table 1) confirmed linkage of the fructofuranosyl component by the 2-position and is consistent with the mannitol being

linked by either its 1- or 6- position.

A higher mass group of ions, *m/z* 653, 681 and 697 (Figure 1, D, E and F) are the respective sodiated pseudomolecular ions $[M + Na]^+$ of the reduced trisaccharides. The most prominent FAB-MS trisaccharides, eluted from paper chromatograms, are represented by the ions *m/z* 653 and 697 (Figure 1, D and F). The ion *m/z* 697 correspond to $[M + Na)^+$ for a molecular weight of 674, which is 47 amu in excess for a permethylated tri-hexose, showing the reduction of one hexose. This ion *m/z* 697 represents, together with the data from the GC-MS relative intensities of mass spectra fragment ions (Table 1), the major trisaccharide 1,6-di-b-D-fructofuranosyl-D-mannitol (two permethylated hexoses plus permethylated mannitol) which is composed of three permethylated hexoses, an A-type fragment ion corresponding to the mass of the non-reducing end terminal group, an additional increment of the reduced-reducing end and mass of the cationising ion, *i.e.* (3 x 204) + 15 + 47 + 23, gives the observed ion *m/z* 697. GC-EIMS data after the standard derivatisation procedure for linkage analysis implied that this trisaccharide contained an alditol linked at positions 1 and 6 and also the same fructofuranose as occurs in sucrose, linked at C_2. Data from GC-CIMS complemented the limited fragment ion data of EIMS and confirmed particularly the molecular mass of derivatives of 1, 6-linked mannitol and 2-linked fructofuranose (Table 1).

FAB-MS data, after permethylation, showed others trisaccharides sodiated with ions *m/z* 653 and 681 (Figure 1, D and E) which equate to 204 amu (hexose) additions to the sodiated molecular ions of the disaccharides m/z 449 and 477, respectively (Figure 1, A and B). This data, together with that of linkage analysis, of each pure compound, showing a 1,6 linked fructose (Table 1), confirms occurrence of 1,5-di-b-D-fructofuranosyl-D-arabitol but also reveals a novel tri-fructose composed of 2-1 and 2-6 linked moieties, termed "inulevan-triose".

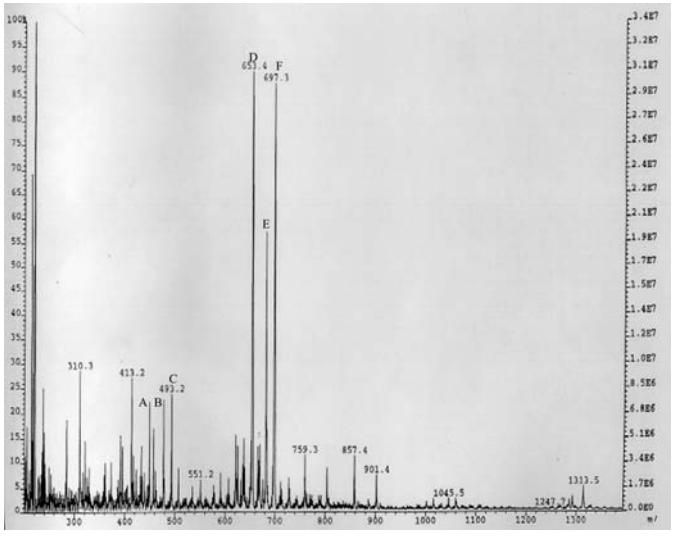

Figure 1 – Fast atom bombardment-mass spectrum of permethylated oligosaccharides in natural honeydew of *C. africana*. A) fructosyl-arabitol, B) di-hexoses, C) fructosyl-mannitol, D) difructosyl-arabitol, E) tri-hexoses, and F) difructosyl-mannitol. Numbers relate to the mass/change value (m/z) of the pseudo molecular íon [M+ Na]+ of each oligosaccharides with identical mass.

Table 1. Gas chromatography retention time of derivatives of purified monosaccharides, which were either free or were released during linkage analysis of oligosaccharides from the honeydew of *Claviceps africana*, together with the relative intensities of mass spectral fragment ions seen in electron impact mode, correlated with molecular ions seen in chemical ionisation mode.

Derivatised monosaccharides	Important fragment ions (GC-EIMS)																		GC-CIMS		LINKAGE
	Rt (min)	59	75	87	89	101	102	117	118	129	133	145	161	162	163	177	189	190	205	$(M+H)^+$	$(M+NH_4)^+$	
1,2,3,4,5-penta-*O*-methylpentitol	11.57	++	++		++	+++	++				++	+++			+					250	267	D-arabitol
1-*O* -acetyl-2,3,4,5-tetra-*O*-methylpentitol	14.18	+		+	+	+++		++					+			+				251	268	5 - arabitol
1,2,3,4,5,6-hexa-*O*-methylhexitol	14.70	++	++		++	+++	++				++	+++				+				294	311	D-mannitol
1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylpentitol	16.60			+		++		+++					+							279	296	1,5 - arabitol
6-*O*-acetyl-1,2,3,4,5-penta-*O*-methylhexitol	17.03	+	+		++	+++		+++				++	++							295	312	5 - mannitol
5-*O*-acetyl-1,(2D),3,4,6-penta-*O*-methylhexitol	17.92			+						+++			+	++	+				+	295	312	D-fructose
2,5-di-*O*-acetyl (2D)-1,3,4,6-tetra-*O*-methylhexitol	17.95			+						+++			+	++	+				+	324	341	2 - fructose
1,5-di-*O*-acetyl(1D) 2,3,4,6 tetra-*O*-methylhexitol	18.64			+		+	+++		++	++		++	+	+					+			1 - glucose
5-*O*-acetyl-1,2,3,4,6 (1D)-penta-*O*-methylhexitol	18.67			++		++	+++		++	++		++	+	++	+				+	295	312	D-glucose
1,6-di-*O*-acetyl-2,3,4,5 tetra-*O*-methylhexitol	19.15					+++		+++					++							323	340	1,6 - mannitol
1,2,5-tri-*O*-acetyl(2D) 3,4,6 tri-*O*-methylhexitol	19.77			+						+++			++					+				1 - fructose
2,5,6-tri-*O*-acetyl(2D) 1,3,4 tri-*O*-methylhexitol	19.77			+						++				++			+					6 - fructose
1,2,5-tri-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol	19.84			++						+++			++					++				1,2 - fructose
1,2,5,6-tetra-*O*-acetyl(2D) 3,4 di-*O*-methyl hexitol	.19.84			+						+++			+					+				1,6 fructose

abbreviations: +++, well-defined in spectrum; ++, middle-defined in spectrum; +, weakly-defined in spectrum

The second most prominent trisaccharide is the ion *m/z* 653 (Figure 1, D) which represent, together with the data from the GC-MS relative intensities of mass spectra fragment ions (Table 1) the 1,5-di-b-D-fructofuranosyl-D-arabitol (two permethylated hexoses plus permethylated arabitol). The minor trisaccharides have the ions *m/z* 681. The ion *m/z* 681 (three permethylated hexoses) covers a group including, theoretically, 1-kestose, 6- kestose, inulintriose or any other tri-hexose.

Concerning the oligosaccharides biological activities, the trisaccharide 1,6-di-b-D-fructofuranosyl-D-mannitol at 1 g/mL inhibited macrospore germination by 94% and at 2 g/mL, the spore germination was completely inhibited. The 1-b-D-fructofuranosyl-D-mannitol disaccharide was completely inhibitory at 5 g/mL but had no effect at 2 g/mL. The 1,5-di-b-D-fructofuranosyl-D-arabitol and 5-b-D-fructofuranosyl-D-arabitol also completely inhibited at 5 g/mL but were 70% inhibitory at 2 g/mL and 40% inhibitory at 1 g/mL. By contrast, pure compounds of raffinose, D-mannitol and D-arabitol at 5 g/mL had no inhibitory effect on spore germination.

DISCUSSION

Some linkage ambiguities in Mower et al. (14) interpretation have been clarified and redefined, and the combined use of FAB-MS and GC-MS techniques, particularly in the chemical ionisation mode, has provided a new firm diagnostic application of these methodologies to natural oligosaccharides not containing glucose. Consequently, evidence for additional minor fructosans has been obtained and this has enabled formulation of a putative biosynthetic metabolic pathway in which the occurrence of the alditol oligosaccharides is seen partly as a rational expression of the pathogen's selective nutritive metabolism of the sucrose supplied by the host plant. It is also recognised that this range of complex biotransformations is not easy to rationalise for the pathogen's benefit on account of their energetic cost.

The proposal is that, whereas the oligosaccharides of the *C. purpurea* honeydew are the classical types (retaining the glucose of the sucrose acceptor) as a function of fructosyl transferase in making some glucose available for fungal nutrition (5), the oligosaccharides of *C. africana* may similarly first arise by fructosyl transferase to link one molecule of mannitol, arising from the pathogen's reduction of fructose by mannitol dehydrogenase, to the fructose moiety of sucrose. However the linkage of mannitol is to a 2-position of fructose, as subsequently detected by linkage analysis. It mimics the similar linkage with the glucose moiety in the sucrose substrate for this biotransformation and occurs without fructose ever being free. Of course, glucose freed by this process is available for direct utilisation in the pathogen's metabolism if the biotransformation is cell-associated. However, if the biotransformation is occurring within exuded honeydew, free glucose is potentially available to fuel conidial metabolism, including secondary sporulation. Thus, the pathogen even has extra cellular mechanisms to promote the secondary sporulation which is a potent factor to establish epidemics of proliferation. As a consequence, the bioactive disaccharide 1-b-D-fructofuranosyl-D-mannitol is formed. From GC-MS linkage analysis of this disaccharide it seems that the mannitol is randomly linked 1-2 or 6-2 to fructose. Further addition of fructose, also linked by its 2-position to the remaining 1 or 6 positions of mannitol in the disaccharide, gives the trisaccharide 1,6-di-b-D-fructofuranosyl-D-mannitol. As in the formation of the disaccharide, mannitol becomes linked to a 2-position of a fructose in another sucrose molecule with concomitant release of glucose, but in this case the mannitol is already linked to a fructose moiety.

In the other ergot pathogens of sorghum, *C. sorghi* McRae and *C. sorghicola* Tsukiboshi, Shimanuki & Uematsu, honeydew saccharides are mainly fructose and glucose, implying that the principal mode of utilisation of the host's sucrose is via an invertase hydrolysing it to its constituent monosaccharides. Probably, *C. africana* also has invertase activity to nutritional requirement as a parasite but its not dominant over saccharide-transferring activity which it is to produce the alditol-containing oligosaccharides. Since secondary sporulation is only an *in vitro* phenomenon in *C. sorghi* (13), the anamorph of which (*Sphacelia sorghi*) is morphologically indistinguishable from that of *C. africana*, it is important not to be confused where *C. africana* is misnamed as *C. sorghi* (1). A reason why *C. sorghi* never significantly exhibited secondary sporulation *in vivo* concerned the less bulky growth habit of its sphacelium (9). This results in less demand on the host's sucrose and a much higher saccharide concentration in honeydew. In this case, probably the high content of fructose is alone sufficient to account for the general failure to produce the white cascade of secondary sporulation on the surface of infected plants. However, it makes the point that it is the ratio between the pathogen's demand and the host's supply which most influences whether or not conidia in honeydew will germinate to release an airborne secondary spore. Even *C. sorghicola*, the anamorph of which is different from that of the other two Sorghum pathogens, can perform secondary sporulation *in vitro* (10), but this has never been reported as an *in vivo* event, again probably because of the dominance and abundance of fructose in the honeydew.

Normally, the developing sphacelium of *C. africana*, which is so bulky that it forces the glumes apart, utilises virtually all of the sucrose supplied by the grain Sorghum floret. This is why the concentration of saccharides in the honeydew is generally so low. The glucose moiety of sucrose is metabolised by the pathogen as a carbon and energy source, and the fructose moiety, partly reduced to mannitol, is incorporated into oligosaccharides. The efficient biosynthetic mechanism of linking mannitol to fructose molecules and the apparent abundance of the enzyme activity both fit and explain the increase in the concentration of bioactive oligosaccharides in honeydew where the sorghum plant has a 'sweet' character. The inevitability of perfusion of phloem leakage through a highly metabolic sphacelium ensures efficient utilisation of the sucrose, and transformation of excess in sweet sorghums to the oligosaccharides. The present mechanistic study therefore reinforces the idea that incorporation of a sweet character into A lines for sorghum hybrid production could provide an effective limitation on the adverse impact of ergot disease by restricting secondary sporulation on honeydew.

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