

Effects of Substrate Composition on Growth and Fructo-Oligosaccharide Production by *Gliocladium virens*

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ABSTRACT

Several species of filamentous fungi isolated from the rhizosphere of Asteraceae from the Brazilian *cerrado* have been shown to produce and metabolise fructose-containing sugars. Among them is *Gliocladium virens*, isolated from the rhizosphere of *Vernonia herbacea*, an inulin-accumulating species. In the present work, we investigated the ability of *G. virens* to produce fructo-oligosaccharides (FOS) when sucrose was used as the carbon source. We also studied the growth and FOS production in cultures of this fungus fed with different nitrogen sources. Significant increases in mycelium dry matter and production of FOS were observed when the sucrose concentration in the culture medium was increased to 3%. 1-Kestose, nystose, and 1-F-fructofuranosyl-nystose were the main FOS detected in fluids of *G. virens* cultured up to 18 d on 3% sucrose-containing media. The addition of complex sources of nitrogen, such as corn and yeast extracts, increased biomass production and reduced the content of extracellular proteins when *G. virens* was cultured in a sucrose-containing medium. Production of FOS was detected during the fungal growth cycle and was not affected by the nitrogen source. Although the production of oligo-fructans has gained tremendous commercial importance, only few microorganisms have the potential for industrial application. In this context, it is worth to find microbes from unexplored environments with the ability to synthesise these products. *G. virens* isolated from the rhizosphere of tropical plants has shown the ability to produce FOS, indicating that the Brazilian *cerrado* represents a profitable environment to search these microbes.

Keywords: filamentous fungus, nitrogen sources, oligofructan production, sucrose

INTRODUCTION

Fructans are fructose-based oligo- and polysaccharides that are the main reserve carbohydrate in about 15% of flowering plants (Hendry and Wallace 1993) and in microbes, where they serve different functions (Heyer and Wendenburg 2001). In plants, these carbohydrates are built up by the successive addition of fructose residues with different linkages to three trisaccharides, 1-kestose (1-F-fructosyl sucrose), 6-kestose (6-F-fructosyl sucrose) and neokestose (6-G-fructosyl sucrose), resulting in structurally different types of fructans, such as inulin, levan, mixed fructans and the so-called neo-series (Carvalho *et al.* 2007). In bacteria, fructans are generally of the levan type and produced by extracellular enzymes that use sucrose as a substrate, synthesising very high molecular mass polymers. Series of inulin-based, low or high molecular mass fructans are also synthesised by fungi, although less information on fungal fructans is available in the literature than for plants and bacteria (Heyer and Wendenburg 2001; Velázquez-Hernández *et al.* 2009). Production of fructans has been reported for example in *Aspergillus sydowi* (Kawai *et al.* 1973), *A. niger* (Hirayama *et al.* 1989), *A. foetidus* (Rehm *et al.* 1998), *A. aculeatus* (Ghazi *et al.* 2007), *Penicillium chrysogenum* (Olah *et al.* 1993), *Fusarium oxysporum* (Patel *et al.* 1997) and *Rhodotorula* sp. (Hernalsteens and Maugeri 2008).

In contrast to plants, which need at least two distinct enzymatic activities to catalyse priming and elongation of the fructan chain, microbes require only a single fructosyltransferase activity for both reactions. Microbial β -fructofuranosidases are able to transfer a fructosyl residue to sucrose or to a fructan acceptor molecule through different glycosidic bonds, depending on the enzyme source (Yun 1996; Velázquez-Hernández *et al.* 2009). Commercial production

of FOS comes mainly from the enzymatic transformation of sucrose by β -fructosyltransferases from microorganisms (Maiorano *et al.* 2008). Currently, *Aspergillus* fructosyltransferase is the main industrial producer of inulin-type FOS, synthesising mixtures of oligosaccharides with 1-kestose, nystose, and 1-F-fructofuranosyl-nystose as the main components (Sangeetha *et al.* 2005; Linde *et al.* 2009).

Interest in fructans and FOS has increased steadily since they are considered to be functional food ingredients acting as prebiotics and therefore being beneficial to human nutrition (Heyer and Wendenburg 2001). Studies have demonstrated that these sugars are low-calorie and non-cariogenic sweeteners that stimulate selectively the growth of bifidobacteria. They have also been claimed to contribute to the prevention of colon cancer and to reduce cholesterol, phospholipids and triglyceride levels in serum (Ritsemá and Smeeckens 2003; Maiorano *et al.* 2008). A number of studies have been carried out on methods of producing fructose syrup or FOS from inulin and sucrose; many of these methods include the use of microbial β -fructofuranosidases (Sangeetha *et al.* 2005). Therefore, the search for microorganisms capable of synthesising oligofructans is relevant for the industrial production of FOS (Linde *et al.* 2009).

Although the production of FOS has been widely reported in microorganisms (Maiorano *et al.* 2008), most research has been carried out with *Aspergillus* and *Penicillium* spp. (Hayashi *et al.* 2000; Sangeetha *et al.* 2004). Little is known about the production of FOS by microbes from the rhizosphere of tropical plants.

The savanna vegetation of the Brazilian *cerrado* includes a number of native Asteraceae species that accumulate inulin-type fructans in their thickened underground organs (Carvalho *et al.* 2007 and refs. therein). The rhizosphere of these species has been used as a source of micro-

organisms that are able to produce β -fructofuranosidases (Figueiredo-Ribeiro *et al.* 2007). Among these organisms, *Gliocladium virens*, a saprophytic and mycoparasitic fungus, have biological control activity (Howell 1982; Papavizas 1985). Many different enzymes, mainly related to the degradation of plant and fungal cell walls, have been observed in cultures of *G. virens* (van Tilburg and Thomas 1993 and refs. therein). Considering that *G. virens* produces a wide range of extracellular enzymes, we investigated its ability to synthesise FOS from sucrose and the effects of different nitrogen sources on growth and FOS production in cultures of this filamentous fungus.

MATERIALS AND METHODS

Organism

Gliocladium virens J.H. Mill., Giddens & A.A. Foster (URM 3333) was obtained from the rhizosphere of *Vernonia herbacea* (Vell.) Rusby (Asteraceae) using the procedure described by Cordeiro-Neto *et al.* (1997). The parent culture was maintained in solid potato dextrose agar medium for an 8-d period at 25-28°C and then stored at 4°C.

Culture conditions

G. virens was subcultured on potato dextrose agar (PDA) medium for 7 d at 28°C. Spores suspended in sterile distilled water containing 0.01% Tween 20 (1×10^7 spores mL⁻¹) were transferred to 125-mL Erlenmeyer flasks that contained 50 mL of a medium composed of the following ingredients (g L⁻¹): NaNO₃ (3), KH₂PO₄ (1), KCl (0.5), MgSO₄·7H₂O (0.5), FeSO₄·7H₂O (0.01), and 0.3% or 3% sucrose as the carbon source. The cultures were incubated up to 18 d on an orbital shaker (140 rpm) at 28°C under dark conditions (Pessoni *et al.* 1999). At the end of each incubation period, the *G. virens* culture fluid was separated from the fungal tissue by vacuum filtration through glass fibre filters (Whatman). Mycelia were exhaustively washed with distilled water, lyophilised and weighed to evaluate growth (dry mass). Filtrates were used for the colourimetric determinations of sugar and protein contents and for measurements of enzymatic activity.

G. virens was also cultured in liquid media containing different nitrogen sources, using disks of mycelium as inocula. Two 9-mm-diameter disks were taken from seven-day-old cultures of *G. virens* grown on PDA. Inocula were transferred to 250 mL-Erlenmeyer flasks containing 100 mL of the medium described above as well as 3% sucrose and the different nitrogen sources listed in **Table 1**. Cultures were incubated up to 5 d on an orbital shaker (140 rpm) at 28°C under dark conditions (Pessoni *et al.* 1999). After the incubation period, culture fluids and mycelia of *G. virens* were separated by filtration as previously described. Fructose-containing sugars were extracted from the mycelia according to Itaya *et al.* (2002). Mycelium extracts and culture fluids were analysed as described below.

Sugar and protein analyses

Total carbohydrate content, reducing sugars, free and combined fructose and total protein content were determined colourimetrically in the culture filtrates at different times up to 18 d, according to Dubois *et al.* (1956), Somogyi (1945), Jermyn (1956) and Bradford (1976), respectively. Glucose, fructose and an equimolar solution of both hexoses were used as sugar standards. Bovine serum albumin (Sigma) was used as standard for protein determination. Culture media without fungal inoculations were used as controls.

Samples (400 μ g fructose equivalents mL⁻¹) of the mycelium extracts and culture filtrates were deionised through ion exchange columns (10 \times 1 cm) containing cationic (Dowex 50 \times 80) and anionic (Dowex 1 \times 8) resins, filtered in 0.45 μ m filters (Spartan-3, Aldrich) and then analysed by high-performance anion-exchange chromatography and pulsed amperometric detection (HPAEC/PAD) in a Dionex DX-300 system, using a CarboPac PA1 column (4 \times 250 mm). Gradients of 150 mM NaOH (eluent A) and 500 mM sodium acetate in 150 mM NaOH (eluent B) were used as eluents, as established by Shiomi *et al.* (1991) and Vieira *et al.* (1995).

Samples (70 μ g of fructose equivalents) of the culture fluid and mycelium extract were also analysed by thin-layer chromatography (TLC) on silica-gel plates developed as described by Cairns and Pollock (1998). Ketoses were stained with urea-phosphoric acid (Wise *et al.* 1955) and identified by comparison with oligosaccharide standards of the inulin series obtained from tubers of *Helianthus tuberosus*.

Enzymatic assays

Samples of the liquid culture medium filtered under vacuum through a 0.45 μ m membrane filter (Millipore) were added to a solution of 0.02% sodium azide, 0.1 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride and 1 mM EDTA to prevent bacterial growth and nonspecific proteolyses. Enzymatic assays were performed as previously described (Pessoni *et al.* 1999). Enzyme activity was determined by the quantification of reducing sugars released after incubation with 2% sucrose (Sigma) in 0.1 M sodium acetate buffer (pH 5.0) at 30°C for 1 h. The reaction was stopped by the addition of 1 mL of Somogyi-Nelson alkaline copper reagent (Somogyi 1945). One unit of invertase activity was defined as the amount of enzyme that hydrolyses one μ mol of sucrose in 1 h. An equimolar mixture of glucose and fructose was used as the standard for the quantification of reducing sugars.

The optimum pH of the enzymes was determined by using 0.1 M citrate-phosphate buffer (pH 3 to 7) at 30°C for 30 to 60 min. The optimum temperature was determined by measuring the enzyme activity at different temperatures (30 to 50°C), at pH 5.0, for 1 h. To examine the patterns of hydrolysis, the products of incubation were analysed on a CarboPac PA-1 anion exchange column using a Dionex DX 300 gradient chromatography system (HPAEC/PAD). The gradient and chromatographic conditions were established according to Shiomi *et al.* (1996).

Table 1 Composition of the liquid culture media used for growing *Gliocladium virens*.

Component (g L ⁻¹)	Medium					
	Basic	Peptone	Yeast extract	Amino acids	Corn extract	Malt extract
Sucrose (30)	x	x	x	x	x	x
NaNO ₃ (3)	x	x	x	x	x	x
KH ₂ PO ₄ (1)	x	x	x	x	x	x
KCl (0.5)	x	x	x	x	x	x
MgSO ₄ (0.5)	x	x	x	x	x	x
FeSO ₄ (0.01)	x	x	x	x	x	x
Peptone (7.5)		x				
Yeast extract (0.5)			x			
Arginine (1.75)				x		
Glutamine (1.53)				x		
NH ₄ SO ₄ (2.0)				x		
Commercial corn extract (30.0)					x	
Malt extract (10.0)						x

RESULTS AND DISCUSSION

In the Brazilian *cerrado*, *Gliocladium virens* occurs together with other filamentous fungi in the rhizosphere of *Vernonia herbacea*, a native Asteraceae species that accumulates 80% of underground organs dry mass as inulin. Although many of these fungi have been reported to produce extracellular inulinases (Cordeiro Neto *et al.* 1997; Pessoni *et al.* 1999, 2007), this activity was not detected in *G. virens* when either inulin or sucrose was used as the carbon source in liquid medium. However, *G. virens* has the ability to produce fructo-oligosaccharides (FOS) when grown on sucrose-containing medium.

Fig. 1 shows the growth curves of *G. virens* in Czapek liquid medium containing 0.3 and 3% sucrose. In the medium containing 3% sucrose, the log phase occurred from 5 to 10 d, followed by a stationary growth phase until the end of the analysed period. A decline in fungal dry mass was not observed under this condition. In contrast, lower sucrose concentration in the culture medium resulted in lower dry mass (**Fig. 1A**). In 3% sucrose medium, protein concentration in culture filtrates increased gradually up to

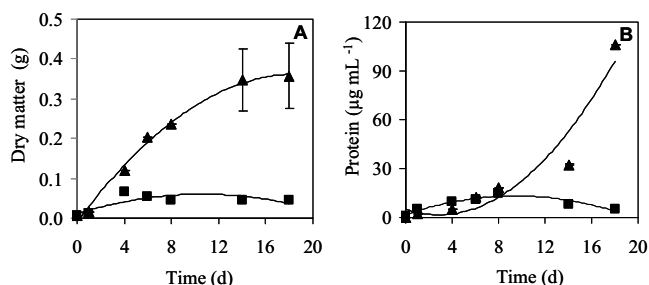


Fig. 1 Growth and extracellular protein production of *Gliocladium virens*. (A) Growth of mycelia, expressed as dry matter mass, in Czapek medium supplemented with 0.3% (■) and 3% (▲) sucrose. (B) Extracellular protein content in the culture fluids. Bars indicate standard error ($n = 3$).

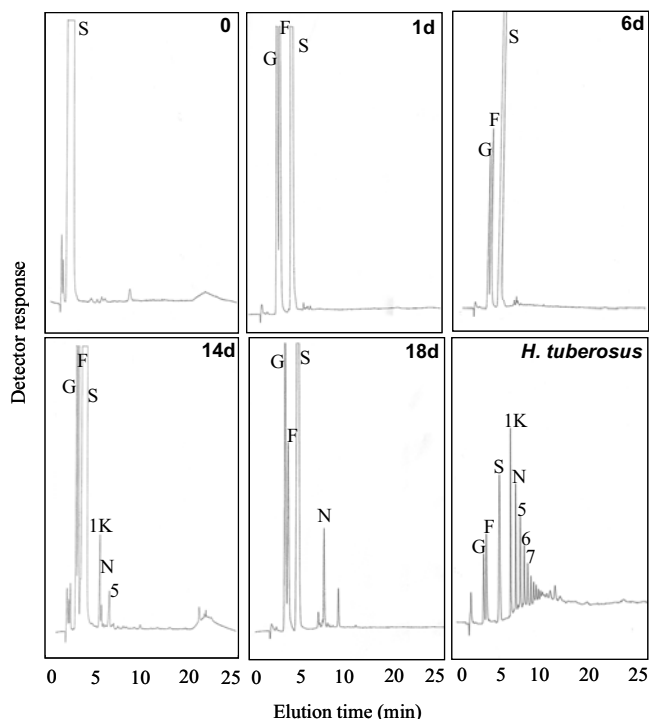


Fig. 2 HPAEC/PAD profiles of culture filtrates of *Gliocladium virens* grown in Czapek medium containing 3% sucrose. The culture filtrates were obtained at different periods of fungal growth (0 to 18 d). The peaks were identified by comparison with the retention times of glucose, fructose and fructo-oligosaccharides obtained as standards from tubers of *Helianthus tuberosus*. G, glucose; F, fructose; S, sucrose; 1K, 1-kestose; N, nystose; numerals indicate the DP of fructo-oligosaccharides.

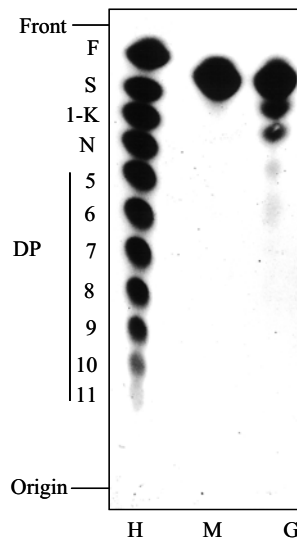


Fig. 3 TLC of fructo-oligosaccharides produced by *Gliocladium virens* grown in Czapek 3% sucrose medium for 5 d. F, fructose; S, sucrose; 1K, 1-kestose; N, nystose. Numerals mark the mobilities of FOS with DP 5-11. Each lane contains 70 µg of fructose equivalents. H, FOS standard obtained from tubers of *Helianthus tuberosus*; M, Czapek medium; G, *G. virens* culture filtrate.

30 µg mL⁻¹ until 14 d, with a 3-fold increase at the end of the growth period. Conversely, extracellular proteins present in the culture fluid of the fungus grown on 0.3% sucrose increased up to 8 d and declined after that (**Fig. 1B**).

HPAEC/PAD profiles of sugars from fluids of *G. virens* cultured up to 18 d on 3% sucrose-containing media showed the presence of FOS (**Fig. 2**), mainly 1-kestose, nystose and 1-F-fructofuranosylnystose, which was confirmed by TLC analysis (**Fig. 3**). The production of these compounds has been extensively reported to other filamentous fungi (**Table 2**).

Fungal cultivation in 0.3% sucrose resulted in lower production of FOS (data not shown) than for 3% sucrose medium. The concentration of carbon source has been one of main studied variable to reach the best condition for FOS production (Maiorano *et al.* 2008). Cultivation of *G. virens* on 3% sucrose by the fermentation method yielded *ca.* 15 mg L⁻¹ FOS, which is very low when compared to other microorganisms. As suggested by Chen and Liu (1996), below 10% sucrose the large portion of the carbon source is used for cell growth, while high sucrose concentrations result in higher induction of FOS-producing enzymes. This could explain the low FOS production by *G. virens* under our experimental conditions. Moreover, increased yields of FOS are usually achieved through enzyme method production using sucrose concentration over 40% (Hidaka *et al.* 2007; Linde *et al.* 2009).

Traces of oligofructans were also detected in the mycelium extracts (data not shown). Hirayama *et al.* (1989) reported the production of fructosyltransferase within the mycelium of *A. niger*, increasing the possibility of industrial production of FOS by direct application of the mycelium. The investigation of the production and properties of fructosyltransferases in the mycelium of *G. virens* would be an alternative to improve FOS production by this fungus.

According to Shiomi (2008), there are more than 500 kinds of oligosaccharides occurring in nature, including microbial FOS produced by enzymatic synthesis from sucrose. The main commercial production of FOS comes from the enzymatic transformation of sucrose by unspecific microbial β-fructofuranosidases with fructosyltransferase activity (Maiorano *et al.* 2008). Recently, Kurakake *et al.* (2008) reported the presence of two β-fructofuranosidases with invertase and fructosyltransferase activities in *Aspergillus oryzae*.

The high proportions of glucose and fructose detected

Table 2 Production of FOS by filamentous fungi.

Microorganism	Carbon source	FOS	Reference
<i>Aspergillus aculeatus</i>	Sucrose, raffinose	1-kestose	Ghazi <i>et al.</i> (2007)
<i>Aspergillus foetidus</i>	Sucrose	1-kestose	Rehm <i>et al.</i> (1998)
<i>Aspergillus niger</i>	Sucrose, nystose	1-kestose, nystose	Goosen <i>et al.</i> (2007)
<i>Aspergillus japonicus</i> TIT-KJI	Sucrose	oligofructans	Chien <i>et al.</i> (2001)
<i>Aureobasidium pullulans</i> CFR77	Sucrose	oligofructans	Sangeetha <i>et al.</i> (2004)
<i>Penicillium citricum</i> FERM P-15944	Sucrose	1-kestose, nystose, neokestose	Hayashi <i>et al.</i> (2000)
<i>Sporotrichum thermophile</i> ATCC 28811	Sucrose	1-kestose, 6-kestose, neokestose	Katapodis <i>et al.</i> (2004)
<i>Rhodoturulula</i> sp.	Sucrose	1-kestose, nystose, fructosyl nystose	Hernalsteens and Maugeri (2008)
<i>Gliocladium virens</i>	Sucrose	1-kestose, nystose, ¹ F-FOS	This work

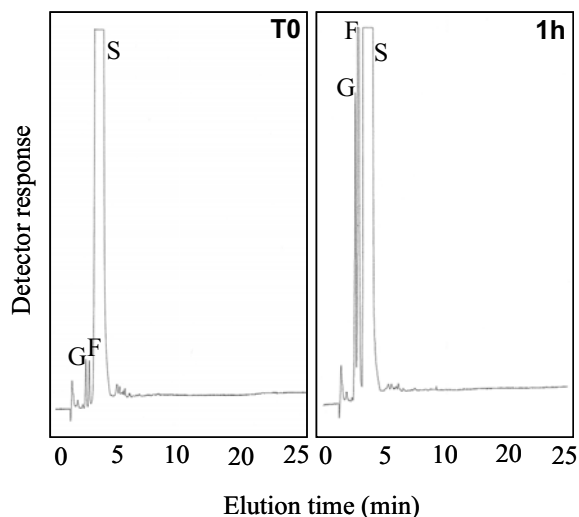


Fig. 4 HPAEC/PAD profiles of products from the assays of the extracellular enzymes extracted from *Gliocladium virens* culture filtrates. The fungus was grown for 4 d in Czapek 3% sucrose medium. T0, mixture of incubation without extracellular enzymes; 1h, mixture with enzymes incubated for an hour; G, glucose; F, fructose; S, sucrose.

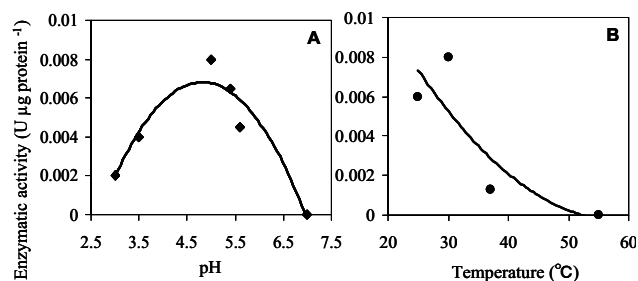


Fig. 5 Effects of pH and temperature on enzymatic activity of culture filtrate extracts of *Gliocladium virens* grown for 4 d in Czapek 3% sucrose medium. (A) Effect of different pH levels on enzyme activity at 30°C. (B) Effect of different temperatures on enzyme activity at pH 5. One unit of invertase activity was defined as the amount of enzyme that hydrolyses 1 µmol of sucrose in an hour.

by HPAEC/PAD in *G. virens* culture fluids after 1 d of incubation (**Fig. 2**) suggests the presence of invertase activity early in the fungal growth cycle. Indeed, culture fluids incubated for 4 d with 2% sucrose showed 0.008 U µg protein⁻¹ of enzymatic activity. The pattern of hydrolysis as examined by HPAEC/PAD after 1 h of incubation was also consistent with the presence of invertase activity (**Fig. 4**). The optimal pH and temperature were determined to be 5.0 and 30°C, respectively (**Fig. 5**). It has been shown that microbial β-fructofuranosidases, including invertases, are active at high sucrose concentrations, and these enzymes have been used industrially to produce FOS for human consumption (Rehm *et al.* 1998).

Enzyme production can be improved by changing culture conditions and substrates. In *G. virens*, it has been shown that the level of enzymatic activity varies with the

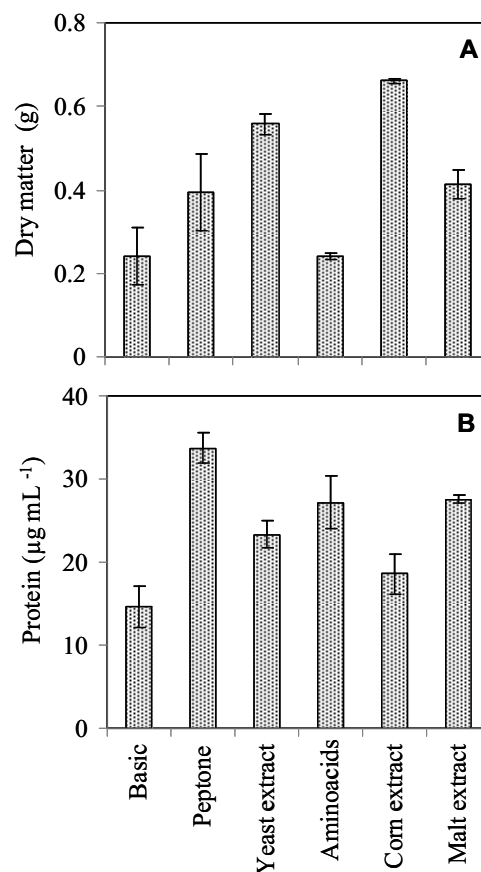


Fig. 6 Fungal growth and extracellular soluble protein production from 5-day-old cultures of *Gliocladium virens* grown in Czapek 3% sucrose medium supplemented with different nitrogen sources. (A) Growth of the mycelia, expressed as dry matter. (B) Protein content in culture fluids. Bars indicate standard error ($n = 3$).

age of the culture and the carbon source (van Tilburg and Thomas 1993). In the present work, growth and extracellular protein production were investigated in *G. virens* grown in media containing different nitrogen sources over a 5-d period (**Fig. 6**). The addition of complex nitrogen sources, mainly corn and yeast extracts, to the culture media increased fungal biomass (**Fig. 6A**). The extracellular production of proteins also increased in the supplemented media, mainly with addition of peptone (**Fig. 6B**). However, oligo-fructan production seemed to be unaffected by the nitrogen source in the culture medium since a higher proportion of these oligomers was found in the unsupplemented medium (**Fig. 7**).

G. virens is a mycoparasitic fungus that has been reported to have biological control activity and a high enzyme production capacity (van Tilburg and Thomas 1993 and refs. therein). Complex extracellular enzymes are produced by this fungus in response to its environment. In the present work, we report that cultures of *G. virens* grown on unsupplemented medium containing 3% sucrose have the ability

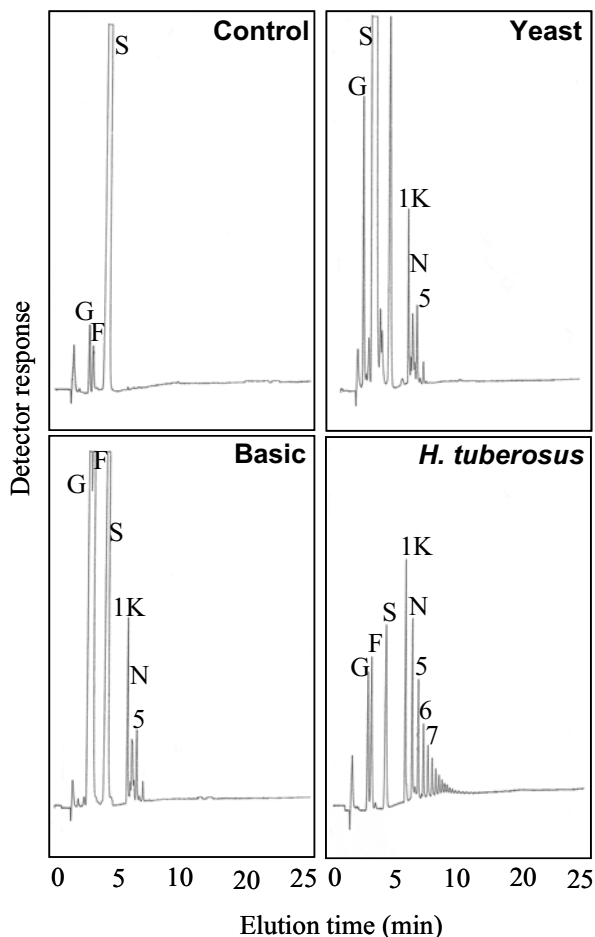


Fig. 7 HPAEC/PAD profiles of the culture filtrates of *Gliocladium virens* grown for 5 d in Czapek media containing 3% sucrose and supplemented with different nitrogen sources. Peaks were identified by comparison with the retention times of glucose, fructose and fructooligosaccharides obtained as standards from tubers of *Helianthus tuberosus*. G, glucose; F, fructose; S, sucrose; 1K, 1-kestose; N, nystose; numerals indicated the DP of oligosaccharides.

to produce FOS and that this production was not affected by the nitrogen source. Therefore, we speculate that the induction of extracellular β -fructofuranosidases and the production of FOS by *G. virens* in field conditions might be influenced by the rhizosphere exudates of *V. herbacea* and control activity of other microbes as part of an ecological strategy to survive in nitrogen-deficient soils such as those in the *cerrado*.

Since the production of FOS and fructosyltransferases has gained tremendous commercial importance on a global scale (Hidaka *et al.* 2007), it is worthwhile to find microbes with the ability to synthesise these products. As reported in the present work, the Brazilian *cerrado* is a profitable environment in which such microbes can be found.

The amounts of FOS produced from sucrose vary greatly, depending on the origin of the enzyme, age of the culture, the substrate concentration and other variables that influence the culture conditions. In our work, we conducted shake-flasks experiments to report FOS production by *G. virens*. However, in order to find out if this filamentous fungus can be considered as a new and efficient source of enzymes for FOS production in large scale, optimization procedures related to changes in culture conditions still need to be addressed. Moreover, the enzymatic activity of extracellular proteins isolated from *G. virens* culture fluids require further characterisation and purification for use in future applications.

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