Mycopathologia (2005) 159: 441–448
DOI: 10.1007/s11046-005-0253-5

Characterization and *in vitro* expression patterns of extracellular degradative enzymes from non-pathogenic binucleate *Rhizoctonia* AG-G

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Received 20 September 2004; accepted in final form 6 January 2005

Abstract

Many filamentous fungi produce an array of extracellular enzymes that acting in cell walls release elicitors of the plant defense response These enzymes may therefore be important in biocontrol applications. The aim of this study was to characterize extracellular degradative enzymes produced by a non-pathogenic binucleate isolate of *Rhizoctonia* AG-G.

The fungus was grown in liquid culture supplemented with pectin, polygalacturonic acid or glucose as a carbon sources and filtrates of the culture media were analyzed for the detection of pectinolytic and glucan hydrolytic enzymes. Using only pectin as a carbon source, secretion of polygalacturonases and methylesterases was found. When the liquid medium was supplemented with polygalacturonic acid, only polygalacturonase activity was detected. However, when glucose was used as carbon source β-1,3 and β-1,6 glucanases activities were detected, using laminarin and pustulan as substrates, but none of the pectinolytic activities were found.

These enzymes were partially purified and characterized. The β-(1,3)(1,6) glucanase and polygalacturonase enzymes showed to be active against cell wall polysaccharides from potato sprouts. These enzymes may have an important role in fungus-plant cell wall interaction. This is the first study about the production of extracellular enzymes by non-pathogenic binucleate *Rhizoctonia* AG-G.

Key words: cell wall polysaccharides, glucanases, pectin methyl esterases, polygalacturonases, potato sprouts

Introduction

Several non-pathogenic isolates of binucleate *Rhizoctonia* (np-BNR) belonging to anastomosis groups AG-A, AG-B, AG-G, AG-I, AG-K and AG-P have been characterized as effective biocontrol agents against several plant diseases caused by *R. solani* Kühn [1–5].

Escande and Echandi protected potato with np-BNR *Rhizoctonia* AG-G from Rhizoctonia canker caused by *Rhizoctonia solani* (teleomorph = *Thanatephorus cucucmeris*) [6].

The above mentioned studies tested the efficacy of np-BNR isolates to suppress and control disease, and only a few demonstrated that this suppression may be attributed to one of the following mechanisms: (i) competition of the np-BNR isolates with the pathogen for infection sites [7, 8] and (ii) systemic induced host resistance [9–12]. It is quite possible that different np-R isolates actually protect the seedling via different mechanisms, and that one isolate might protect the plants through more than one mechanism.

Plant cell walls are the first barrier in plant–microorganisms interactions and one of the most significant effects of microorganisms on cell walls is their enzymatic degradation. Plant cell walls are mainly constituted by carbohydrates such as

pectin and glucans. Polygalacturonases (PGs) and other pectinolytic enzymes, such as pectin methyl esterase (PME) and pectate lyase (PL), have been isolated and studied in a wide variety of bacterial and fungal plant pathogens [13–16]. Pectolytic enzymes have been suggested to be responsible for plant tissue maceration, cell death and putative pathogenicity factors [17–19]. Moreover, pectic fragments of the host cell wall released by action of fungal hydrolases can act as 'endogenous elicitors' which can activate diverse defense responses, such as phytoalexin accumulation and increase of pathogenesis-related (PR) proteins [20–22].

Fungi produce many other plant cell wall degrading enzymes, such as β-1,3-glucanases [23]. For instance, a fungal gene encoding an exo-β-1, 3-glucanase which cleaves callose, a plant polysaccaharide associated with defense responses [24].

In bean, np-BNR isolates penetrated and colonized epidermal cells [25]. These results imply that the np-BNR species produces cell wall degrading enzymes. Evidence for *in vitro* production of cellulolytic and pectolytic enzymes by np- *Rhizoctonia* species has been provided by Marcus et al. [26] and Sneh et al. [27]. Pectinolytic enzymes and glucanhydrolases such as polygalacturonases, pectin methyl esterases and glucanases from np-R isolates, may hydrolize potato cell walls releasing oligogalacturonides and glucans that may act as inducers of the defense response in potato plants.

Therefore, the objective of this study was to characterize the extracellular hydrolytic enzymes produced by non-pathogenic binucleate *Rhizoctonia* species (BNR) belonging to the anastomosis group AG-G. This way carried out by studying the induction of these enzymes in liquid media with different carbon sources and measuring the hydrolytic activities in the presence of various substrates, including potato cell walls.

Materials and methods

Organisms and culture conditions

Rhizoctonia AG-G and potato tubers (*Solanum tuberosum* cv. Kennebec) were obtained from the Experimental Station of the INTA Balcarce, Argentina. This cultivar was selected because it is moderately resistant to Rhizoctonia disease. Stock cultures were maintained on potato 2% glucose agar, at 18 °C in the dark. For induction of glucanases and pectinolytic enzymes, *Rhizoctonia* AG-G was grown in 2% potato-glucose medium and Maxwell liquid medium [28] supplemented with 0.5% (w/v) pectin or 0.5% (w/v) polygalacturonic acid, respectively. The cultures were grown at 18 °C.

Enzymes production during growth

Rhizoctonia AG-G isolate was grown on potato 2% glucose liquid medium. At different times of growth the mycelium was harvested by filtration. The culture filtrates were used for determination of glucanase activity and the mycelium was dried at 40 °C for 2 days for dry weight determination.

Extraction and enzyme purification

For glucanase purification, the mycelium was harvested after 8 days by filtration through filter paper Whatman 3 mm and Millipore (0.2 μm) filters. The culture filtrate was concentrated by ultrafiltration with Amicon P10 membranes and precipitated with two volumes of cold acetone and then used as a source of glucanases. The acetone-precipitate was applied to a Superdex 75 column (FPLC, Pharmacia, Uppsala, Sweden) equilibrated with 50 mm sodium acetate buffer, pH 5.2. Only a peak with glucanase activity was eluted from the column.

For partial purification of pectinolytic enzymes, the mycelium grown on pectin 2% Maxwell liquid medium was harvested after 8 days of culture, and processed as described above. The acetone pellet was resuspended in 50 mm sodium acetate, pH 5.2.

Polygalacturonase activity

Polygalacturonase activity was determined by measuring the release of reducing groups using polygalacturonic acid (PGA, Sigma, St. Louis, USA) and pectin as substrates. Reducing sugars content was measured by the method described by Milner and Avigard [29] using D-galacturonic acid (Sigma, St. Louis, USA) as a standard.

The activities were determined in 0.1 ml reaction mixtures containing 0.5% (w/v) polygalacturonic acid as substrate, 50 mm sodium acetate (pH 5.2) and suitable amounts of culture filtrates. Samples were maintained at 37 °C for 60 min. One

unit of polygalacturonase activity was defined as the amount of enzyme required to release 1 μmol of reducing groups per minute.

Glucanase activity and substrates specificity

Glucanase activity was estimated by measuring the rate of reducing sugar release using laminarin (β-1,3-glucan) from *Laminaria digitata* (Sigma St. Louis, USA), and pustulan (β-1,6-glucan) from *Umbilicaria papulossa* (Calbiochem), as substrates. This activity was routinely assayed by incubating 0.8 ml of 5 mg.ml^{-1} substrate in 50 mM potassium acetate buffer, pH 5.5, with 0.2 ml of enzyme solution appropriately diluted in the same buffer. The mixture was incubated at 37 °C for 30 min, reactions were stopped by boiling for 5 min, and the amount of reducing sugars released determined by the Somogyi–Nelson method [30,31], using glucose as standard. Enzyme and substrate blanks were included. One unit of glucanase activity was defined as the amount of enzyme that released 1μmol of reducing sugar equivalents, expressed as glucose, per hour, under the standard assay conditions.

For substrates specificity assay, glucanase activity was measured on laminarin, pustulan or carboxymethylcellulose as was described above.

Isolation of plant cell wall polysaccharides

Potato sprouts cv. Kennebec (10–15 g) were homogenized in 40 ml of 0.5 M potassium phosphate, pH 7.2 in a blender (Omnimixer). The homogenate was filtered by vacuum on glass fiber filters (Millipore type 1 from Sigma). The solid residue was washed with $CHCl_3$–MeOH (1:1) and dehydrated with acetone. The dried cell walls (5–6 g) were incubated with *Streptomyces griseus* protease (Sigma) at 35 °C for 45 min. The solid residue was filtered off, resuspended in distilled water and autoclaved at 120 °C for 3 h. The supernatant was filtered and centrifuged at 8000 *g* for 10 min. The supernatant was lyophilized and suspended in distilled water. Total sugar content was determined by the phenol–sulfuric method [32].

Hydrolysis of cell wall polysaccharides from potato sprouts

To monitor cell wall hydrolytic activity, the glucanase or polygalacturonase fractions (0.5 U) were incubated with 6 mg of lyophilized cell walls from potato sprout cv. Kennebec in 50 mM potassium acetate buffer, pH 5.5, in 1 ml of total volume. Mixtures were incubated at 37 °C for 16 h with occasional shaking. The reactions were stopped by centrifugation (10,000 $g \times 10$ min) and the reducing sugars released were determined in the supernatants by the Somogyi–Nelson method [30, 31], using glucose as standard. Enzymes and cell wall polysaccharides blanks were included. One unit of glucanase activity was defined as the amount of enzyme that released 1 μmol of reducing sugar equivalents, expressed as glucose, per hour, under the standard assay conditions.

Glucanase and pectinolytic activity in polyacrylamide gel electrophoresis (PAGE)

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on slab gels using 12% (w/v) acrylamide [33]. Samples were treated only with SDS, before SDS-PAGE. Gels were stained with silver nitrate [34]. Pectinolytic activity was measured in gels according to Ried and Collmer [35]. Activity bands were visualized by staining the gel for 30 min in 0.03% (w/v) Ruthenium red (Sigma St. Louis, USA) staining [36].

Glucanase activity in the gels was carried out using pustulan 10% as described by Soler et al. [37]. The active bands were visualized by staining the gel with 0.03% Congo Red.

Protein concentration was measured by the of bicinchoninic acid method [38], using bovine serum albumin (BSA) as standard. Equal amounts of proteins were loaded in each lane of the gels.

Results

Production of enzymes and substrate specificity

When non-pathogenic binucleate *Rhizoctonia* AG-G was grown in potato-glucose medium as the sole carbon source, maximum glucanase yield was recorded at 8 days of incubation, when the culture media was in logarithmic growth stage (Figure 1). Beyond this time, the enzyme yield decreased. At 10 days, the yield was only 25% of the yield found at 8 days. This decrease of glucanase yield after an optimum period of time was probably due to the

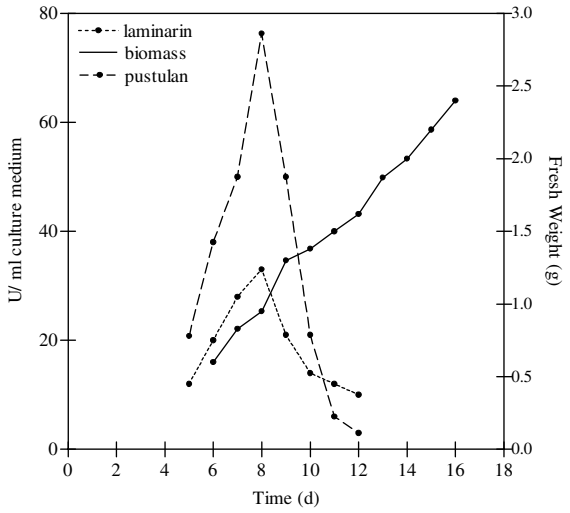

Figure 1. Production of β-(1,3)(1,6)-glucanase activities during the growth of *Rhizoctonia* AG-G. The non-pathogenic *Rhizoctonia* AG-G isolate was grown in potato glucose (2%) medium at 18 °C. Glucanase activity of the liquid culture, on laminarin and pustulan as substrates, were measured at different times of grow. Dry weight of mycelium was determinated simultaneously.

reduced nutrient levels of the medium affecting the enzyme synthesis and also enzyme denaturation by proteases secreted by the fungus. Smith and Grula [39] and Sandhya et al. [40] reported a sharp drop of extracellular enzymes after a maximal peak in *B. bassiana* and *T. harzianum* in liquid medium, respectively.

The substrate specificity of the glucanase was assayed with a variety of glucan substrates (Table 1). The maximal activity was detected against pustulan, which is a linear β-1,6-glucan. Approximately 50% of the maximal activity was observed with laminarin, which is a glucan mainly composed of β-1,3 and β-1,6-glycosidic linkages as branches at the ratio 7:1 of linkage type. No activity was detected neither with cellulose as substrate, which is a linear polymer composed exclusively of β-1–4-linked glucose units. We conclude that the enzyme is a specific β-1,6- β-1, 3-glucanase. (Figure 1 and Table 1).

The purified glucanase preparation migrated in a non-denaturing SDS-PAGE as a single sharp band with no trace of contaminants, indicating purification of the enzyme to electrophoretical homogeneity. (Figure 2, lane a). This band showed β-1,6-glucanase activity as demonstrated by zymogram using pustulan as substrate (Figure 2, lane b).

In the presence of pectin as carbon source, *Rhizoctonia* AG-G showed two different activities in the medium: a band with polygalacturonase activity and several bands with lower Mr with pectin methyl esterase activity, as shown in the zymogram of concentrated culture medium of *Rhizoctonia* AG-G (Figure 3, lane b). Maximum pectinolytic yield was recorded at 8 days of incubation (Figure 1). Beyond this time, the activity yield decreased.

Induction of pectinolytic enzymes was detected, by zymograms, at earlier stages in culture media with a higher concentration of pectin and polygalacturonic acid (PGA) (Figure 4, panel a and b). A different degree of induction of these enzymes was observed depending on the concentration of the carbon source. Besides, a

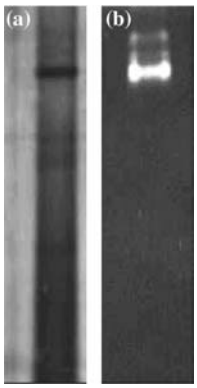

Figure 2. SDS-PAGE and β-1,3-glucanase zymogram of the proteins eluted from Superdex 75 column. (a) SDS-PAGE. Proteins were stained with silver nitrate. (b) β-1,3-glucanase activity of purified proteins on pustulan (10%). Equal amounts of proteins were loaded in each lane.

Table 1. Substrate specificity of β-(1,3)(1,6)-glucanases from *Rhizoctonia* AG-G

Substrate (0.5%)	Main linkage/momomer	Activity (μg glucose/h/μg protein)	Relative activity (%)
Pustulan	β-(1,6) glucose	300	100
Laminarin	β-(1,3) glucose	225	75
Carboxymethylcellulose	β-(1,4) glucose	0	0

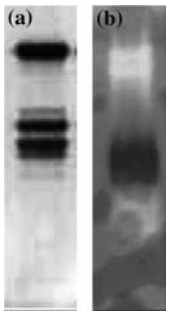

Figure 3. SDS-PAGE and zymogram of pectinolytic activity of culture filtrate from *Rhizoctonia* AG-G grown with pectin as carbon source. (a) SDS-PAGE. Proteins were stained with silver nitrate. (b) Pectinolytic activity of proteins on pectin (0.2 %). The white bands indicate polygalacturonase activity, the dark bands indicate pectin methyl esterase activity. Equal amounts of proteins were loaded in each lane of the gel.

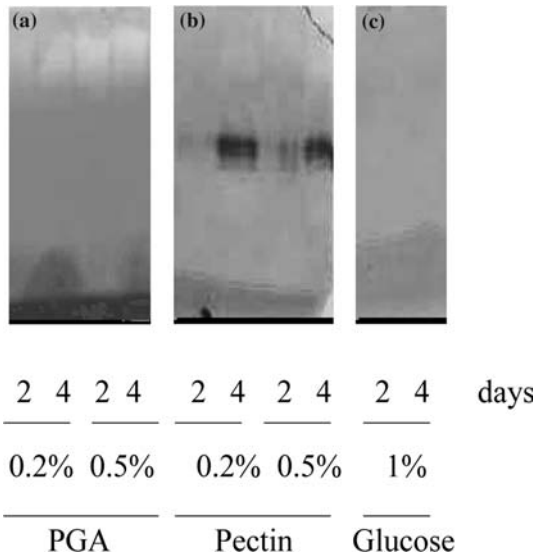

Figure 4. Pectinolytic isozime patterns from culture filtrates of *Rhizoctonia* AG-G at earlier stages of grown with different carbon sources: (a) PGA. (b) pectin. (c) glucose. 0.25 mg of micelium dry weigth were loaded in each line of the gel.

quantitative and qualitative difference was evident when were compared. When pectin was used as a carbon source, both pectin methyl esterase and polygalacturonase activities were detected. However, in the presence of PGA only polygalacturonase activity was observed (Figure 4, panel a). The culture media of AG-G grown in presence of glucose did not show induction of these activities (Figure 4, panel c).

Action on plant cell walls

High hydrolytic activity (glucanase and pectinolytic) was detected on cell wall polysaccharides from potato sprouts (Figures 5 and 6, respectively). The presence of these activities suggests that AG-G may be able to degrade both β-1,6-glucans and β-1,3-glucans, polygalacturonic acid and pectin, which are components of plant cell walls.

Discussion

The study of potato-associated non-pathogenic binucleate *Rhizoctonia* is important not only for understanding their ecological role in the interaction with plants, but also for any biotechnological applications, e.g. biological control of potato plant pathogens. Numerous studies have demonstrated the ability of np-BNR *Rhizoctonia* species to suppress diseases caused by fungal potato pathogens. Attempts to exploit the np-BNR isolates as potential biological control agents have recently led to the proposal that they could be strong elicitors of plant defense reactions [9, 10, 25]. The results obtained by Jabaji-Hare et al. [25] support the view that induced protection of young bean seedlings by np-BNR colonization is associated

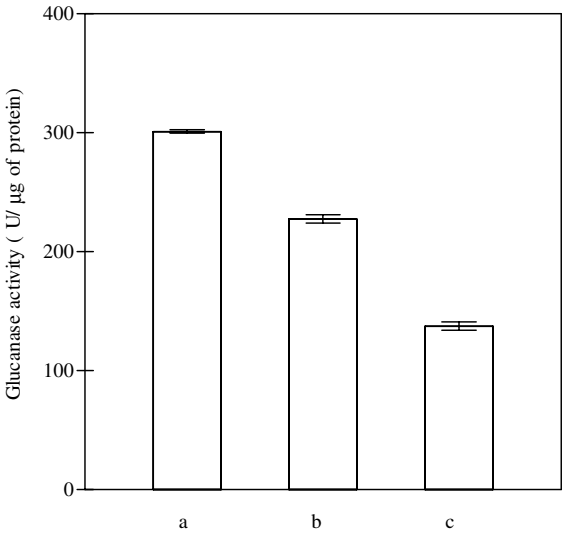

Figure 5. Glucanase activity of the proteins eluted from Superdex 75 column on different substrates: (a) pustulan, (b) laminarin and (c) potato sprouts cell wall polysaccharides. Bars represent the mean values ± SD of three independent experiments with five replicates per experiments.

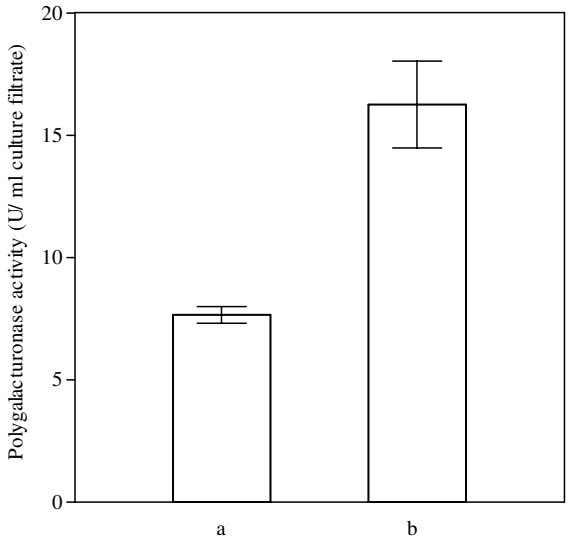

Figure 6. Activity of polygalacturonase of filtrate culture of *Rhizoctonia* AG-G, on (a) 0.5% PGA and (b) potato sprouts cell wall polysaccharides as substrates. Bars represent the mean values ± SD of two independent experiments with three replicates per experiments.

with striking anatomical modification of outermost layers of epidermis and cortex, and the accumulation of an electron dense barrier rich in suberin, phenolic compounds and pectin substances. One possible mechanism of np-BNR species antagonism towards potato pathogens includes the production of host cell-wall-degrading enzymes.

There are reports about the production of extracellular enzymes by non-pathogenic *Rhizoctonia* [26, 27] but it has not yet been demonstrated that this enzymes are produced during plant colonization and its role in biocontrol.

In the present work, we report the first study related with the production of extracellular enzymes by non-pathogenic binucleate *Rhizoctonia* AG-G. We investigated the production of glucanases, polygalacturonases and pectin methyl esterases in cultures supplemented with different sources of carbon. We also showed hydrolytic activity of these enzymes on cell walls of potato sprouts, suggesting that these enzymes may play an important role in signalling phenomena underlying plant–microorganism interactions.

Cultures of non-pathogenic *Rhizoctonia* were analyzed for glucanase activity. *Rhizoctonia* AG-G extracellular glucanases hydrolyzed pustulan, laminarin and cell wall oligosaccharides from potato sprouts, indicating the presence of enzymatic activity on β-(1,6) and β-(1,3) glucans (Figure 5). β-(1,6) glucans do not occur in plants as commonly as glucans composed by β-(1,4) or β-(1,3) glucosidic linkages [41]. In fact, only a few linear β-(1,6) glucans have been identified. The best known are pustulan from *Umbilicaria* species [42] and lutean from *Penicillium luteum* [43]. However, β-(1,6) linkages frequently occur in yeast and filamentous-fungal cell walls [44] and, not surprisingly, enzymes hydrolysing β-(1,6) glucosidic linkages are common in these organisms [45, 46]. On the other hand, the presence of the β-1, 3-glucanase activity suggests that this enzymes could release oligosaccharides of plant cell wall with the ability to induce the defense system of plant.

The secretion of glucanases is dependent on the carbon source or the glucose limitation of the culture medium [34]. Preliminary studies have shown that different glucanases are required at different stages of the development of fungi [35, 36] and may have specific functions in the organization of the cell wall during morphogenesis [47, 48]. However, other roles have been assigned to these enzymes in the biocontrol of microorganisms. For example, chitinases, β-1,6-glucanases and β-1, 3-glucanases have been proposed as key enzymes in the lysis of phytopathogenic fungal cell walls during the antagonistic action of *Trichoderma* [49, 50].

We have studied the production of pectinolytic enzymes by *Rhizoctonia* AG-G. Two different pectinolytic enzymes have been identified: polygalacturonases and pectin methylesterases. Both enzymes are produced during growth *in vitro* on pectin as the sole carbon source. They hydrolyzed PGA, pectin and cell wall oligosaccharides from potato sprouts pectin methyl esterases demethylate pectin to pectate, which can subsequently be degraded by polygalacturonases. The presence of two activities suggest that they may have an important role in the interaction with plant cell walls.

Villajuan-Abgona [51] observed that a protective binucleate np-R isolate (AG-A) degraded the cuticular surface in cucumber. The epidermal surface became covered with a thin layer of a viscous material which is positively stained with phenyl thionin, a specific stain for pectins. Such a layer is

not formed by a virulent *R. solani*. The authors suggested that the viscous layer could be formed by pectin degradation of plant cell wall by the np-R isolate. Pectin oligomers released by the activity of pectolytic enzymes of the np-R isolate may elicit plant defense reactions. Hyphal lysis of both virulent and hypovirulent isolates was also observed in seedlings inoculated with both types of isolates. Hyphal lysis were probably caused by the plant's cell wall lytic enzymes induced by the np-R isolate.

In conclusion, non-pathogenic binucleate *Rhizoctonia* produces at least two different extracellular glucanase activities (β-(1,6) and β-(1,3) activities) and two pectinolytic enzymes with activity of polygalacturonases and pectin methylesterases, which may explain, at least in part, the protection observed when potato plants are inoculated with binucleate *Rhizoctonia* AG-G prior to being infected with *Rhizocotonia solani*. However, further study will be required to determine if these enzymes are involved in the hydrolysis of plant cell walls during the interaction, and their capability to induce defense responses in plants.

Acknowledgements

This research was supported by the National University of Mar del Plata, the Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC) and the Consejo Nacional de Investigaciones Científicas y Técnológicas (CONICET). We thank Claudia Oliva for critical review of the manuscript and for excellent assistance. Daleo GR is an established researcher of Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC) Argentina.

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