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Short communication

Water soluble exo-polysaccharide from *Syncephalastrum racemosum*, a strong inducer of plant defence reactions

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ABSTRACT

This study examines the production, characterization and bioactivity on plant cell cultured in vitro of exopolysaccharides (EPS) from *Syncephalastrum racemosum* CBS 443.59. Firstly, the influence of the fungus culture condition in shake flasks (pH, temperature and different carbon and nitrogen sources) on EPS and biomass production was evaluated. In order to enhance EPS production, a new protocol based on two-stage pH fermentation in a 3 L stirred fermentor was developed. Under this condition, EPS production increased by 3.55 times, compared to a constant pH process, reaching a maximal EPS concentration of 2.62 g/L. Structurally, the EPS contains a polyglucuronic acid backbone, linked essentially with mannose and fucose units and some galactose and glucose units. The bioactivity of EPS as inducer of defence reactions in plant suspension-cultured cells was also studied. Our results show, for first time, that EPS from *S. racemosum* CBS 443.59 induces, depending on the concentration, PAL activation and H_2O_2 synthesis in *Arabidopsis thaliana* cell suspensions.

1. Introduction

In recent decades, polysaccharides are arousing a renewal industrial interest. These biopolymers are currently used in the production of sustainable fuel and an emerging polysaccharides based materials industry (such as biomedical and biomimetic materials, bio-inspired membranes and polymer electrolytes) is rising, in addition to their traditional uses like food ingredients. Besides, due to their relevant role in a wide spectrum of physiological activities, numerous formulations for human and plant care are including polysaccharides like bioactive components.

In particular, β-(1–4)-D-glucuronans have attracted the interest due to their immune stimulating activity on human blood monocytes (Courtois-Sambourg & Courtois, 2000) and role in tissue regeneration activation (Petit et al., 2004). β-(1–4)-D-Glucuronans can be chemically produced by regioselective oxidation of cellulose with the use of 2,2,6,6-tetramethylpyperidine-1-oxy (TEMPO)/NaBr/NaOCl (De Nooy, Besemer, & van Bekkum, 1995; Isogai & Kato, 1998). Unfortunately, this method required the solubilization of cellulose in more than 10% aqueous sodium hydroxide to enable the accessibility of primary alcohols (Elboutachfaiti, Delattre, Petit, & Michaud, 2011) and the use of NaBr whose presence in the waste stream is unwanted due to the environmental and toxicological problems (Chang, Park, Shin, Suh, & Kim, 2008).

A more "greener" process for β-(1–4)-D-glucuronans production includes the fermentation of the bacteria *Sinorhizobium meliloti* M5N1CS (Courtois et al., 1993; Heyraud, Courtois, Dantas, Colin-Morel, & Courtois, 1993) or fungi belonging to mucorales (De Ruiter, Josso, Colquhoun, Voragen, & Rombouts, 1992; Tavernier, Delattre, Petit, & Michaud, 2008). In this study, we have considered the production of β-(1–4)-D-glucuronans by the fungus *Syncephalastrum racemosum* (strain CBS 443.59).

S. racemosum is a fungus isolated from soil and known to excrete a polysaccharide containing more than 40% of glucuronic acid (De Ruiter, van der Lugt, Voragen, & Rombouts, 1991). The β-(1–4)-D-glucuronan is the backbone of this extracellular polysaccharide (EPS) which is found to be very resistant toward acid hydrolysis and has a molecular weight of 9–10 kDa (De Ruiter, Josso, et al., 1992; De Ruiter, Schols, Voragen, & Rombouts, 1992). Moreover, this strain also produces chitosan which could be extracted from the mycelial biomass after the EPS production (Amorin, Melo, Carneiro-da-Cunha, Ledingham, & Campos-Takaki, 2003) contributing to the feasibility of the process. Despite these interesting capacities, to our knowledge, nothing has been done to improve the production of mycelial biomass and EPS during the fermentation of this fungus.

Since the mycelial growth rate and EPS production might vary with the temperature, pH, stirring rate, carbon and nitrogen source (Metz & Kossen, 1977; Papagianni, 2004), we have studied the influence of these parameters in shake flasks on *S. racemosum*. In order to enhance EPS production, two-stage pH fermentation in a 3 L stirred fermentor was developed. Finally, to complete this study and search for others practical applications, the ability of the

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β-(1–4)-D-glucuronan EPS from *S. racemosum* to induce plant defence reaction as PAL activation and H_2O_2 accumulation was studied. PAL is a crucial enzyme with regulator activity in the phenylpropanoid pathway dealing with precursors of secondary metabolites in plant, playing therefore a key role in a range of plant–pathogen interactions (Morrison & Buxton, 1993). H_2O_2 are produced in one or more bursts of oxidative activity during resistance expression in a wide range of plant host/pathogen interactions and have been implicated in stimulation of the hypersensitive response, a mechanism used to prevent the spread of infection by microbial pathogens (Apel & Hirt, 2004).

2. Materials and methods

2.1. Microorganism

The strain of *S. racemosum* CBS 443.59 was purchased from Centraalbureau voor Schimmelcultures of Utrecht (The Netherlands). It was maintained on a medium consisted of (g/L): yeast extract, 4; soluble starch, 15; K_2HPO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5; agar, 15. The slant was inoculated and incubated at 30 °C for 7 days, than stored at 4 °C for about a month.

The seed solution was prepared by scraping spores off the slant surface after addition of sterile physiologic water on it.

2.2. Culture conditions study in shake flasks

The liquid medium used to find the optimum pH and temperature was as follows (g/L): glucose, 30; yeast nitrogen base (Sigma–Aldrich, Buchs, Switzerland), 6.7. Medium was adjusted to the required pH after sterilization by addition of sterile 0.5 M HCl or 0.5 M NaOH before inoculation. To determine the optimum temperature, the inoculated flasks were incubated at 32, 34, 37, 40 and 42 °C, respectively. The media used to find the best C and N substrate are described in Section 3. These studies were all made in 250 mL shake flasks containing 50 mL of medium inoculated with 500 μL spores solution at 100 rpm during 10 days.

2.3. Batch fermentation in the 3 L stirred fermentor

Fermentations were carried out in a 3 L fermentor (Biostat A plus, Sartorius, Melsungen, Germany) with 2 L fermentation medium. The agitation speed and aeration rate were controlled at 250 rpm and 0.5 vvm, respectively. pH was regulated by addition of either 1 M NaOH or 1 M HCl. The culture medium was inoculated with 4% (v/v) of the seed solution.

2.4. Isolation of extracellular polysaccharides (EPS)

Fermented media were centrifuged for 30 min at 7000 rpm and the resulting precipitates were removed. The EPS-containing water fraction was poured into four volumes of isopropanol and stored 16 h at 4 °C. The precipitate was separated by centrifugation (30 min, 7000 rpm), washed with ethanol 75% then with ethanol 98%, dialyzed and finally lyophilized.

2.5. Analytical methods

2.5.1. Dry cell weight determination

The mycelial biomass of *S. racemosum* CBS 443.59 was determined by weighting the dry cell amount. Samples collected at different times were centrifuged for 20 min at 7000 rpm, and then resulting precipitate was dried at 105 °C during 24 h to get the dry cell weight (DCW) measurement.

2.5.2. Monosaccharide analysis

Monosaccharide composition was determined by hydrolysis of the polysaccharide samples (50 mg) with H_2SO_4 1 M at 100 °C for 3 h. The hydrolyzed samples were converted to alditol acetates by successive $NaBH_4$ reduction and acetylation with anhydride acetic in presence of 1-methylimidazole following the slightly modified method described by Blakeney, Harris, Henry, and Stone (1983).

The resulting alditol acetates were analyzed by gas chromatography-flame ionization detection (GC-FID) using a Shimadzu model GC-2010A gas chromatograph, using a DB-225 capillary column (30 m × 0.25 mm i.d.), with helium as carrier gas. The analysis was carried out from 40 to 220 °C at 20 °C min^{-1}, maintaining the temperature constant to the end of analysis (30 min). The products were identified by their typical retention times.D-Glucuronic acid content was determined according to the method of *m*-hydroxydiphenyl using D-glucuronic acid as the standard (Filisetti-Cozzi & Carpita, 1991).

2.5.3. HPLC analysis

The evolution of fermentation components was monitored by HPLC using a Shodex Sugar SH1011 column. The system used is a Waters Alliance separation module 2695 coupled with a Waters 2410 RI detector. Fermentation samples were centrifuged and filtered through a 0.2 μm membrane filter prior to analysis. A 50 mM sulfuric acid solution was used as the mobile phase at a flow rate of 1 mL/min. Samples of sucrose, glucose, fructose and *S. racemosum* CBS 443.59 EPS at the concentration of 1 mg/mL were previously and separately injected to determine their retention times.

2.5.4. Infrared spectral analysis

FT-IR analyses of EPS were carried out by the potassium bromide (KBr) pellet method with a Perkin-Elmer Spectrum One FT-IR spectrometer (Norwalk, USA) in the range 400–4000 cm^{-1}.

2.5.5. NMR analysis

Polyglucuronic acid was isolated from EPS by a method according to De Ruiter, Josso, et al. (1992). The EPS preparation (200 mg) was dissolved completely in 10 mL of 2 M HCl and heated for 4 h at 100 °C. The polyglucuronic acid containing precipitate was isolated by decanting the supernatant after centrifugation (15 min, 4400 rpm). The precipitate was extracted twice with 1 M NaOH (3 mL) at room temperature. The combined extracts were dialysed against running tap water (24 h) and distilled water (48 h) followed by lyophilization. The ^1H NMR spectrum of the polyglucuronic acid was recorded at 25 °C in D_2O at 600 MHz with a Varian instrument. Chemical shifts are listed in parts per million downfield from TMS and are referenced by the residual solvent peaks.

2.6. Bioassays for elicitor activity

2.6.1. Cell culture

Suspension-cultured cells of *Arabidopsis thaliana* strain L-MM1 ecotype *Landsberg erecta* were grown in Murashige and Skoog medium (4.43 g/L) with sucrose (30 g/L) and 0.5 μg/mL of NAA and 0.05 μg/mL of Kinetin, pH 5.7. Cultures were maintained under dark, at 24 °C, on a rotary shaker at 100 rpm. Cells were diluted 10-fold in fresh medium every 7 days.

2.6.2. Bioassays

EPS was dissolved in distilled water, filtered through a 0.22 μm membrane filter (Millipore) and 500 μL (at different concentrations in EPS) aseptically added to 4.5 mL of 7 days-old suspension-cultured cells and incubated 24 h at 24 °C under mild agitation. The reaction mixture was centrifuged for 5 min at 1000 rpm and 4 °C to collect the cells (PAL activity measurement) and supernatant (H_2O_2 measurement). Biological data reflect the mean of at least

Table 1
Effect of carbon and nitrogen source on EPS production and mycelial growth.

Variable	Final pH	Mycelial dry weight (g/L)	EPS (mg/L)
Glucose	2.2 ± 0.1	2.1 ± 0.03	124 ± 55
Sucrose	2.2 ± 0.1	2.0 ± 0.02	124 ± 29
Lactose	2.6 ± 0.1	0.8 ± 0.02	25 ± 4
$(NH_4)_2SO_4$	2.9 ± 0.1	0.56 ± 0.00	201 ± 42
Peptone	4.5 ± 0.3	0.24 ± 0.03	46 ± 36
Tryptone	3.3 ± 0.1	0.28 ± 0.00	116 ± 10
Yeast extract	4.1 ± 0.3	0.19 ± 0.02	170 ± 37
Glutamic acid	4.1 ± 0.1	0.39 ± 0.02	85 ± 22

Mean ± standard error; $n = 3$.

three independent experiments. In each case, negative (water) and positive (chitosan) controls were used to ensure repeatability of the plant cells across subcultures.

PAL activity: Cells were homogenized at 4 °C in 1 mL of 0.1 M borate buffer (pH 8.8) containing 2 mM mercaptoethanol. The homogenate was centrifuged at 4000 rpm for 10 min at 4 °C. PAL (EC 4.3.1.5) activity was determined in 0.125 mL supernatant in the presence of 1.37 mL 0.1 M borate buffer (pH 8.8) supplemented with 60 mM L-phenylalanine as described by Beaudoin-Eagan and Thorpe (1985). Protein concentration of the extracts was determined by the Bradford protein assay (Bio-Rad).

H_2O_2 *measurement:* H_2O_2 concentration was measured in the supernatant using the Amplex Red hydrogen peroxide/Peroxidase Assay Kit (Molecular Probes) according to the supplier's instructions.

3. Results and discussion

3.1. Optimization of culture conditions in shake flasks

The effect of carbon source on mycelial growth and EPS production by *S. racemosum* CBS 443.59 was studied in a medium containing 6.7 g/L yeast nitrogen base and 30 g/L glucose, sucrose or lactose as the C source. Sucrose and glucose proved to be the most efficiently converted carbon substrates with both 124 mg/L EPS produced (Table 1).

The influence of the N source on EPS production and mycelial growth by *S. racemosum* CBS 443.59 was studied in a medium containing 30 g/L of glucose, 1 g/L of K_2HPO_4 and 0.5 g/L of $MgSO_4$ with five kinds of nitrogen sources (peptone, tryptone, yeast extract, glutamic acid and ammonium sulphate).

Among the various sources tested, ammonium sulphate was the best source by far for biomass and EPS production expressed in mg/L. Glutamic acid gives the second best value for mycelial growth but in counterpart the EPS production does not follow. Yeast extract is a good substrate for EPS production but the fungus does not grow very well on this medium (Table 1).

S. racemosum CBS 443.59 mycelial growth and EPS production was investigated in the pH range of 3.0–8.0 to find out the optimal pH. Maximum EPS production (241 mg/L) was obtained at an initial pH 6.0, whereas maximum biomass concentration (0.65 g/L) was obtained for the initial pH 4.0 (Fig. 1). Thus pH will be an important parameter to regulate in fermentor depending on whether we would to have mycelial growth or EPS production.

Previous studies have been done at a determined temperature of 37 °C (De Ruiter et al., 1991; De Ruiter, Van Bruggen-van der Lugt, et al., 1992). This data has been taken as central point to test the effect of temperature on mycelial growth and EPS production. The range of temperatures chosen was 32–42 °C. The temperature of 32 °C give better results for mycelial growth and EPS production than 37 °C which in his turn was better than 42 °C (data not shown).

As could be seen in the above results, the EPS production yields reached are very low (maximum 241 mg/L) compared to other

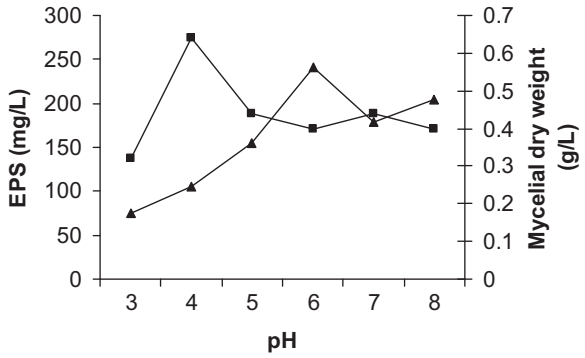

Fig. 1. Effect of initial pH on mycelial growth (■) and EPS production (▲) by *S. racemosum* CBS 443.59.

fungi likes, for examples, *Agrocybe cylindracea* which are between 200 mg/L and 1.1 g/L (Kim et al., 2005), *Phellinus baumii* which are between 590 mg/L and 2.38 g/L (Luo et al., 2009) or *Fusarium solani* which are approximately 960 mg/L (Mahapatra & Banerjee, 2013). But we also noticed that a lower pH promotes mycelial growth, while a higher pH gives rise to a better EPS production. Based on these observations and previous studies using the two-stage pH fermentation to increase the EPS synthesis of fungi strains (Kim, Park, & Yun, 2006; Wu, Ding, & Zhang, 2006), we have developed a new protocol for *S. racemosum* CBS 443.59 fermentation using a similar strategy.

3.2. Two-stage pH fermentation to improve EPS production

The two-stage pH fermentation studied was as follows: in medium containing 6.7 g/L yeast nitrogen base and 30 g/L sucrose, pH was kept at 4.0 during 5 days, time previously determined to reach a maximal mycelial biomass, and then switched to 6.0 the rest of the time to improve EPS synthesis.

The dynamics of EPS production and sucrose consumption in a 3 L stirred fermentor under different controlled-pH conditions is shown in Fig. 2: (A) pH 4.0 during the entire fermentation period, (B) pH change from 4.0 to 6.0 after 5 days of fermentation.

After 8 days of fermentation there was no more sucrose regardless of the culture pH applied.

The maximum EPS production (2.62 g/L) was achieved when culture pH was changed from 4.0 to 6.0. As expected, using fermentation with pH shift, EPS synthesis was enhanced by more than three fold compared to that of the culture with pH controlled to 4.0 (EPS concentration was of 0.76 g/L). We could also notice that the transition from shake flasks to fermentor combined with controlling the pH to 4.0 results in improving the EPS production of more than three times from 241 mg/L to 760 mg/L.

The observation of the mycelial morphologies of the two different culture conditions shows a smooth pellet growing with time to be of 3–4 mm of diameter at the end of fermentation process.

3.3. Partial characterization of EPS

3.3.1. Monosaccharide composition

The EPS was composed of glucose, galactose, glucuronic acid, fucose and mannose in relative percent of 2.82, 8.86, 22.06, 27.12 and 39.11, respectively. These results fit pretty well with previous published values on this EPS (De Ruiter, Schols, et al., 1992) which were for glucose, galactose and glucuronic acid in relative percent of 3, 9 and 21, respectively. But the relative percent of fucose and mannose were slightly different with 35 and 32, respectively. Total sugar content was 92.16%.

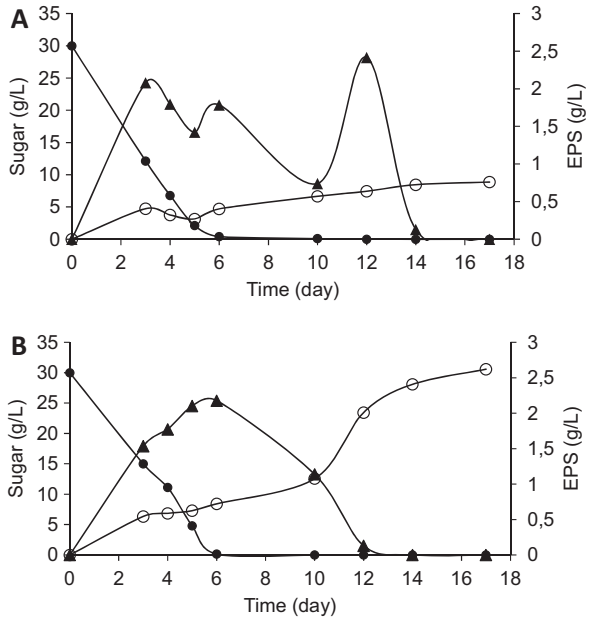

Fig. 2. Time profiles of sugar consumption (sucrose: (●) and glucose + fructose: (▲) and EPS production (○) of filtered culture medium of *S. racemosum* CBS 443.59 grown in a stirred fermentor at pH 4.0 (A) and at pH 4.0 during 5 days and 6.0 the rest of time (B).

3.3.2. FTIR analysis

The FT-IR spectrum of the EPS (Fig. 3) showed a strong and broad absorption band at 3332 cm^{-1} characteristic of OH stretching vibrations assigned to OH groups from monosaccharides and residual water, a band at 2934 cm^{-1} indicating the presence of CH groups and a strong and broad absorption band in the region of 900–1200 cm^{-1} assigned to the C—O stretching and OH bending vibrations. The two bands at 1646 and 1417 cm^{-1} were assigned to the asymmetric and symmetric stretching modes of the carboxyl groups confirming the presence of glucuronic acid. This FT-IR spectrum was found to be in good agreement to previously reported results for polyuronic acids (Deiana, Palma, Premoli, & Senette, 2007; Muzzarelli et al., 2000; Park, Khan & Jung, 2006; Pau-Roblot et al., 2002).

3.4. NMR analysis of the EPS backbone

The backbone of *S. racemosum* EPS was obtained by acid hydrolysis then analyzed by ^1H NMR spectroscopy in D_2O. The signals at 3.33, around 3.60, 3.84 and 4.49 ppm were attributed to H-2, H-3 and H-4, H-5 and H-1, respectively (Fig. 4). Previous work on glucuronan has nearly the same values for the signals (Delattre, Michaud, Lion, Courtois, & Courtois, 2005; De Ruiter, Josso, et al., 1992) confirming that the backbone of our EPS is constitute of polyglucuronic acid as demonstrated by De Ruiter, Josso, et al. (1992).

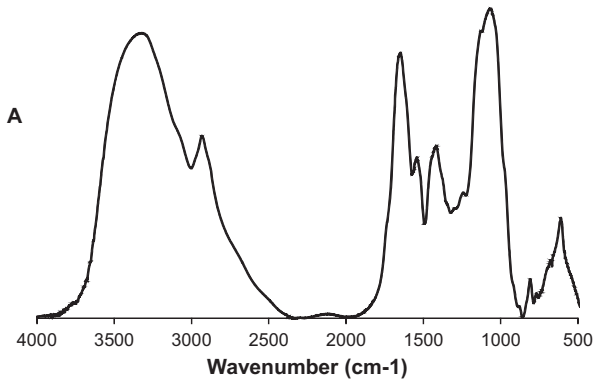

Fig. 3. FTIR spectrum of the EPS produced by *S. racemosum* CBS 443.59.

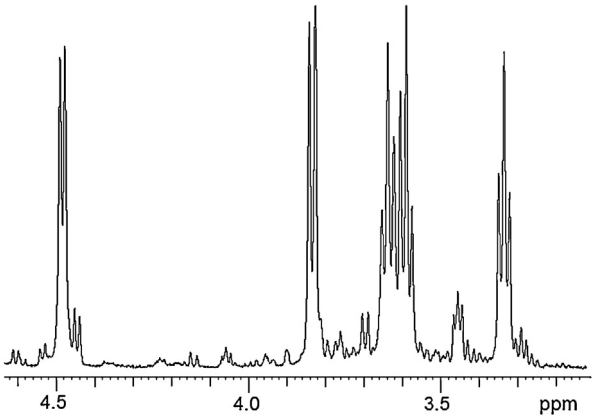

Fig. 4. ^1H NMR of polyglucuronic acid extracted from EPS produced by *S. racemosum* CBS 443.59.

3.5. Bioactivity

The ability of EPS produced by *S. racemosum* to induce plant defence reactions was evaluated using *A. thaliana* suspension-cultured cells. Plant cell suspension cultures are widely used in plant pathology as a useful tool for the screening of molecules acting as elicitors of a wide range of plant defence responses. The homogeneity of an in vitro plant cell population, the satisfactory reproducibility of conditions, the direct contact of the molecule under study with the plant cell surface and the effortlessness in the measurement of some plant defence reactions, make suspension-cultured plant cells suitable for these studies at the cellular and molecular levels.

We have examined the effect of EPS concentration on PAL activation and H_2O_2 accumulation, two widely used biochemical marker of plant resistance activation.

PAL activity in EPS-treated *Arabidopsis* suspension-cultured cells was transiently induced reaching a maximum after 20–24 h of plant cell contact with the elicitor and then decreased to control level (data not shown); consequently we measured the influence of increasing amounts of EPS produced by *S. racemosum* after 24 h (Fig. 5). The *A. thaliana* suspension-cultured cells significantly respond to the increase in EPS dose exhibiting a bell-shaped curve response with a maximal PAL activation at 100 mg/L of EPS in the cultivation media. EPS dose-H_2O_2 accumulation curve show a similar shape but with a maximum at 10 mg/L of EPS.

Increased levels of these biochemical markers of defensive responses, were accompanied with a significant browning of the elicited cells (not shown), a cellular response previously reported using other elicitor (Cabrera, Messiaen, Cambier, & Van Cutsem, 2006) and probably due to the accumulation of phenolic compounds (Hahlbrock & Scheel, 1989).

Usually, H_2O_2 production is one of the earliest defensive responses observed in plant cell challenged with elicitor molecules. However, any significant changes in H_2O_2 level in the *Arabidopsis* cell culture media during the first hours after adding EPS was detected (data not shown).

To the best of our knowledge, this is the first report showing that EPS produced by *S. racemosum* induces plant defence reactions. Since the biological activity of β-(1,4)-polyglucuronic

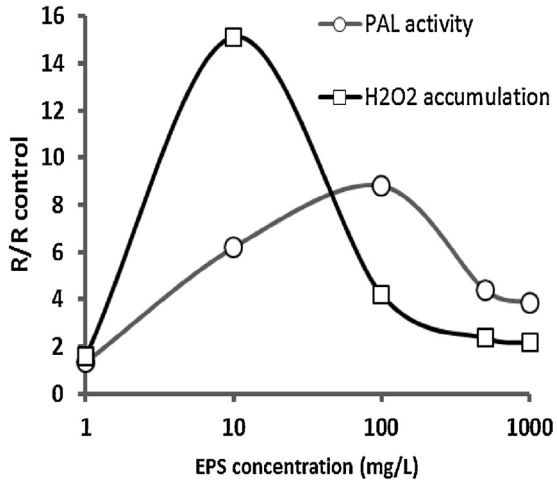

Fig. 5. Dose–response curve of PAL activity and H_2O_2 accumulation in *Arabidopsis* cell suspensions 24 h after application of EPS. The data were expressed as response of each sample divided by the response induced by a control treatment (water) in that experiment (R/R control): R control: PAL activity = 0.1 ± 0.2 μmol cinnamic acid mg^{-1} protein h^{-1} and H_2O_2 accumulation = 100 ± 70 nmol H_2O_2 g^{-1} fresh weight. Data are means ± SD of triplicate samples from one representative of three independent experiments.

acid as inducer of plant defence has been previously reported (Lienart, Heyraud, & Sevenou, 1999), it could be possible that EPS backbone is a major contributor on the *S. racemosum* EPS bioactivity.

4. Conclusions

In the present study, the effect of pH, temperature and C/N source on mycelial growth and EPS production of *S. racemosum* CBS 443.59 were investigated. Sucrose and ammonium sulphate were proved to be the best C and N source, respectively. An improvement of the EPS production with 2.62 g/L was reached with a two-stage pH fermentation. We have also shown that EPS from *S. racemosum* induces, depending on the concentration, PAL activation and H_2O_2 synthesis in *A. thaliana* cell suspensions. This noteworthy result states the possibility of using this polysaccharide as plant biostimulant in agronomic practices.

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