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Original Article

MODULATING THE BIOSYNTHESIS OF A BIOACTIVE STEROIDAL SAPONIN, CHOLESTANOL GLUCOSIDE BY *LASIODIPLODIA THEOBROMAE* USING ABIOTIC STRESS FACTORS

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ABSTRACT

Objective: The present study investigates the modulation of cholestanol glucoside (CG) biosynthesis by *Lasiodiplodia theobromae* in response to oxidative, osmotic and heat shock stresses.

Methods: The fungal cultures were subjected to oxidative stress by supplementing the culture media with menadione or H_2O_2 to the desired final concentrations. Osmotic stress was implemented by the addition of the desired concentrations of NaCl or sorbitol. For heat-shock treatments, the fungal cultures were subjected to required temperature variations. Each stress treatment was carried out at different time points so as to include different stages of fungal growth.

Results: Oxidative stress enhanced CG yield by the fungus by 1.8-fold (88.3±0.6 mg/l) where as osmotic and heat shock stresses proved to be poor enhancers of CG production.

Conclusions: Our findings enable a cost-effective, large scale production of CG by *L. theobromae* and more over throws light on the possible antioxidant activity of the compound in the organism.

Keywords: Secondary metabolites, Cholestanol glucoside, Filamentous fungi, *Lasiodiplodia theobromae*, Abiotic stress.

INTRODUCTION

Filamentous fungi are a major group of industrially important microorganisms [1]. Large scale industrial fermentation of fungi for the production of valuable secondary metabolites is widely practiced. Fungal secondary metabolism gene clusters are controlled by a complex regulatory network that responds to various environmental stimuli [2]. Hence alterations in an external environment can be employed for the improved production of desired secondary metabolites by fungi. An increase in production of β-carotene upon induction of oxidative stress by H_2O_2 supplementation has been reported in *Blakeslea trispora* [3]. Menadione, a well known oxidant, increased riboflavin production in a filamentous fungi *Ashbya gossypii* [4].

In most cases, it is difficult to predict the specific environmental stimuli that would trigger the biosynthesis of a particular metabolite unless the actual physiological role of the metabolite is known. There exists a crosstalk regulation between gene clusters, so that stimulation of a particular biosynthetic pathway can result in the production of even more compounds [5]. *Lasiodiplodia theobromae*, an endophytic fungus isolated from *Saraca asoca*, produced a novel cholestanol sugar, cholestanol glucoside (CG) which exhibited significant antioxidant and anticancer potentialities *in vitro* [6]. The development of CG as a chemotherapeutic drug candidate requires its cost effective production. In the present study, we have attempted to enhance the production of CG by exogenous supplementation of oxidative, osmotic and heat-shock stress factors in the fermentation medium.

MATERIALS AND METHODS

Chemicals used in the study

Menadione sodium bisulphate was procured from Sigma Aldrich. Sorbitol was purchased from Hi-Media. H_2O_2 and NaCl were purchased from Merck Millipore.

Fermentation

L. theobromae was grown in 25 ml M1D (modified medium 1) broth by transferring a 9 mm agar plug (containing actively growing fungal mycelia) for 3 days at 25±2 °C in dark, after which a 2 % inoculum was transferred into 200 ml optimized production medium [that contained (in g/l) D-glucose, 70.0; ammonium sulphate, 0.6; L-asparagine, 2.2; KH_2PO_4, 1.5; $MgSO_4$. $7H_2O$, 0.66 and (in mg/l) $FeSO_4.7H_2O$, 1.0; $MnSO_4$. H_20, 1.0; $CuSO_4.5H20$, 1.0; $ZnSO_4$. 7H20, 1.0; in distilled water] and incubated in dark at 25±2 °C for 16 days.

Stress treatments

H_2O_2 and menadione were used for oxidative stress treatments. Stock solution of menadione was prepared and added to *L. theobromae* cultures on days 3, 6, 9 and 12 so as to obtain final concentrations of 0.5, 1, 2.5, 5 and 50 µmol of menadione in the culture medium. Similarly H_2O_2 was also added to the fungal cultures on days 3, 6, 9 and 12 to obtain final concentrations of 5, 10, 25 and 50 mmol in the culture media. For osmotic stress conditions, cultures of *L. Thrombose was* grown in optimized production medium and supplemented with NaCl or sorbitol on days 3, 6, 9 and 12, so that the final concentrations of each of the stress factors in the fungal culture media were 0.5, 1, 1.5 and 2 mol. For heat-shock treatments, the flasks containing 40 ml cultures on days 3, 6, 9 or 12 after inoculation were mixed with pre-heated (32 °C, 35 °C or 42 °C) 160 ml medium in 500 ml conical flasks and partially immersed in a water bath at 32 °C, 35 °C or 42 °C for 1 h. After heat shock treatments, the flasks containing heat shock treated and untreated cultures were cooled immediately on ice and returned to 25 °C static conditions during rest of the experiment. In each of the stress treatment experiments, triplicates of untreated fungal cultures served as controls. All treated and untreated cultures were incubated at 25±2 °C for a period of 16 days.

Quantification of biomass and CG yield

At the end of incubation period, mycelia were separated from the culture filtrate, dried overnight at 40 °C and the dry weight was determined. Filtrates were extracted with two volumes (v/v) of dichloromethane and condensed using rotary evaporator at 40 ºC. CG contents of the organic extracts were analyzed by high performance liquid chromatography (HPLC). The organic extracts were was re suspended in 0.5 ml of HPLC grade methanol, filtered through 0.22 µ filters (Hi-media) and subjected to reverse phase HPLC analysis in Agilent compact 1120 liquid chromatography equipped with a photodiode array detector. The HPLC column used

was a C18 column of particle size 5 µ and length 150 mm (Agilent Technologies, CA, USA). The mobile phase consisted of acetonitrile: methanol: 2-propanol (95:3.5:1.5) and the flow rate was 1 ml/min.

Effluent was monitored at 288 nm for detection of compound and quantified against peak area calibrations calculated from standard curves. Reference standard for CG was obtained by chromatographic purification from *L. theobromae* dichloromethane extracts.

Statistical analysis

Graphpad prism program (version 6.0) was used for statistical analysis and preparation of graphs. Mean and standard deviation values were calculated from three independent experiments performed in triplicate.

Analysis of variance was used to compare the treatment means. Standard student's t-test was used to compare the values (control vs. treated). A probability less than or equal to 0.05 was considered statistically significant.

RESULTS

Effect of oxidative stress

In order to study the effect of oxidative stress on CG production by *L. theobromae*, the cultures were supplemented with different concentrations of two oxidants, menadione and H_2O_2, on 3rd day, 6th day, 9th day and 12th day post-inoculation. The accumulation of CG in culture media and biomass yields in the presence of stress factors were monitored on 16th day. The results revealed the growth inhibitory effects of higher concentrations of both oxidants (menadione ≥ 2.5 µmol and H_2O_2 ≥ 25 mmol) as evident from the decline in biomass yields. The higher concentrations of oxidants did not enhance CG production by the fungus either. The lowest concentrations of menadione (0.5 µmol) and H_2O_2 (25 mmol) did not hamper the growth of the fungus, but were poor enhancers of CG biosynthesis. Mild oxidative stress by the addition of 1 µmol menadione or 10 mmol H_2O_2 on day 9 significantly enhanced CG biosynthesis compared to the untreated controls (*P<0.05). In both cases, a 1.8-fold increase in CG yield was obtained compared to the controls.

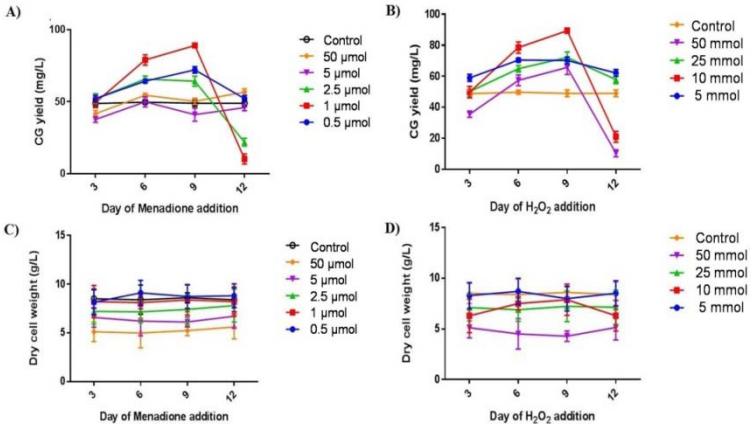

Fig. 1: Effect of oxidative stress on CG production and biomass yields of *L. theobromae*

The fungus was grown in optimized production medium and menadione and H_2O_2 were added to the cultures on day 3, 6, 9 and 12 post-inoculation to the desired final concentrations. CG content and dry biomass were quantified on day 16 post-inoculation. The results were obtained from three independent experiments and expressed as mean±SD. A) Effect on menadione on CG yield by *L. theobromae.* B) Effect of H_2O_2 on CG yield by *L. theobromae.* C) Effect of menadione on biomass yield by *L. theobromae.* D) Effect of H_2O_2 on biomass yield by *L. theobromae*

Effect of osmotic stress

The effect of osmotic stress on CG production by *L. theobromae* was investigated by supplementing the fungal cultures with different concentrations of NaCl or sorbitol on day 3, 6, 9 and 12. Both NaCl and sorbitol when added at a concentration of 1 mol on day 6 was found to enhance the yield of CG by 1.2-fold compared to controls (62.15±1 mg/l and 60.15±1.2 mg/l, respectively). Osmotic stress, except at the lowest concentration (0.5 mol) of NaCl and sorbitol was found to strongly inhibit the growth of *L. theobromae* as evident from the biomass yields (fig. 2C and 2D). Along with the inhibition of fungal growth, these concentrations considerably inhibited the synthesis of CG. The results indicate that CG yield is dependent on the growth of *L. theobromae.*

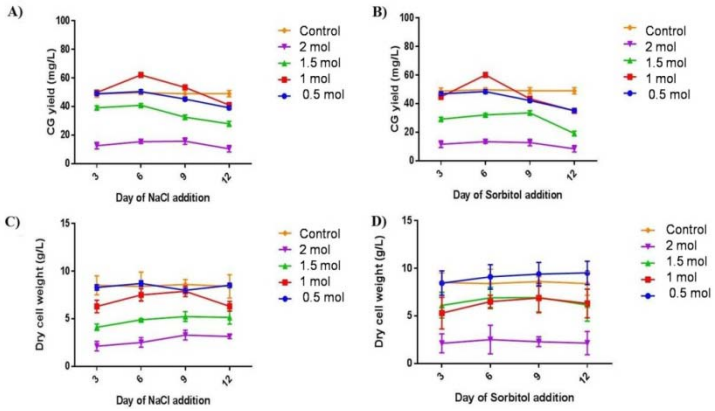

Fig. 2: Effect of oxidative stress on CG production and biomass yields of *L. theobromae*

The fungus was grown in optimized production medium and NaCl and sorbitol were added to the cultures on day 3, 6, 9 and 12 post-inoculation to the desired final concentrations. CG content and dry biomass were quantified on day 16 post-inoculation. The results were obtained from three independent experiments and expressed as mean±SD. A) Effect of NaCl on CG yield by *L. theobromae.* B) Effect of sorbitol on CG yield by *L. theobromae.* C) Effect of NaCl on biomass yield by *L. theobromae.* D) Effect of sorbitol on biomass yield by *L. theobromae*

Effect of heat-shock stress

To test the effect of heat shock stress on CG biosynthesis, the cultures were treated at 32 °C, 37 °C and 42 °C for 60 min on day 3, 6, 9 and 12 post-inoculation. As can be seen in fig. 3B, biomass production was not largely affected by heat shock treatments. Heat shock stress at 32 °C and 42 °C was found ineffective in enhancing the biosynthesis of CG (fig. 3A). Heat shock at 37 °C given on day 6 was found to produce 1.2-fold increase in CG production (62.16±1.6 mg/l) compared to the controls. However, there was no decrease in biomass upon heat-shock stress.

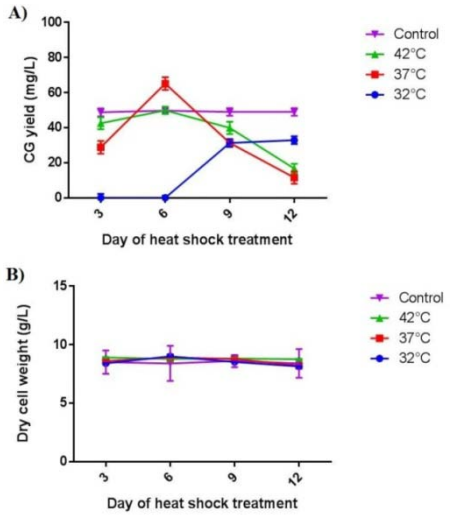

Fig. 3: Effect of heat shock stress on CG production and biomass yields of *L. theobromae*

L. theobromae was grown in optimized production medium and heat shocked at 32 °C, 37 °C and 42 °C for a time period of 60 min, on day 3, 6, 9 and 12 post-inoculation. CG yield (A) and biomass (B) were determined on day 16 post-inoculation. The results were obtained from three independent experiments and expressed as mean±SD

DISCUSSION

Endophytic fungi are versatile sources of bioactive secondary metabolites [7]. The regulation of secondary metabolism in filamentous fungi is largely dependent on the availability of nutritional factors, culture conditions as well as environmental factors [8-14]. Secondary metabolites are known to mediate stress tolerance mechanisms in organisms they occur [15]. This knowledge has been exploited in plant cell culture systems for the enhanced production of secondary metabolites by exogenous supplementation of stress factors [16-19]. There have been few reports of enhanced production of particular secondary metabolites by fungal cultures in response to various stress conditions [20-22]. However, the selection of appropriate stress factor for the enhanced yield of a particular metabolite becomes onerous due to the complex regulatory network involved in fungal secondary metabolism [23-24]. The increase in biosynthesis of a particular metabolite can be a direct response to the imposed stress or an indirect effect of cross talk between biosynthetic pathways.

Cholestanol glycosides are steroidal sugars with promising anticancer potentialities [25-27]. CG produced by an endophytic fungus, *L. theobromae* was found to possess *in vitro* anticancer and antioxidant activities. CG, owing to its bioactivity can be a potential lead structure for the development of new chemotherapeutic agents. Low cost production of bioactive compound is a major criterion determining its development as a drug. Since the physiological role of CG in the fungus was unknown, we assessed the production of CG by *L. theobromae* in response to three different abiotic stress conditions-heat shock stress, osmotic stress and oxidative stress. Fungi generally synthesize secondary metabolites on completion of their initial growth phase [28]. Hence we subjected the fungal cultures to stress treatments at different stages of growth (lag phase, log phase and stationary phase).

The supplementation of fungal cultures on day 9 with H_2O_2 (10 mmol) as well as menadione (1 µmol) resulted in 1.8-fold increase in CG production by the fungus. Menadione and H_2O_2 are known to induce oxidative stress in filamentous fungi [29-30]. Osmotic and heat-shock treatments produced no significant increase in CG yield. It was also observed that CG production by the fungus was hampered when growth of the fungus was affected. There are several reports of enhanced production of antioxidant secondary metabolites by fungi in response to oxidative stress [3-4, 31]. Oxidative stress has also been reported to enhance the biosynthesis of toxic secondary metabolites such as trichothecenes and aflatoxins by fungi [32-33]. CG exhibited antioxidant activities *in vitro* [6]. The enhancement of CG yield upon mild oxidative stress treatment suggests the compound to be a part of the non enzymatic oxidative stress tolerance mechanism in *L. theobromae*. Thus our findings not only support the development of low cost, sustained fermentation of CG but also throw light on the physiological role of the compound in the organism.

CONCLUSION

An enhanced biosynthesis of cholestanol glucoside was observed in *L. theobromae* cultures were subjected to oxidative stress treatments. A mild oxidative stress treatment on day 9 significantly stimulated the biosynthesis of CG giving a yield of 88.3±0.6 mg/l. This appreciable yield of the compound encourages its development as an economic therapeutic agent. The *in vitro* antioxidant capacities of CG has already been reported. Increased biosynthesis of the compound in response to oxidative stress suggests the compound to have similar roles within the organism.

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CONFLICT OF INTERESTS

Declared None

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