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Chitosan activates a MAP-kinase pathway and modifies abundance of defense-related transcripts in calli of *Cocos nucifera* L.

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Abstract

As the study of coconut defense responses against pathogenic microorganisms is hampered by the absence of suitable model systems, we investigated if imbibition of coconut calli with chitosan could be used *in vitro* to simulate the molecular interactions that occur with pathogens. Our results showed that calli imbibition with 10 mg mL^{-1} chitosan caused the accumulation of hydrogen peroxide, and stimulated a β-1,3-glucanase activity with an Rf near 0.1. In addition, in-gel kinase assay and specific immunoblotting showed that a ~46 kDa MAPK-like protein was activated shortly after elicitation, and remained in this state for at least 80 min. Chitosan addition also differentially modified the expression of some genes, whose DNA sequence showed high similarities to receptor-like kinases (RLKs), *Verticillium*-like protein, and mitochondrial alternate oxidase 1b. Addition of salicylic acid to the calli also modified transcript abundance for these genes, while methyl jasmonate did not seem to influence their expression, implying that they could be involved in defense responses. These results strongly suggest that elicitation of coconut tissues cultivated *in vitro* constitutes a suitable alternative to characterize both biochemical and molecular interactions that occur between the coconut palm and its associated pathogens.
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1. Introduction

Coconut palm (*Cocos nucifera* L.) is an ecologically and economically important species in the tropics. Unfortunately, this palm is subject to attack by several disease-producing agents, including viruses [1], viroids [2], mollicutes [3], protozoa [4], fungi [5], and nematodes [6]. Some diseases become epidemic, e.g., in the recent years, the "Atlantic Tall" variety has been almost totally eliminated from the coasts of the Yucatan Peninsula and its abundance diminished along the Gulf of Mexico because of the Lethal Yellowing (LY) disease [7]. Even though there are many epidemiological studies for this and other coconut diseases, the basic physiology of coconut–pathogen interaction is poorly understood. In addition, there are no data regarding the coconut's biochemical resistance/susceptibility to pathogens, nor have the genes involved in the establishment of disease resistance been identified. Some factors that traditionally restrict these analyses include the coconut's long life cycle, the large size of the whole palm, and the lack of an efficient *in vitro* propagation system.

Abbreviations: MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; AOX, mitochondrial alternative oxidase; SA, salicylic acid; MeJA, methyl jasmonate; NO, nitric oxide; ROS, reactive oxygen species; AC, activated charcoal; TTC, 2, 3, 5-triphenyl tetrazolium chloride; dp, degree of polymerization; SN, supernatant; RT, room temperature.

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In vitro plant tissue cultures have shown its feasibility as alternative models to the study of intact plants, as they maintain the basic physiology to respond to the addition of both elicitors [8] and signaling intermediates [9] that induce the expression of genes involved in the defense against plant pathogens. In this way, *in vitro* elicitation of defense responses using general elicitors have been successfully applied to several plant species including *Oryza sativa* [10], *Triticum aestivum* [11], *Lycopersicon peruvianum* [12], *Pinus sylvestris* [13], etc., and could be attempted in coconut. Unfortunately, working with *in vitro* tissue cultures of coconut and other palms has proven to be extremely difficult, to our knowledge only cell suspension cultures from other palms like *Elaeis guineensis* Jacq. [14] and *Phoenix dactylifera* L. have been established [15].

In spite of this, protocols for coconut regeneration from *in vitro* embryogenic calli have been optimized recently with the use of activated charcoal [16,17]. Thus, *in vitro* coconut calli culture has emerged as a potential model to gain insights into the coconut–pathogen interactions. As the use of *in vitro* tissue cultures is a reasonable way to work aseptically with susceptible or endangered plant species, the main goal of this study was to evaluate whether elicitation of coconut calli could be a suitable alternative to study plant defense mechanisms, including the activation of signaling cascades and the induction of defense-related genes.

2. Materials and methods

2.1. Plant materials and treatments

Calli from *Cocos nucifera* L., var. "Atlantic Tall" were cultured *in vitro* according to Chan et al. [16]. Three-month-old calli were used in all experiment treatments, which were repeated at least three times with three replications each. To determine the optimal chitosan (Sigma-Aldrich, St. Louis, MO) concentration for elicitation, calli were vacuum infiltrated (650 mm Hg, negative pressure) for 20 min with different concentrations of chitosan (0, 0.5, 1.0, 5.0, and 10.0 mg mL^{-1}), then total proteins were extracted at different periods (0, 4, 12, 16, and 24 h after treatment) and activity of β-1,3-glucanases was evaluated as described below. The activation of MAP kinases was evaluated after calli were vacuum infiltrated for 20 min with control buffer or 10.0 mg mL^{-1} chitosan, then total proteins were extracted at different periods (0, 5, 10, 20, 40, 60, 70, and 80 min), and MBP-kinase activity was determined as described below.

To evaluate the effects of elicitation on gene expression, calli were exposed for 20 min to different concentrations of either chitosan (10.0 mg mL^{-1}), salicylic acid (SA)(0, 5, 50, and 500 mM) or methyl jasmonate (MeJA)(0, 50, and 500 μM). After these periods, calli were rinsed with control buffers and incubated further for 2 and 4 h at room temperature (RT), and then total RNA was extracted, pooled and analyzed by reverse northern blotting as described below. For corroboration of the gene expression patterns, two negative controls were applied: first, "untreated" calli were neither vacuum infiltrated nor exposed to any solution (labeled "U" in Figs. 7 and 8); second, "control" calli were vacuum-infiltrated for 20 min with the correspondent control buffers (labeled "C" in Figs. 7 and 8).

Chitosan stock solution was prepared according to Benhamou and Thériault [18], and the chitosan working solutions were prepared from this stock after adjusting the pH at 5.5 using 2.5 N NaOH. Dilutions of SA (Sigma) were prepared from a 500 mM stock solution prepared in water. MeJA stock solution (50 mM) was prepared in ethanol and working solutions were obtained by final dilution in 1% (v/v) ethanol.

2.2. Protein extraction

Coconut calli that were subjected to the different chitosan treatments were frozen in liquid nitrogen and homogenized in 1 mL of extraction buffer per g of tissue (50 mM HEPES-Tris pH 7.5, 5 mM EDTA, 5 mM EGTA, 50 mM β-glycerophosphate, 10 mM sodium orthovanadate, 10 mM sodium fluoride, 10% glycerol, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 μM aprotinin, 1 μM leupeptin, 5 mM DTT, 20% transcinnamic acid, 20% polyvinylpolypyrrolidone (PVPP)). Cell extracts were centrifuged at 19 500 × *g* for 30 min at 4 °C. The supernatant (SN) was recovered, quickly frozen in liquid nitrogen and stored at −80 °C until used. The protein concentration in the samples was measured by the method of Bradford [19], using bovine serum albumin (Sigma) as standard.

2.3. β-glucanase assays

β-glucanase assays were conducted according to Pan et al. [20]. Briefly, protein extracts (35 μg) from calli exposed to different chitosan treatments were fractionated by native polyacrylamide gel electrophoresis. After a 5 min wash with 0.05 M sodium acetate, gels were incubated for 1 h at 40 °C in the same solution supplemented with 13.3 mg mL^{-1} laminarin (SIGMA); then enzymatic activity was revealed by addition of 0.075% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma) in 1 M NaOH. Gels were heated in a microwave oven for 10 s and photographed. Software from a Kodak EDAS 290 system was used to determine the relative band intensities.

2.4. Quantification of hydrogen peroxide production

Coconut calli were vacuum-infiltrated for 20 min with 10 mg mL^{-1} chitosan, then calli samples were collected at different periods (0, 2, and 4 h) and ground in liquid nitrogen. After homogenization with 1 mL of 10 mM 2-morpholinoethanesulfonic acid (MES), pH 6.5, per g of tissue, samples were centrifuged at 14 000 × *g* for 5 min at

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