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## **Antimicrobial compounds as side products from the agricultural processing industry**

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## Chapter 5

### *Induction of fungal cell wall stress*

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#### **Abstract**

The effect of *Humulus lupulus* and *Tectona grandis* extracts on a transgenic *Aspergillus niger* was studied, in order to learn more about the possible mode of action. The transgenic strain that was used is a cell wall damage model. It shows induction of 1,3- $\alpha$ -D-glucan synthase gene by coupling it to a green fluorescent protein (GFP) marker encoding sequence. Induction of the gene encoding the glucan synthase is detected as fluorescence in the fungal cells. The results show that *T. grandis* extract, fraction 87 (hemitectol + tectol) and deoxylapachol, which were derived from this plant extract, induce fungal cell wall stress.

### 5.1 Introduction

The fungal cell wall is an attractive target for the development of new antifungal agents because it is essential for the viability of fungal cells, and the fungal cell wall has no counterpart in mammalian cells. The fungal cell wall is composed 80-90% of carbohydrates. The main structural polysaccharides include cellulose, chitin, mannan and glucan [Hugo and Russell, 1992]. 1,3- $\alpha$ -D-glucan synthase is a prominent component in the cell walls of many fungal species such as *Schizosaccharomyces pombe* [Bacon et al., 1968; Sietsma and Wessels, 1990], *Aspergillus nidulans* [Bull, 1970, Zonneveld, 1972] and *Aspergillus niger* [Horisberger et al., 1972; Johnston, 1965]. The first 1,3- $\alpha$ -D-glucan synthase gene, named *ags1*, was identified and analyzed in *S. pombe* [Hochstenbach, et al., 1998]. Damveld and coworkers [2005] identified a family of five 1,3- $\alpha$ -D-glucan synthase-encoding genes in *A. niger*.

To further study the antifungal activity of *Humulus lupulus* and *Tectona grandis* extracts shown in Chapter 3 the mode of action was tested. A transgenic *Aspergillus niger* is used as a model for the induction of fungal cell wall stress. By coupling a green fluorescence protein marker encoding sequence to the 1,3- $\alpha$ -D-glucan synthase gene, this cell line will show fluorescence when cell wall synthesis is induced after cell wall damage.

### 5.2 Materials and Methods

*Humulus lupulus* flowers and *Tectona grandis* sawdust were extracted with chloroform-methanol (CHCl<sub>3</sub>-MeOH, 1:1) as described in Chapter 3. The isolation of active compounds from *T. grandis* extract is described in Chapter 4.

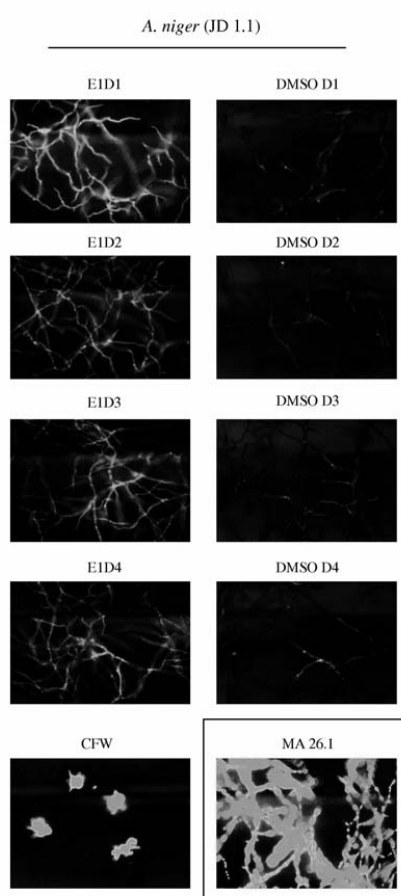
Three transgenic *A. niger* strains were developed by the Fungal Genetics Group, Clusius Laboratories, Institute of Biology, Leiden University, Leiden, The Netherlands. *Aspergillus niger* MA 26.1 (PgpA-H<sub>2</sub>B-GFP) was used as a GFP positive control (the GFP gene is continuously expressed) while *A. niger* N402 was used as a GFP negative control (not containing the GFP gene). *Aspergillus niger* RD 6.47 (PagsA-H<sub>2</sub>B-GFP) and *A. niger* JD 1.1 (PagsA-GFP-Mc) were used for measuring the induction of fungal cell wall stress by coupling a green fluorescence protein marker encoding sequence to the glucan synthase gene (expression of the reporter gene resulting in the presence of GFP in the nucleus and cytoplasm, respectively) [Damveld, et al., 2005]. These fungi were grown and the fungal spores were harvested as previously described in Chapter 3. The detection of green fluorescence was done in a 96 well optical Etm Plt PolymerBase (Nalge Nunc International, New York, USA). A 200  $\mu$ L solution of plant extract or compound was added and a two fold dilutions with sterile water were made to the concentrations of 100, 50, 25, 12.5 ppm. Dimethylsulfoxide (DMSO) was used as the

negative control ( the concentrations of 5.0, 2.5, 1.25 and 0.625 %) while 100 ppm calco fluor white (CFW) (Sigma, Steinheim, Germany) and 50 ppm sodium dodecyl sulfate (SDS) (MP Biomedicals, Eschwege, Germany) were used as the positive control. *Aspergillus niger* spore suspension was diluted to the concentration of  $2 \times 10^4$  CFU/mL in the complete media (CM) [Bennett and Lasure, 1991]. One hundred  $\mu$ L of *A. niger* (RD 6.47 or JD 1.1) was added to each well after adding the plant extracts, compounds and controls. *Aspergillus niger* N402 was used to indicate the GFP non-expression while *A. niger* MA 26.1 was used to indicate the expression of GFP. Spores of this latter strain was only diluted in CM (concentration of  $2 \times 10^4$  CFU/mL) and added to the wells containing sterile water only. The final volume in each well was 200  $\mu$ L. The microplate was incubated at 30 °C in the dark for 16 hours. A fluorescence microscope (Zeiss, Germany) was used to determine the induction of fungal cell wall stress by measuring the fluorescence caused by expression of the GFP genes via the *agsA* promoter. The liquid in the microplate was removed by turning the microplate upside down onto thick absorption paper. Pictures were taken of the fungal mycelium. The growth shapes were observed and the intensity of green fluorescence in each treatment was compared with the control.

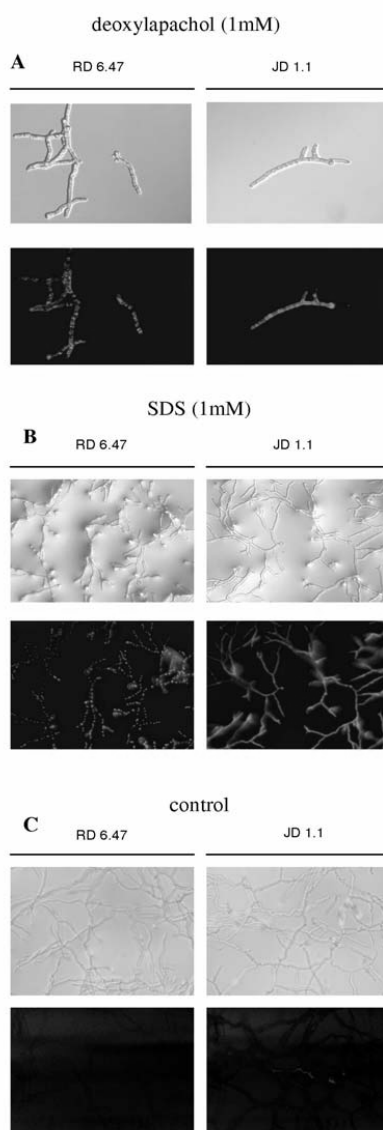
### 5.3 Results and discussion

*Humulus lupulus* and *Tectona grandis* (CHCl<sub>3</sub>-MeOH, 1:1) extracts were used to test the induction of fungal cell wall stress using *A. niger* RD 6.47 and *A. niger* JD 1.1 with expression of the reporter gene resulting in the presence of GFP in the nucleus and cytoplasm, respectively. The results showed that *H. lupulus* extract did not induce fungal cell wall stress in either strain up to the concentration of 100 ppm. *Tectona grandis* extract at the concentrations of 100, 50, 25 and 12.5 ppm induced fungal cell wall stress in *A. niger* JD 1.1 (Figure 5.1) while concentrations of 100 and 50 ppm induced *A. niger* RD 6.47. Positive controls (SDS and CFW) induced fungal cell wall stress while a negative control (DMSO) did not induce the fungal cell wall stress. *Aspergillus niger* MA 26.1 (GFP positive control) expressed GFP while *A. niger* N402 (GFP negative control) did not express GFP. After isolating the compounds from *T. grandis* sawdust extract, the induction of fungal cell wall stress was tested with the isolated compounds deoxylapachol, tectoquinone and fraction 87 (hemitectol + tectol). It was found that deoxylapachol induced fungal cell wall stress in *A. niger* JD 1.1 and RD 6.47 at the concentrations of 100, 50, 25 and 12.5 ppm, while tectoquinone precipitated and quenched fluorescence, making it difficult to observe the fluorescence. Fraction 87 (hemitectol + tectol) induced fungal cell wall stress in *A. niger* RD 6.97 and JD 1.1 at concentrations of 100, 50, 25 and 12.5 ppm, but some precipitation and quenching of fluorescence was observed as well.

Apparently, from the work on the isolation and structure elucidation described in Chapter 4, hemitectol in fraction 87 is unstable. Deoxylapachol at high concentration (100 ppm) gave a similar result as SDS. The effects of deoxylapachol and SDS on fungal cell wall stress were compared at the same concentration (1mM). The results show that deoxylapachol induces fungal cell wall stress and inhibits fungal growth more than SDS. Moreover, an unusual *A. niger* mycelium growth with branching was observed in deoxylapachol treatment after spore germination (Figure 5.2).



**Figure 5.1** GFP expression in *Aspergillus niger* (JD 1.1) mycelium after treatment with *Tectona grandis* extract (E1) at the concentrations of 100 ppm (D1), 50 ppm (D2), 25 ppm (D3) and 12.5 ppm (D4), compared with the negative controls; DMSO 5% (D1), 2.5% (D2), 1.25% (D3) and 0.625% (D4) and the positive controls CFW and *Aspergillus niger* (MA 26.1).



**Figure 5.2** The growth of *Aspergillus niger* (RD 6.47 and JD 1.1) and GFP expression in their mycelium after treatment with deoxylapachol (1 mM) (A), SDS (1 mM) (B) and control (5% DMSO) (C).

#### **5.4 Conclusion**

From the results, we conclude that deoxylapachol might be an interesting natural compound which can be isolated from *Tectona grandis* sawdust for application as an antifungal compound. *Tectona grandis* wood is one of the most valuable materials for house construction and furniture in South-East Asia. The plant material used for this study was sawdust, which is a waste material from wood factories. Deoxylapachol could become a new product from this waste material, adding new value to the waste. The transgenic *Aspergillus niger* cell lines proved to be an excellent system to confirm antifungal activity of compounds and learn more about their the mode of action.