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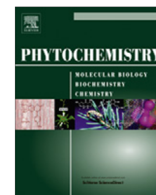

Phytotoxic dioxolanone-type secondary metabolites from *Guignardia bidwellii*

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ABSTRACT

Phenguignardic acid was recently described as a phytotoxic secondary metabolite from submerged cultures of the grape black rot fungus *Guignardia bidwellii*. Since the production rate of this natural product in submerged culture is very low, fermentation optimisation was carried out. The optimisation of cultivation conditions led to the identification of seven secondary metabolites, structurally related to guignardic acid, a known secondary metabolite from *Guignardia* species containing a dioxolanone moiety. All metabolites presented here have not been described to date and are presumably biosynthesised via deamination products of amino acids, such as phenylalanine, valine, tyrosine, and alanine. Four of the seven compounds showed phytotoxic activity. Based on the structures determined by NMR spectroscopy a preliminary structure activity relationship indicated a free carboxyl group as presumably required for the phytotoxic activity.

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1. Introduction

The causal agent of black rot on grapes is the phytopathogenic fungus *Guignardia bidwellii*. In Germany, infections were progressively observed since the beginning of the present century in abandoned vineyards, constantly expanding to cultivated vineyards (Ullrich et al., 2009). It is believed that climate changes like increased atmospheric humidity and spring temperatures promote the spread of the disease (BMELV, 2004). *G. bidwellii* is able to infect all green expanding tissues (Kuo and Hoch, 1996) of grapevine. However, infections on leaves and young berries are of major importance for the epidemic. Berries are highly susceptible for direct infection between fruit set and the beginning of bunch closure (Molitor and Berkelmann-Löhnertz, 2011). Such berry infections can result in significant crop losses ranging from 5% to 80% (Ramsdell and Milholland, 1988). All commercially important *Vitis vinifera* cultivars were reported to be susceptible to the disease (Hoffman and Wilcox, 2002).

Plants can be infected by a multitude of plant pathogenic organisms, such as phytopathogenic fungi (Sugawara, 2000). These microorganisms infect and colonise their host plants by specialised strategies in order to overcome plant defence mechanisms. Part of main infection strategies is the synthesis and release of phytotoxins, low-molecular secondary metabolites which are often involved in disease symptom formation. In general, many necrotrophic fungi were found to produce toxic metabolites in order to facilitate infection (Stierle et al., 1992). The relevance of phytotoxins became apparent in 1970, when significant crop losses were recorded in Canada and the USA during corn harvest caused by the leaf spot disease (Tatum, 1971). The causal agent was found to be *Helminthosporium maydis*, a fungus producing phytotoxins involved in formation of disease symptoms (Karr et al., 1974). Ever since many metabolites produced by phytopathogenic fungi, toxic to the plant but not necessarily required for disease symptom formation, were identified. Phytotoxic microbial secondary metabolites were classified according to the following criteria: (i) disease symptoms can be reproduced with purified toxin, (ii) the toxin concentration correlates with pathogenicity, (iii) the toxin must be produced during active growth of the pathogen *in planta* and (iv) reduced virulence or lack of virulence in non-toxigenic strains. Additionally, phytotoxins can be distinguished into host-specific and non-host-specific toxins. In the latter case the metabolite shows activity

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against a wide range of plants which are not necessarily host plants (Bender et al., 1999).

In a previous study, *G. bidwellii* was found to produce two phytotoxic secondary metabolites which are believed to be involved in the pathogenicity. These metabolites, guignardic acid and phenguignardic acid, show a strong phytotoxic activity on vine leaves as well as on leaves of non-host plants, e.g. *Oryza sativa*. The study revealed that phenguignardic acid is produced by *G. bidwellii* in lower amounts compared to guignardic acid (Molitor et al., 2012). Due to the small amounts of phenguignardic acid obtained from submerged cultures of the fungus the biological characterisation of the compound was hampered. In order to obtain a higher yield of the favoured compound culture conditions were improved. Generally, an increased production rate can be achieved by a strain-improvement using mutation and selection (Schmid, 2006) or by improved fermentation conditions and supplementation of induction factors (Metz, 1971).

In the present study, the fermentation yield of phenguignardic acid was increased by variation of parameters such as temperature, pH and nutrient composition as well as supplementation of different precursors of the natural compound. During fermentation optimisation, seven secondary metabolites were detected, isolated, and characterised regarding their chemical structures and biological properties.

2. Results and discussion

In order to increase the production rate and thereby the yield of phenguignardic acid of the fungus *G. bidwellii* variations in fermentation parameters and cultivation conditions in submerged cultures, respectively, were applied. Apart from variations in nutrient composition, pH, and temperature the culture was supplemented with putative biochemical precursors of the phytotoxic secondary metabolite. Since it is believed that the compound is synthesised via deamination of amino acids, adequate precursors, such as phenylalanine and phenylpyruvic acid were added. Furthermore, pyridoxal phosphate, a prosthetic group of some enzymes involved in amino acid metabolism, was supplemented.

2.1. Optimisation of fermentation conditions

Several media were used for submerged cultivation of *G. bidwellii* in order to increase the synthesis rate of bioactive compounds (Fig. 1), since it is known that the production rate of secondary metabolites is influenced by the composition of the nutrients in the medium (Pöhlmann, 2004). WEM was used as control. Cultivation of the fungus in this medium resulted in a very low phenguignardic acid concentration of 5.8 mg l^{-1} at day 9, which is surprising due to the fact that many phytopathogenic organisms do not synthesise phytotoxins in the absence of host plant tissues (Stierle et al., 1992). However, it was found that the highest production rate of the metabolite was obtained in MEM (45.2 mg l^{-1} at day 28). These results indicate that the production of phenguignardic acid is not triggered by constituents of the host plants. This medium was used for further submerged cultivations of the fungus.

The fermentation in CM led to comparable amounts of the phytotoxin (7.8 mg l^{-1} at day 12) as in WEM. Moderate increases in phenguignardic acid concentration were detectable in the media HMG, HA, and DM (23.8 mg l^{-1} at day 9, 14.2 mg l^{-1} at day 12 and 21.7 mg l^{-1} at day 33). The media HMG, HA and WEM are composed of malt extract, glucose and a nitrogen source. Since these media differ only in the glucose concentration, a comparable production rate was expected. The data indicate that the production rate increases with the amount of glucose, probably caused by osmotic stress. The delayed biosynthesis of phenguignardic acid in DM is attributed to the required cleavage of maltose in order to release glucose by the fungus. In order to examine whether the production of phenguignardic acid is temperature-sensitive, the cultivation of *G. bidwellii* was performed at varying temperature (Fig. 2). The cultivation temperatures were chosen in respect to specific growth requirements of *G. bidwellii*. For a successful penetration of the host cell, *G. bidwellii* needs a sufficient leaf wetness period, which depends on the temperature. The temperature optimum for such an infection is 26.5 °C (Spotts, 1977). It is supposed that the secondary metabolites produced by the fungus are significantly involved in a successful infection process. A temperature of 30 °C conforms to the optimal temperature for conidial germination (Spotts, 1980). Surprisingly, the highest production rate was found at 20 °C (28 mg l^{-1} at day 15) in the fermentation experiments. Incubation at higher temperatures led to a lower production rate.

In order to assess the influence of the pH value of the medium on the production rate, *G. bidwellii* was cultivated at different pH values (Fig. 3). It was found that the pH is of importance for the production rate of phenguignardic acid. The highest concentrations in MEM were found in cultures incubated at pH 8 (20.0 mg l^{-1} at

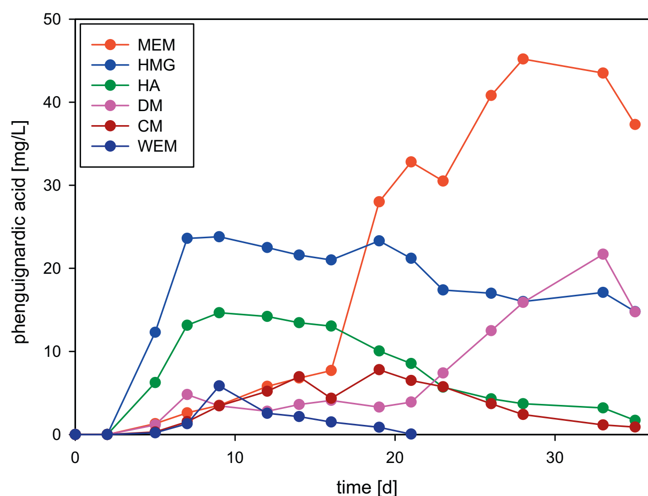

Fig. 1. Media optimisation to identify the best media for phenguignardic acid production by *G. bidwellii*. The media MEM, HMG, HA, DM, CM, and WEM were used for submerged cultivation of *G. bidwellii*. As control WEM was used. The fermentation was performed in 1 l medium in 2 l Erlenmeyer flasks at 20 °C and 120 rpm.

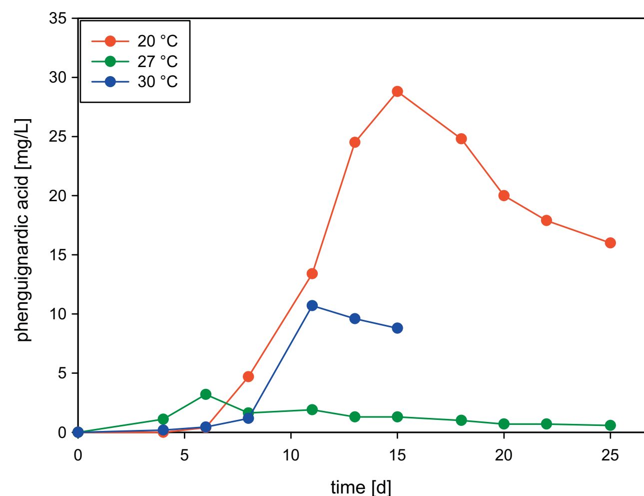

Fig. 2. Temperature optimisation to identify the optimal temperature for phenguignardic acid production by *G. bidwellii*. For the fermentation of *G. bidwellii* different temperatures were chosen (20, 27, and 30 °C). The fermentation was performed in 1 l medium in 2 l Erlenmeyer flasks at 120 rpm.

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