Plant Science 175 (2008) 818–825

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Phyllostictine A, a potential natural herbicide produced by *Phyllosticta cirsii*: *In vitro* production and toxicity

Maria Chiara Zonno [a,*], Maurizio Vurro [a], Sergio Lucretti [b], Anna Andolfi [c], Carmen Perrone [c], Antonio Evidente [c]

[a] *Institute of Sciences of Food Production, National Research Council, via Amendola 122/O, 70125 Bari, Italy*
[b] *Plant Genetics and Genomics Section, Biotechnologies, Agro-industries and Health Protection Department, ENEA Casaccia Research Centre, Via Anguillarese 301, 00123 Rome, Italy*
[c] *Department of Soil, Plant, Environmental and Animal Production Sciences, University of Naples Federico II, via Università 100, 80055 Portici, Italy*

ARTICLE INFO

Article history:
Received 7 May 2008
Received in revised form 31 July 2008
Accepted 5 August 2008
Available online 19 August 2008

Keywords:
Phyllostictine A
Flow cytometry
Cirsium arvense
Nicotiana tabacum
HPLC analytical method
Protoplasts

ABSTRACT

Phyllostictine A is a powerful toxin produced by *Phyllosticta cirsii*, a potential mycoherbicide of *Cirsium arvense*. To support its potential use as a natural herbicide, toxin production has been studied using different media and cultural conditions. The toxin content in the crude extracts has been determined by using a HPLC method set up for this purpose. Furthermore, its phytotoxicity has been evaluated on tobacco protoplasts by flow cytometric analysis, and on *C. arvense* protoplasts, by fluorescence microscopy. The best cultural conditions found allowed to produce more than 28 mg ml^{-1} of toxin in culture filtrate. The pure metabolite proved to have rapid dose-dependant toxic effects on host and no-host plant protoplasts.

1. Introduction

Weed pathogens are considered interesting potential sources of novel natural herbicides. Recently *Phyllosticta cirsii* has been proposed as a potential mycoherbicide for the biological control of the noxious and widespread weed, *Cirsium arvense*, commonly known as Canada thistle [1]. Species belonging to the genus *Phyllosticta* are known to produce bioactive metabolites, including non-host phytotoxins, e.g.: phyllosinol, brefeldin and PM-toxin, isolated by cultures of *Phyllosticta* sp., *P. maydis* and *P. medicaginis*, respectively [2–4]. This led us to investigate the production of toxins by *P. cirsii* [5]. From the fungal liquid culture phyllostictine A (**1**, Fig. 1) was purified and chemically characterized as a new oxazatricycloalkenone. This toxin, isolated together with other 3 related metabolites (named phyllostictines B–D) (**2**–**4**, Fig. 1), in preliminary bioassays showed interesting biological properties. In particular, it proved to have a noteworthy phytotoxicity, no antifungal activity and an antibiotic and zootoxic activity at high concentrations. Considering its interesting and promising properties, phyllostictine A was proposed as a potential natural herbicide deserving further studies on its phytotoxicity. Three main objectives were defined for the present study: (a) finding the best conditions for phyllostictine A *in vitro* production, in order to obtain sufficient amounts of the toxin to perform the biological assays, and to evaluate the capability of the fungus to produce this metabolite *in vitro*; (b) setting up a rapid analytical method to determine the phyllostictine A content in the culture filtrates, in order to easily compare the influence of the different cultural conditions on the toxin production; (c) assaying the phytotoxic activity of phyllostictine A, to better evaluate its potential as natural herbicide.

2. Material and methods

2.1. Optimizing fungal growth and phyllostictin A production

A strain of *Phyllosticta cirsii* was supplied by Alexander Berestetskiy, Russian Research Institute of Plant Protection, Saint-Petersburg, Russia. It was stored in the mycological collection of Institute of Science of Food Production (ITEM N. 8964) in 20% glycerol at −80 °C. For inoculum production, it was transferred to potato-dextrose-agar (PDA) plates and grown at 25 °C under near UV lights for 2 weeks in order to obtain a sufficient production of picnidia. Plates were then used to prepare

* Corresponding author. Tel.: +39 080 5929332; fax: +39 080 5929374.
E-mail address: mariachiara.zonno@ispa.cnr.it (M.C. Zonno).

0168-9452/$ – see front matter © 2008 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.plantsci.2008.08.003

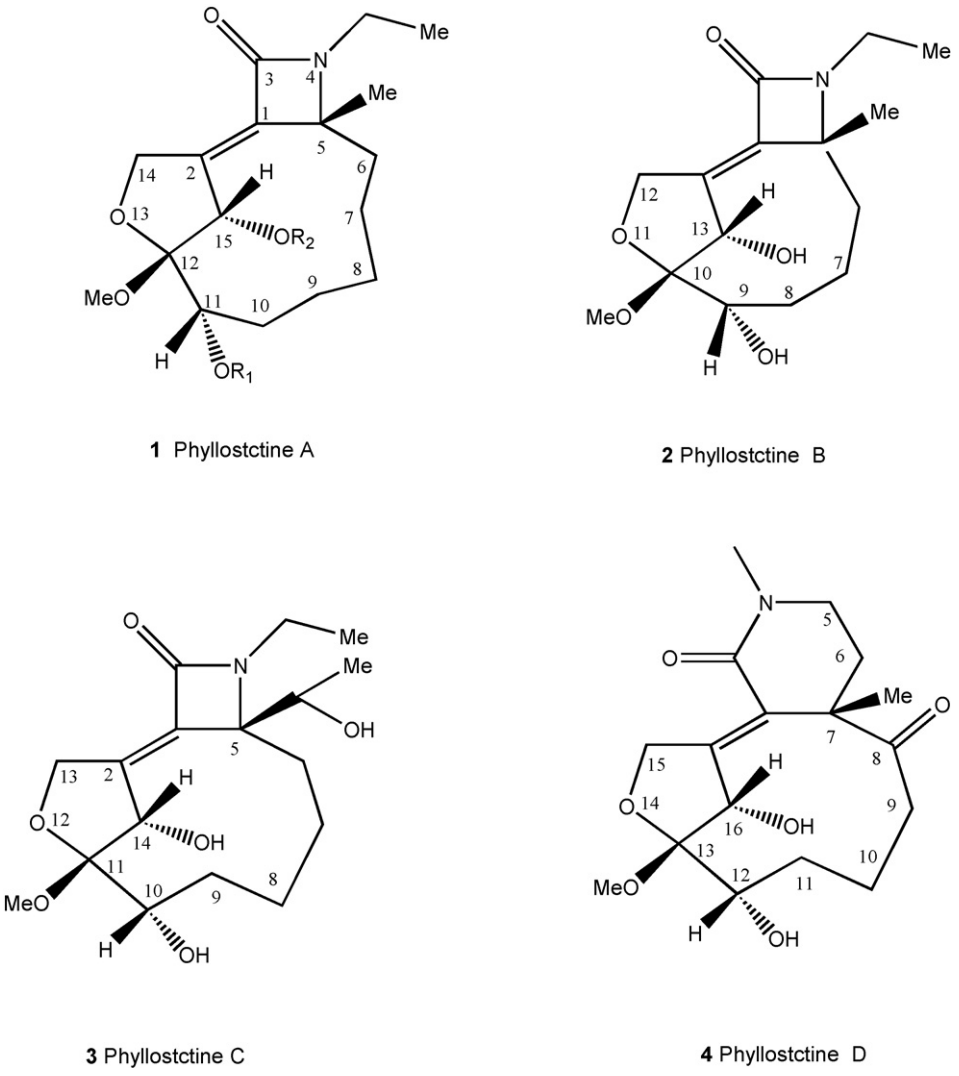

Fig. 1. Structures of phyllosticnines A–D (**1**–**4**).

conidial suspensions. In order to find the best conditions for fungal growth and toxin production, the fungus was grown in four mineral liquid media, namely Malt extract [6], Fries modified [7], M-1-D [8] and Kent-Strobel [9]. For each medium, both Erlenmeyer flasks (containing 400 ml of medium) and Roux bottles (containing 200 ml medium) were used, for shaken and static conditions, respectively.

Aliquots of suspensions (approximately 10^6 conidia ml^{-1}) were used to inoculate flasks and bottles (0.1 and 0.2 ml respectively) that were then incubated in shaken (100 rpm) or static conditions, respectively, at the dark and at 25 °C.

Static cultures were harvested at week intervals, up to 9 weeks, whereas the shaken cultures were harvested at four-day intervals, up to 20 days. All the cultures were produced in duplicate.

The fungus was also grown on M-1-D medium with reduced saccharose content (1/2, 1/5 or 1/10 of the standard recipe, i.e. 28.25 g l^{-1}), or with glucose (28.25 g l^{-1}) as carbon source, or even with the addition to the standard M-1-D medium [10] of minced fresh leaves of *C. arvense* (2 g l^{-1}), collected from naturally infested fields near Bari. These inoculated media were incubated for 7 weeks in static condition at 25 °C at the dark. After incubation, the cultures were filtered on filter paper (Whatman N.4), mycelium fresh and dry weights were determined and pH of culture filtrates was measured. All culture filtrates were tested for the toxicity by using the leaf puncture assay described below. Aliquots (100 ml) were lyophilized and stored until the determination of the phyllostictin A content, as described below.

2.2. Leaf puncture bioassay

To compare the biological activity of the culture filtrate to the phyllostictine A content, the toxicity of all the cultured filtrates obtained under various conditions and media was tested by a puncture assay on thistle leaves. Young leaves were cut from *C. arvense* plants grown in the greenhouse, obtained from seeds collected from wild plants in a field near Bari.

Droplets (20 μl) of solutions were placed on punctured leaves that were kept in a moist chamber at room temperature for 72 h. The diameter of the necrotic area was measured.

2.3. HPLC analysis—toxin content

The lyophilised fungal culture filtrates (corresponding to 50 ml for the static cultures and to 100 ml for the shaken cultures), obtained as reported above, were extracted tree times by $CHCl_3$–*iso*-propanol (9:1, v/v) solution (15 and 30 ml for static and shaken