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Review

The search for new natural herbicides – Strategic approaches for discovering fungal phytotoxins

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ABSTRACT

Plant pathogens produce toxic metabolites which may play a role in affecting their plant hosts by the development of diseases. Such compounds present considerable potential as models for developing herbicides with new modes of action unrelated to those in current use. This study provides an overview on the chemical ecology of plant-pathogenic fungi interactions as a promising approach for discovering new molecules possessing herbicide activity, along with the main research strategies currently employed for the isolation and identification of such compounds. The steps involved in: a) isolating fungal phytotoxic metabolites; b) the factors affecting *in vitro* biosynthesis of phytotoxins, extraction and fractionation methods; c) the bioassay-guided fractionation procedure, and d) the bioassays most commonly used for monitoring isolation processes, are discussed.

1. Introduction

The mid-1940's witnessed the revolutionary prospect of weed control through the discovery of 2,4-dichlorophenoxyacetic acid (2,4-D), a potent herbicide (Wood and Olson, 1946). Thenceforward, the large scale and random synthesis, followed by biological tests employing screening synthetic compounds for phytotoxicity, has been the traditional method adopted by the agrochemical industry in the search for new herbicides (Kudsk and Streibig, 2003). This simple research and development (R & D) strategy has granted the perfected by the agrochemical industry considerable success in identifying new herbicides (Cole et al., 2000; Lein et al., 2004).

However, since the 1980's, an increase in regulatory requirements has significantly increased the investment required for the development of new herbicides. This has discouraging maintenance of extensive synthetic programs further depressing the development of new herbicides (Copping, 2003; Rüegg et al., 2007; Duke, 2012). Also, the widespread adoption of herbicide-resistant crops (HRC) led to a substantial reduction of non-HRC herbicides market (Duke and Abbas, 1995; Gast, 2008).

As a result, the rate of new herbicide output substantially decreased, and no major herbicides with mode of action unrelated to those discovered so far have been introduced in the market during the last 20 years (Duke, 2012). Following the widespread adoption of herbicide resistant crops (Dill, 2005; Duke, 2005; Duke and Powles, 2008, 2009), indiscriminate, repetitive and continuous use of known herbicides, with the same mechanism of action, on the same acreage. This practice has exerted a selective pressure and led to a shift in the spectrum of weeds in several areas, where weed biotypes resistant to herbicides with certain mechanisms of action have been replaced the sensitive biotypes (Trezzi et al., 2005; Vidal et al., 2007; Vila-Aiub et al., 2008; Powles, 2008; Cerdeira et al., 2011; Beckie, 2011). Today, around 380 weed biotypes are resistant to herbicides worldwide (Heap, 2012).

The increasing demand of food for a growing world population coupled with allocation of arable lands for biofuels production necessitates increasing global agricultural yields (Edgerton, 2009; OECD-FAO, 2009). Since weeds are responsible for the major part of agricultural losses worldwide, maintenance of world agricultural production is directly dependant upon chemical control of weeds (Hall et al., 2000; Neve et al., 2009; Oerke, 2006). Thus, the search for new herbicides with modes of action different from those exploited by herbicides already available is of increasing importance. Inexpensive alternative strategies to large scale synthetic programs are therefore urgently required.

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2. Fungal phytotoxins as sources of new herbicides

The use of evidence-based ecological chemistry, for instance using knowledge of plant–pathogen interactions, constitutes a promising strategy for discovering new herbicides. Evidence that biological sources can provide natural products with phytotoxic activity that is also herbicidal has been published (Barbosa et al., 2009a; Cantrell et al., 2012; Dayan et al., 2009; Demuner et al., 2006; Duke et al., 2000, 2002; Passos et al., 2010).

Phytopathogenic fungi produce toxins which may play a role in development of plant diseases, adversely affecting their hosts (Berestetskiy, 2008; Möbius and Hertweck, 2009; Horbach et al., 2011). These phytotoxins are mostly low molecular weight secondary metabolites capable of producing specific symptoms such as wilting, suppression of growth, chlorosis, necrosis and leaf spots (Abbas et al., 1998; Demuner et al., 2006; Evidente et al., 2005a, 2005b, 2008a, 2008b, 2008c; Yuzikhin et al., 2007; Pedras et al., 2009). Considering their broad spectrum of phytotoxic activities, fungal phytotoxins are divided into host-selective (HSTs) and non-host selective (NHSTs) toxins. Typically, HSTs are active only towards plants that are hosts of the toxin-producing fungus, and are essential for pathogenicity (Walton, 1996; Strange, 2003; Horbach et al., 2011). In some cases, host sensitivity is mediated by gene-for-gene interactions, and toxin sensitivity is mandatory for disease development (Jackson and Taylor, 1996; Wolpert et al., 2002). Contrarily, NHSTs are not primary determinants of host range and not essential for pathogenicity, although they may contribute to virulence. These toxins have a broader range of activity, causing symptoms not only on hosts of the pathogenic fungus but also on other plant species (Walton, 1996; Strange, 2007).

Known fungal phytotoxins have rarely had their mechanisms of action identified. However, studies have shown that in many cases the mechanisms of action do not overlap those of synthetic herbicides (Brosch et al., 1995; Hensens et al., 1995; Fields et al., 1996; Vurro and Ellis, 1997; Briquet et al., 1998; Meiss et al., 2008; Dayan et al., 2011). Consequently, the great structural diversity, high potency and exclusive mechanisms of action (compared to synthetic herbicides) make fungal toxins highly attractive for discovering herbicidal activity. Even if natural phytotoxins are not necessarily suitable for direct use as a commercial herbicide, the identification of new sites of herbicidal action can prove valuable for developing novel herbicides (Duke et al., 2000, 2002; Zhang et al., 2011).

Some reviews covering the structural diversity, biological activities (Strange, 2007; Berestetskiy, 2008; Barbosa et al., 2009a) and mode of action of fungal phytotoxins (Dayan et al., 2000; Duke and Dayan, 2011), as well as their potential role in the development of new herbicides (Amusa, 2006; Hüter, 2011; Zhang et al., 2011) have been published.

Herein, the basic approaches adopted in the search for phytotoxins produced by pathogenic fungi are presented, focussing on the different strategies and factors that may influence each stage of the research.

3. Strategies in the search for fungal phytotoxins

3.1. The fungal culture and factors influencing in vitro production of phytotoxins

The isolation of phytopathogenic fungi may be direct, through the transfer of pathogen structures (spores, hyphae, rhizomorphs, sclerotia) immediately from infected tissues of the host plant, or indirectly by formation of colonies from selected portions of the infected plant tissue host after surface sterilization (Robeson and Strobel, 1982; El-Sayed, 2000; Smith, 2001; Chen and Swart, 2002; Zerroug et al., 2007). Pure isolates can be stored under different conditions. However, many microorganisms are genetically unstable and the production of secondary metabolites is non-constitutive, so that successive subcultures and prolonged storage may adversely affect the production of toxins (Smith, 2001).

For production and isolation of metabolites, fungi are initially transferred to Petri dishes containing a solid media and cultured for a period of time sufficient for the occurrence of spore and mycelial biomass production. Next the fungus is subcultured in a growth medium by transferring fragments of the solid culture (Evidente et al., 2008c; Andersen et al., 2008; Berestetskiy et al., 2010; Löffler and Mouris, 1992) or suspension of mycelium and spores (Cimmino et al., 2008; Li et al., 2008). This cultivation is generally performed in liquid culture medium, although solid media can also be used (Li et al., 2008). However, media with complex composition may hinder the processes of isolation of phytotoxins, therefore, the use of synthetic (artificial) or semi-synthetic media is preferable.

Given the potential influence of various factors (e.g. medium composition, temperature, light, time of incubation, shaken or static cultivation etc.) on *in vitro* biosynthesis of phytotoxins, the initial culturing of the fungus in various culture media and under different conditions is recommended, followed by filtration and assessment of phytotoxicity of the filtrate via biological tests. Thus, the conditions for fungal growth resulting in higher phytotoxicity can be used for mass production of the fungus and subsequent isolation of phytotoxic compounds (Pedras and Ahiahonu, 2004; Berestetskiy et al., 2010).

The following sections present a brief discussion of the influence of modulating factors on the amount of phytotoxins produced by fungi:

a) **Composition of the medium**. By culturing *Fusarium oxysporum* f. sp. *lili* in various media, the fungus failed to produce fusaric acid **(1)** when grown under nutritional stress. The highest production of the toxin was obtained in Czapek–Dox medium (Löffler and Mouris, 1992). The fungus *Ascochyta rabiei* produced solanopyrones A, B and C **(2–4)** in Czapek–Dox medium supplemented with amino acids, vitamins and inorganic salts. Although the fungus has grown in Czapek–Dox without addition of supplements, toxin concentration was below detectable limits. Systematically elimination of components from the growth medium led to identification of Zn^{2+}, Ca^{2+}, Mn^{2+} and Cu^{2+} cations as essential constituents for toxin the production (Chen and Strange, 1991). Rao et al. (2010) showed that fumonisin B_1 **(5)** production by *Fusarium moniliforme* was also greatly influenced by media composition, being stimulated by increasing malt, yeast extract and peptone concentrations in the culture media. Fig. 1.

Metabolites produced by the host plant may act as necessary chemical signals to biosynthesis of phytotoxins. The HC toxin **(6)** was synthesized by *Cochilobolus carbonum* only after addition provision of a sugarcane extract into the medium. The compound serinol was identified as the factor required for activating toxin biosynthesis (Pinkerton and Strobel, 1976). *Septoria cirsii* produced greater amounts of the phytotoxic compound β-nitropropionic acid **(7)** when thistle leaves, the host plant of the fungus, were added to the M-1-D medium (Hershenhorn et al., 1993). The phytotoxicity of *Bipolaris euphorbiae* was recovered after re-inoculation of the fungus on leaves of *Euphorbia heterophylla* (Barbosa et al., 2002).

b) **Time of cultivation**. The maximum toxin production depends on the incubation time, and can be achieved by incubating fungal cultures for periods ranging from a few days (Barbosa et al., 2002; Evidente et al., 1995) to several weeks (Evidente et al., 2003, 2006a, 2006b, 2006c) after the onset of fungus