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Short communication

Aspergillus flavus transformation of glucosinolates to nitriles by an arylsulfatase and a β-thio-glucosidase

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ABSTRACT

Biofumigation is a biocontrol method that uses volatile compounds to combat soil pathogens. We investigated a biofumigation process based on the green manure of *Brassicaceae*. These plants contain the glucosinolate-myrosinase system which releases inhibitory compounds such as isothiocyanate into the soil. However, the biocontrol effectiveness is often lower than expected, possibly due to microbial transformation of the isothiocyanates. In order to identify the possible function of microorganisms, their interaction with glucosinolates and glucosinolate-derived products was investigated. We report the ability of a soil isolate of *Aspergillus flavus* to grow in liquid culture and convert 2-propenyl and 2-phenylethyl glucosinolate and their desulfo-derivatives, to nitriles. This finding would suggest the existence of an arylsulfatase and a β-thio-glucosidase, different from myrosinase, which could direct glucosinolate conversion in soil towards nitriles rather than isothiocyanates. If confirmed in soil, our observations could at least partially explain the low concentrations of isothiocyanates after biofumigation.

Biofumigation is a biological technique used in agriculture against soil-borne pathogens. One example is based on the incorporation into the soil of *Brassicaceae* crops, containing the glucosinolate-myrosinase system (Kirkegaard et al., 1998). The mechanical disruption of the plant tissues during incorporation into the soil allows myrosinase to come into contact with glucosinolates (GLs) and, if water is present, convert them to volatile products. Isothiocyanates (ITCs) are commonly produced from GL degradation and have a toxic effect on several plant pathogens (Fig. 1) (Brown and Morra, 1997). The effectiveness of this method for controlling phytopathogens is variable, and will depend on a number of soil biological, chemical and physical properties. In particular, the role of soil microorganisms in this process needs to be investigated. Low values or rapid decreases of GL hydrolysis derived products (GHDPs) or GLs without a simultaneous increase of ITCs in the soil after biofumigation have been reported (Borek et al., 1995; Gimsing and Kirkegaard, 2006). This phenomenon has been ascribed to the action of microorganisms (Smelt et al., 1989; Warton et al., 2003; Leoni et al., 2004). Some *Aspergillus* isolates possess myrosinase activity (Reese et al., 1958; Sakorn et al., 1999). However, *Aspergillus* species, which are widespread in the environment and commonly found in soil, are well-known to produce arylsulfatase (EC 3.1.5.6) (Hanson et al., 2004). This enzyme is able to hydrolyze GLs producing desulfo-glucosinolates (DSGLs) (Thies, 1979), which can then be transformed to nitriles by β-glucosidases (EC 3.2.1.21) (Wathelet et al., 2001).

All the available studies on the interaction of microorganisms and GLs are based on GHDP analysis as a marker of myrosinase activity, whereas sulfatase activity has never been considered even though high concentrations of nitriles have occasionally been detected (Smits et al., 1993; Gardiner et al., 1999). The present work investigated the enzymatic interaction between a selected isolate of *Aspergillus* and two GLs, namely 2-propenyl (the most effective GL of the aerial apparatus and seeds of *Brassicaceae*) and 2-phenylethyl (the most abundant GL in the roots) (Brown and Morra, 1997). The GLs were prepared in pure form from seeds of *Brassica carinata* A. Braun (2-propenyl) and *Barbarea verna* (Miller) Ascherson (2-phenylethyl) (Barillari et al., 2001).

Aspergillus flavus Link ex Fries, isolate BAM-AS9, was recovered from an Italian soil sample after serial dilution on *Trichoderma* selective medium (Elad et al., 1981). This isolate was chosen from several *Aspergillus* spp. isolates based on its ability to release sulfate from 2-propenyl GL, according to a method described by Sakorn et al., 2002. Four agar-plugs (10 mm diam.) of an actively growing colony on Potato Dextrose Agar of BAM-AS9 were inoculated in

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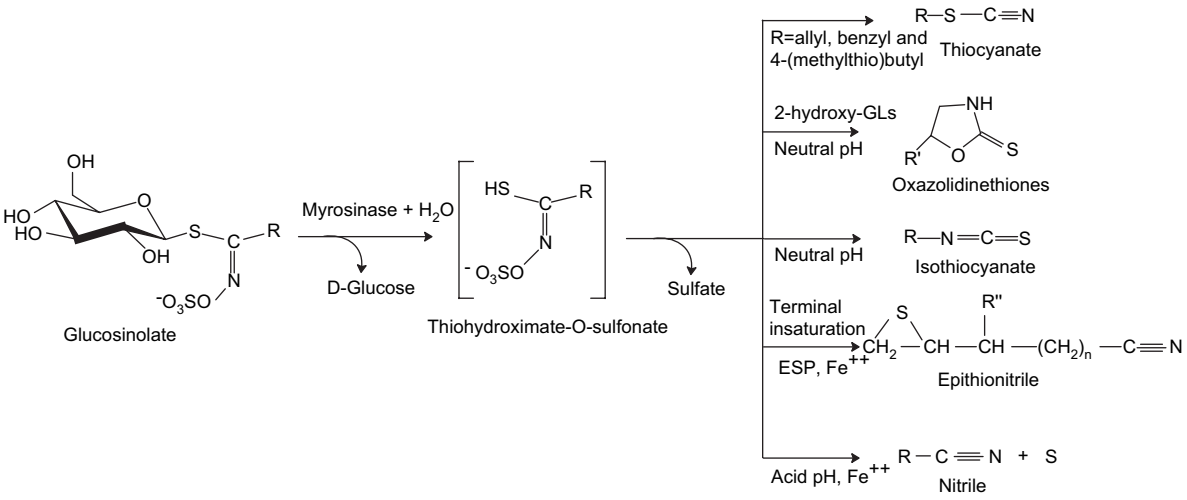

Fig. 1. General scheme of glucosinolate hydrolysis by myrosinase.

a 1 l-glass flask (2 replicates) containing 5 mM ammonium chloride and 4.0 mM GL or 6.0 mM DSGL in 0.1 M sodium phosphate buffer pH 6.5, as the sole carbon source, then incubated at 30 °C (Sakorn et al., 2002). Controls without GL or DSGL addition were included. Aliquots of 2 ml were taken daily for 5 d or more, filtered (0.22 μm) and stored at −20 °C until HPLC or GC–MS analysis. In addition, the headspace of the flask was analyzed daily using a gas-tight microsyringe or SPME (Polyacrilate, 85 μm) for sampling. The experiment was done twice.

DSGLs were prepared by hydrolyzing pure GLs by arylsulfatase immobilized on nylon (Leoni et al., 1998) or in free form. The solution of hydrolyzed GL was loaded on a column containing an anion exchange resin (DEAE-Sephadex A-25, Pharmacia) conditioned with 50 mM acetate buffer pH 5.6. DSGLs were eluted with water. Purity and concentration of the DSGL solution and of GLs were checked by HPLC, using an Agilent 1100 Instrument equipped with a Inertsil ODS 3 column (250 × 3 mm, 5 μm) and a diode array as detector ($\lambda = 229.5$ nm). Analysis was carried out at 30 °C using 10% acetonitrile in water as eluant (flow rate 0.8 ml min^{-1}). Quantitation was performed on the basis of calibrations defined before the analysis (range of concentration 0–1 mM) using titred solutions of pure GLs or DSGLs (Barillari et al., 2005).

Extraction of GHDPs from the liquid substrate was done using CH_2Cl_2 (1:1 v/v). Nitriles and ITCs were analyzed by GC–MS using a Hewlett–Packard GCD system, model G1800A, equipped with a J&W DB23 capillary column (30 m, I.D. 0.32 mm widebore, film 0.25 μm), at a flow rate of 1 ml min^{-1} (splitless mode injection). The column temperature was set at 60 °C for 3 min followed by a linear increase (10 °C min^{-1}) to 200 °C. Injector and detector temperatures were 220 °C and 280 °C, respectively. The MS spectra were scanned at 70 eV from 10 to 425 amu.

BAM-AS9 cultured in the presence of pure GL was able to grow by 24 h after inoculation, and within 5–7 d almost completely covered the surface of the liquid substrate. In the absence of GL the growth stopped after 2 d. GL consumption occurred gradually, reaching undetectable levels on the 4th day (Fig. 2a and b). Traces of the corresponding ITCs were detected only in the initial phases, indicating weak myrosinase activity. As GLs disappeared, increasing amounts of DSGLs and nitriles were found in the liquid culture (starting from day 1–2). This finding indicates the existence of an arylsulfatase which can degrade GL to DSGL (Thies, 1979). GC–MS detection of nitriles in the liquid culture as the only GHDP formed (Fig. 3a and b) also excluded a myrosinase-mediated reaction, since myrosinase usually produces nitriles at more acidic pH values than that of the liquid substrate (6.5) (Fig. 1). Addition of myrosinase to the medium of pH 6.5 containing the GLs studied resulted in production of ITCs but not of nitriles (data not shown). When GLs had almost disappeared (by the 4th day), the amount of DSGLs began to decrease, whereas the concentration of nitriles remained stable or continued to increase slowly (Fig. 2a and b).

The nitrile formation by BAM-AS9 can be attributed to the ability of *Aspergillus* to produce β-glucosidases (Seidle et al., 2004). Nitrile production could thus be consistent with the existence of a β-thio-glucosidase that is able to release glucose from DSGLs (Wathelet et al., 2001). This hypothesis seems to be supported by the observation that BAM-AS9 was able to grow in the presence of

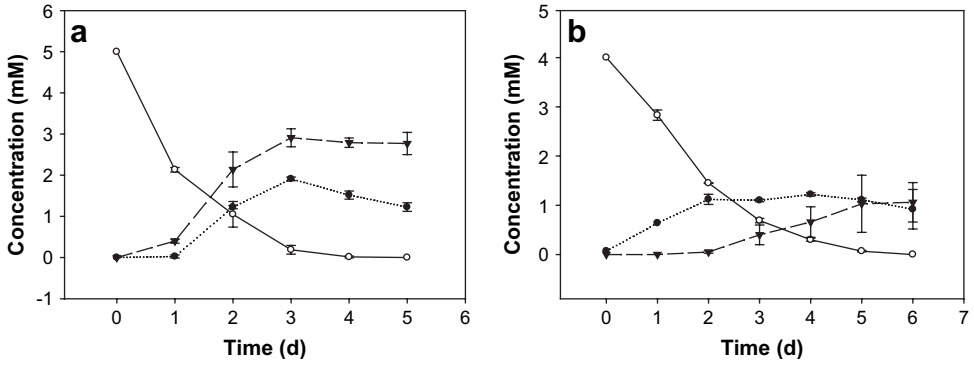

Fig. 2. Glucosinolate (—○—), desulfo-glucosinolate (···●···) and nitrile (- - - ▲- - -) concentration over time in different *Aspergillus flavus* BAM-AS9 growing media: (a) 2-propenyl glucosinolate; (b) 2-phenylethyl glucosinolate. Bars indicate standard deviation ($n = 4$).