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# In vitro metabolism of ginsenosides by the ginseng root pathogen *Pythium irregulare*

Lina F. Yousef, Mark A. Bernards *

*Department of Biology, University of Western Ontario, London, ON, Canada N6A 5B7*

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Dedicated to Dr. Rod Croteau on occasion of his 60th birthday.

**Abstract**

The role of ginseng saponins (ginsenosides) as modulators or inhibitors of disease is vague, but our earlier work supports the existence of an allelopathic relationship between ginsenosides and soilborne microbes. Interestingly, this allelopathy appears to significantly promote the growth of the important ginseng pathogen, *Pythium irregulare* while inhibiting that of an antagonistic non-pathogenic fungus, *Trichoderma hamatum*. Herein we report on the apparent selective metabolism of 20(*S*)-protopanaxadiol ginsenosides by an extracellular glycosidase from *P. irregulare*. Thus, when *P. irregulare* was cultured in the presence of a purified (>90%) ginsenoside mixture, nearly all of the 20(*S*)-protopanaxadiol ginsenosides ($Rb_1$, $Rb_2$, Rc, Rd, and to a limited extent G-XVII) were metabolized into the minor ginsenoside $F_2$, at least half of which appears to be internalized by the organism. No metabolism of the 20(*S*)-protopanaxatriol ginsenosides ($Rg_1$ and Re) was evident. By contrast, none of the ginsenosides added to the culture medium of the non-pathogenic fungus *T. hamatum* were metabolized. The metabolism of 20(*S*)-protopanaxadiol ginsenosides by *P. irregulare* appears to occur through the hydrolysis of terminal monosaccharide units from disaccharides present at C-3 and/or C-20 of ginsenosides $Rb_1$, Rc, $Rb_2$, Rd and G-XVII to yield one major product, ginsenoside $F_2$ and one minor product (possibly G-III). A similar transformation of ginsenosides was observed using a crude protein preparation isolated from the spent medium of *P. irregulare* cultures.


*Keywords:* *Panax quinquefolius*; Araliaceae; *Pythium irregulare*; *Trichoderma hamatum*; Ginseng; Ginsenosides; Extracellular glycosidase

## 1. Introduction

Saponins are a diverse group of preformed secondary metabolites produced by many plants (Hostettmann et al., 1991), comprised of glycosylated steroids, steroidal alkaloids and triterpenoids that often exhibit antifungal activity against potential pathogens of saponin producing plants. Consequently, saponins are presumed to play a phytoprotective role in disease resistance (Olsen, 1973a,b; Mordekhai et al., 1986; Carter et al., 1999; Osbourn, 1999; Nicol et al., 2002). For example, oat mutants not capable of producing the triterpenoid saponin avenacin A-1 are more susceptible to ''take-all disease'', caused by *Gaeumannomyces graminis*, than wild type plants (Papadopoulou et al., 1999). The exact mechanism of saponin fungitoxicity is not completely understood, but it is believed to take place through the formation of complexes between saponins and sterols present in the membranes of fungi, eventually causing the disruption of membrane integrity (Olsen, 1973a; Keukens et al., 1992; Armah et al., 1999; Morrissey and Osbourn, 1999).

Fungi that attack saponin-containing plants are less sensitive to their host plant's saponins in vitro than non-pathogens of these plants (Osbourn et al., 1995; Papadopoulou et al., 1999; Roldàn-Arjona et al., 1999; Nicol et al., 2002, 2003), supporting a requirement for saponin resistance for successful disease development. Fungal resistance to saponins may vary, but often involves enzymatic deglycosylation of some or all of the monosaccharide units

* Corresponding author. Tel.: +1 519 661 2111; fax: +1 519 661 3935.
*E-mail address:* bernards@uwo.ca (M.A. Bernards).

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from the parent aglycone (sapogenin) to produce less toxic derivatives (Morrissey and Osbourn, 1999). For example, fungal detoxification of the tomato saponin, α-tomatine, by *Botrytis cinerea* (Quidde et al., 1998), *Septoria lycopersici* (Osbourn et al., 1995) and *Verticillium albo-atrum* (Pegg and Woodward, 1986) involves the cleavage of a terminal monosaccharide from the tetrasaccharide present on the sapogenin, while *Fusarium oxysporum* f. sp. *lycopersici* (Ford et al., 1977) and *F. solani* (Lairini and Ruiz-Rubio, 1998) cleave the entire tetrasaccharide chain.

*Panax* spp. produce dammarane-type triterpenoid saponins called ginsenosides (Fig. 1). The major ginsenosides are further classified as either 20(*S*)-protopanaxadiol- or 20(*S*)-protopanaxatriol-types, based on the hydroxylation pattern of the parent sapogenin. Importantly, ginsenosides are bisdesmosidic, with sugar units attached at either C-3 and C-20 (20(*S*)-protopanaxadiols) or C-6 and C-20 (20(*S*)-protopanaxatriols). Essentially, nothing is known about the metabolism of ginsenosides by soilborne microorganisms, even though these organisms are differentially affected by the presence of ginsenosides in their culture medium in vitro (Nicol et al., 2002, 2003).

*Pythium* spp. belonging to the Pythiaceae (Oomycota) are a major cause of damping-off of 1- to 2-year old seedlings of American ginseng (*Panax quinquefolius*) grown in Canada, accounting for a substantial loss in the final yield of the harvested crop (Howard et al., 1994; Reeleder and Brammall, 1994; Punja, 1997). The reason for the high susceptibility of American ginseng to diseases caused by *Pythium* spp. is unclear, but our earlier work supports a role for ginsenosides, since these compounds significantly promote the in vitro growth of the important ginseng root pathogen *Pythium irregulare*, while inhibiting that of leaf pathogens and the antagonistic, non-pathogenic fungus *Trichoderma hamatum* (Nicol et al., 2002, 2003).

Two mechanisms have been proposed to explain the apparent insensitivity of Oomycetes to saponins: (1) natural or innate resistance due to the lack of sterols in the

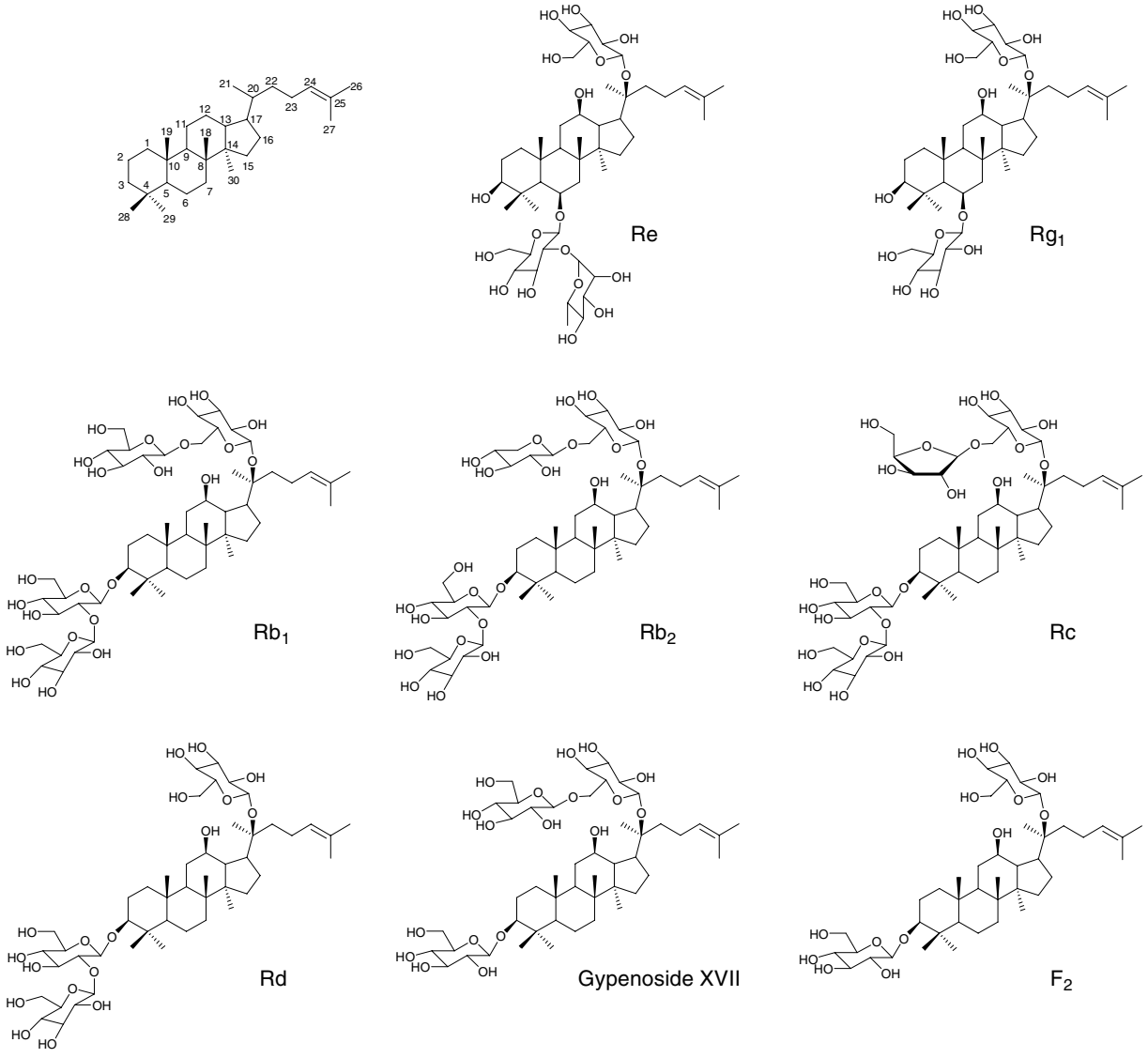


Fig. 1. Common ginsenosides of *Panax quinqufolius*. The parent dammarane carbon skeleton that forms the basis of ginsenosides is shown at top left. Ginsenosides are shown with 20(*S*)-stereochemistry, according to Teng et al. (2002).

membranes of Oomycetes (e.g., Olsen, 1973b), and (2) detoxification, via enzymatic degradation by glycoside hydrolases such as BGX1 described for *Phytophthora infestans* (Brunner et al., 2002). The purpose of the present work, therefore, was to examine the possibility that *P. irregulare* could enzymatically degrade ginsenosides added as a supplement to their culture medium in vitro.

## 2. Results and discussion

### 2.1. Growth of test organisms and recovery of ginsenosides from liquid broth

In order to track the metabolic fate of ginsenosides used to supplement the culture medium of test organisms (e.g., *P. irregulare*, *T. hamatum*), their reliable recovery from the culture medium is important. Preliminary experiments revealed that only <25% of ginsenosides added to PDA or other agar-based media could be recovered (data not shown). Consequently, a liquid culture system was tested, from which individual ginsenosides could be recovered with greater efficiency, e.g., $Rg_1$/Re, 59.8%; $Rb_1$, 88.2%; $Rb_2$, 53.1%; Rc, 50.8%; Rd, 63.0%. However, since organisms can differ in their growth habit when cultured under different conditions, it became important to determine whether the ginsenoside-based growth stimulation observed for *P. irregulare* on solid medium (Nicol et al., 2003) would also occur in liquid culture. Table 1 shows the mean biomass accumulation of *P. irregulare* and *T. hamatum* grown in Czapek–dox mineral medium in the absence (control) and presence of ginsenosides (at 0.1% w/v) with and without exogenously supplied sucrose. Consistent with our earlier observations using solid culture media (Nicol et al., 2003), the growth of *P. irregulare* in the presence of ginsenosides was >2-fold of that in control flasks (no ginsenoside). Under the same conditions (i.e., Czapek–dox mineral broth ± ginsenosides) *T. hamatum* growth was slightly (but not significantly) reduced when ginsenosides were added to the Czapek–dox mineral broth (Table 1). Interestingly, the growth stimulation observed when *P. irregulare* cultures were grown in the presence of ginsenosides was dependent on the absence of exogenous sugar. That is to say that, when sucrose was added to the culture medium (up to 15 g $L^{-1}$), no growth differential was observed between cultures grown on ginsenoside-supplemented and control media (Table 1).

Table 1
Growth of *Pythium irregulare* and *Trichoderma hamatum* in liquid culture

| Treatment | Biomass (mg)[a] | |
|---|---|---|
| | *P. irregulare* | *T. hamatum* |
| Control | 2.67 ± 0.32a[b] | 2.20 ± 0.32a |
| Sucrose (7.5 g $L^{-1}$) | 11.47 ± 0.50b | 5.93 ± 0.23b |
| Sucrose (15 g $L^{-1}$) | 14.20 ± 1.16c | 7.43 ± 0.43b |
| Ginsenoside (no sucrose) | 5.87 ± 0.62d | 1.97 ± 0.37a |
| Ginsenoside + sucrose (7.5 g $L^{-1}$) | 12.47 ± 1.17bc | 5.46 ± 0.08b |
| Ginsenoside + sucrose (15 g $L^{-1}$) | 15.03 ± 0.58c | 7.03 ± 1.67b |

*Pythium irregulare* and *Trichoderma hamatum* were cultured in vitro in Czapek–dox mineral broth alone (control) or supplemented with sucrose (7.5 or 15 g $L^{-1}$), ginsenosides (1 mg $mL^{-1}$), or a combination of both sucrose and ginsenosides. At the end of the culture period, (7 days for *P. irregulare* and 6 days for *T. hamatum*), mycelia were collected by filtration on a nylon filter (0.22 μm), dried in an oven (72 °C) and weighed (see Section 4).

[a] Values are the mean ($n = 3$) ± standard error.

[b] Within each column, values followed by the same letter are not significantly different from each other by general ANOVA followed by an LSD comparison of means ($P = 0.0001$).

### 2.2. Recovery of ginsenosides from spent broth

With an appropriate liquid culture system established (i.e., one in which ginsenosides could be readily recovered and a ginsenoside-based growth differential observed), the fate of exogenous ginsenosides administered to either *T. hamatum* or *P. irregulare* cultures in vitro was investigated. Thus, when ginsenosides were re-extracted from the spent broth of either *T. hamatum* or *P. irregulare* cultures that had been grown for five to six days in Czapek–dox mineral broth supplemented with 0.1% w/v ginsenosides, and subjected to HPLC analysis, three observations were made. First, nearly all of the ginsenosides initially added to the culture medium were recovered from the spent broth of *T. hamatum* (compare Fig. 2(a) and (b)). Second, the main 20(*S*)-protopanaxadiol ginsenosides ($Rb_1$, $Rb_2$, Rc, Rd) but not G-XVII, were substantially, if not completely depleted from the spent broth of *P. irregulare*, while the 20(*S*)-protopanaxatriol ginsenosides Re and $Rg_1$ were almost completely recovered (Fig. 2(c)). Third, the apparent disappearance of $Rb_1$, $Rb_2$, Rc and Rd, was coincident with the appearance of two new peaks in the HPLC trace of ginsenosides recovered from the spent medium of *P. irregulare*: a minor peak (RT = 38.7 min) and a major peak (RT = 43.9 min) (Fig. 2(c)).

When monitored over time, the inability of *T. hamatum* to metabolize ginsenosides and the difference in 20(*S*)-protopanaxatriol and 20(*S*)-protopanaxadiol metabolism by *P. irregulare* became more evident. That is to say, the amount of 20(*S*)-protopanaxatriol ginsenosides remained constant over the five (*P. irregulare*) or six (*T. hamatum*) day culture period (Fig. 3(a)) as did the amounts of all 20(*S*)-protopanaxadiol ginsenosides in the culture broth of *T. hamatum* (Fig. 3(b)). By contrast, the decline in 20(*S*)-protopanaxadiol ginsenosides in the spent broth of *P. irregulare* was essentially linear over the five day culture period (Fig. 3(c)), with the apparent rate of $Rb_1$ metabolism (ca 0.44 mg $day^{-1}$) being more than double that of $Rb_2$, Rc or Rd (ca. 0.09, 0.02 and 0.18 mg $day^{-1}$, respectively). Interestingly, the amount of G-XVII increased slightly during the first four days of culture before declining again (Fig. 3(c)). At the same time, the amount of the new major peak (labeled II in Fig. 2(c)) in the HPLC chromatogram of the ginsenosides recovered from the spent broth of

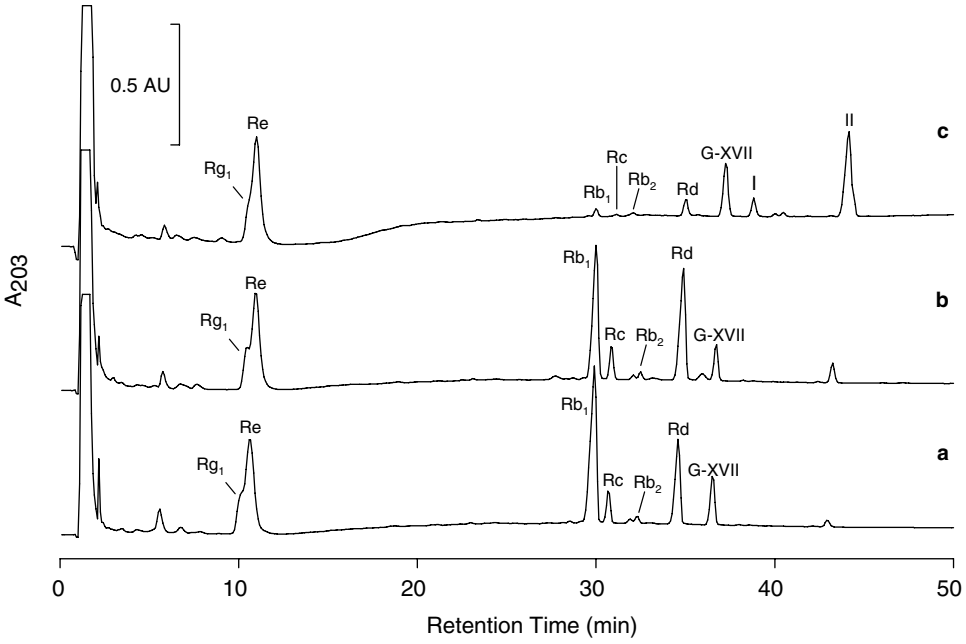


Fig. 2. HPLC analysis of ginsenosides. Ginsenosides were recovered by n-BuOH extraction of Czapek–dox mineral broth supplemented with 1 mg $mL^{-1}$ ginsenosides, in which either (a) no organism (control), (b) *Trichoderma hamatum*, or (c) *Pythium irregulare* had been incubated for 5–6 days, and analyzed by HPLC as described in Section 4. Peaks were identified by co-chromatography with authentic standards as well as LC-MS. Peaks labelled I and II (trace c) represent new ginsenosides resulting from the metabolism of the 20(*S*)-protopanaxadiols $Rb_1$, $Rb_2$, Rc and Rd. Representative chromatograms are shown. See text for details.

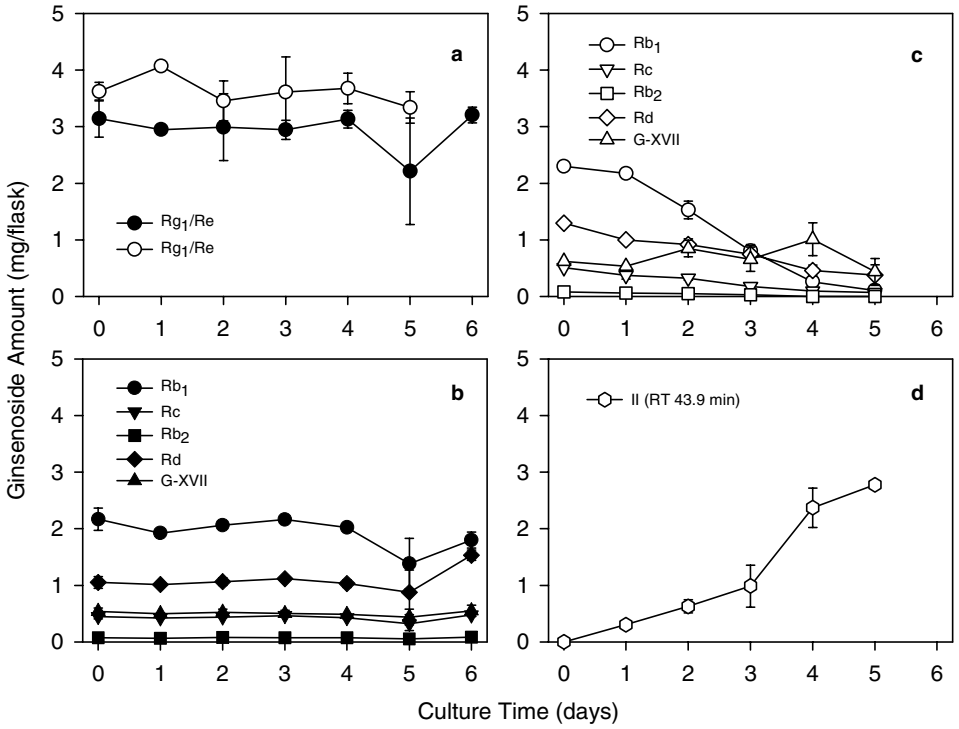


Fig. 3. Time course of ginsenoside metabolism. Ginsenosides were recovered by n-BuOH extraction of Czapek–dox mineral broth supplemented with 1 mg $mL^{-1}$ ginsenosides, in which either *Trichoderma hamatum* (closed symbols) or *P. irregulare* (open symbols) had been incubated for 5–6 days, and quantified by HPLC as described in Section 4. For clarity, the 20(*S*)-protopanaxatriol ginsenosides Re and Rg1 (a), 20(*S*)-protopanaxadiols $Rb_1$, $Rb_2$, Rc, Rd and G-XVII (b and c) and the major metabolite (RT 43.9) (d) are plotted separately. Data points represent the mean of triplicate measurements (after correction for individual ginsenoside recovery efficiency) ± standard error.

*P. irregulare* increased steadily (ca. 0.56 mg day$^{-1}$) (Fig. 3(d)). The decline in 20(*S*)-protopanaxadiols and coincident increase in less polar compounds in the HPLC chromatograms of the ginsenosides recovered from the spent broth of *P. irregulare* suggested that selective deglycosylation could be occurring. However, definitive proof required identifying the new metabolites.

### 2.3. Structural identification of new metabolites

To identify the new metabolites, the ginsenoside fraction recovered from the spent broth of *P. irregulare* was first subjected to LC-MS analysis. At low cone voltage (to minimize collision-induced fragmentation and maximize parent ion formation) the minor metabolite (RT = 38.7 min) showed an *m*/*z* of 915, while the major metabolite (RT = 43.9 min) showed an *m*/*z* of 784. These *m*/*z* values are consistent with a 20(*S*)-protopanaxadiol skeleton with three (i.e., two glucose and one arabinose) and two (i.e., two glucose) attached monosaccharides, respectively. Next, the major metabolite (RT = 43.9 min) was isolated by reversed-phase HPLC in sufficient quantity for FAB-MS, and NMR spectroscopic analysis. In the negative ion FAB-MS spectrum, the parent ion (*m*/*z* 784) was evident, as well as major fragments at *m*/*z* 621 ($[M-163]^-$) and 458 ($M-163-163]^-$), indicative of the sequential loss of two glucose molecules, resulting in the 20(*S*)-protopanaxadiol aglycone. All signals in the $^1$H NMR spectrum were consistent with the major metabolite being a 20(*S*)-protopanaxadiol ginsenoside (Fuzzati et al., 1999; Lin et al., 2001; Teng et al., 2002; Haijiang et al., 2003). Of particular importance were two doublets [$\delta$ 4.51 ($J = 7.5$ Hz) and $\delta$ 4.22 ($J = 7.5$ Hz)], each representing the proton from the anomeric carbon of a glucose moiety, confirming the presence of only two glucoses in the structure.

Examination of the structures of the major 20(*S*)-protopanaxadiol ginsenosides (Fig. 1) reveals two possible ways that a single 20(*S*)-protopanaxadiol ginsenoside metabolite with two attached glucose moieties could arise from $Rb_1$, $Rb_2$, Rc and Rd. First, the complete cleavage of sugar units from C-20 would yield a common product with a glc(1 → 6)glc disaccharide at position C-3 of the 20(*S*)-protopanaxadiol parent structure. Alternatively, cleavage of terminal sugars from all disaccharides would yield the same 3,20-*bis*(*O*-β-D-glucose)-substituted 20(*S*)-protopanaxadiol (e.g., ginsenoside $F_2$) from all 20(*S*)-protopanaxadiols. To distinguish between these two possibilities, the position of the two glucose units on the major metabolite was determined using $^{13}$C and 2-D NMR data analysis. Using the published data for ginsenoside Rd (Lin et al., 2001) as a reference, all signals in the $^{13}$C NMR spectrum could be assigned to a 3,20-*bis*(*O*-β-D-glucose)-substituted 20(*S*)-protopanaxadiol ginsenoside. For example, the presence of a signal at $\delta$ 84.9 ppm, with no attached protons evident in the gHMQC spectrum, confirmed the attachment of glucose at C-20. Similarly, the signal at $\delta$ 90.6 ppm confirmed the presence of glucose at C-3. Other supporting data for the 3,20- *bis*(*O*-β-D-glucose)-substituted 20(*S*)-protopanaxadiol structure include signals for anomeric carbons C-1′ at $\delta$ 106.7 ppm (single glucose unit attached at C-3) and C-1″at $\delta$ 98.3 ppm (single glucose unit attached at C-20), and a shift in the signal for C-2′ from $\delta$ 81.1 ppm (as for the disaccharide in Rd) to $\delta$ 75.4 ppm in the major metabolite. Consequently, the major metabolite was identified as ginsenoside $F_2$. The minor metabolite (*m*/*z* 915) is consistent with ginsenoside G-III (Karikura et al., 1991), and is likely derived from either Rc or $Rb_2$ through the loss of a terminal glucose from the disaccharide at C-3. However, this remains to be confirmed.

### 2.4. Accumulation of ginsenoside $F_2$ in the spent medium and mycelia of P. irregulare

The isolation and identification of ginsenoside $F_2$ (>89% pure) allowed for the calibration of our HPLC and subsequent accurate quantification of $F_2$. Thus, while the decline in 20(*S*)-protopanaxadiol ginsenosides in the spent broth of *P. irregulare* was accompanied by an accumulation of ginsenoside $F_2$ (Fig. 3(d)), only two thirds of the total amount of protopanaxadiol ginsenosides lost from the spent medium (ca. 3.9 mg) were accounted for by the accumulation of ginsenoside $F_2$ (ca. 2.8 mg). Since oomycetes have been reported to incorporate sterols and terpenoids from their environment into their membranes (Olsen, 1973a; Nes and Stafford, 1983; Nes, 1987), we investigated the possibility that *P. irregulare* had taken up ginsenosides from the culture medium. In a separate experiment, HPLC analysis of an extract prepared from the mycelia of *P. irregulare* collected and washed after five days culture in ginsenoside-supplemented Czapek–dox mineral broth revealed one major metabolite, confirmed to be ginsenoside $F_2$ by LC-MS (data not shown). Interestingly, the amount of 20(*S*)-protopanaxadiol ginsenosides lost from the culture medium (ca. 6.8 mg in this experiment) was almost completely accounted for by the cumulative amounts of ginsenoside $F_2$ in the spent media (ca. 2.8 mg) and that associated with the mycelia of *P. irregulare* (ca. 3.7 mg).

We initially thought that *P. irregulare* partially deglycosylated ginsenosides in order to incorporate them as 'sterol' triterpenoids into their membranes. However, the bisdesmosidic nature of the main metabolite found, ginsenoside $F_2$, makes it seem unlikely that it is incorporated into the membrane. Instead, ginsenoside $F_2$ could be taken up intracellularly, possibly through association with a specific elicitin, where it may act as a component in the recognition between host and pathogen (reviewed in Ponchet et al., 1999; Lascombe et al., 2000; Osman et al., 2001) and result in the stimulation of growth associated with attempted infection. Alternatively, ginsenoside $F_2$ may mimic an as yet unidentified growth hormone in the Pythiaceae (Nes, 1987). These interpretations remain speculative, and require further experimentation.

### 2.5. Extracellular glycosidase activity and the sequential metabolism of ginsenosides

A crude extracellular protein preparation obtained from the spent broth of *P. irregulare* catalyzed a similar biotransformation of exogenous ginsenosides as found in living cultures (Fig. 4). That is, even after two weeks of incubation, the level of 20(*S*)-protopanaxatriols ($Rg_1$/Re) remained unchanged, while the 20(*S*)-protopanaxadiols were essentially depleted (except for Rd and G-XVII) with the concomitant accumulation of ginsenoside $F_2$. The combined accumulation of Rd, G-XVII, the minor metabolite (RT 38.7) and $F_2$ (ca. 0.07 mg) accounted for the combined decrease in the initial amount of $Rb_1$, Rc and $Rb_2$ added as substrate (ca. 0.07 mg). No inter-conversion of 20(*S*)-protopanaxadiols occurred when the protein preparation was boiled at 100 °C for 10 min prior to the addition of the ginsenoside substrate (data not shown).

The structural relationships between the five major 20(*S*)-protopanaxadiol ginsenosides ($Rb_1$, $Rb_2$, Rc, Rd and G-XVII) and the major metabolite ($F_2$) found in the spent medium of *P. irregulare* grown in ginsenoside-supplemented medium are depicted in Fig. 5. Note that Rd is positioned as a central metabolite in the conversion of $Rb_1$, $Rb_2$, and Rc into $F_2$. This is supported by the slower rate of Rd metabolism (compared to $Rb_1$) in *P. irregulare* cultures (Fig. 3(c)) and the accumulation of Rd in in vitro assays using crude protein preparations (Fig. 4). Taken together, the main pathway of 20(*S*)-protopanaxadiol ginsenoside metabolism appears to proceed with the initial cleavage of the terminal glc/ara from the disaccharide at C-20 of $Rb_1$, $Rb_2$ and Rc, to yield Rd, followed by the slower cleavage of the terminal glc from the disaccharide at C-3 of Rd. The alternative conversion of $Rb_1$ into $F_2$ via G-XVII appears to be a minor route since the amount of G-XVII remains fairly constant in the spent medium of *P. irregulare* (Fig. 3(c)) and does not accumulate to any significant amount in in vitro assays (Fig. 4).

While the biotransformation of 20(*S*)-protopanaxadiol ginsenosides appears to be selective, it is not clear whether 20(*S*)-protopanaxatriol ginsenosides are excluded as substrates. In contrast to the 20(*S*)-protopanaxadiol ginsenosides, the two main 20(*S*)-protopanaxatriol ginsenosides used in this study (Re and $Rg_1$) have only one glucose unit attached at C-20, and only Re has a disaccharide unit, albeit attached at position C-6. Since Re and $Rg_1$ are poorly resolved by HPLC it is difficult to determine whether Re is converted into $Rg_1$ by the extracellular glycosidase(s) produced by *P. irregulare*. However, LC-MS analysis indicates that even after five days of culture, a significant amount of Re remains in the spent medium of *P. irregulare* (data not shown).

### 2.6. Response of P. irregulare to ginsenosides, ergosterol and glucose

Our initial observations using liquid cultures revealed that the growth stimulation of *P. irregulare* associated with exogenously supplied ginsenosides depended on the absence of sucrose from the culture medium. That is, with increasing sucrose content, there was a corresponding decrease in the effect of ginsenosides on growth (Table 1).

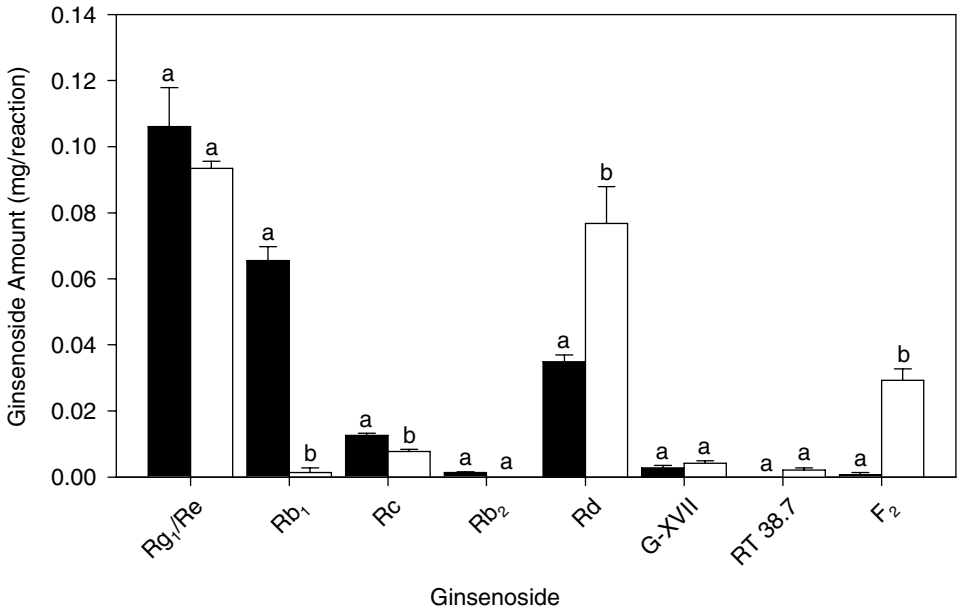


Fig. 4. In vitro modification of ginsenosides by a crude extracellular protein preparation from *P. irregulare*. Ginsenosides isolated from *Panax quinquefolius* were incubated in either 50 mM Tris–HCL, pH 8 (closed bars) or with a crude protein preparation from the spent medium of *P. irregulare* reconstituted in 50 mM Tris–HCL buffer pH 8 (open bars), for up to two weeks. At the end of the incubation period, samples were acidified, centrifuged and analyzed directly by HPLC (see Section 4). Data values represent the mean of triplicate measurements (after correction for individual ginsenoside recovery efficiency) ± standard error. Within pairs, bars labelled with the same letter are not significantly different from each other by general ANOVA followed by an LSD comparison of means ($P = 0.0001$).

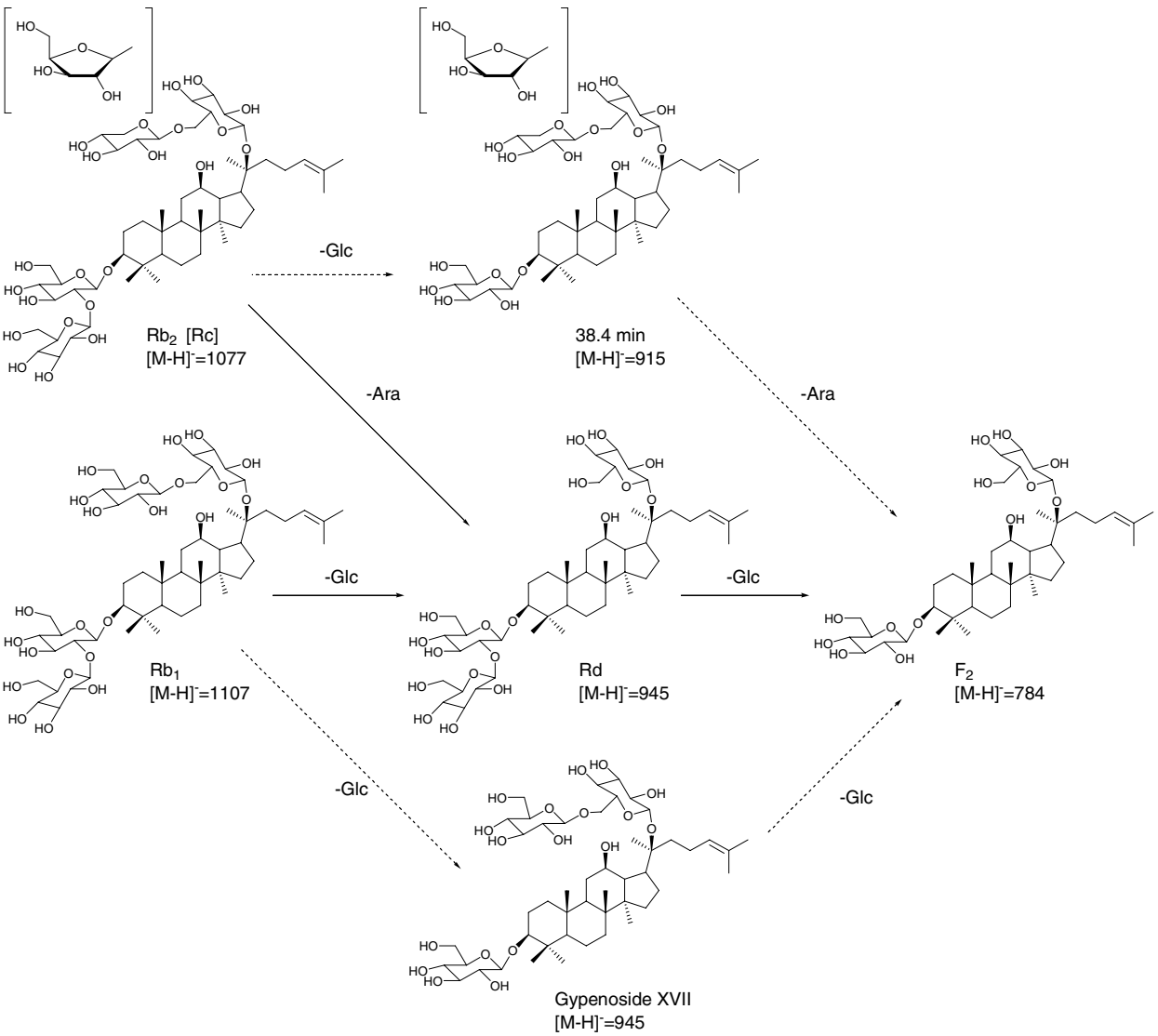


Fig. 5. Tentative scheme depicting the sequential deglycosylation of 20(*S*)-Protopanaxadiol ginsenosides by an extracellular glycosidase(s) from *P. irregulare*. The inter-conversion of 20(*S*)-protopanaxadiol ginsenosides is depicted based on the disappearance of $Rb_1$, $Rb_2$, Rc and Rd from the growth medium of *P. irregulare*, and the accumulation of ginsenoside $F_2$. Rd is shown as a central intermediate since the removal of either one glucose or arabinose from the disaccharide at C-20 of $Rb_1$, $Rb_2$ or Rc lead directly to it, while subsequent loss of a glucose from the disaccharide at C-3 of Rd yields $F_2$. The solid arrows trace the (presumed) dominant route to $F_2$. Alternative routes to $F_2$ are shown with dashed arrows. The structure shown for the 38.7 min peak (i.e., G-III) is tentative, based solely on its LC-MS-derived *m*/*z* of 915. See text for details.

This observation implies that *P. irregulare* may be using the sugars released from ginsenosides as a carbon source. However, when Czapek–dox mineral broth was supplemented with an amount of glucose equimolar to that released into the medium by the metabolism of $Rb_1$, Rc, $Rb_2$ and Rd no increase in *P. irregulare* biomass was observed (Table 2). Interestingly, broth supplemented with both the fungal sterol ergosterol and glucose resulted in a growth increase similar to that observed when broth was supplemented with ginsenosides (Table 2), while the addition of ergosterol alone to the broth resulted in only a moderate increase in biomass. The stimulation of mycelial growth observed with the addition of ergosterol to the growth medium is consistent with earlier literature (Nes et al., 1983) where the addition of cholesterol to the growth medium of *Phytophthora cactorum* (Pythiaceae) resulted in the stimulation of mycelial growth. Interestingly, *Phytophthora cactorum* has also been shown to incorporate steroidal alkaloids into its membrane (Nes et al., 1983) as well as to discriminate between different sterols in vitro and even biotransform them into forms it can use for growth and reproduction (Nes and Stafford, 1983). In keeping with this, our results suggest that while *P. irregulare* may be using the monosaccharides hydrolyzed from ginsenosides as a carbon source, the observed increase in biomass when it is cultured in the presence of ginsenosides could also be due to its selective formation of ginsenoside $F_2$, which may serve to stimulate growth as if a host root were nearby. Alternatively, it could be mimicking a growth hormone (Nes, 1987).

Table 2
Growth of *P. irregulare* in liquid culture

| Treatment | Biomass (mg)[a] |
|---|---|
| Control | $2.93 \pm 0.12$a[b] |
| Ginsenoside | $6.10 \pm 0.70$b |
| Ergosterol | $4.13 \pm 0.17$c |
| Glucose | $2.97 \pm 0.07$a |
| Ergosterol + glucose | $5.33 \pm 0.34$b |

*Pythium irregulare* was cultured in vitro in Czapeks–dox mineral broth alone (control) or supplemented with either ginsenosides (1 mg $mL^{-1}$), ergosterol (20 μg $mL^{-1}$), glucose ($1 \times 10^{-6}$ mol $mL^{-1}$) or a combination of both ergosterol (20 μg $mL^{-1}$) and glucose ($1 \times 10^{-6}$ mol $mL^{-1}$). At the end of the culture period (5 days), mycelia were collected by filtration on a nylon filter (0.22 μm), dried in an oven (72 °C) and weighed (see Section 4).

[a] Values are the mean ($n = 3$) ± standard error.

[b] Within each column, values followed by the same letter are not significantly different from each other by general ANOVA followed by an LSD comparison of means ($P = 0.0001$).

## 3. Conclusions

Previously we demonstrated that the addition of both ergosterol and ginsenosides to the culture medium of *P. irregulare* resulted in a cumulative biomass increase (Nicol et al., 2003), suggesting that the resistance of *P. irregulare* to ginsenosides was not simply due to a lack of sterol content in its membranes. Now, we have shown that the ginseng root pathogen *P. irregulare*, and not the non-pathogenic antagonist *T. hamatum*, has the ability to biotransform a mixture of 20(*S*)-protopanaxadiol ginsenosides via an extracellular glycosidase(s) into a common metabolite, ginsenoside $F_2$, and possibly internalize it. It remains to be determined whether the metabolism of ginsenosides, especially 20(*S*)-protopanaxadiols, by *P. irregulare* results in their detoxification, conversion into growth regulating compounds and/or conversion into host recognition factors. Nevertheless, the production of extracellular glycosidases by *P. irregulare* could be an important factor in explaining its pathogenicity on ginseng.

## 4. Experimental

### 4.1. Plant and microbial materials

*P. irregulare* (CCFC 000245) and *T. hamatum* (DAOM 215955) were obtained from the Canadian Collection of Fungal cultures (Agriculture and Agri-Food Canada, Ottawa, ON), and maintained on potato dextrose agar (PDA) in the dark at 25 °C. Three-year old American ginseng roots were obtained from a commercial farm near Delhi, Ontario.

### 4.2. Ginsenoside extraction and chromatography

Ginsenosides were extracted from the dried roots of three-year old ginseng plants with 80% MeOH and purified using C-18 and aminopropyl Extract-Clean™ solid phase extraction tubes (Alltech Canada, Guelph, ON) as described (Nicol et al., 2002). The final extract (>90% ginsenosides) contained $Rg_1$/Re (37%), $Rb_1$ (36%), $Rb_2$ (1.5%), Rc (1.2%), Rd (14%) and G-XVII (6.2%).

Ginsenosides were analyzed by HPLC as described (Nicol et al., 2002) using a Microsorb® C-18 (5 μm, 150 mm × 4.6 mm; Varian Canada, Inc) column. Authentic standards of ginsenosides $Rg_1$, Re, $Rb_1$, Rc, $Rb_2$ and Rd were obtained from INDOFINE Chemical Company Inc (Somerville, NJ). Ginsenoside G-XVII was quantified using the Rd calibration curve, while the minor metabolite (RT = 38.7 min) was quantified using an average curve derived from the calibration curves of $Rb_2$ and Rc. For LC-MS, the eluent from the HPLC column was split, with 1/20 (50 μL $min^{-1}$) mixed with 1% aqueous $NH_3$ (10 μL $min^{-1}$) before entering the electrospray ionization source of a Micromass Triple Quad-T mass spectrometer operating in the negative ion mode (desolvation temperature of 200 °C). NaI was used as the internal standard. Mass spectra were recorded between 200 and 1500 *m/z* with a 20 V cone voltage.

Ginsenoside $F_2$ (10.8 mg) was isolated from the filtered (0.45 μm nylon membrane), spent medium of several large scale (100 mL) *P. irregulare* cultures using water-saturated n-BuOH. The n-BuOH extract was concentrated in vacuo, reconstituted in MeOH and multiple aliquots injected separately into a Microsorb® C-18 HPLC column as above. The $F_2$ peak was collected manually and the solvent removed in vacuo.

### 4.3. Ginsenoside $F_2$: white powder

$^1$H NMR (599.53 MHz, $CD_3OD$): δ 5.10 (1H, *t*, $J = 6.7$ Hz, H-24), 3.54 (1H, *m*, H-12), 2.98 (1H, *m*, H-3), 2.19 (1H, *m*, H-17), 1.97 (2H, *m*, H-23), 1.72 (1H, *m*, H-13), 1.58 (3H, *s*, H-26), 1.52 (3H, *s*, H-27), 1.24 (3H, *s*, H-21), 0.94 (3H, *s*, H-28), 0.92 (3H, *s*, H-18), 0.82 (6H, *s*, H-19 and H-30), 0.76 (3H, *s*, H-29); 3-O-glc, δ 4.22 (1H, *d*, $J = 7.5$ Hz, H-1′), 3.74 (1H, *dd*, $J = 1.5$, 12.0 Hz, H-6a′), 3.54 (1H, *m*, H-6b′), 3.06–3.26 (4H, *m*, H-2′- H-5′); 20-O-glc, δ 4.51 (1H, *d*, $J = 7.5$ Hz, H-1″), 3.68 (1H, *dd*, $J = 1.5$, 12.0 Hz, H-6a″), 3.54 (1H, *m*, H-6b″), 3.06–3.26 (4H, *m*, H-2″- H-5″); $^{13}$C NMR (125.77 MHz, $CD_3OD$): δ 16.2 (C-18), 16.8 (C-19 and C-29), 17.2 (C-30), 17.9 (C-27), 19.2 (C-6), 24.2 (C-21 and C-23), 25.8 (C-26), 27.2 (C-2 and C-16), 28.4 (C-28), 31.0 (C-11), 31.6 (C-15), 35.9 (C-7), 36.7 (C-22), 37.9 (C-10), 40.3 (C-1 and C-4), 41.0 (C-8), 49.8 (C-13), 51.1 (C-9), 52.5 (C-14), 53.1 (C-17), 57.6 (C-5), 71.7 (C-12), 84.9 (C-20), 90.6 (C-3), 125.8 (C-24), 132.3 (C-25); 3-*O*-glc, δ 62.8 (C-6′), 71.2 (C-4′), 75.4 (C-2′), 77.7 (C-5′), 78.3 (C-3′), 106.7 (C-1′); 20-O-glc, δ 62.8 (C-6″), 71.2 (C-4″), 75.7 (C-2″), 77.9 (C-5″), 78.2 (C-3″), 98.3 (C-1″); FAB-MS (negative ion mode, xenon gas, 8 kV ion current, thioglycerol matrix) (*m/z*) 784 ($[M]^-$), 621 ($[M - 163]^-$), 458 ($[M - 163 - 163]^-$).

### 4.4. Bioassays

All bioassays were conducted using a mineral broth based on Czapek–dox minimal salts (Difco) pH 7.6. The

broth was used as a minimal medium or supplemented with sucrose (up to 15 g $L^{-1}$), 1 mg $mL^{-1}$ ginsenoside, $1 \times 10^{-6}$ mol $mL^{-1}$ glucose and/or 20 μg $mL^{-1}$ ergosterol (see legends to Tables 1 and 2). Ginsenosides were filter sterilized into the broth through a 0.2 μm PVDF syringe tip membrane. For experiments, 50 mL Erlenmeyer flasks containing 10 mL of the experimental media were inoculated with 5.6 or 9 mm diameter plugs removed from the edge of actively growing cultures (from PDA) and incubated without shaking in the dark at 25 °C for five (*P. irregulare*) or six (*T. hamatum*) days.

For the recovery of ginsenosides from spent medium, mycelia were filtered through pre-weighed nylon membranes (0.45 μm) and extensively washed with 50 mM Tris–HCL buffer, pH 8. The recovered spent medium was extracted with water-saturated n-BuOH with the n-BuOH phase evaporated in vacuo. The residue was dissolved in MeOH and analyzed by HPLC and LC-MS as described above.

For the recovery of ginsenosides associated with the mycelia of *P. irregulare*, nylon membranes containing mycelia were dried overnight in a conventional oven at 72 °C, placed into screw top culture tubes (25 mm × 150 mm), sealed with teflon-lined screw caps and subjected to a microwave-assisted extraction method developed for sterols (Young, 1995). The extractives were dissolved in MeOH and analyzed by HPLC and LC-MS as described above.

### 4.5. Extracellular glycosidase assays

For in vitro enzyme assays, the spent broth from several large scale (100 mL) cultures of *P. irregulare*, incubated in the dark at 25 °C for two weeks in the presence of ginsenosides (0.01% w/v), was collected by filtration (0.45 μm nylon membranes), pooled separately and concentrated with an Amicon® ultrafiltration unit using regenerated cellulose membranes (MW cut off: 10,000 Da), and reconstituted in 50 mM Tris–HCL buffer pH 8. Protein was quantified using a microassay based on the Bradford Protein Assay (Bradford, 1976), using commercially available dye concentrate (BioRad®) and γ-globulins as standards. Enzyme assays were carried out at 25 °C in the dark over a two week period, using 3 mg $mL^{-1}$ ginsenosides as substrate, and 1 mg $mL^{-1}$ crude protein preparation in an 80 μL reaction mixture. At the end of the incubation, the reaction mix was acidified, extracted with n-BuOH, and the n-BuOH phase recovered. After removal of the 1-n-BuOH and reconstitution in MeOH, samples were centrifuged (19,000*g*) and 30 μL analyzed by HPLC as above.

### 4.6. Statistical analysis

All experiments were independently replicated at least twice, with a minimum of 3 sample replicates per experiment. Representative data are shown. Biomass measurements and biotransformation assays were analyzed via an ANOVA followed by an LSD comparison of means ($P = 0.05$) using STATISTIX™ (analytical software, P.O. Box 12185, Tallahassee, FL 32317).

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## References

Armah, C.N., Mackie, A.R., Roy, C., Price, K., Osbourn, A.E., Bowyer, P., Ladha, S., 1999. The membrane-permeabilizing effect of avenacin A-1 involves the reorganization of bilayer cholesterol. Biophys. J. 76, 281–290.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Brunner, F., Wirtz, W., Rose, J.K.C., Darvill, A.G., Govers, F., Scheel, D., Nürnberger, T., 2002. A β-glucosidase/xylosidase from the phytopathogenic oomycete, *Phytophthora infestans*. Phytochemistry 59, 689–696.

Carter, J.P., Spink, J., Cannon, P.F., Daniels, M.J., Osbourn, A.E., 1999. Isolation, characterization, and avenacin sensitivity of a diverse collection of cereal-root-colonizing fungi. Appl. Environ. Microbiol. 65, 3364–3372.

Ford, J.E., McChance, D.J., Drysdale, R.B., 1977. The detoxification of α-tomatine by *Fusarium oxysporum* f. sp. *lycopersici*. Phytochemistry 16, 544–546.

Fuzzati, N., Gabetta, B., Jayakar, K., Pace, R., Peterlongo, F., 1999. Liquid chromatography-electrospray mass spectrometric identification of ginsenosides in *Panax ginseng* roots. J. Chrom. A 854, 69–79.

Haijiang, Z., Yongjiang, W., Yiyu, C., 2003. Analysis of 'SHENMAI' injection by HPLC/MS/MS. J. Pharm. Biomed. Anal. 31, 175–183.

Hostettmann, K., Hostettmann, M., Manston, A., 1991. In: Dey, P.M., Harborne, J.B. (Eds.), Methods in Plant Biochemistry, vol. 7. Academic Press, London, pp. 435–471.

Howard, R.J., Garland, J.A., Seaman, W.L., 1994. Diseases and pests of vegetable crops in Canada. Canadian Phytopathological Society and Entomological Society of Canada, Ottawa, pp. 11–15.

Karikura, M., Miyase, T., Tanizawa, H., Taniyama, T., Takino, Y., 1991. Studies on absorption, distribution, excretion and metabolism of ginseng saponins. VII: Comparison of the decomposition modes of ginsenoside $Rb_1$ and $Rb_2$ in the digestive tract of rats. Chem. Pharm. Bull. 39 (9), 2357–2361.

Keukens, E.A.J., Vrije, T.D., Fabrie, C.H.J.P., Demel, R.A., Jongen, W.M.F., Kruijff, B.D., 1992. Dual specificity of sterol-mediated glycoalkaloid induced membrane disruption. Biochim. Biophys. Acta 1110, 127–136.

Lairini, K., Ruiz-Rubio, M., 1998. Detoxification of α-tomatine by *Fusarium solani*. Mycol. Res. 102 (11), 1375–1380.

Lascombe, M-B., Milat, M-L., Blein, J.P., Panabières, F., Ponchet, M., Prangé, T., 2000. Crystalization and preliminary X-ray studies of oligandrin, a sterol-carrier elicitor from *Pythium oligandrum*. Acta Chrystallograph. D 56, 1498–1500.

Lin, M.C., Wang, K.C., Lee, S.S., 2001. Transformation of ginsenosides $Rg_1$ and $Rb_1$ and crude sanchi saponins by human intestinal microflora. J. Chin. Chem. Soc. 48, 113–120.

Mordekhai, L., Zehavi, U., Naim, M., Polacheck, I., 1986. An improved procedure for the isolation of medicagenic acid 3-*O*-β-D-glucopyranoside from alfalfa roots and its antifungal activity on plant pathogens. J. Agric. Food Chem. 34, 960–963.

Morrissey, J.P., Osbourn, A.E., 1999. Fungal resistance to plant antibiotics as a mechanism of pathogenesis. Microb. Mol. Biol. Rev. 63, 708–724.

Nes, D.W., 1987. Biosynthesis and requirement for sterols in the growth and reproduction of Oomycetes. In: Fuller, G., Nes, W.D. (Eds.), Ecology and Metabolism of Plant Lipids. American Chemical Society, Washington, DC, pp. 304–328.

Nes, W.D., Stafford, A.E., 1983. Evidence for metabolic and functional discrimination of sterols by *Phytophthora cactorum*. Proc. Natl. Acad. Sci. 80, 3227–3231.

Nes, W.D., Saunders, G.A., Heftmann, E., 1983. A reassessment of the role of steroidal alkaloids in the physiology of *Phytophthora*. Phytochemistry 22, 75–78.

Nicol, R.W., Traquair, J.A., Bernards, M.A., 2002. Ginsenosides as host resistance factors in American ginseng (*Panax quinquefolius*). Can. J. Bot. 80, 557–562.

Nicol, R.W., Yousef, L., Traquair, J.A., Bernards, M.A., 2003. Ginsenosides stimulate the growth of soilborne pathogens of American ginseng. Phytochemistry 64, 257–264.

Olsen, R.A., 1973a. Triterpeneglycosides as inhibitors of fungal growth and metabolism. 5. Role of sterol contents of some fungi. Physiol. Plant. 28, 507–515.

Olsen, R.A., 1973b. Triterpeneglycosides as inhibitors of fungal growth and metabolism. 6. The effect of aescin on fungi with reduced sterol contents. Physiol. Plant. 29, 145–149.

Osbourn, A.E., 1999. Antimicrobial phytoprotectants and fungal pathogens: A commentary. Fungal Genet. Biol. 26, 163–168.

Osbourn, A.E., Bowyer, P., Lunness, P., Clarke, B., Daniels, M., 1995. Fungal pathogens of oat roots and tomato leaves employ closely related enzymes to detoxify different host plant saponins. MPMI 8 (6), 971–978.

Osman, H., Vauthrin, S., Mikes, V., Milat, M.L., Panabières, F., Marais, A., Brunie, S., Maume, B., Ponchet, M., Blein, J.P., 2001. Mediation of elicitin activity on tobacco is assumed by elicitin-sterol complexes. Mol. Biol. Cell 12, 2825–2834.

Papadopoulou, K., Melton, R.E., Leggett, M., Daniels, M.J., Osbourn, A.E., 1999. Compromised disease resistance in saponin-deficient plants. PNAS 96, 12923–12928.

Pegg, G.F., Woodward, S., 1986. Synthesis and metabolism of α-tomatine in tomato *Lycopersicon esculentum* isolines in relation to resistance to *Verticillium albo-atrum*. Physiol. Mol. Plant Pathol. 28, 187–202.

Ponchet, M., Panabières, F., Milat, M.-L., Mikes, V., Montillet, J.-L., Suty, L., Triantaphylides, C., Tirilly, Y., Blein, J.-P., 1999. Are elicitins cryptograms in plant–Oomycete communications? CMLS 56, 1020–1047.

Punja, Z.K., 1997. Fungal pathogens of American ginseng (*Panax quinquefolium*) in British Columbia. Can. J. Plant Pathol. 19, 301–306.

Quidde, T., Osbourn, A.E., Tudzynski, P., 1998. Detoxification of α-tomatine by *Botrytis cinerea*. Physiol. Mol. Plant Pathol. 52 (3), 151–165.

Reeleder, R.D., Brammall, R.A., 1994. Pathogenicity of *Pythium* species, *Cylindrocarpon destructans*, and *Rhizoctonia solani* to ginseng seedlings in Ontario. Can. J. Plant Pathol. 16, 311–316.

Roldàn-Arjona, T., Pérez-Espinosa, A., Ruiz-Rubio, M., 1999. Tomatinase from *Fusarium oxysporum* f.sp. *lycopersici* defines a new class of saponinases. MPMI 12 (10), 852–861.

Teng, R., Li, H., Chen, J., Wang, D., He, Y., Yang, C., 2002. Complete assignment of $^{1}H$ and $^{13}C$ NMR data for nine protopanaxatriol glycosides. Magn. Reson. Chem. 40, 483–488.

Young, J.C., 1995. Microwave-assisted extraction of the fungal metabolite ergosterol and total fatty acids. J. Agric. Food Chem. 43, 2904–2910.