Use of a ^{15}N-tracer Method as a Tool to Indicate the Assimilation of Elicitin-Sterol Complexes by *Phytophthorasojae*

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Abstract

The mechanism of sterol acquisition and transport in oomycetes of the genus *Phytophthora* is suspected to involve secreted class-I elicitins, but little information is available to confirm this. The objective of this study was to determine if class-I elicitins of *Phytophthorasojae*promote assimilation into this organism when a phytosterol is amended into its growth medium using a qualitative ^{15}N tracer method. Two *P. sojae* growth experiments were conducted involving ^{15}N labeled mycelium or ^{15}N spent broth containing putative *P. sojae*elicitins in the absence or presence of a phytosterol (stigmasterol). Mycelium was harvested after 0, 2, 3 or 6 days of growth and analyzed for $\delta^{15}N$ by Elemental Analysis-Isotopic Ratio Mass Spectrometry (EA-IRMS). Results from the two experiments demonstrated that *P. sojae* assimilated putative elicitins from the spent broth more rapidly when stigmasterol was present in the growth medium. These results provide evidence for the involvement of secreted class-I elicitins in sterol acquisition by *P. sojae*.

Keywords: ^{15}N stable isotope labeling, Elicitin, Oomycetes, *Phytophthora*, Receptor, Sterol carrier protein

1. Introduction

Oomycetes in the genus *Phytophthora* are fungal-like organisms in the kingdom Stramenopiles(Tyler 2006). *Phytophthora spp.* constitute a large number of destructive pathogens that cause disease on a wide range of herbaceous and woody plants (Erwin and Ribiero 1996), and all are reported to secrete large amounts of low molecular weight proteins (10 kDa) belonging to class-I elicitins(Ponchet*et al*. 1999). The intrinsic function of class-I elicitins remains largely unknown but is presumed to involve sterol acquisition because *in vitro* experiments showed they bind sterols and catalyze their transfer across artificial membranes (Mikes et al., 1998) and plasma membrane preparations (Osman *et al.* 2001b; Vauthrin *et al.*1999).

Phytophthora spp. are sterol auxotrophs because they lack key enzymes such as squaleneepoxydase and the 14α-demethylase that are essential in converting sterol precursors to active sterols (Tyler *et al.* 2006). Because of the auxotrophic requirement for sterols combined with the *in vitro* sterol binding activity of class-I elicitins, it has been hypothesized that elicitins act as shuttles that transport sterols from the environment to the pathogen (Ponchet *et al.* 1999). Yousef *et al.* (2009) found that nanomolar concentrations of sterols (stigmasterol and cholesterol) caused a down-regulation of class-I elicitin gene expression. This also coincided with a reduction in

class-I elicitin protein secretion into the spent broth. These findings suggested class-I elicitins are involved in sterol acquisition by *P. sojae*.

Elicitins were first characterized on the basis of their ability to induce the hypersensitive response (HR) when infiltrated into tobacco leaves (Ponchet *et al.* 1999). The HR-eliciting property of elicitins was attractive to plant pathologists because it suggested that elicitins secreted by *Phytophthora*spp. may function as determinants of host range for selected plant-*Phytophthora* interactions (Ponchet*et al.* 1999). Efforts were made to determine the mechanisms by which elicitins trigger the HR in tobacco. This lead to the discovery that elicitins interact with receptors present in tobacco plasma membrane preparations that exhibit a high affinity for elicitins(Blein*et al.* 1991). Interestingly, ligand binding studies determined that the affinity of these receptors in tobacco was higher for elicitins when they were bound to a sterol than when alone, which was also a requisite step to trigger the HR (Osman *et al.* 2001b). Surprisingly, however, there have been no attempts to identify if a similar mechanism of elicitin-sterol uptake involving receptors exists in *Phytophthora*, the discovery of which would add to our knowledge of the intrinsic roles of elicitins and the mechanisms of sterol uptake in this important group of plant pathogens.

The use of enriched stable isotopes in tracer experiments is a valuable tool for investigating the fate of elements and biomolecules in biological (Stuurup *et al.* 2008) and natural ecosystems (e.g. Bohlke *et al.* 2004; Peterson 1999). Experiments utilizing stable isotopes are very similar to radioisotope tracer experiments, except they have the advantage of being nontoxic and can be used to study active pathways in living organisms (Stuurup *et al.* 2008). The objective of this study was to utilize a 15N tracer method as a tool for providing evidence for the involvement of ^{15}N-elicitins in *P. sojae* sterol acquisition. The hypothesis is that if receptors for elicitins are present on the mycelia of *P. sojae*, and only become activated to internalize elicitins when they are bound to sterol, then δ^{15}N signature shifts will be observed only when stigmasterol is added to the growth medium of *P. sojae*.

2. Materials and Methods

2.1 Culture Source and Growth Media

The *Phytophthorasojae* culture (race 1) was obtained from Dr. A. Dorrance (OARDC, Wooster OH, USA) and maintained on non-clarified V8 agar (18% v/v V8 juice, 0.3% $CaCO_3$, and 2% agar) at 25^o C in the dark. A defined minimal broth similar to (Wu et al., 2003) was made by dissolving the following in one L of distilled water: 0.2 g K_2HPO_4, 0.1 g $MgSO_4$, 0.1 g $CaCl_2$, 0.1 g L-asparagine, 10 g glucose and 1 mL trace elements. The trace element solution was prepared by dissolving 200 mg FeEDTA, 10 mg $CuSO_4$, 10 mg $MnCl_2$, 10 mg Na_2MoO_4, 10 mg $Na_2B_4O_7$, and 20 mg $ZnSO_4$, and 100 mg thiamine hydrochloride in 100 mL distilled water. All chemicals were obtained from Sigma Aldrich (St. Louis, MO). To obtain ^{15}N labeled or unlabeled mycelium or broth for Experiments 1 and 2, described below, 0.1g $Na^{14}NO_3$ or $Na^{15}NO_3$ (98+ atom% ^{15}N), was added to the above growth medium.

The growth medium of *P. sojae*cultures that received $NaNO_3$-^{15}N (98% atom) was enriched by 37 % atom ^{15}N. This means that 37 % of all the N atoms present in the growth medium are ^{15}N. This was calculated as follows: A total of 0.1g L^{-1} L-asparagine (representing 28.7 mg total N) and 0.1 g L^{-1} $Na^{15}NO_3$ -98% atom (representing 17 mg total N) were used as the sole N source in the growth medium. N enrichment was then calculated as follows:

^{15}N enrichment (%) = (17 mg) (0.98; this is % atom enrichment in $Na^{15}NO_3$)/ 45.7 mg (100).

2.2 Preparation of ^{15}N Labeled and Unlabelled Mycelium and Broth

Four 500 mL Erlenmeyer flasks containing 200 mL each of growth media (as described in section 2.1) that included $Na^{15}NO3$ (98+ atom% ^{15}N) were inoculated with three mycelial plugs (9 mm diameter). These were incubated in the dark for 4 weeks after which the mycelial plugs were separated from the combined four flasks containing the spent broth using vacuum filtration through a sterile 250 mL Stericup unit having a 0.22 μm PES membrane (Millipore Billerica, Ma). The mycelia was washed with and maintained in sterilized distilled water. The labeled ^{15}N-labelled mycelium and broth were saved and stored for the subsequent growth incubation treatments. The broth contains the presumed labeled sterol-binding elicitins.

The same growth conditions as described in the section above for preparation of ^{15}N-labeled mycelium and broth were imposed, except that the $NaNO_3$ was not enriched in ^{15}N. Again after 4-weeks of incubation, the combined spent broth from each replicate was vacuum-filtered and separated from the mycelium with the mycelium preserved in sterilized distilled water. The unlabeled mycelium and broth were saved and stored for subsequent growth incubation treatments. The broth contains the presumed unlabelled sterol-binding elicitins.

2.3 ^{15}N Tracer Incubation Growth Experiments

Two incubation experiments were conducted that each had four treatments and three replications. The starting materials (^{15}N-labelled and unlabelled mycelium and spent broth) for the two experiments were obtained from the incubations described in section 2.2. These two experiments were designed to have treatments that isolate the availability of N (either unlabeled or ^{15}N-labeled N sources) in the presence or absence of a phytosterol during the growth of *P. sojae*. The hypothesis was that if elicitins form complexes with sterols and are taken up by *P. sojae*, then this will be reflected in a change in ^{15}N content in mycelium. In the first experiment in which the starting material is unlabelled mycelium of *P. sojae* growing in the presence of ^{15}N-labelled spent broth containing labeled elicitins, the 15N content will increase as mycelium growth proceeds. In the reverse incubation experiment, in which the starting material is ^{15}N-enriched -mycelium of *P. sojae* growing in spent broth containing unlabelledelicitins, a decrease in ^{15}N content in the mycelium will occur.

2.3.1 Incubation Experiment 1 (non-labeled mycelium transferred into spent broth containing ^{15}N-labelled elicitins).

Fifty ml of spent broth containing ^{15}N -putative elicitins originating from the 4-week ^{15}N-labeled incubation cultures, were transferred into 75 mL Erlenmeyer flasks and inoculated with a 9mm plug of unlabeled mycelium of *P. sojae* that was recovered from the 4-week unlabelled incubation cultures (see section 2.2). Then the following treatments were established in the flasks (Table 1):.These treatments were (1) labeled spent broth control (no amendment); (2) labeled spent broth amended a plant sterol which was 1 µM stigmasterol (final concentration); (3) labeled spent broth amended with 10 g L^{-1} glucose and 0.1 g L^{-1} unlabelled $NaNO_3$; and (4) labeled spent broth amended with 10 g L^{-1} glucose, 0.1 g L^{-1} unlabelled $NaNO_3$ and 1 µM stigmasterol. Flasks were incubated in the dark at 25 ^oC and mycelia sampled at days 0, 2, 3 and 6.

2.3.2 Incubation Experiment 2 (15N-labeled mycelium transferred into spent broth containing unlabeled 14N-labelled putative elicitins).

Fifty mL of spent broth containing unlabelledputative elicitins originating from the 4-week incubation cultures were transferred into 75 mL Erlenmeyer flasks and inoculated with a 9mm plug of ^{15}N-labeled mycelium of *P. sojae* that was recovered from the 4-week labeled incubation cultures (see section 2.2). Then four different treatments were established in the flasks (Table 1). These treatments were (1) unlabelled spent broth control (no amendment); (2) unlabelled spent broth amended with 1 µM of the plant sterol stigmasterol (final concentration); (3) unlabelled spent broth amended with 10 g L^{-1} glucose and 0.1 g L^{-1} $Na^{15}NO_3$; and (4) unlabelled spent broth amended with 10 g L^{-1} glucose, 0.1 g L^{-1} $Na^{15}NO_3$ and 1 µM stigmasterol. Flasks were incubated in the dark at 25^o C and mycelia sampled at days 0, 2, 3 and 6.

2.4 Analytical Procedures

2.4.1 Mycelia Harvest

At each sampling day, mycelia were harvested from the treatments using a sterilized glass pipette in a non-destructive fashion by suctioning out a portion of the mycelia mass, and repeated washing with sterilized distilled water over a nylon filter. The mycelia were then recovered from the filter using toothpicks and placed on the surface of glass plates and air-dried overnight. The dried mycelia on the glass plates were ground using a mortar and a round glass rod. Approximately 150-250 µg were weighed into tin (Sn) capsules (Costech, Valencia, CA) in preparation for combustion and determination of the ^{15}N to ^{14}N isotopic ratio.

2.4.2 Stable Isotope Measurements

The ^{15}N to ^{14}N isotopic ratio values of the tissue samples were determined using an elemental analyzer (EA) (Carlo Erba CHN EA 1108, now Thermo Fisher Scientific, Waltham, MA) coupled to an isotope ratio mass spectrometer (IRMS) (FinniganConflo III Interface and a ThemoFinnigan Delta V Advantage mass spectrometer, Bremen, Germany). Samples weighed in Sn capsules were combusted at 1600 ºC in the elemental analyzer under a stream of oxygen. A standard N_2 gas of known 15N/14N ratio was introduced into the IRMS with every sample run. Acetanilide (purchased from Arndt Schimmelmann, Indiana University, Bloomington, IN,) was calibrated against IAEA-N-1 and IAEA-N-2 and was used as the instrumental reference material after every 20 samples. Isotope ratios are expressed as δ ^{15}N values per mille [‰] relative to an established N_2 reference gas, and were calculated using software integrated in the EA-IRMS instrument via the following equation:

$$(\delta \text{ in } ‰) = (R_{sample}/R_{standard} - 1)1000$$

Where R = ratio of the heavy (^{15}N) to light (^{14}N) isotope in the sample and reference determined by mass spectrometry.

3. Results

3.1 Base-line $\delta^{15}N$ values on Day 0

The growth medium of *P. sojae* cultures from experiments that received $NaNO_3$-^{15}N was enriched by 37% atom ^{15}N. The $\delta\ ^{15}N$ values of non-enriched mycelia typically ranged from 4 to 6 ‰ on day 0 of the experiment (Fig. 1). In comparison, $\delta\ ^{15}N$ values of the 37% ^{15}N enrichment of *P. sojae* ranged from 2160 to 3122 ‰ on day 0 of the experiment (Fig. 2).

3.2 Shifts in $\delta\ ^{15}N$ over time for unlabeled mycelia transferred into ^{15}N spent broth

In Experiment 1, the $\delta\ ^{15}N$ values of mycelia increased over-time when unlabeled mycelia were transferred into enriched [1]spent broth containing ^{15}N-enriched elicitins (Fig. 1). The same was true for mycelia recovered from the same spent broth that had been amended with 1 μM stigmasterol (Fig. 1), but on day 6 the $\delta\ ^{15}N$ values were approximately 2-fold greater than the $\delta\ ^{15}N$ value of mycelia recovered from spent broth that did not have stigmasterol (Fig. 1 top). Thusthe presence of stigmasterol caused a greater incorporation of the ^{15}N-label into *P. sojae* than if no stigmasterol was present.

The addition of glucose and $^{14}NO_3$ increased the $\delta\ ^{15}N$ values of mycelia by day 6 (Fig. 1 bottom). These $\delta\ ^{15}N$ values on day 6 were approximately 45-fold higher for mycelia recovered from spent broth containing stigmasterol than the $\delta\ ^{15}N$ values of mycelia recovered from spent broth not containing stigmasterol (Fig. 1 bottom).

3.3 Shifts in $\delta\ ^{15}N$ over time for ^{15}N-labeled mycelia transferred into unlabelled spent broth

In Experiment 2, the $\delta\ ^{15}N$ values of harvested mycelia decreased over time when enriched ^{15}N mycelia were transferred into unlabelled spent broth suggesting the uptake and assimilation of unlabelled material (Fig. 2). The $\delta\ ^{15}N$ values of mycelia in spent broth that contained stigmasterol were approximately 2-fold and 30-fold lower on days 2 and 3, respectively, when compared to the $\delta\ ^{15}N$ values of mycelia recovered from non-stigmasterol supplemented broth (Fig. 2 top). Thus, the presence of stigmasterol enhanced the incorporation of the unlabelled material from the spent broth by *P. sojae*. However, the $\delta\ ^{15}N$ values on day 3 began to increase and by day 6 had reached $\delta\ ^{15}N$ values similar to those on day 0.

Up to day 3, the addition of glucose and $^{15}NO_3$ to the spent broth resulted in a $\delta\ ^{15}N$ profile (Fig. 2 bottom) similar to the one observed for mycelia recovered from spent broth that had no glucose or nitrate (Fig. 2 top). After day 3, however, the $\delta\ ^{15}N$ values of mycelia recovered from spent broth containing stigmasterol continued to decline over time when glucose and ^{15}N labeled nitrate were present (Fig. 2 bottom) but increased when they were not present (Fig. 2 top).

4. Discussion

In this study, elicitins were labeled with ^{15}N by adding $Na^{15}NO_3$ to the growth medium of *P. sojae*. The ^{15}N-labelled elicitins that are synthesized are then secreted into the spent culture broth. There is the possibility that *P. sojae* produces and secretes other proteins than just class-I elicitins. However, we previously determined that the most abundant protein in spent filtrates of the organism were class-I elicitins using 2D-PAGE followed by LC-MS analysis (Yousef *et al.* 2009). This was also confirmed in this study by visually inspecting spent filtrates on PAGE (data not shown), in which class-I elicitins were the only proteins detected in spent filtrates after silver staining. Therefore, the spent broth was assumed to contain predominantly ^{15}N-labelled elicitins (Experiment 1, Fig. 1) or unlabelledelicitins (Experiment 2, Fig. 2).

Shifts in $\delta^{15}N$ values could also result if *P. sojae* assimilates nitrate present in the spent broth that had been carried over from the initial 4-week incubation. To determine if this was significant, unlabelled $NaNO_3$ was added to the spent broth in Experiment 1(Figure 1 bottom) and ^{15}N-labelled NaNO3 was added to the spent broth in Experiment 2 (Figure 2 bottom). Since *P. sojae* is a heterotrophic organism that derives energy from the oxidation of organic carbon (Tyler 2006), glucose was also amended into the spent broth to avoid possible cannibalism of spent elicitins by *P. sojae* as a carbon source.

Data from these treatments indicate that in the absence of stigmasterol, some of the $\delta^{15}N$ changes in the mycelium of Experiment 1 (Figure 1) could be due to the assimilation of 'left-over' ^{15}N-nitrate from the spent broth for nutritional purposes. For example, on day 6 the average $\delta^{15}N$ value of mycelia from control treatment (non-stigmasterol amended) was approximately 900 (Figure 1 top), whereas it was 200 for the same treatment that had been amended withunlabelled (i.e. $^{14}NaNO_3$) (Figure 1 bottom). However, this phenomenon was only observed in Experiment 1 and not in the reverse experiment (Experiment 2, Fig. 2). This suggests that there was a low amount of residualunlabelled NO_3 remaining in the spent broth in experiment 1 (Fig. 1 top) which was

comparable to the residual amount oflabeled NO_3 remaining in the spent broth in the reverse experiment (Fig. 2 top).

The addition of stigmasterol to the spent broth caused up to a 51-fold difference in measured $\delta^{15}N$ values of mycelia when compared to controls not receiving stigmasterol (Figures 1 and 2). This is a clear indication that stigmasterol is causing a rapid assimilation by *P. sojae*of elicitins from the spent broth. Furthermore, the addition of nutrients (glucose and nitrate) to the spent broth caused a much larger increase (Fig. 1 bottom) or decrease (Fig. 2 bottom) in $\delta^{15}N$ values over time when compared to when these nutrients were not present (Fig. 1 top and 2 top). This could possibly be due to the energy requirement of biological assimilation/sequestration, and therefore the depleted level of nutrients in the spent broth was insufficient to support bioaccumulation of elicitin-sterol complexes from the surrounding environment.

The conclusion from this study is consistent with other observations from the literature. First, it is known that class-I elicitin genes in *P. sojae* become down-regulated over time when nanomolar concentrations of stigmasterol are present in the growth medium (Yousef *et al.* 2009), which also coincided with reduced detection of elicitins in the spent broth. Second, previous studies have shown that elicitins exhibit sterol carrier activity *in vitro*(Mikes *et al.* 1998; Vauthrin *et al.* 1999). Structurally, elicitins are composed of an α-helix fold stabilized by three disulfide bonds, which provides a hydrophobic cavity able to bind sterols in a 1:1 sterol:elicitin stoichiometry (Osman *et al.* 2001b).

There are two other physiological mechanisms for how *P. sojae* might take up an elicitin-sterol complex. One possibility is that the elicitin-sterol complex binds to receptors present on *P. sojae* membranes that have a higher affinity for an elicitin-sterol complex than when the elicitin binds alone. The higher affinity of receptors for an elicitin-sterol complex would insure that only elicitins carrying a sterol are sequestered by *P sojae*. While there are no direct studies in *Phytophthora* to support this hypothesis, putative elicitin receptors have been identified in membrane preparations of tobacco which are the sites responsible for activation of hypersensitivity in tobacco (Bourque *et al.* 1999; Bourque *et al.* 1998; Ponchet *et al.* 1999; Svozilova *et al.* 2009). These receptor sites also exhibited stronger binding characteristics for an elicitin-sterol complex than when the elicitin was alone (Bourque *et al.* 1998; Ponchet *et al.* 1999). It is possible that orthologs of elicitin receptors are present in *Phytopthora*, which would act as recognition sites in the organism that preferentially bind and capture from the environment an elicitin-sterol complex over an elicitin alone. This would explain why, in this experiment, the $\delta^{15}N$ values changed more rapidly when stigmasterol was present than when it was not. The gene sequences of the elicitin receptors present in tobacco membranes have not yet been delineated, the sequences of which may be used to search for orthologs in the genome of *P. sojae* that is available on the Joint Genome Institute web site (http://genome.jgi-psf.org).

The second hypothetical mechanism is that the binding of an elicitin-sterol complex to elicitin receptors leads to receptor-mediated endocystosis of the elicitin-sterol complex. This could be because biological membranes are composed of an amphipathic bilayer (i.e. a hydrophilic exterior and a sandwiched hyrodphobic layer), and the kinetics of moving hydrophic molecules, such as sterols, across biological membranes would be very slow without the assistance of transport proteins (Prinz 2007). Organisms in the kingdom, protozoa, are similar to *Phytophthora*in that they universally lack the ability to synthesize cholesterol and must acquire it from the environment (Lige *et al.* 2009). For example, the protozoan organism *Toxoplasma gondii,* which is an opportunisticparasite under immunosuppressive conditions, has been reported to sequester cholesterol from its host presumably via receptor-mediated endocytosis of cholesterol using a sterol carrier protein (Lige *et al.* 2009).

If sterol-uptake by *Phytophthora*involves the endocytosis of an elicitin-sterol complex, then the internalization of ^{15}N-labelled elicitin would also cause a shift in the natural abundance of ^{15}N in *P. sojae* mycelia as was observed in Figure 1. Similar experiments utilizing pure elicitin protein will be necessary to validate this proposed mechanism. However, results in this study support the conclusion that secreted elicitins mediate sterol acquisition by *Phytophthora* spp. to support their growth and reproduction.

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Table 3.1 Treatments for 6-day incubation Experiments 1 and 2. Growth medium was with amendment (+) or without amendment (-)

Treatment groups	**Stigmasterol 1 µM final**	**$Na^{15}NO_3$ (Labelled) 0.1 g L^{-1}**	**$Na^{14}NO_3$ (Unlabelled) 0.1 g L^{-1}**	**Glucose 10 g L^{-1}**
Experiment 1 (^{15}N-labelled spent broth supporting growth of mycelium that had not previously been labeled)				
(1) No amendment	-	-	-	-
(2) Stigmasterol amendment	+	-	-	-
(3) Nutrient amendment	-	-	+	+
(4) Nutrient + Stigmasterol amendment	+	-	+	+
Experiment 2 (Unlabelled spent broth supporting growth of mycelium that had been previously labeled with ^{15}N.				
(1) No amendment	-	-	-	-
(2) Stigmasterol amendment	+	-	-	-
(3) Nutrient amendment	-	+	-	+
(4) Nutrient + Stigmasterol amendment	+	+	-	+

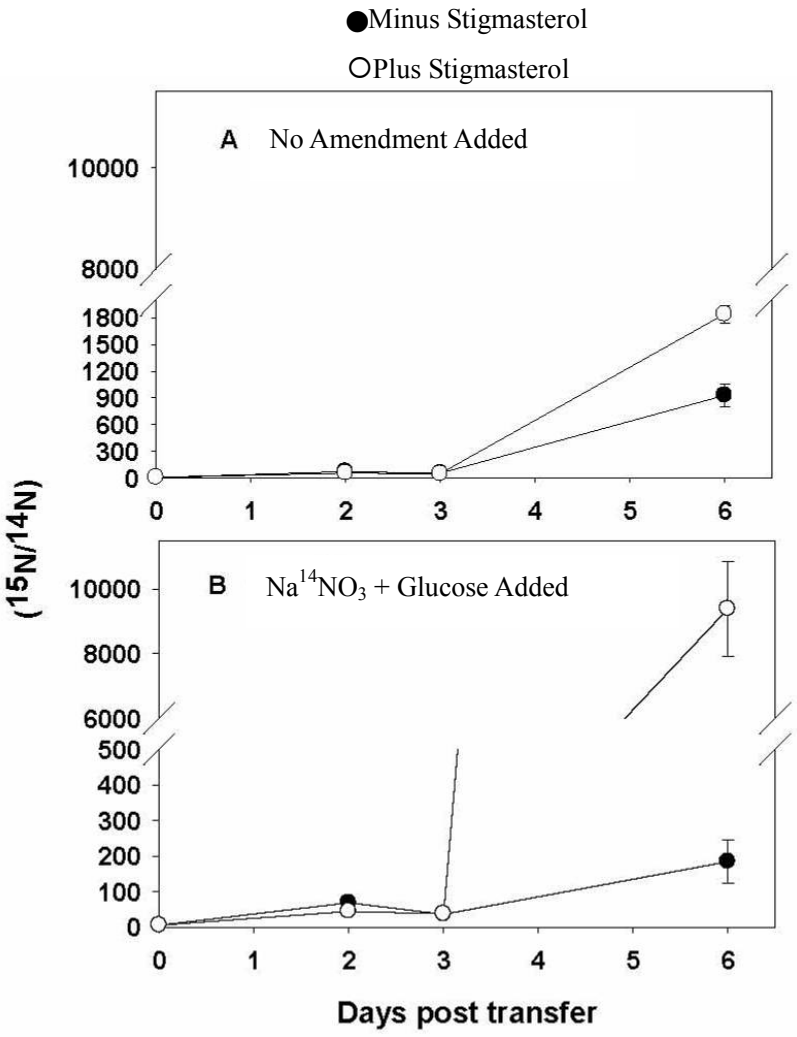

Figure 1. Change of $^{15}N/^{14}N$ ratios in non-enriched *P. sojae* mycelia growing in spent broth containing putative ^{15}N-labelled elicitins. Mycelia from ^{15}N-labeled spent broth without amendment (top) and mycelia from the same spent broth that was amended with 0.1 g L^{-1} $Na^{14}NO_3$ (i.e. unlabelled nitrate) plus 10 g L^{-1} glucose (bottom)

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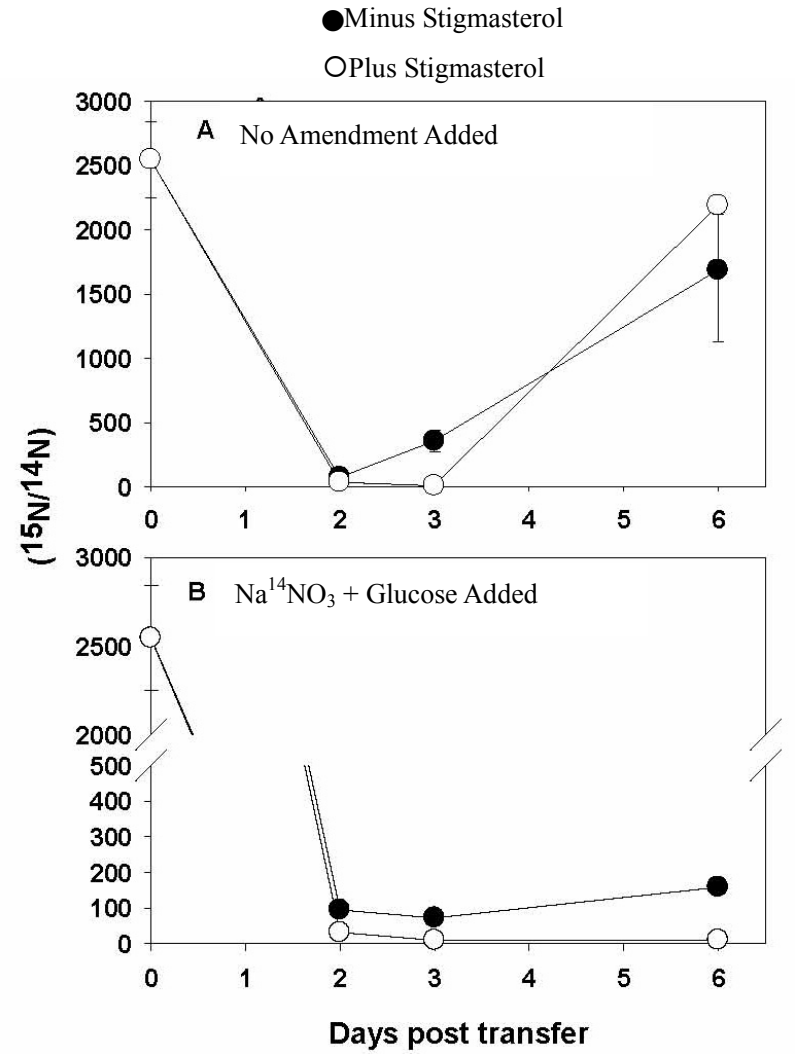

Figure 2. Change of $^{15}N/^{14}N$ ratios in ^{15}N-enriched *P. sojae* mycelia growing in spent broth containing putative unlabelledelicitins. Mycelia from unlabelled spent broth without amendment (top) and mycelia from the same spent broth that was amended 0.1 g L^{-1} $Na^{15}NO_3$ (i.e. labelled nitrate) plus 10 g L^{-1} glucose (bottom).