# HIGH VARIABILITY IN BIOMARKER GENE RESPONSES TO *SIREX NOCTILIO* VENOM IN FIELD-GROWN PINES

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## Introduction

*Sirex noctilio* is an exotic forest pest in North America and is capable of causing significant economic damage in plantation and naturally-grown pines (*Pinus* spp.) (Borchert et al., 2006). Hazard maps for *S. noctilio* are essential to predicting high-risk areas for establishment, and should account for such factors as stand density, edaphic effects, and host preference. Patterns in *S. noctilio* woodwasp host preference for southeastern pine species have previously been documented; the woodwasp preferred Virginia (*P. virginiana* Mill.) and white (*P. strobus* L.) pines to four other southeastern U.S. *Pinus* species in bolt assays (Dinkins, 2011). Though useful, these data are not sufficient to estimate host tree susceptibility. A susceptibility assay for trees in the field would have utility for predictions of *S. noctilio* establishment.

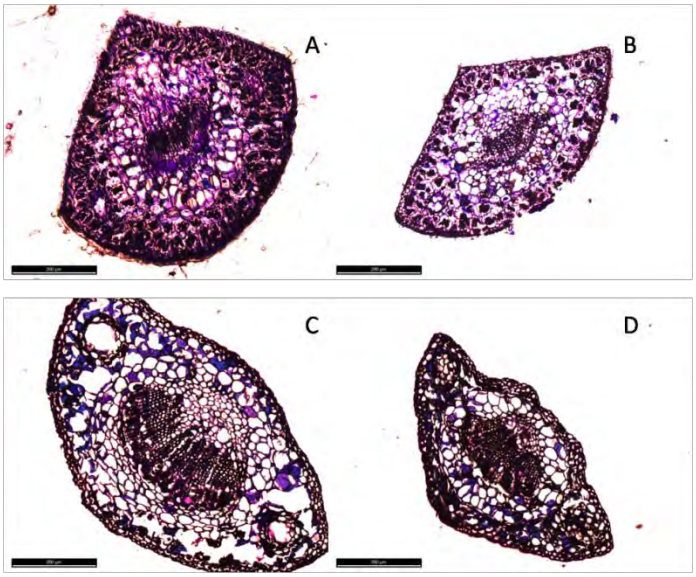


**Figure 1: Cross-sections of Monterey pine (*P. radiata*) needles exposed to fresh *S. noctilio* venom, 10X. Treated needles were fixed in 4% formaldehyde and sections were stained with toluidine blue. Needles were treated as follows: Panel A, 24 hrs water; B, 24 hrs fresh venom; C, 48 hrs water; D, 48 hrs fresh venom. The predominant cellular phenotype caused by venom treatment was collapse of phloem cells as described by Fong and Crowden (1973).**

Early observations of *S. noctilio* attack on pines noted that needles showed a characteristic wilt phenotype ('flagging'), which on a cellular level was correlated with collapsed phloem cells (Fong and Crowden, 1973). We were able to reproduce this phenotype in the laboratory (Fig. 1). Previously, we demonstrated that laboratory-grown seedlings and cuttings from potted trees can be used to gauge tree susceptibility to *S. noctilio* venom by measuring gene expression changes in two biomarker genes (PR4 and TLP) (Bordeaux et al., 2012). Biomarker gene response was detectable well before phloem collapse and wilt in needles. The assay has shown utility for measuring gene responses in several species of pine exposed to *S. noctilio* venom.

The purpose of the present study was to assess whether this biomarker assay would be similarly useful for detecting responses in a variety of field-grown *Pinus* species. We hypothesized that changes in biomarker gene expression in response to *S. noctilio* venom would be similar in field-grown pines of various species compared with seedlings grown under controlled conditions. We further hypothesized that these responses could be correlated with susceptibility or resistance to *S. noctilio* attack in a species-specific manner. If successful, such an assay would enable us to more efficiently study insect preference and host tree responses to *S. noctilio* under field conditions, and should permit development of better predictive models and incursion risk maps prior to woodwasp establishment in southeastern U.S.

## Materials and Methods

### Gene Expression Assays

A biomarker assay of pine gene responses to *S. noctilio* venom using quantitative real-time polymerase chain reaction (qRT-PCR) was previously described for use in laboratory-grown loblolly (*P. taeda*) and Monterey (*P. radiata*) tissues (Bordeaux et al., 2012). The assay measures changes in transcription levels for two defense-response genes in pine (PR4, pathogenesis-related protein; TLP, thaumatin-like protein) in comparison to several housekeeping (control) genes. For this study, actin (ACT1, ACT2), ubiquitin-conjugating factor (UBCF), and glyceraldehyde phosphate dehydrogenase (GAPDH) were used as control genes.

It has been widely recognized that pine gene sequences are highly conserved between species and as a consequence oligonucleotide primers developed for one species are often directly transferrable to other pine species (Barbara et al., 2007; Krutovsky et al., 2007; Lesser et al., 2012). To test whether the primer pairs for the biomarker and control genes listed above would amplify genes from species to be tested, needles were collected from field-grown loblolly, longleaf (*P. palustris* Mill.), Scots (*P. sylvestris* L.), shortleaf (*P. echinata* Mill.), table mountain (*Pinus pungens* Lamb.), Virginia, and white pines. One to three needle fascicle bundles from a mature tree yielded sufficient RNA to perform reverse-transcriptase PCR (RT-PCR) assays in replicate. Needle sampling was minimally invasive and deemed unlikely to affect gene expression in needles elsewhere in the tree. RT-PCR amplification produced products (amplimers) using RNA from each test species as template, and all products were the same size (Fig. 2), which

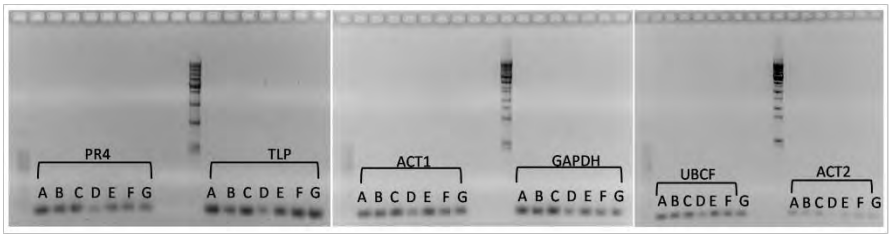


**Figure 2: RT-PCR products amplified from seven species of *Pinus* using primer pairs based on *P. taeda* genes. Template: cDNA prepared from RNA isolated from needles incubated in water (control conditions). Initial concentrations of template were not standardized, which accounts for variations in spot density. Amplification ran for 30 cycles of conditions described in (Bordeaux et al., 2012). Lanes on far left and in the middle contain 100bp and 1kbp standards, respectively. Amplified genes are noted above the braces. Pine species were as follows: lane A, *P. sylvestris*; B, *P. strobus*; C, *P. pungens*; D, *P. virginiana*; E, *P. echinata*; F, *P. taeda* (positive control); G, *P. palustris*.**

strongly suggested acceptable conservation of sequence between genes within these species of *Pinus* (Fig. 2).

**Site selection and sampling plan**

Trees were sampled from a common garden (The Pinetum at Thompson Mills Forest) near Braselton, Georgia (34°07'22.82"N, 83°47'48.95"W). The Pinetum contains examples of all conifer species native to Georgia. Sampling took place in February 2012 with a complete replicate collected four weeks later (March 2012). Six pine species native to Georgia -- loblolly, longleaf, shortleaf, table mountain, Virginia, and white – as well as a European species that is a natural host to *S. noctilio* (Scots pine) were sampled for this study. Samples were taken from six trees of each species (N=6), except for table mountain and Scots pines (N=3). Four samples (fascicle bundles) were taken from each sampled tree, two of which were used for venom treatment, and two for water treatment (control). After exposure to venom or water for 24 h, needles were flash-frozen in liquid nitrogen, and RNA was extracted as previously described (Lorenz et al., 2010; Bordeaux et al., 2012). Using protocols from the same studies, cDNA was synthesized from RNA and qRT-PCR was performed.

**Results**

Relative expression data for the biomarkers in venom- versus water-treated needles showed high variability between trees within species as well as between species (for example, between *P. strobus* vs. *P. palustris*). There was also high variability between the two biomarkers with respect to species response (Fig. 3A, PR4; Fig. 3B, TLP). Presenting the data in terms of fold-change (Fig. 3C, 3D) was useful for identifying general trends in the responses of some species, but did not provide for a reliable estimate of biomarker gene expression responses within a given species due to the high variability of response within species (see for example *P. sylvestris* and *P. palustris*). Variations between biomarkers in different species were especially pronounced (Fig. 3C, 3D).

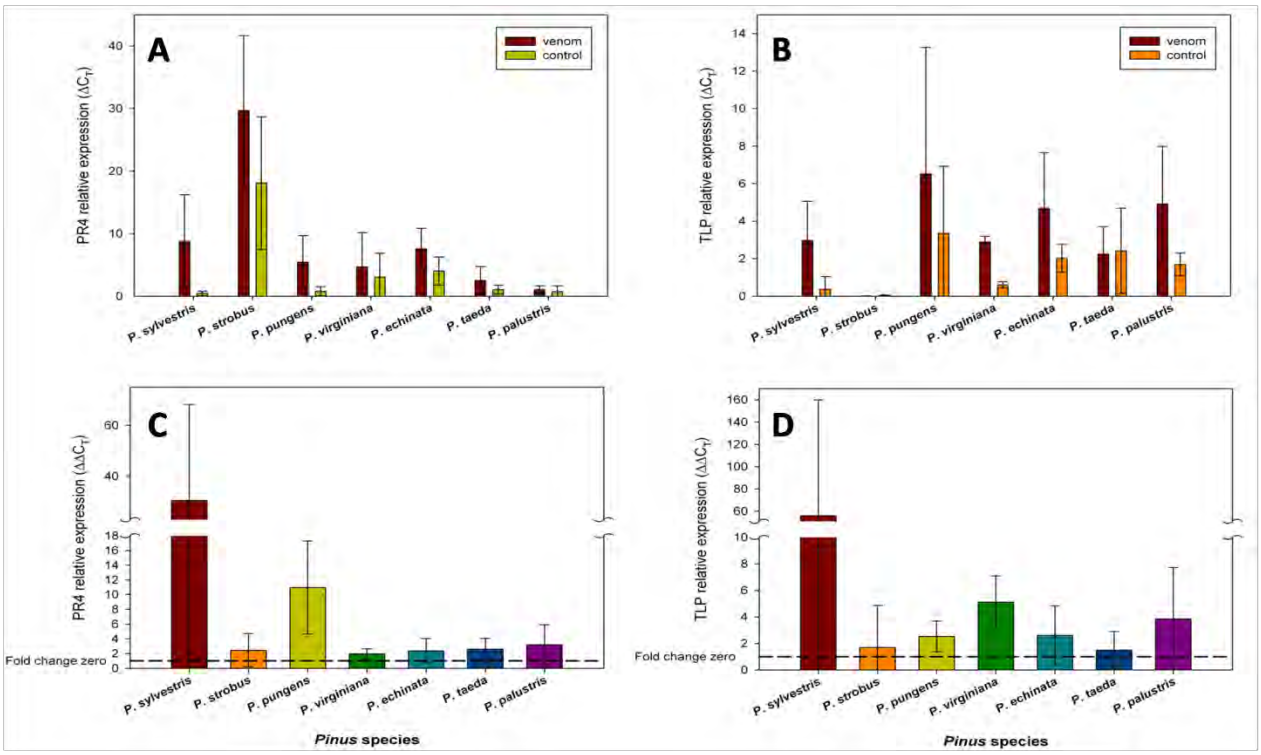


**Figure 3: Expression levels of biomarker genes in pine needles treated with *S. noctilio* venom. Needles from the same tree were treated either with an aqueous solution of *S. noctilio* venom or water, and gene expression levels were quantified using qRT-PCR. Panels A and B show relative expression for both venom-treated and water control needles. Panels C and D use fold change (ratio of treatment response to control response) to display the same data. In these figures the dashed line represents a ratio of one, signifying no change in relative expression. Panels A, C, PR4 expression; panels B, D, TLP expression. Note differences between y-axis scales in all panels.**

Comparing biomarker gene responses in two southeastern U.S. pine species to Scots pine, a natural host of *S. noctilio*, in the North American species the two biomarkers showed a similar magnitude of response, but did not agree in direction. In *P. palustris*, for example, PR4 expression changed very little in response to venom, whereas TLP was strongly up-regulated by *S. noctilio* venom exposure (Fig. 4). These results were not consistent with previous results using seedlings grown under controlled conditions, in which case expression was up-regulated for both biomarkers following venom exposure. Comparisons of biomarker expression changes in needles collected on different dates (Fig. 4A, 4B versus Fig. 4C, 4D) were somewhat more consistent, but any attempt to generalize from these data was confounded by high variability within species and between trials.

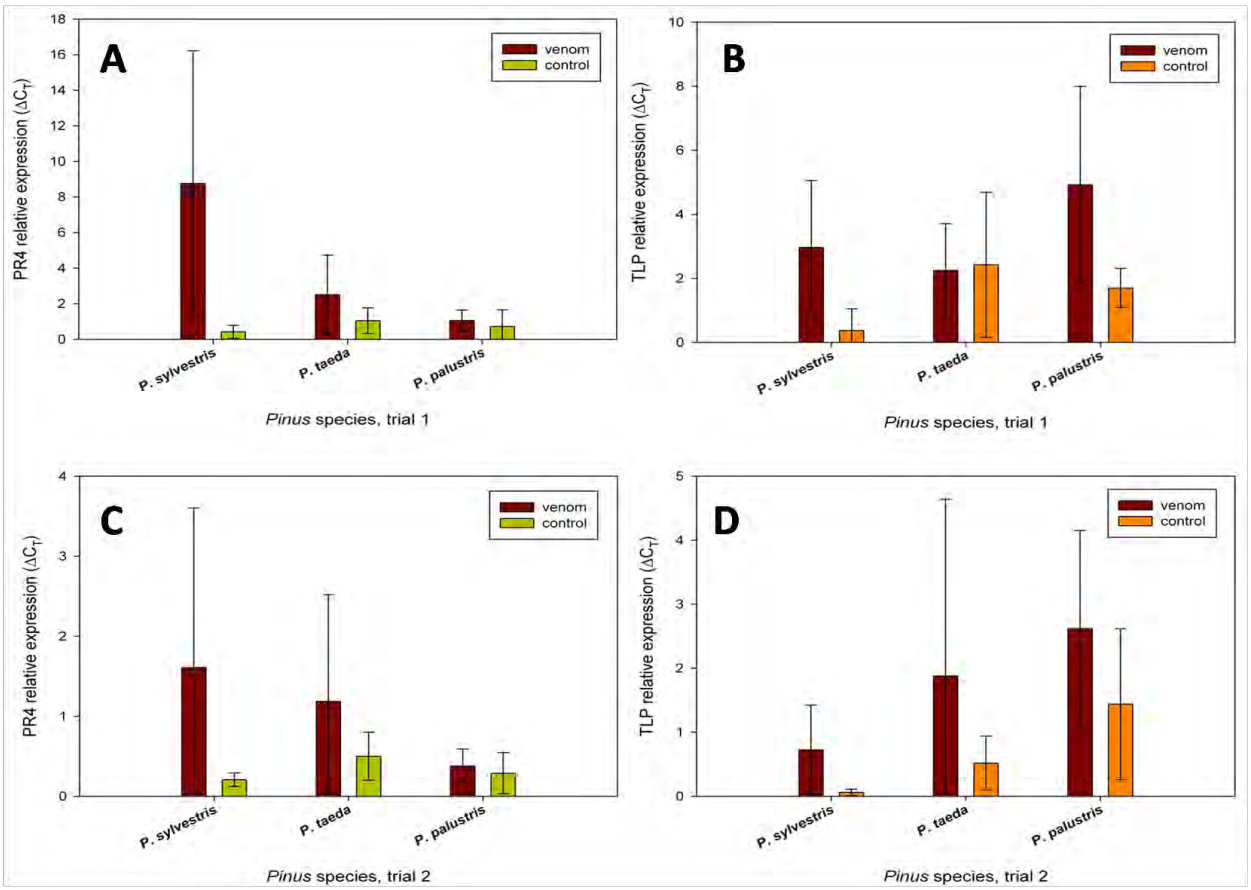


**Figure 4: Apparent biomarker gene expression responses to *S. noctilio* venom in needles collected on different dates. Results from the first collection date (February 2012) appear in panels A and B, while results from the second collection date (March 2012) appear in panels C and D. Data are shown only for the three species (Scots, loblolly, and longleaf pines) that yielded fully-replicated data at both time points, and the data shown in panels A and B are a subset of the data in Fig. 3A, 3B. Trees sampled were N=5 in Trial 1, and N=6 in Trial 2 for *P. taeda* and *P. palustris*, respectively, and N=3 for *P. sylvestris* for both trials. Panels A and C show PR4 expression relative to a water control, while panels B and D show relative TLP expression. Note differences between y-axis scales in all panels.**

One observation of note from these experiments was that up-regulation of the biomarker genes was consistently stronger in Scots pine species compared to either of the southeastern native pines (Fig. 5). Scots pine assays were particularly variable, which might be related to the small sample size (N=3 trees). While it was not possible to generalize broadly from these data, the magnitude of difference in biomarker expression (especially for TLP) in Scots pine relative to loblolly or longleaf pine was profound. Our previous studies showed that elevated levels of biomarker gene expressions was strongly correlated with the degree of wilt symptoms in needles (Bordeaux et al., 2012).

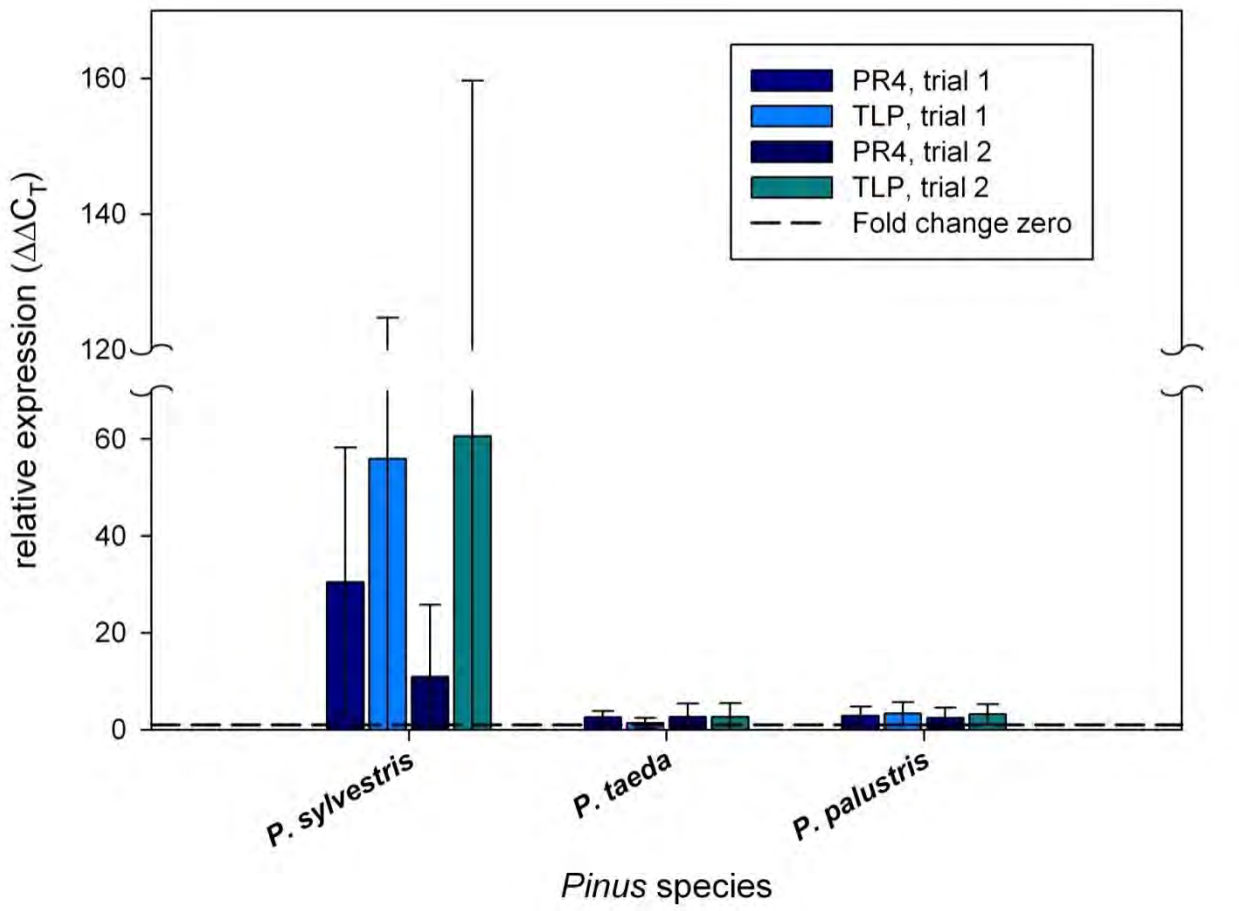


**Figure 5: Fold-change in expression of biomarker genes in three pine species responding to *S. noctilio* venom. The dashed line represents a ratio of one, signifying no fold-`change difference in relative expression. Trees sampled were N=5 in Trial 1, and N=6 in Trial 2 for *P. taeda* and *P. palustris*, respectively, and N=3 for *P. sylvestris* for both trials.**

**Discussion**

As sessile organisms that must cope with highly variable environmental conditions, plants monitor their growth conditions closely and respond quickly to changes by varying the expression levels of many different genes (Atkinson and Urwin, 2012). Thus, it is not surprising that gene expression patterns observed when plants are grown under controlled conditions in a laboratory differ significantly from the expression patterns in field grown plants. Among the more responsive genes in this regard are those involved with plant defense responses (Richards et al., 2012). Such variations in gene expression are problematic for studies within species and even within clonal or isogenic lines, not to mention between the highly heterozygous genotypes that are typical of pine populations or between species. Inherent variability in expression of some genes may be dampened in such situations by using trees grown in a common garden, but various microclimatic conditions, including differential lighting, soil chemistry and drainage, and water supply, will still lead to variations between samples. Despite these issues, this study

clearly demonstrated the utility of the PCR primer pairs developed on the basis of sequence information from loblolly pine for detecting and quantifying cognate transcripts in a wide variety of other pine species. Also, while the high degree of variability in gene expression from sample to sample indicate that these biomarker genes would be unreliable for detecting and assessing the degree of *S. noctilio* attack under field conditions, general trends across all experiments indicate that woodwasp venom tends to lead to increased expression of these genes to varying extents in the species tested.

Discoordinate expression of the two biomarker genes, PR4 and TLP, was not seen previously in venom-treated loblolly or Monterey pines grown under laboratory conditions, but was seen in several instances in this study. Thus, we observed that expression of the two biomarker genes differed not only in magnitude of relative expression (Fig. 3C, D) but in direction, as well (Fig. 4). Clearly field-grown trees offer a special challenge for the selection of useful biomarker genes, and experiments to identify potential biomarkers useful under these conditions will likely need to sample venom-treated field-grown trees.

One additional important observation from this study was that Scots pine, a European species that co-evolved with *S. noctilio*, consistently demonstrated stronger induction of biomarker gene expression in response to venom compared to all of the North American species we tested. Strong up-regulation of defense genes correlates with increased resistance in many plant-pest systems; however, it will take significantly more effort to establish whether or not this is the case for the *Sirex-Pinus* pathosystem. The degree of biomarker induction seen here indicates that Scots pine recognizes *S. noctilio* venom quickly and responds strongly. Further work with this species pairing could identify breeding targets for efforts to develop pines that are more resistant to this pest.

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