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# Virulence, sporulation, and elicitin production in three clonal lineages of *Phytophthora ramorum*

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ABSTRACT

*Phytophthora ramorum* populations are clonal and consist of three lineages. Recent studies have shown that the clonal lineages may have varying degrees of aggressiveness on some host species, such as *Quercus rubra*. In this study, we examined virulence, sporulation and elicitin production of five *P. ramorum* isolates from each of the three clonal lineages. Virulence (lesion size) and sporulation (sporangia production) were determined on wound-inoculated detached leaves of *Rhododendron catawbiense* 'Nova Zembla'. Lesion area differed between the clonal lineages ($p < 0.001$) with the EU1 and NA2 isolates producing significantly greater lesion areas than did NA1 isolates on inoculated leaves (approx. 4.2, 3.6, and 0.8 $cm^2$ respectively). Similarly, lineages EU1 and NA2 produced significantly more sporangia per leaf ($p < 0.001$) than did lineage NA1 (approx. 800, 1000, and 300 sporangia per leaf respectively). Real-time PCR assays detected expression of the class I elicitins (ram-α1 and ram-α2) in all 15 isolates. Of the two elicitins, only the ram-α2 differed between lineage ($p < 0.0001$) with nearly 2-fold higher levels of expression in the EU1 and NA2 lineages as compared to the NA1 lineage. Ram-α2 expression showed a positive linear relationship with isolate virulence or lesion size ($R^2 = 0.707$). A significant, positive, linear relationship was also observed between ram-α2 expression and sporulation although it was not as strong ($R^2 = 0.209$). In summary, isolates belonging to clonal lineages EU1 and NA2 are generally more virulent, produce more sporangia, and produce more ram-α2 elicitin *in vitro* than isolates belonging to lineage NA1.

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## 1. Introduction

Sudden Oak Death caused by the plant pathogenic oomycete, *Phytophthora ramorum*, contributes to significant mortality in various oak species from central coastal California to southern Oregon [1]. Currently, three clonal lineages of *P. ramorum* have been identified in the United States [2]; the North American lineage (lineage NA1, mating type A2) is responsible for infections in CA and OR forests [3]. The European lineage (lineage EU1, predominantly A1) is responsible for infections in Europe, but has also been found in nurseries in OR and WA [4]. A third lineage (NA2, mating type A2) has only been isolated in a few instances from nurseries in WA and CA [5].

Phenotypic and adaptive differences have been observed between NA1 and EU1 isolates of *P. ramorum* [6–8]. NA1 isolates were slower growing, and varied more between isolates, than their EU1 counterparts [7]. Within the NA1 isolates, Brasier et al. [7] noted two general colony morphologies, wild type (fast-growing) and non-wild type (typically slow-growing). Interestingly, subcultures from individual NA1 isolates gave rise to both the wild type and non-wild type morphologies, although the factors responsible for this variability are unknown. Virulence of the EU1 and NA1 isolates was also examined by phloem inoculations of mature *Quercus rubra* stems, a rigorous test of comparative pathogenic ability. In three different experiments, Brasier et al. [7] found that populations of EU1 isolates produced greater lesion areas as compared to populations of NA1 isolates. The lower virulence in the NA1 isolates, as compared to the EU1 isolates, was present for both the wild type and non-wild type NA1 morphologies, although the slow-growing non-wild type cultures were the least virulent.

Elicitins are 10 kDa proteins produced by most *Phytophthora* and *Pythium* spp. and are thought to aid in sterol uptake from the environment, an absolute requirement for sporulation [9]. *Phytophthora* spp. can discriminate between sterols and differentially utilize sterols from their growth media, affecting growth and sexual reproduction [10,11]. In addition, *Phytophthora* elicitin production

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has been shown to vary by isolate [12] and the host species [13,14]. In this study, we compared two measures of fitness, sporulation and virulence, of *P. ramorum* isolates from its three clonal lineages on *Rhododendron catawbiense* 'Nova Zembla'. Elicitin gene expression was also measured in these same isolates *in vitro* and examined for possible correlations with virulence and sporulation.

## 2. Materials and methods

### 2.1. P. ramorum elicitin production

Fifteen isolates, including five isolates from each of the three clonal lineages NA1, NA2, and EU1, were included in the experiments (Tables 1 and 2). Although NA1 isolates have been noted to degenerate upon sub-culturing to become morphologically irregular [7], all NA1 isolates used in this study appeared to be 'wild type' and had a uniform culture morphology. Three replicate cultures (50 ml) of each of the 15 *P. ramorum* isolates were grown in V8 broth (100 ml clarified V8 juice, 1.65 g $CaCO_3$, 1200 ml $dH_2O$) for 30 days at 18 °C, at which point, mycelia and culture filtrates were separated by vacuum filtration (0.45 μm) and stored at −20 °C. Two independent trials were conducted.

#### 2.1.1. Elicitin gene expression

Total RNA was extracted from the stored mycelia (50 mg) of the 15 isolates ($n = 3$) using the RNeasy Total RNA Extraction kit (Qiagen, Germantown, MD, USA) and cDNA synthesized using the RETROscript kit (Applied Biosystems/Ambion, Austin, TX, USA) using the manufacturer's recommendations. TaqMan chemistry was used to determine gene expression for two elicitin genes: ram-α1 [GenBank accession no. DQ680026] and ram-α2 [GenBank accession no. DQ680027]. For each sample, a single multiplex reaction was run and analyzed using the BioRad iCycler machine and software (Hercules, CA, USA). All PCR reactions had a final volume of 20 μl and contained 2 μl cDNA template, 10 μl of 2× Sigma Jumpstart Reaction mix (Sigma–Aldrich, St. Louis, MO, USA), 2.8 μl 25 mM $MgCl_2$, and 3.2 μl $dH_2O$. All three probe/primer sets (Table 1) were added at 0.8 μl 10 μM forward primer, 0.8 μl 10 μM reverse primer, and 0.4 μl 10 μM probe. Amplification conditions were as follows: an initial denaturation of 95 °C 2 min followed by 40 cycles of 95 °C 15 s and 60 °C for 45 s. Gene expression for each primer set was determined using a four-point external curve (0.1–100 ng $\mu l^{-1}$) using cDNA pooled from all 15 isolates, and reported elicitin gene expression values are relativized to β-tubulin.

**Table 1**
*Phytophthora ramorum* isolates.

| Lineage | Isolate[a] | Original name | Source | Location | Year |
|---|---|---|---|---|---|
| NA1 | 4313 | – | *Rhododendron* | OR | 2002 |
| | 4353 | – | *Lithocarpus* | OR | 2003 |
| | 9488 | – | *Lithocarpus* | OR | 2006 |
| | 9650 | – | *Lithocarpus* | OR | 2006 |
| | 9770 | – | *Lithocarpus* | OR | 2006 |
| NA2 | PR-05-002 | RHCC-4 | *Rhododendron* | CA | 2005 |
| | PR-05-166 | MR31 | *Rhododendron* | WA | 2004 |
| | PR-07-031 | 15-WA-M | soil | WA | 2006 |
| | PR-07-057 | 43-WA-SU | *Rhododendron* | WA | 2005 |
| | PR-07-058 | 44-WA-SU | *Rhododendron* | WA | 2005 |
| EU1 | CSL-1727 | 2 028 528 | *Pieris* | UK | 2002 |
| | CSL-2026 | 20 302 104 | *Kalmia* | UK | 2003 |
| | CSL-2065 | 20 304 595 | *Leucothoe* | Ireland | 2003 |
| | CSL-2066 | 20 305 086 | *Syringa* | UK | 2003 |
| | CSL-2097 | 20 309 382 | *Hamamelis* | UK | 2003 |

[a] Isolates were obtained from E.M. Hansen, Oregon State Univ (4313, 4353); C.D. Nelson, USDA-FS-SRS (9488, 9650); J. Laine, Oregon Dept Foresty (9770); D.M. Rizzo, UC Davis (PR-05-002); M. Garbelotto, UC Berkeley (PR-05-166); G.A. Chastagner, Washington State Univ (PR-07-031, PR-07-057, PR-07-058); The Food and Environment Research Agency (formerly CSL), UK (CSL-1727, CSL-2026, CSL-2065, CSL-2066, CSL-2097).

**Table 2**
TaqMan probe/primer sets used for *Phytophthora ramorum* elicitin gene expression.

| Target | Probe/primer | Sequence (5′ − 3′) |
|---|---|---|
| ram-α1 | RAMA1_102F1[a] | GCTCGTGAGCATCCT |
| | RAMA1_123R1[b] | CCGTCAGCATCGAGTAG |
| | RAMA1_172P[c] | CTCGTCGTTCAACCAGTGCGC |
| ram-α2 | RAMA2_102F4[a] | TACGTGGCGCTCGTG |
| | RAMA2_118R2[b] | GTCAGCATCGAGTAGC |
| | RAMA2_170P[c] | TCGGAATCGTCCTTCTCGACGT |
| β-tubulin | PGBT308F[a,d] | GGTACAATGGCACGTCTGATCTC |
| | PGBT429R[b,d] | GGACGCCTATATCGCAAGTCA |
| | PG336BTUBP[c,d] | CGAGCGCATGAACGTCTACTTCAACG |

[a] Forward primer.
[b] Reverse primer.
[c] TaqMan probes: RAMA1_172P is labeled with the reporter dye ROX, RAMA2_170P is labeled with the reporter dye HEX, PG336BTUBP is labeled with the reporter dye 6-FAM.
[d] Winton et al. [30].

#### 2.1.2. ELISA assay

The concentration of elicitin proteins secreted into the culture filtrates by the 15 isolates ($n = 3$) was determined using a custom, indirect ELISA assay using rabbit anti-elicitin polyclonal antibodies (Covance Research, Denver, PA, USA). The ELISA assay was performed as follows. Three technical replicates of each isolate's culture filtrate (200 μl each) were added to individual wells of a Grenier Bio-One Microlon 96-well ELISA plate (San Francisco, CA, USA) and incubated at 37 °C for 1hr. After removal of the antigen solution, the plate was then washed 3× with 300 μl of PBST (0.05% tween 80 in 10 mM PBS) and blocked with 300 μl of PBST plus 3% BSA Fraction V for 1 h at 37 °C. After removal of the blocking solution, 100 μl of the diluted (1:5000 with PBST plus 3% BSA) primary antibody (rabbit anti-elicitin polyclonal antibody) was added to each well and incubated at 37 °C for 1 h. After washing 5 times with 300 μl PBST, 100 μl of the diluted (1:5000 with PBST plus 3% BSA) secondary antibody (HRP-conjugated goat anti-rabbit IgG antibody, Jackson Immuno Research, West Grove, PA, USA) was added to each well and incubated at 37 °C for 1 h. The plate was then washed 5× with 300 μl of PBST and reacted with 100 μl of 1 mg $ml^{-1}$ ABTS (2,2′-azino-bis [3-ethylbenzthiazoline-6-sulphonic acid]) plus 0.03% hydrogen peroxide. Absorbance at 650 nm was recorded every 30 s for 15 min with shaking using a Biotek ELx808 microplate reader (Winooski, VT, USA). Elicitin concentrations were determined using an external standard curve (100, 50, 25, 12.5, 6.3, 3.1, 1.6, 0.8, 0.4, 0.2 μM) using the purified recombinant *P. ramorum* elicitin obtained with a *Pichia* expression system (Supplementary methods).

### 2.2. Fitness experiments: inoculation and incubation

Five isolates of *P. ramorum* from each of three different clonal lineages (Table 1) were collected and grown on V8 agar (100 ml clarified V8 juice, 23.4 g Bacto-agar, 1.65 g $CaCO_3$, 1200 ml water) for 10 days to establish viable mycelia. Ten healthy detatched *R. catawbiense* 'Nova Zembla' leaves were subsequently inoculated with each of the 15 isolates by first wounding the leaf with a sterile push pin and then placing an agar plug containing the isolate directly on the wound, mycelium side toward the leaf. Leaves were incubated at room temperature (19–20 °C) in Rubbermaid containers lined with paper towels saturated with sterile water. There were two trials; Trial 1 was conducted in March and Trial 2 was conducted in April, 2008.

After four days of incubation, the agar plug was removed from the leaf. In trial 1, leaves were misted on the fourth, seventh, and