A rapid infection assay for Armillaria and real-time PCR quantitation of the fungal biomass in planta

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ABSTRACT

Slow and unreliable infection in the greenhouse has been a barrier to research on Armillaria root disease. The existing infection assay takes 7–18 months for detectable infection, during which time the inoculum often dies, resulting in unequal challenge among plants. Because symptom expression and mortality are rare, presence or absence of infection, determined by culturing, is the only datum derived from the existing infection assay. This limits both routine comparisons of strain virulence and complex investigations of pathogenesis, neither of which have been done for Armillaria mellea. We tested a new infection assay, in which grape rootstocks growing in tissue culture medium are inoculated, and compared to rootstocks previously characterized from the existing infection assay as tolerant (Freedom) or susceptible (3309C). Culture media of 25 plants per rootstock was inoculated and five plants per rootstock were harvested 0, 2, 4, 6, and 8 weeks postinoculation; the experiment was completed twice. Confocal microscopy and quantitative PCR (Q-PCR) were used to quantify infection. Roots were treated with WGA-AlexaFluor488, hyphae and roots were scanned on green and red channels on a confocal microscope, and percent root colonization was quantified. A fungal gene (EF1a) was determined to have a single copy in A. mellea, and both EF1a and a single-copy grape gene (UFGT) were amplified by Q-PCR; fungal DNA: plant DNA served as a measure of fungal biomass. Armillaria was detected by culture, microscopy, and Q-PCR starting 2 weeks postinoculation from all inoculated plants, demonstrating that the new infection assay is rapid and plants do not escape infection. Our findings of higher percent root colonization (as measured by microscopy) of 3309C than Freedom at all harvests (P < 0.0001), consistently higher fungal biomass (as measured by Q-PCR) of 3309 than Freedom, and a significant positive correlation between percent root colonization and fungal biomass (P = 0.01) suggests that the quantitative methods of our new assay give similar results to the qualitative method of the existing infection assay.

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Introduction

Armillaria root disease is a serious threat to fruit and nut crops, timber trees, and ornamentals in temperate and tropical regions of the world (Hood et al. 1991). The causal pathogens are species of the genus Armillaria (Basidiomycota, Physalacriaceae), the most virulent of which include Armillaria mellea, Armillaria ostoyae, and Armillaria tabescens. A. mellea is
known for its virulence among fruit and nut crops throughout the northern hemisphere [e.g., Citrus, Juglans, Malus, Prunus, Pyrus, and Vitis in western Europe (Guillaumin 1977; Guillaumin et al. 1989) and the US (Baumgartner & Rizzo 2001; Proffer et al. 1987)]; annual yield losses of Vitis vinifera (grape), the highest value fruit crop in the US (Anonymous 2007), are 10–40% in California vineyards (Baumgartner 2004). A. ostoyae is one of two root pathogens responsible for the majority of timber losses in North America and Europe (Bendel et al. 2006; Kile et al. 1991; Lung-Escarmant & Guyon 2004); estimates of annual timber losses in western North America alone are 2–3 million m³ (Morrison & Mallett 1996). A. tabescens is one of the most destructive pathogens of Prunus persica (peach) in the southeastern US (Cooley 1943; Rhoads 1954; Savage et al. 1953; Schnabel et al. 2005); annual yield losses are estimated at $1.5 to $4 million per individual peach-producing state (Miller 1994; Williams-Woodward 2002).

Among the most virulent Armillaria species is A. mellea, which occurs not only in orchards and vineyards, but also in forests (Rishbeth 1982, 1991) and urban areas (Coetzee et al. 2001; Motta & Korhonen 1986; Rishbeth 1983). Replanting with another host after removing an infected one is unlikely to eliminate attack from A. mellea because of its broad host range (300+ species; Raabe 1962). The pathogen spreads vegetatively when host roots grow to contact residual roots and are subsequently colonized by mycelium (Redfern & Filip 1991). Fallowing infected soil is not cost-effective for fruit crops because A. mellea lives as a saprobe on residual roots for decades (Baumgartner & Rizzo 2002; Rizzo et al. 1998). Cultural and biological methods improve yields, but only temporarily (Baumgartner 2004; Baumgartner & Warnock 2006). A. mellea is recalcitrant to soil fumigation (Bliss 1951; Gubler 1992; Munnecke et al. 1981), a common means of controlling root pathogens, and resists other conventional fungicide applications (Adaskaveg et al. 1999; Aguin et al. 2006), as well.

Little is known about infection and pathogenesis of A. mellea, despite its broad host and geographic ranges, and the lack of effective controls. For example, it is not known where on the roots the pathogen first colonizes, if it extensively colonizes the root surface before penetration, what the mechanism of root penetration is, if it grows inter- or intracellularly, in which root tissues it colonizes, or which of these steps coincide with symptom expression. It is not known if virulence varies among strains or if they exhibit host-specificity. It is not known why A. mellea is a virulent pathogen on 300+ species of woody plants (Raabe 1962), but also colonizes achorophyllous orchids, Galeola septentrionalis and Gastrodia elata, that are myco-heterotrophs of A. mellea [i.e., the plant parasitizes the fungus (Leake 1994)]. The gene encoding an antifungal protein from the orchid, gastrodianin (Xu et al. 1998), has been cloned and transformed into Prunus domestica [e.g., plum (Nagel et al. 2008)], but there is no in planta assay to determine if transgenic plum is more resistant to A. mellea than the wild-type.

An infection assay is needed to examine the infection biology of A. mellea, to better understand the disease cycle, to learn how A. mellea can function as a parasite in some hosts and as a beneficial symbiont in others, and to identify resistant plant material. Inoculation techniques that have been developed for other important root pathogens have helped advance the understanding of the plant diseases that they cause [e.g., Phytophthora lateralis (Oh & Hansen 2007), Fusarium oxysporum (Zhou & Everts 2004)]. Such advances are outside our reach for A. mellea, however, because the existing infection assay is time-consuming, unreliable, and qualitative.

The existing infection assay involves cultivating mycelium on autoclaved wood for 3–6 months and, afterwards, tying the resulting inoculum to root collars of potted plants with string (Mansilla et al. 2001; Raabe 1979; Raziq & Fox 2003, 2005; Singh 1980). This infection assay has several limitations. Detectable infection, based on culture from the inoculation site, occurs 4–12 months postinoculation (Entry et al. 1991; Mallet & Hiratsuka 1988; Thomas 1934). Inoculum often dries out and dies in the 4–12 months before infection. Because foliar symptoms (e.g., stunted shoots, chlorotic leaves) and mortality are rare, treatments are compared in terms of number of plants from which the pathogen is cultured out of total inoculated (percent infection), rather than symptom severity or percent mortality. After 7–18 months, an Armillaria researcher is, thus, left with two groups of plants: 1) noninfected plants that are either resistant to infection or escaped infection, and 2) infected plants that cannot be further characterized by symptom expression, mortality, or degree of infection. Although replication can help distinguish resistant plants from escapes, albeit in an additional 7–18 months, simply repeating the experiment cannot address the fact that there is no means of distinguishing plants with limited versus extensive colonization. With its host range of hundreds of species, we expect most plants to become infected by A. mellea. However, presence/absence of infection as the only response variable gathered from the existing infection assay is not a useful measure for answering research questions about infection biology and pathogenesis, or for comparing variation in strain virulence or host resistance.

The goal of our research was to develop a new infection assay for Armillaria that is rapid, that equally challenges all inoculated plants, and provides a quantitative measure of infection. Our approach was to inoculate grapes grown in tissue culture medium with A. mellea, and to quantify root colonization by confocal microscopy and quantitative real-time PCR (Q-PCR) at regular intervals. We tested the ability of the new assay to differentiate grape rootstocks, the susceptibility of which was previously characterized using the existing infection assay (Baumgartner & Rizzo 2006; De Luca et al. 2003; Mansilla et al. 2001; Raabe 1979). Grape was selected over other hosts because it is one of the most economically important hosts of A. mellea and it is susceptible to infection throughout the pathogen’s geographic range [e.g., Italy (Pertot et al. 2008), Spain (Aguin-Casal et al. 2004), Switzerland (Prospero et al. 1998), and California (Baumgartner & Rizzo 2002)]. Q-PCR primers were tested on several Armillaria species, in order to evaluate the new infection assay for use in multiple Armillaria pathosystems.

**Materials and methods**

**Plant propagation and inoculation**

Plant material consisted of grape rootstocks grown in magenta boxes (75 × 75 × 100 mm), which were propagated...
from green cuttings rooted in grape tissue culture medium (Golino et al. 1998). The approach of co-cultivating the pathogen and host on an agar-based medium has been used for Armillaria mellea on grape (Perazzoli et al. 2007), and for other root pathogens [e.g., Thielaviopsis basicola (Hood & Shew 1996)]. The research was carried out in two independent experiments, with plants for each experiment propagated 2 months apart, and incubated in the same Convirion MTR30 growth chamber (Controlled Environments Limited, Winnipeg, Manitoba, Canada). Stem cuttings (two nodes; ϥ6 cm length × 0.5 cm diam.) from actively growing greenhouse plants of each rootstock were surface-sterilized in 30 % bleach + 0.1 % Tween20 for 15 min, then rinsed six times in sterile dH₂O for 5 min per rinse. One end of each cutting was inserted in the center of each magenta box, to a depth of 2 cm in the tissue culture medium, and magenta boxes with lids atop were placed in the growth chamber. Plants were grown under two F72T12/CW/VHO (cool white, very high output) fluorescent lamps (Philips Lighting Company, Somerset, NJ), with 140 μmol m⁻² s⁻¹ light intensity, 16 h daylight, at 25–27 °C.

There are no reports of grape rootstock performance in infested vineyards. Therefore, we inoculated the most tolerant rootstock, Freedom, and the most susceptible rootstock, 3309C, as identified from a consensus of the only four published studies on grape (Baumgartner & Rizzo 2006; De Luca et al. 2003; Mansilla et al. 2001; Raabe 1979). As all four studies used the existing infection assay, this was the best basis for comparison available from a review of the literature. Our experiment was not meant to be an exhaustive screen of germplasm, but rather a test of the new assay. The priority was to evaluate replicate plants over multiple time periods, within each quantitation method, rather than to evaluate many different plant species. Our main criterion for selecting rootstocks was their phenotype, in terms of infection by A. mellea, and a secondary criterion was their rooting capacity.

In contrast, much of the germplasm screened by Raabe (1979) is not used as rootstock (e.g., Vitis arizonica, Vitis doaniana, Vitis longii, Vitis rubra, and Vitis treleasei), due to poor rooting (Pongracz 1983).

The diploid strain of A. mellea (Son3) used in our new infection assay was also used in the existing infection assay in a previous study (Baumgartner & Rizzo 2006). For our new assay, inoculum was prepared by homogenizing a culture grown in potato dextrose broth with 2.5 mM sodium acetate (14 d, 25 °C, 100 rpm) for 30 s, then spreading 100 μl homogenate on 1 % malt extract agar and incubating at 25 °C for 7 d. Each plant was inoculated after 2 months of growth by placing four agar plugs from the 7-d A. mellea culture on the tissue culture medium at positions approximately equidistant between the plant stem and each corner (Fig 1). Each experiment was restricted to 4 months (including growth preinoculation), which is when rootstocks outgrow magenta boxes and develop nutrient deficiencies. We did not want symptoms of nutrient deficiency to confound symptoms of infection.

**Root sampling**

For each of two independent experiments, five inoculated and two noninoculated plants per rootstock were harvested on the day of inoculation and at four two-week intervals postinoculation (five harvests × seven plants per rootstock × two rootstocks = 70 plants per experiment). At each harvest, shoots were severed, dried at 70 °C for 7 d, and weighed. Roots were carefully separated from tissue culture medium by submerging the root system embedded within the medium in sterile water to break the medium into smaller pieces, then using a forceps to gently pull roots from pieces of medium. Roots were separated into three subsets: 1) root tips (three per plant) were stored at 4 °C in 0.1 M PIPES buffer (Sigma; with 4 % glutaraldehyde, pH 6.8) for confocal microscopy, 2) root tips plus larger root fragments (150 g per plant) were

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*Fig 1 – Inoculation of grape rootstocks propagated in tissue culture medium. The surface of the tissue culture medium for (A), inoculated plants, was inoculated with agar plugs (arrows) from 7-d cultures of Armillaria mellea. For noninoculated plants, (B), plugs (arrows) of noninoculated malt extract agar were used.*
stored at −20 °C for Q-PCR, and 3) root tips plus larger root fragments (three per plant) were plated on water agar (incubated at 25 °C, 10 d) for confirmation of root infection by culturing. Root tips in contact with Armillaria mellea hyphae were scarce on plants at 2 and 4 weeks postinoculation (i.e., plants had a total of three to four root tips), thus necessitating prioritization of root tips for confocal microscopy, which is limited to tissues < 180 μm in diam.

Microscopy

Percent root colonization by Armillaria mellea was quantified by confocal microscopy. Root tips were used because they were within the scanning limit of the microscope (−180 μm), compared to root fragments, which were >180 μm in diameter. Root tips were removed from storage vials, washed three times with 0.1 M phosphate buffer, pH 6.8 (1 h per wash), soaked for 1 h in 20-μg ml⁻¹ Alexa Fluor® 488 – wheat germ agglutinin conjugate (WGA-AF 488, Invitrogen, Molecular Probes, Inc., Eugene, OR) diluted in phosphate buffered saline, washed in 0.1 M phosphate buffer, pH 6.8 (30 min.), then mounted on microscope slides and covered with a glass coverslip. Root tips were imaged with a Leica TSP SP2 confocal microscope (Leica Microsystems, Heidelberg, GmbH), with excitation at 488 nm and detection at 520–552 nm and 664–996 nm, for the fungus (green) and root (red), respectively. Root tips (three per plant) were scanned at 100×, a sequential series of images was collected from upper to lower root surfaces, and images were combined as a maximum projection. Percent root colonization was quantified by converting separate fungal and root maximum projection series to black and white, measuring the area of image occupied by fungus or root tissue, then calculating root colonization as a percentage of root area occupied by fungus (Fig 2). Images were analyzed with ImageJ (v1.4, National Institutes of Health, Bethesda, MD; rsb.info.nih.gov/ij/index.html). Average percent root colonization of three root tips per plant was used for statistical analyses.

Q-PCR

Fungal biomass of Armillaria mellea in roots was quantified by Q-PCR. Genomic DNA was extracted from a 150-mg (fresh weight) subsample of a combination of root tips plus root fragments of each plant (Bhat & Browne 2009). It is important to

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Fig 2 – Confocal microscopy of grape root tips colonized by Armillaria mellea. Fungal hyphae were visualized by WGA-AF 488. Maximum projection series for (A), hyphae, and (B), roots, were compiled from 65 sections. For calculation of percent root colonization by hyphae, images were converted to black and white separately for (C), hyphae, and (D), roots, and the proportion of black pixels that occupied the total area of each image (262 144 pixels) was measured to give fungal area and root area. Percent root colonization was calculated as fungal area/root area × 100. Magnification × 100, bar = 100 μm.
reiterate that there were not a sufficient number of root tips in contact with the hyphae on all plants at 2 and 4 weeks post-inoculation, in order to both examine three root tips per plant under the confocal scope and to extract DNA from 150-g root tips per plant for Q-PCR. Q-PCR quantitation of A. mellea biomass in grape was based on a procedure for quantifying Magnaporthe oryzae biomass in rice (Berruyer et al. 2006). The approach was to amplify fungal and plant genes, in separate Q-PCR reactions with separate primers, from genomic DNA extracted from roots, and to calculate fungal biomass per sample as follows (assuming equal efficiency of extracting fungal and plant DNA):

\[
\text{Fungal biomass} = \left( \frac{\text{ng of fungal gene}}{\text{gene copy number in fungal genome}} \right) \\
\times \left( \frac{\text{ng of plant gene}}{\text{gene copy number in plant genome}} \right)
\]

This approach allowed for quantification of fungal biomass regardless of root biomass or DNA concentration, the latter being particularly difficult to measure in DNA extractions from grape because persistent phenolic compounds have the same absorbance spectrum as DNA and, thus, confound spectrophotometer readings (Loomis 1974; Watson & Blackwell 2000).

The fungal gene amplified from A. mellea in Q-PCR was the nuclear elongation factor subunit 1-alpha (EF1α). Primers EF1α-F1 (5′GGATGGCACCGTGATAACAT) and EF1α-R1 (5′AGTCTTGCCCCTGAGCAC) were designed to amplify a 150-bp section of EF1α (EF150), based on alignments of EF1α from throughout the pathogen’s geographic range (Table 1). Alignments of EF1α also included other northern hemisphere Armillaria species: Armillaria cepistipes, Armillaria gallica, Armillaria gemina, Armillaria nabsnona, Armillaria ostoyae, and Armillaria tabescens. EF150 was internal to the ~600-bp section amplified with primers EF595F and EF1160R (Kauserud & Schumacher 2001) by Maphosa et al. (2006). Q-PCR primers were tested on two grape strains of A. mellea from our collection (Son3, Napa275), on all Armillaria species reported from California [A. gallica, A. mellea, A. nabsnona, North American

| Table 1 – Armillaria (EF1α) and Vitis (UFGT) sequences used for primer design, and collections of both used for Q-PCR primer testing. |
|-----------------|-----------------|-----------------|-----------------|
| Species          | Geographic origin | GenBank ID      | Reference       |
| Armillaria species (strain) |                    |                  |                 |
| A. cepistipes    | British Columbia, Canada | DQ435630 | Maphosa et al. (2006) |
| A. gallica       | Washington, USA | DQ435628 | Maphosa et al. (2006) |
| A. gallica       | New Hampshire, USA | DQ435629 | Maphosa et al. (2006) |
| A. gallica (Napa025)* | California, USA | FJ911684 | This study |
| A. gemina        | New York, USA | DQ435626 | Maphosa et al. (2006) |
| A. gemina        | Vermont, USA | DQ435627 | Maphosa et al. (2006) |
| A. mellea        | South Korea | DQ435640 | Maphosa et al. (2006) |
| A. mellea        | Massachusetts, USA | AY881023 | Metheny et al. (unpub.) |
| A. mellea (Napa186ssb) | California, USA | EU434714 | This study |
| A. mellea (Napa275)* | California, USA | EUJ53259 | This study |
| A. mellea (Son3)* | California, USA | EUJ53258 | This study |
| A. nabsnona      | Washington, USA | DQ435631 | Maphosa et al. (2006) |
| A. nabsnona (Men004)* | California, USA | FJ911685 | This study |
| A. ostoyae       | Vermont, USA | DQ435625 | Maphosa et al. (2006) |
| A. ostoyae (OR001)* | Oregon, USA | FJ911685 | This study |
| A. tabescens     | Ohio, USA | DQ435641 | Maphosa et al. (2006) |
| A. tabescens     | France | DQ435642 | Maphosa et al. (2006) |
| A. tabescens (SC001)* | South Carolina, USA | FJ911687 | This study |
| NABSX (Modo007)* | California, USA | FJ911688 | This study |
| Vitis species     |                    |                  |                 |
| V. vinifera ‘Muscat of Alexandria’ | Hiroshima, Japan | AB047096 | Kobayashi et al. (2001) |
| V. vinifera ‘Muscat of Alexandria’ | Hiroshima, Japan | AB047097 | Kobayashi et al. (2001) |
| V. vinifera ‘Muscat of Alexandria’ | Hiroshima, Japan | AB047098 | Kobayashi et al. (2001) |
| V. vinifera ‘Muscat of Alexandria’ | Hiroshima, Japan | AB047099 | Kobayashi et al. (2001) |
| V. vinifera ‘Italia’ | Hiroshima, Japan | AB047092 | Kobayashi et al. (2001) |
| V. vinifera ‘Italia’ | Hiroshima, Japan | AB047093 | Kobayashi et al. (2001) |
| V. vinifera ‘Italia’ | Hiroshima, Japan | AB047094 | Kobayashi et al. (2001) |
| V. vinifera ‘Italia’ | Hiroshima, Japan | AB047095 | Kobayashi et al. (2001) |
| V. vinifera ‘Lambrusco Foglia Frastagliata’ | Milano, Italy | X75968 | Sparvoli et al. (1994) |
| V. vinifera × V. labrusca ‘Kyoho’ | Hiroshima, Japan | AB047091 | Kobayashi et al. (2001) |
| Freedom*b        | California, USA | EUJ908018 | This study |
| 3309C*b          | California, USA | EUJ908019 | This study |

a Armillaria strains from our collection (all diploid, except for Napa186ssb) that were tested with Q-PCR primers EF1α-F1 and EF1α-R1, and confirmed to give a single PCR product of expected size (150 bp).
b Vitis rootstocks from our collection that were tested with Q-PCR primers UFGT118F and UFGT118R, and confirmed to give a single PCR product of expected size (118 bp).
Biological Species (NABS X), and on a virulent Armillaria species found outside of California, A. ostoyae (Baumgartner & Rizzo 2001). Genomic DNA was extracted from mycelium using the DNA extraction procedure of Porebski et al. (1997). For PCR amplification of EF150, 2 μl of DNA extract was added to 23 μl of the following mixture: 1X colorless buffer (Promega), 1 M Betaine (Sigma), 4 mM MgCl₂ (Promega), 5 μg BSA (Invitrogen), 200 μM dNTPs (Promega), 1 μM per primer (Operon Biotechnologies, Inc., Huntsville, AL), 0.625 units GoTaq(R) Flexi DNA Polymerase (Promega), and sterile molecular biology grade water (GIBCO). PCR cycling parameters were 95 °C for 2 min, 40 cycles at 95 °C for 1 min, 58 °C for 1 min, and 72 °C for 1.5 min, and final extension at 72 °C for 5 min. EF150 from Son3 and Napa275 was sequenced, to verify that primers EF1a-F1 and EF1a-R1 amplified the target region. Primers EF1a-F1 and EF1a-R1 gave no PCR products from Freedom or 3309C.

Gene copy number of EF1α in the A. mellea genome was determined by southern blot hybridization. There are no genes of the following mixture: 1X colorless buffer (Promega), 1 M Betaine (Sigma), 4 mM MgCl₂ (Promega), 5 μg BSA (Invitrogen), 200 μM dNTPs (Promega), 1 μM per primer (Operon Biotechnologies, Inc., Huntsville, AL), 0.625 units GoTaq(R) Flexi DNA Polymerase (Promega), and sterile molecular biology grade water (GIBCO). PCR cycling parameters were 95 °C for 2 min, 40 cycles at 95 °C for 1 min, 58 °C for 1 min, and 72 °C for 1.5 min, and final extension at 72 °C for 5 min. EF150 from Son3 and Napa275 was sequenced, to verify that primers EF1α-F1 and EF1α-R1 amplified the target region. Primers EF1α-F1 and EF1α-R1 gave no PCR products from Freedom or 3309C.

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On each Q-PCR plate, we included known amounts of EF150 or UFGT118 (PCR products amplified from plasmid preps of cloned EF150 and UFGT118) to generate a standard curve, from which total amounts of EF150 or UFGT118 in the samples were estimated based on their cycle threshold (C_T) values. Standards were run in triplicate on each plate. A standard curve was constructed by plotting mean C_T values of 0, 2 x 10⁻⁹, 2 x 10⁻⁸, 2 x 10⁻⁷, 2 x 10⁻⁶, and 2 x 10⁻⁵ total ng to undiluted DNA from noninoculated plants. Dilution of DNA to 1:100 with sterile water was necessary to avoid inhibition. Dilution to 1:10 or 1:50 was insufficient, as was addition of bovine serum albumin (0.6 μg). In addition, Q-PCR products of EF150 and UFGT118 reactions from four samples from inoculated plants (two Freedom, two 3309C) were sequenced, to verify that Q-PCR primers amplified the target regions.

The plant gene amplified from Freedom and 3309C rootstocks in Q-PCR was a 118-bp section of UDP-glucose: flavonoid 3-O-glucosyl transferase (UFGT). UFGT is a single-copy gene, 37 bp section of UFGT (UFGT118), based on alignments of UFGT sequences from Vitis vinifera and V. vinifera × Vitis labrusca (Table 1). UFGT118 was internal to the region amplified with primers 355U and 465L by Goto-Yamamoto et al. (2002). Q-PCR primers were tested on Freedom and 3309C. Genomic DNA was extracted from a 150-μg (fresh weight) subsample of a combination of root tips plus root fragments (Bhat & Browne 2009). PCR amplification of UFGT118 was identical to that of EF150 and EF785, in terms of PCR program and mixture.

UFGT118 from Freedom and 3309C was sequenced, to verify that primers UFGT118f and UFGT118r amplified the target region. Primers UFGT118f and UFGT118r gave no PCR products for A. mellea strains Son3 or Napa275.

For Q-PCR, 1 μl of DNA extract (100–500 ng template DNA, as determined by visualization on a gel, diluted 1:100 in sterile water) was added to 24 μl of the following mixture: 1X Brilliant SYBR Green Q-PCR Master Mix (Stratagene, La Jolla, CA), 150 nM per primer (Operon Biotechnologies), 30 nM ROX Reference Dye (Invitrogen), and sterile molecular biology grade water (GIBCO). All reactions were performed in 200-μl, 96-well plates in an Mx3000p Real-time PCR Thermalcycler (Stratagene). PCR cycling parameters were 95 °C for 10 min, and 40 cycles at 95 °C for 30 s, 58 °C for 30 sec, and 72 °C for 1 min. Samples were run in triplicate reactions, once on each of three 96-well plates. Prior to running all samples, we confirmed the presence of PCR inhibitors in DNA extractions by adding known concentrations of EF150 or UFGT118 (2 x 10⁻⁹, 2 x 10⁻⁸, 2 x 10⁻⁷, 2 x 10⁻⁶, and 2 x 10⁻⁵ total ng) to undiluted DNA from noninoculated plants. Dilution of DNA to 1:100 with sterile water was necessary to avoid inhibition. Dilution to 1:10 or 1:50 was insufficient, as was addition of bovine serum albumin (0.6 μg). In addition, Q-PCR products of EF150 and UFGT118 reactions from four samples from inoculated plants (two Freedom, two 3309C) were sequenced, to verify that Q-PCR primers amplified the target regions.

Statistical analyses

Analysis of variance (ANOVA) was used to determine the effects of independent experiment (1 or 2), rootstock (Freedom or 3309C), harvest interval (2, 4, 6, or 8 weeks postinoculation), and their interactions on percent root colonization, fungal biomass, root tip diameter, and dry shoot weight. The latter two measurements were analyzed as possible indirect measures of infection. ANOVA was performed using the MIXED procedure in SAS, with Kenward-Roger as the denominator degrees of freedom method (Littell et al. 1996). All main and interaction effects were considered fixed effects, and F values with P < 0.05 were considered significant. Prior to ANOVA, the presence of homogeneity of variance across all treatments for percent root colonization, fungal biomass, root tip diameter, and dry shoot weight was determined (Box et al. 1978). To satisfy
assumption of homogeneity of variance, a log_{10} transformation was applied to percent root colonization and fungal biomass, the only parameters for which errors were not normally distributed. A log_{10} transformation was selected based on the slope of the regression line of the log_{10} of the treatment means plotted against the log_{10} of their standard deviations (Box et al. 1978). After ANOVA, for significant effects \( P < 0.05 \), differences among treatment means were assessed by comparison of 95% confidence intervals, such that means without overlapping intervals were considered significantly different (Westfall et al. 1999). Back-transformed geometric means and 95% confidence intervals are presented for percent root colonization and fungal biomass, for ease of interpretation. The CORR procedure of SAS was applied to a data set that combined percent root colonization (dependent variable) and fungal biomass (independent variable). A linear correlation was conducted to measure the intensity of association between percent root colonization, as measured by confocal microscopy, and fungal biomass, as measured by Q-PCR.

**Results**

Q-PCR primers EF1a-F1 and EF1a-R1, which were designed to amplify a 150-bp section of EF1\( a \) from *Armillaria mellea* (EF150), were found to amplify identical sequences from the strain of *A. mellea* that was used for grape inoculations in our new infection assay (Son3) and from a second strain that colonizes grape (Napa275; Table 1). Primers EF1a-F1 and EF1a-R1 were also confirmed to amplify a single 150-bp product from other northern hemisphere *Armillaria* species from our culture collection (*Armillaria gallica*, *Armillaria nabsnora*, *Armillaria ostoyae*, *Armillaria tabescens*, and NABS X). Q-PCR primers UFGT118f and UFGT118r, which were designed to amplify a 118-bp section of UFGT from *Vitis* (UFGT118), were found to amplify identical sequences from both rootstocks inoculated in our new infection assay (Freedom and 3309C; Table 1).

Southern hybridization demonstrated that EF1\( a \) is a single-copy gene in *A. mellea*, based on the presence of single bands in both the EcoRI and BamHI restriction digests of Son3, after exposure of 2.5 h at \( -80 \) °C (Fig 3). UFGT118 from Freedom and 3309C had a single match in a BLAST search of the *Vitis vinifera* cv. Pinot noir genome (Jailion et al. 2007). Because both EF1\( a \) and UFGT are single-copy genes, and because both grape and *A. mellea* are uninucleate, diploid organisms (Pongracz 1983; Ulrich & Anderson 1978), the divisor of both fungal and plant genes, in our calculation of fungal biomass, was 1. Therefore, fungal biomass was calculated as follows:

\[
\text{Fungal biomass} = \left( \frac{\text{ng EF150}}{1} \right) \times \left( \frac{\text{ng UFGT118}}{1} \right)
\]

Hyphae proliferated in the tissue culture medium within days, and were in direct contact with roots at 2 weeks postinoculation (Fig 1). Starting at the first postinoculation harvest and continuing throughout the experiment, both confocal microscopy (Fig 4A and E) and Q-PCR (Fig 5) confirmed the presence of *A. mellea* hyphae within roots of both rootstocks. The pathogen was recovered in culture from roots of all inoculated plants at each harvest interval after inoculation (data not shown). The pathogen was absent from all plants harvested on the day of inoculation and was also absent from all noninoculated plants at all harvest intervals, based on no visual evidence of hyphae with confocal microscopy, no C\( r \) values with Q-PCR, and no recovery of *A. mellea* from roots plated on water agar. No plants died during the 4-months course of the experiment, nor did they show foliar symptoms of infection (stunted shoots, chlorotic leaves).

ANOVA identified significant differences in percent root colonization between rootstocks, as measured by confocal microscopy (Table 2). Rootstock 3309C had significantly higher root colonization than Freedom, with means of 8.77% versus 3.28% \( (n = 40, \text{averaged across both independent experiments and all four postinoculation harvests}) \), respectively (Fig 4). Higher percent root colonization in 3309C was consistent in both independent experiments and at all four harvests, based on the fact that ANOVA showed that the main effects of independent experiment and harvest time on percent root colonization were not significant (Table 2). Percent root colonization, as measured by confocal microscopy, varied between rootstocks, but did not vary over time; means were consistently higher in 3309C than in Freedom (Fig 4). There were no confounding interaction effects of time or independent experiment on the significant main effect of rootstock (Table 2).

ANOVA identified significant temporal increases in fungal biomass, as measured by Q-PCR (Table 2). Fungal biomass increased significantly for both rootstocks over time in both independent experiments (Fig 5). However, there were no significant
Fig 4 – Armillaria mellea colonization of inoculated 3309C [(A), 2 weeks, (B), 4 weeks, (C), 6 weeks, and (D), 8 weeks postinoculation] and Freedom [(E), 2 weeks, (F), 4 weeks, (G), 6 weeks, and (H), 8 weeks postinoculation], as determined by confocal microscopy. Mean percent root colonization is shown (n = 10, averaged across both independent experiments) for each rootstock at each harvest; means followed by different letters are significantly different within each harvest, based on lack of overlap of their 95% confidence intervals. Magnification × 100, bar = 100 μm.
differences in fungal biomass between rootstocks (Table 2), although mean fungal biomass was higher for 3309C than for Freedom at all four postinoculation harvests and in both independent experiments (Fig 5). There was a significant positive correlation between percent root colonization, as measured by confocal microscopy, and fungal biomass, as measured by Q-PCR ($r = 0.96$, $P = 0.01$). Therefore, plants with high percent root colonization also had high levels of fungal biomass.

There was a significant effect of the harvest × rootstock interaction on root tip diameter (Table 2). Means comparisons within each rootstock, based on overlap of 95% confidence limits and reported here for the sake of being thorough, showed that root tip diameter of 3309C increased from the first to the last postinoculation harvest, whereas Freedom remained unchanged (Table 3). There were significant effects of independent experiment and harvest on shoot weight (Table 2), but means comparisons showed no differences between the two experiments or among harvests (Fig 6). Therefore, despite the fact that 3309C had significantly higher percent root colonization, as measured by confocal microscopy, and consistently higher fungal biomass, as measured by Q-PCR, both rootstocks had similar shoot growth among harvests throughout both independent experiments.

### Discussion

Our finding of significantly higher percent colonization, as measured by confocal microscopy, of 3309C compared to Freedom at each of four postinoculation harvests and in both independent experiments, suggests that Freedom supports less extensive colonization by *Armillaria mellea*. Based on Q-PCR, 3309C had greater fungal biomass than Freedom. Although the Q-PCR results were not statistically significant, the trend of higher fungal biomass in 3309C versus Freedom was consistent at each of four postinoculation harvests and in both independent experiments. In addition, linear correlations showed a significant positive relationship between percent root colonization, as measured by confocal microscopy, and fungal biomass, as measured by Q-PCR. These findings are consistent with those of our previous observation of a higher frequency of infection (i.e., a greater proportion of infected plants out of the total inoculated) of 3309C relative to Freedom, as determined using the existing infection assay (Baumgartner & Rizzo 2006). Other authors determined that 3309C is a susceptible *Vitis* rootstock, also using the existing infection assay (De

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**Table 2 – F values for analyses of variance for percent root colonization (measured by confocal microscopy), fungal biomass (measured by Q-PCR), root tip diameter, and dry shoot weight of inoculated grape rootstocks, Freedom and 3309C.**

<table>
<thead>
<tr>
<th>Source</th>
<th>Num df</th>
<th>Den df</th>
<th>$F$ values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Percent root colonization</td>
</tr>
<tr>
<td>Experiment</td>
<td>1 64</td>
<td>64</td>
<td>0.20</td>
</tr>
<tr>
<td>Harvest</td>
<td>3 35</td>
<td>35</td>
<td>0.81</td>
</tr>
<tr>
<td>Experiment × harvest</td>
<td>3 35</td>
<td>35</td>
<td>0.89</td>
</tr>
<tr>
<td>Rootstock</td>
<td>1 64</td>
<td>64</td>
<td>19.33***</td>
</tr>
<tr>
<td>Experiment × rootstock</td>
<td>1 64</td>
<td>64</td>
<td>0.05</td>
</tr>
<tr>
<td>Harvest × rootstock</td>
<td>3 35</td>
<td>35</td>
<td>1.07</td>
</tr>
<tr>
<td>Experiment × harvest × rootstock</td>
<td>3 35</td>
<td>35</td>
<td>0.32</td>
</tr>
</tbody>
</table>

* *, **, *** Indicate significance at $P \leq 0.05$, 0.01, and 0.0001, respectively.

a Percent root colonization was measured by confocal microscopy and was calculated as fungal area/root area × 100. Fungal biomass was measured by Q-PCR and was calculated as (total fungal DNA/gene copy number in the fungal genome): (total plant DNA/gene copy number in the plant genome). Root tip diameter was measured by confocal microscopy. Dry shoot weight was determined by harvesting shoots at the surface of the tissue culture medium, drying at 70 °C for 7 d, and weighing. Num = numerator, Den = denominator.

b Source of variation: independent Experiment 1 or Experiment 2 (experiment), 2, 4, 6, or 8 weeks postinoculation (harvest), Freedom or 3309C (rootstock).
3309C and Freedom. Mean shoot weight is shown (Fig 6 – Temporal changes in shoot weight of inoculated grape rootstocks. Means (n = 10, averaged across two independent experiments) followed by different letters within the same rootstock are significantly different, based on lack of overlap of their 95 % confidence intervals.

Table 3 – Temporal changes in root tip diameter of two grape rootstocks. Means (n = 10, averaged across two independent experiments) followed by different letters within the same rootstock are significantly different, based on lack of overlap of their 95 % confidence intervals.

<table>
<thead>
<tr>
<th>Rootstock</th>
<th>Harvest (weeks postinoculation)</th>
<th>Root tip diameter (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3309C</td>
<td>2</td>
<td>84.47a</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>79.63a</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>99.10ab</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>108.07b</td>
</tr>
<tr>
<td>Freedom</td>
<td>2</td>
<td>92.30a</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>73.37a</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>77.79a</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>87.10a</td>
</tr>
</tbody>
</table>

Luca et al. 2003; Mansilla et al. 2001). Therefore, our new infection assay gives comparable results to the existing infection assay, at least with respect to differentiating infection of 3309C and Freedom by A. mellea.

With 7–18 months from the start of inoculum production to first infection (Entry et al. 1991; Mallet & Hiratsuka 1988; Thomas 1994), replicating inoculations over time with the existing infection assay is cumbersome. The characteristics of unequal chance of infection (due to inoculum death) and qualitative measure of infection by means of culturing (presence/absence) seriously limit the capacity of the existing infection assay to differentiate resistant versus susceptible hosts and avirulent versus virulent strains, for example. Such research questions for Armillaria are still unanswered. The new infection assay is rapid and reliable, based on our confirmation of infection as early as 2 weeks postinoculation from all inoculated plants, using both the qualitative method from the existing infection assay (culturing) and two new methods (microscopy and Q-PCR). The new infection assay also provides for quantification of infection on a per plant basis. Cytological observations of infection can be made in a more rapid and repeatable manner, in order to adequately address questions on infection and pathogenesis. Cytological observations can be further combined with investigation of the numerous cell-wall degrading enzymes produced by Armillaria (Billard & Thurston 1996; Curit et al. 1997; Mwenje & Ride 1997; Robene-Soustrade et al. 1992; Wahlstrom et al. 1991), not to mention the melleolides (Donnelly et al. 1985; Donnelly et al. 1986; Midland et al. 1982), a unique class of bioactive small molecules that have been shown to inhibit other root pathogens, e.g., Heterobasidion annosum, and host cells (Peipp & Sonnenbichler 1992). The new infection assay is adaptable to other hosts, albeit those that root well in tissue culture medium, such as Prunus spp. (Mante et al. 1989), Rosa spp. (Foroozabady et al. 1994), Quercus spp. (Manzanera & Parados 1990), and Ficus spp. (Gupta & Durzan 1985). Also, given that our EF150 primers amplify all Armillaria species in California and all virulent North American Armillaria species, Q-PCR can also be adapted to disease diagnostics.

Our efforts to identify a single-copy gene in A. mellea are notable, in terms of Armillaria research, at least, as single-copy genes are extremely useful for various aspects of comparative genomics, systematics, and evolutionary biology. EF1α is now the only gene of confirmed copy number in any Armillaria species. It is true that other PCR primers are available for the following regions: EF1α (~600 bp section of southern hemisphere Armillaria species [Kauzerud & Schumacher 2001]), rDNA intergenic spacer 1 (IGS-1) [all Armillaria species (Coetzee et al. 2000a; Coetzee et al. 2000b; Duchesne & Anderson 1990; Smith-White et al. 2002; Veldmen et al. 1981)], rDNA internal transcribed spacer (ITS) [all Armillaria species (Smith-White et al. 2002; White et al. 1990), and natural product genes [A. mellea (Misiek & Hoffmeister 2008)]. These other PCR primers did not suit our study because the regions they amplified were too variable among species (e.g., ~600 bp section of EF1α), they amplified multicopy genes (e.g., IGS-1 and ITS), or they were likely to be too species or strain-specific (e.g., natural product genes of A. mellea). Microsatellite markers are also available for Armillaria gallica (LeFrancois et al. 2002), A. mellea (Baumdgartner et al. 2009), and Armillaria ostoyae (Langrell et al. 2001; Prospero et al. 2008; Worrall et al. 2004), but there was little utility for loci of unknown function in our study. The utility of having a gene of known copy number extends beyond this study to other applications, such as determining the number
of transgene inserts in transformants of Armillaria using Q-PCR, instead of using the standard, and more cumbersome, method of southern hybridization.

There was a discrepancy, based on ANOVA, between the results of Q-PCR—no significant differences between rootstocks—and microscopy, which did show significant differences. We could not use the same root pieces for both microscopy and Q-PCR, and it is possible that the greater proportion of root fragments in the Q-PCR samples, relative to root tips, affected the results. Due to the fact that there were very few root tips in contact with mycelium at 2 and 4 weeks postinoculation, there were not sufficient root tips for both microscopy and Q-PCR (six per plant). If Q-PCR was based only on root tips at each harvest, as was the case for confocal microscopy, differences between rootstocks might then have been apparent. Nonetheless, rootstock differences in fungal biomass, as measured by Q-PCR, are greatest at 6 and 8 weeks postinoculation, which is when Q-PCR samples did consist of three root tips per plant.

Similar to our previous study using the existing infection assay (Baumgartner & Rizzo 2006), plants inoculated using the new infection assay neither developed foliar symptoms nor died from infection. Furthermore, shoot and root growth of the rootstocks was not differentially affected, as might be expected based on our finding of differential levels of infection. It is possible that 2-months postinoculation are not sufficient for development of foliar symptoms or for mortality. However, grape rootstocks tend to outgrow their magenta boxes after 4 months, and with insufficient root growth within the first 2 months of propagation, it was not possible to inoculate plants earlier. Of course, it is possible that other rootstocks are more susceptible than 3309C and would have outgrown the new infection assay (Baumgartner & Rizzo 2006), plants inoculated using the standard, and more cumbersome, method of southern hybridization.

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REFERENCES


