12-2014

# Evaluation of Medicago truncatula Accessions for Triterpene Saponins and their Potential Impacts on Plant Pests

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A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Plant Pathology

by

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University of Central Arkansas
Bachelor of Science in Biology, 2011

December 2014
University of Arkansas

This thesis is approved for recommendation to the Graduate Council.

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**Abstract**

Plants have developed numerous ways to protect themselves from microbes and insects, including producing secondary metabolites that negatively impact an invading pathogen or pest. Saponins are specialized metabolites found in many plant species and may play a role in protecting the plant. Though saponins are found in many plant species, triterpene saponins are found primarily in dicotyledons, including legumes. *Medicago truncatula* is a model system for studying legume biology and some accessions accumulate high concentrations of saponins. Accessions of *M. truncatula* known to have differing levels of saponins in both foliar and root tissues were tested for their suitability as hosts for the necrotrophic fungus, *Phoma medicaginis*, the pea aphid, *Acyrthosiphon pisum*, and the root-lesion nematode, *Pratylenchus penetrans*. Fungal disease measured by whole-plant ratings or via localized development were highest on ESP105, an accession with very low foliar saponins, and A17, which is derived from a commercial cultivar compared to accessions containing high concentrations of saponins. A trend of accessions with high saponin levels in foliar tissue being more resistant to *P. medicaginis* infection was found. Only one accession, GRC43, limited population growth of aphids compared to other lines. No significant differences were observed in nematode populations among the accessions tested. An isolate of *P. medicaginis* expressing green fluorescent protein was used to visualize fungal infection and growth on the same accessions of *M. truncatula*. Spread of GFP-expressing hyphae was reduced on lines PRT178 and GRC43, in strong agreement with disease ratings. Finally, plant responses to pests were assessed at a sub-cellular level, and accumulation of several transcripts encoding enzymes of the saponin biosynthetic pathway were affected post inoculation with *P. medicaginis*, pea aphid and *P. penetrans*. This research validates the importance of using accessions of *M. truncatula* to study saponin effects on pests and suggests potential promise of saponins as biological controls for various pests.

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# Introduction

## Plant Defenses

Plants have evolved to defend themselves against unfavorable environments, harmful pests and lethal pathogens. Plant immune responses include but are not limited to secreting antimicrobial compounds, detecting pathogens, and defense signaling which can lead to transcriptional reprogramming (Kwon and Yun, 2014). Plants have pattern-recognition receptors (PRRs) like the *Arabidopsis* FLS2 (flagellin receptor) that recognizes bacterial flagellin (Zipfel et al., 2004). Transcriptional reprogramming during innate immunity responses in plants is brought about by an interaction between PRRs and pathogen-associated molecular patterns (PAMPs). Downstream events then occur through intracellular signaling by MAPK (mitogen-activated protein kinase) cascades and transcription factors like WRKY/TGA (Kwon, 2010). Another defense plants have at combating pests are resistance (R) proteins that respond to effectors transported from the pathogen or pest into the plant cells to disrupt plant immune responses (Jones and Dangle, 2006).The R proteins are part of effector-triggered immunity (ETI). Effector-triggered immunity can act in concert with PAMP immunity, prompting the plant to undergo cell death and a localized hypersensitive response to prevent the spread of a pathogen (Dodds and Rathjen, 2010). Plants also utilize systemic acquired resistance (SAR), where the infestation or infection on one leaf leads to increased resistance throughout the plant (Glazebrook, 2005). Crosstalk, whereby induction of one signaling pathway can impact another signaling pathway, is very important in the immune responses in plants to both pathogens and insects. Wound-induced protein kinase, WIPK, has a crosstalk between salicyclic acid (SA) and jasmonic acid (JA) pathways, in tobacco. By increasing WIPK activity in tobacco JA levels

increase, however if WIPK is decreased through gene-silencing there is an increased SA production after wounding (Seo et al., 1995; Seo et al., 1999). Other crosstalk events that are relevant to plant defense are MAPK crosstalk with pathogen responses, to osmotic stress, hormone signaling and cytokinesis (Jonak et al., 2002).

Most plant defenses fit into three major categories of defense strategies, and these are structural defenses, protein defenses, and chemical defenses. Examples of structural defenses are the waxy cuticle covering exterior parts of the plant, and trichomes and thorns. Structural defenses are the first line of defense and provide physical barriers between the plant and harmful pests. An interesting structural defense is glandular trichomes which contain various oils and chemical compounds to inhibit biotic stresses such as insects. For example, wild-tomato glandular trichomes entrap potato aphid, and reduce both probing and feeding by the aphid (Goffreda et al., 1988).

Proteins and enzymatic activities can also be utilized by the plant to defend itself. Among the wide variety of defensive proteins are protease inhibitors, chitinases and defensins. Another example of defensive proteins is the attachment arabinogalactan protein (attAGP), in tomato, which are released when the parasitic plant dodder infects (Albert et al., 2006).

Plant chemical defenses include toxins active against pathogens and insects, insect anti-feedants and repellants, and other specialized metabolites. Some types of defenses, such as a thick cuticle or chemical defenses, are constitutively expressed, meaning they are produced at all times in the plant. Other defense compounds are typically not expressed under non-stressed conditions, but can be induced following exposure to a biotic stress. Plants have developed many

effective adaptations for detecting pests, and also the signaling and biosynthetic pathways that are triggered to produce these defensive compounds.

**Saponins**

An example of chemical defensive compounds is a class of molecules known as saponins, which are secondary metabolites found in many plant species such as soybeans, garlic, and spinach (Carelli et al. 2011). Well over 450 plant species in more than 90 families contain saponins (Hostettmann and Marston, 2005; Kofler, 1972).

The word "saponin" is derived from the Latin "sapo" meaning frothing (Naoumkina et al. 2010). Saponins are glycosidic compounds with base structures of triterpene aglycones called sapogenins (Naoumkina et al. 2010). The saponins found in *Medicago* spp. are a complex mixture of pentacyclic triterpene glycosides (Carelli et al. 2011). In *M. truncatula* the triterpene saponins are derived from β-amyrin and can be separated into two classes described as hemolytic, e.g., hederagenin and zanhic acid, which are considered the most bioactive of the saponins, and the other class is non-hemolytic, e.g., soyasapogenol A and B, which are less bioactive (Tava et al., 2011 and figure 1). Saponins in *M. truncatula* are glycosides of the triterpene aglycones zahnic acid, medicagenic acid, hederagenin, bayogenin and soyasapogenol A, B and E (Argentieri et al. 2008, Suzuki et al. 2002, Confalonieri et al. 2009 and figure 1). The total saponin profile of hosts is influenced by many factors such as the environment, genotype, stage of growth and plant tissue (D'Addabbo et al. 2011). The aglycone, the nature and the location of the sugar moieties, and the overall profile all appear to influence the biological properties of saponins (Argentieri et al. 2008).

Saponins are found primarily in dicotyledonous plants (D'Addabbo et al., 2010). In addition to the more common triterpene saponins, steroidal saponins are also found in the plant kingdom. There are two groups of steroidal saponins, spirostanol glycosides and furostanol glycosides, they do not have the same properties as the triterpene saponins but they are found mostly in monocots such as rice, wheat, and corn (Hostettmann and Marston, 2005).

Saponins can be used for a variety of industrial and medical uses. Some are the preparatory material for semi-synthesis of drugs, some are used as emulsifiers, foaming agents (Tava and Avato 2006), and have been used as adjuvants in vaccines (Sun et al. 2009).

To date, saponins have been associated with all of the following properties: anti-inflammatory, antiviral, antitumor, antibacterial, cytotoxic activities, fungicidal, mollusicidal, antibacterial, insecticidal, nematicidal, anti-palatability, antipalatability factors, and phytotoxic (reviewed in Tava and Avato 2006 and table 1).

Among the most studied saponins with activity against biotic pests is avenacin found in oat roots. This saponin is unique in its ability to fluoresce under UV light without any treatments to the roots. Avenacin saponins have antifungal properties and provide the oat roots protection against the soilborne pathogen, *Gaeumannomyces graminis* var *tritici*, the causal agent of Take-all disease (Osbourn 1996, Mylona et al. 2008).

Triterpenes are produced in plants predominantly via the cytosolic mevalonic acid pathway (Suzuki et al. 2002). Three enzymes are responsible for the final steps in the formation of the precursor triterpene leading to the saponins, β-amyrin. These enzymes act sequentially and are squalene synthase, squalene epoxidase and β-amyrin synthase (Sadeghi, et al. 2009).

The first committed step in the synthesis of saponin triterpenes in *Medicago* is the catalyzation by a specific oxidosqualene cyclase known as β-amyrin synthase (Suzuki et al., 2002). Transgenic *M. sativa* transformed with a novel β-amyrin synthase gene, AsOXA1, accumulated increased amounts of some triterpenic compounds indicating that this enzyme controls a key regulatory step in the pathway (Confalonieri et al., 2009).

**Biological Pests**

Herbivorous insects, nematodes and pathogens are known to trigger plant defense responses and cause economic and production losses on crops. The work described in this thesis will focus on host plant responses and interactions with a broad spectrum of plant pests, specifically a fungal pathogen, aphids and a parasitic nematode.

The fungal pathogen *Phoma medicaginis* infects *Medicago* species causing spring black stem and leaf spot disease on alfalfa, an important forage crop (Jasinski et al. 2009). The pathogen is a necrotroph, as it completes part of its life cycle on dead host tissue. Disease symptoms seen with this pathogen are stem canker, leaf spot and seedling blight. Infected plants will show leaf chlorosis and defoliation, stem and petiole lesions that can be severe, and crown and root rot (Ellwood et al. 2006). The conditions that favor this disease are cool and wet environments (Ellwood et al. 2006). The conidia are dispersed via splashing rain.

Aphids are widespread phloem-feeding insects that are a major pest in the agricultural world. In temperate regions, one in four plant species can be infested by at least one species of aphid (Dixon, 1998). Aphids feeding on sieve elements, part of the phloem, can cause severe damage to the plant by draining it of nutrients, and can infect the plant with pathogenic viruses

(Dixon, 1998). The feeding style of aphids generally circumvents the plant's wound defense system, because aphids do not cause severe wounding and therefore they sometimes avoid detection by the host. The octadecanoid signaling pathway has been linked to resistance to pea aphid in *M. truncatula*; resistant plants expressed 10 out of 13 genes that are involved in the octadecanoid pathway after an infestation with blue-green aphid, *Acyrthosiphon kondoi* (Gao et al., 2007). The octadecanoid pathway can function in defense against aphids via jasmonic acid-regulated responses. Following aphid infestation there is an up-regulation of both salicylic acid and jasmonic acid (Thompson and Goggin, 2006); the salicylic acid pathway is important for the resistance to potato aphid in tomato for *Mi*-1-mediated resistance (Li et al., 2006). Induction of salicylic acid (SA)-mediated defenses is effective against aphids. Salicylic acid has been shown to be increased upon aphid feeding and application of comparable concentrations of SA in affected plants decreased fecundity of aphids fed an artificial diet (Donovan et al., 2013). Induction of SA-mediated signaling includes many downstream events such as production of triterpene saponins and other secondary metabolites (Gao et al., 2007).

A root-lesion nematode known for its destructive ability in many crops, such as red raspberry, is *Pratylenchus penetrans* (Zasada et al., 2010). This nematode is a migratory endoparasite, meaning it freely moves in the soil in search of nutrients, and will they feed and move within plants, mainly the roots. The nematode feeding causes necrotic lesions in the roots and hinders the plant's ability to provide for its own nutritional needs, which results in stunting of the plant. A severe infestation of *P. penetrans* can result in plant death. *Pratylenchus penetrans* is known to infect numerous plant species making it difficult to control through crop rotation. Recent changes to regulations eliminate the use of effective nematicidal products such

as methyl bromide, limiting options available to control nematodes. Therefore, growers must rely more on natural host defenses and forms of resistance found within a plant species.

***Medicago truncatula***

The barrel medic, *M. truncatula* is grown as an annual forage crop in Australia, northern Africa and Mediterranean countries. In Western Australia, *M. truncatula* is grown on over one million hectares in pasture dry-land farming and as a rotation crop (Barbetti et al., 2006). *Medicago truncatula* is also a model system for studying legume biology. There are several attributes that make *M. truncatula* a useful system for biological research, including a rapid germination time, a small diploid genome, self-fertile reproduction, distinctive secondary metabolites and availability of well described accessions (Tivoli et al. 2006, Confalonieri et al. 2009). This species also serves as a host organism of necrotrophic pathogens like *Phoma medicaginis.* Although *Arabidopsis thaliana* has been used as a host plant to study a few necrotrophic pathogens (Dickman and Mitra, 1992; Koch and Slusarenko, 1990), it has limited potential as a host for necrotrophic pathogens compared to *Medicago* spp. (Tivoli et al. 2006). Current work on plant defenses using *M. truncatula* includes studies using soilborne and foliar pathogenic fungi, insects, and mycorrhizal fungi (Young et al., 2011; Salzer et al., 2000; Leitner et al., 2005).

Alfalfa, *M. sativa*, is a close relative to *M. truncatula* that is commonly grown in the United States primarily as a forage crop (Tivoli et al. 2006). In 2013 the United States had 7.16 million hectares of alfalfa with an economic value of over 10.8 billion dollars (http://www.nass.usda.gov/Publications/Ag_Statistics/2013/chapter06.pdf). Because *M.*

*truncatula* is closely related to alfalfa, advances in knowledge of plant defense using this species have potential to be transformative in an important forage crop.

## Objectives

### Hypothesis

We propose that accessions of *M. truncatula* with varying levels of saponins can be used to determine whether high levels of these secondary metabolites play an important role in defense against selected aphids, nematodes, and fungi. The hypothesis is that accessions with the highest overall levels of hemolytic saponins will be less suitable hosts for the biotic pests tested.

### Objective 1.

To test the hypothesis stated above, accessions of *M. truncatula* known to have differing levels of saponins in both foliar and root tissues will be tested for their suitability as hosts for the pea aphid, *Acyrthosiphon pisum*, the root-lesion nematode *Pratylenchus penetrans*, and the necrotrophic fungus *Phoma medicaginis*.

### Objective 2.

An isolate of *P. medicaginis* expressing green fluorescent protein will be used to visualize fungal infection and growth on accessions of *M. truncatula* known to have differing levels of saponins.

### Objective 3.

To assess plant responses to pests at a sub-cellular level, accumulation of transcripts will be measured for genes encoding enzymes of the terpene and saponin biosynthetic pathways, following inoculation with pea aphid, root-lesion nematode, or *P. medicaginis*.

## Materials and Methods

### Plant, aphid, nematode and fungal maintenance

The *M. truncatula* isoline A17 is derived from a commercial cultivar Jemalong and seed was provided by the Samuel Roberts Noble Foundation. Seeds for accessions ESP105, GRC43 and PRT178 were obtained from the Institut National de la Recherche Agronomique (INRA), Toulouse, France. For germination, seeds were scarified in concentrated sulfuric acid for five minutes and then rinsed with $dH_2O$. Scarified seeds were surface sterilized by soaking for one minute in 95% ETOH, thoroughly rinsed with $dH_2O$ and further treated for five minutes in a solution of 20% commercial bleach and 0.1% Tween 20, followed by rinsing with water. The surface-sterilized seeds were germinated on moist filter paper in the dark for at least 24 hours before planting into autoclaved Redi-Earth potting medium. After two weeks the seedlings were transplanted into 4-inch square pots with autoclaved Sunshine LC1 potting mix. The plants were treated weekly with Miracle-Gro© tomato plant food (N-P-K ratio 18-18-21), 15 g per 3.78 liters of water, and Gnatrol©, 1.7 g per 3.78 liters. The plants were maintained in a growth chamber set to 16 hr light:8 hr dark daily regimen and 22.5°C.

*Phoma medicaginis* was obtained from Drs. Richard Dixon and Bettina Devours, Samuel Roberts Noble Foundation, Ardmore, OK. The fungal isolate was maintained on V8 agar (100 ml V8 juice, 8.45 g agar per liter) at room temperature in dark conditions. Fresh cultures were started on V8 agar from spores stored in 30% glycerol at -80 $^{o}$C.

Transgenic isolates of *P. medicaginis* expressing green fluorescent protein (GFP) were maintained initially on 0.2X PDA agar media with hygromycin B as the selective antibiotic agent and then on V8 agar.

Pea aphids, *A. pisum*, were provided by Dr. John Reese, Kansas State University. Aphids were maintained on 6- to 12-week-old *Vicia faba*, Fava bean, in a mesh cage in a growth chamber set to 21°C, and transferred to fresh plants as needed to prevent overcrowding.

Root-lesion nematodes, P. *penetrans*, were provided by Dr. Antoon T. Ploeg, University of California – Riverside on corn, tomato and rose roots. Additional cultures were provided by Dr. Paula Agudelo, Clemson University on alfalfa plated in Petri dishes. Nematodes were maintained in clay pots on alfalfa roots with 60:40 river sand: play sand.

### ***P. medicaginis*** **preparation and disease assays**

To test host suitability of the four accessions of *M. truncatula* to *P. medicaginis,* whole plants were inoculated with a spore solution of *P. medicaginis*, and rated for disease progression. The spore suspension was prepared immediately before inoculation by dispensing 3 ml of sterile $dH_2O$ onto a Petri plate containing a sporulating culture (at least two weeks old) and dislodging spores with a flame-sterilized glass rod. The resulting spore suspensions were each transferred into a 15 ml conical tube and diluted 1:5 in sterile $dH_2O$. Spore suspensions were placed on ice and spore concentration was quantified with a hemocytometer. Spores were further diluted to a final concentration of $10^6$ spores/mL in a solution of 0.1% Tween 20. Plants were inoculated with the spore suspension in a chemical fume hood using a mist sprayer until run-off. Mock-inoculated control plants were treated in the same fashion with a solution of 0.1% Tween 20. Inoculated plants were held at 100% relative humidity for 48 hr, in a dew chamber with the following settings: 19°C wall, 30°C water and 26°C air. After 48 hr, plants were transferred to a growth chamber, and each was fitted with a clear plastic bag covering the plant to maintain the

relative humidity at 100% to promote disease. The plants were rated for disease progression 7 to 10 days after inoculation.

Plants were rated for disease with a scale adapted from Salter and Leath (1991). Plants were rated with whole numbers from 1 to 5: 1, resistant with healthy, symptom-free growth; 2, resistant with small (≤ 2mm) brown or black lesions or flecks and no defoliation; 3, rated susceptible with larger (2 to 3 mm) discrete lesions on either leaves or petioles and no defoliation; 4, susceptible with large (≥ 3mm) lesions and no defoliation; and 5, susceptible with lesions ≥ 3mm, visible fungal fruiting, with dead leaves and/or defoliation. Data was compiled and analyzed using the statistical software, Prism with a non-parametric t-test to determine significance.

Trifoliates on the whole plant were wounded with a tracing wheel and immediately spray-inoculated with a spore solution as described above. The plants were placed in a dew chamber with the same settings as described previously. Trifoliates were observed and photographed to record the symptoms of the infection in the early stages at three and five days post-inoculation. The images were analyzed with Image J© and the mean percent area of disease was quantified for each image. This is performed by first adjusting the hue saturation and brightness to highlight the non-diseased area of each leaf, followed by measurement with the "analyze particles" command, which generates a measure of diseased area. Data was compiled and analyzed using Prism with an ANOVA and Tukey's multiple comparison test to determine significance.

**Aphid bioassays on accessions of *M. truncatula***

Pea aphids were staged for plant feeding assays by placing 20 fecund females onto a caged four-week-old fava bean plant for 11-13 days. This insured that resulting offspring used for bioassays were relatively young and of similar age. Aphids were transferred with a fine, camel hair brush. For aphid bioassays, three 11- to 13-day-old staged aphids per plant were placed onto leaves of 6-week-old *M. truncatula*. Eight plants of each *M. truncatula* accession were infested with three aphids per plant inside an 8 L clear plastic bucket with fine netting on top secured with a rubber band. The buckets were placed on a greenhouse bench. Aphid offspring were quantified after 12 days. The aphids were removed by cutting the plant at the soil line and submerging all above-ground tissue in a solution of 0.1% liquid dish detergent at approximately 30ºC. The liquid was then strained through miracloth to collect the aphids, and aphids were manually counted. Plant tissue was blotted dry with paper towels, and fresh weight of the foliar tissue was recorded 10 minutes after the aphids were removed. The population of aphids and the plant fresh weights were analyzed with Prism statistical software with an ANOVA and Tukey's multiple comparison test to determine significance among accessions.

**Nematode bioassay on accessions of *M. truncatula***

To evaluate the host suitability of the four accessions of *M. truncatula* to *P. penetrans*, all accessions were inoculated with a suspension of 100 nematodes per pot. The accessions were grown in 4-inch clay pots containing a ratio of 60:40 river sand:play sand, topped with a thin layer of Sunshine LC1 mix to promote a favorable environment for the nematodes. There were eight replications per each accession, and nematodes were quantified 50 days after inoculation.

For nematode extraction, soil from the pots was removed and placed into a 2 gallon bucket and filled with tap water and mixed thoroughly by hand. The soil-water mixture was poured over a #60 sieve, 250 mm mesh size, to remove debris, and collected into a 2 gallon bucket. The collected liquid was poured through a #400 sieve, 38 mm mesh size, and nematodes were collected on the screen surface. Nematodes were collected by lightly spraying water across the top of the screen, and the resulting suspension was centrifuged at 1500 rpm for 5 min. The pellet was resuspended in 5 ml of 2.0 M sucrose and centrifuged for 2 min. The nematode-containing supernatant was decanted over a #500 sieve, 25 mm mesh size, and the filtrate was discarded. The contents held on the #500 sieve were collected by rinsing with a mild stream of distilled water and the nematodes collected in a final volume of 20 ml. Nematodes were quantified in 1 ml of the suspension by placing them onto a sectored disk and visualizing via microscopy. Individuals were counted in four sections and the numbers were averaged over the whole disk to determine final concentration.

To initiate the exit of the nematodes that were feeding internally in the plant, roots were lightly rinsed with water and rough chopped with sterile razor blades to segments ranging from 1 cm - 2.5 cm. Roots were placed onto a sheet of facial tissue over window screen mesh of 1 x 1 mm, and placed in water so that the surface of the water just covered the roots, allowing nematodes to leave the roots and move through the screen into the water. After five days the resulting suspension was filtered through a 500 sieve and nematodes from the sieve surface were collected and quantified as described above.

**GFP-*P. medicaginis* bioassays on accessions of *M. truncatula***

A gene expressing GFP was introduced into the necrotrophic fungal pathogen, *P. medicaginis* via *Agrobacterium tumefaciens*-mediated transformation. The *A. tumefaciens* strain AGL1 was provided by Dr. Stephen Marek, Oklahoma State University. The plasmid vector pBHt2_sGFP, provided by Dr. Thomas Mitchell, The Ohio State University, was introduced into *Agrobacterium* via the method of Utermark and Karlovsky (2008). The binary vector pBHt2_sGFP contains the sGFP gene driven by the *ToxA* promoter and a hygromycin resistance gene, *HPH*, driven by the *trpC* promoter (Li, 2013). The *A. tumefaciens* strain with the vector was prepared from a glycerol stock streaked onto LB agar medium containing 50 µg/ml carbenicillin and 50 µg/ml of kanamycin and grown at 30°C. A single colony was placed into 5 ml of LB medium amended with kanamycin, rifampicin and carbenicillin at 50, 50, and 25 µg/ml, respectively, in a 50ml conical tube and shaken for two days at 250 rpm at 30°C. The saturated culture was centrifuged at 4000 x g for 5 minutes at room temperature; the supernatant was quickly removed and 1 ml of induction medium (25 M potassium phosphate buffer, MN buffer( $MgSO_4$, NaCl), 10 mg $ml^{-1}$ $CaCl_2$, 1 mg $ml^{-1}$ $FeSO_4$ , IM-salts( $H_3BO_3$, $ZnSO_4 \cdot 7H2O$, $CuSO_4$, $MnSO_4$, $Na_2MoO_4$), 200 mg $ml^{-1}$ $NH_4NO_3$, 50% glycerol, 1 M MES(pH 5.5), and 200 mg $ml^{-1}$ glucose) was added to the pellet and the tube was vortexed to uniformly resuspend the cells. The suspension was transferred to a 2 ml microcentrifuge tube and centrifuged at 4000 x g for 5 minutes at room temperature. The bacterial pellet was resuspended in 150 µl of induction medium and diluted further with induction medium supplemented with 200 µM acetosyringone, to activate *vir* genes, to an $OD_{600}$ of approximately 0.15. Cells were transferred to an Erlenmeyer flask with LB media and grown with vigorous aeration at 28°C until the culture reached an $OD_{600}$ of 0.3. The culture was stored at 4 $^oC$ until the co-cultivation step. A 200 µl

1:1 mixture of the *P. medicaginis* spore suspension ($10^6$ per ml) and the induced *A. tumefaciens* were spread onto a surface of cellophane on induction medium agar supplemented with 200 µM acetosyringone. Plates were incubated in the dark for 60 hours at 23°C. The cellophane membranes were then transferred from the plate using sterile forceps onto new selection plates containing 200µM cefotaxime and hygromycin B. After 7 – 10 days transformed colonies were visible as white mycelial growth, and the green GFP expression was visible under UV light. Positive colonies were transferred onto fresh selection plates as described above. After three transfers the transformed cultures were maintained on V8 agar plates without antibiotic selection.

Spore solutions were collected by dispensing 3 ml of sterile dd$H_2O$ onto a fungal culture plate and dislodging spores by agitating the culture with a flame-sterilized glass rod. The resulting spore suspension was collected with a sterile transfer pipette and Tween 20 was added (0.01% final concentration). Spores were counted with a hemocytometer and diluted to $10^6$ spores/ml in sterile dd$H_2O$ with 0.01% Tween 20. For inoculation, trifoliates were removed from the plant as branches holding 3-5 fully expanded leaves. Spores were applied to a leaf in 2 µl with mild wounding from the pipette tip. Branches were incubated in a Petri plate with a water-saturated sterile filter paper and wrapped in cellophane to maintain high humidity. The trifoliates were then sectioned with a razor blade and vacuum infiltrated with microscopic immersion oil, and observed via fluorescence microscopy on a Nikon 90i upright confocal microscope at 24, 36, 48, 72 and 96 hours after inoculation. The GFP fluorescence was observed via excitation at 470 nm and light emission at 509 nm. At 100x magnification the entire wound site and the fungal growth around and in the wound was visible. A confocal image was taken and the Z-stack images were composed of 40 to 60 stacks. The images were analyzed using NIS-Elements AR Analysis software. Non-GFP fluorescent layers were removed from the images and the final

image was converted to a jpg file. All Z-stack layers were compressed into a single image using Max Intensity projection analysis. The experiment was repeated three times, and in each case at least eight inoculation sites were observed per accession. The images were analyzed as described above with Image J© and the mean percent area of fluorescence was quantified for each image. Data was compiled and analyzed using Prism with an ANOVA and Tukey's multiple comparison test to determine significance.

**Evaluation of saponin content in *M. truncatula* accessions**

The levels of saponins in leaf tissue in the four individual accessions of *M. truncatula* were evaluated after extraction and analysis. The detached leaves were freeze-dried with a lypholizer for at least 48 hours. The weight was recorded and used to calculate how much 85% MeOH, 25 ml of 85% MeOH to 1 g of tissue, to use when pureeing the tissue. A hand-held electric blender was used to puree the sample into slurry that was then poured into a 1000 ml Erlenmeyer flask using a glass funnel. The flask was sealed with parafilm and placed at room temperature for 3.5 hours with shaking at 150 rpm. The slurry was filtered through 4 layers of cheesecloth into 200 ml plastic conical tubes and centrifuged for 15 min at 5000 rpm to pellet large fragments. The supernatant was transferred to a round-bottom flask and the methanol was removed with a rotary evaporator. The remaining sample, in water, was transferred to a 50 ml conical tube and stored at -20°C. Saponins were purified from the crude extract via solid phase extraction with Waters Sep-pak Vac 35cc C18 columns. The columns were prepared with two washes of 35% MeOH and 10 ml of the extract in 35% methanol was loaded onto the column. Each column was rinsed twice with ddH2O followed by two rinses with 15% MeOH. Saponin

fractions were eluted with 100% MeOH and collected. Saponin samples were stored at -20° and later dried to powder using rotary evaporation and a stream of nitrogen gas.

Aliquots of the saponin extracts were sent to the Samuel Roberts Noble Foundation. Samples were analyzed by gradient elution with reverse-phase HPLC with on-line UV and mass selective detection. The separation on HPLC was done using a 250 x 4.6 mm i.d., 5µm, reverse-phase, C18 column (J.T. Baker). Samples were eluted with a linear $H_2O$/acetonitrile gradient at a flow rate of 0.8ml/min. The saponins were identified using HPLC retention times, mass spectra, and tandem mass spectral data and with the additional information of authentic standards for known saponins. Known standards included, 3-Glc-medicagenic acid, 3-Glc-Glc-Glc-23-Ara-28Ara-Rha-Xyl-Api-zanhic acid from alfalfa and soyasaponin I from soybeans. The relative total saponin content of each of the four accessions was compiled (Huhman et al. 2005).

**Assessment of transcripts of triterpene and saponin biosynthetic pathway in accessions of *M. truncatula***

To assess plant responses to pests, accumulation of transcripts for genes encoding enzymes of the terpene biosynthetic pathways was measured following inoculation with *P. medicaginis,* pea aphid, root-lesion nematode and beet armyworm. Tissue was collected for RNA analysis when pest evaluations in each case where completed. Leaf tissue inoculated with *P. medicaginis* was collected after six days. Foliar tissue was collected on the 12$^{th}$ day of feeding for the aphid experiment. The roots were collected 24 days after inoculation with *P. penetrans* nematode. Three to four trifoliates were removed 24 hours after beet armyworm infested and feed on the different accessions of *M. truncatula.* Tissue, either root or leaf, was collected

following each treatment and RNA was isolated with Tri-reagent (Molecular Research Center, Inc. Cincinnati, OH) according to the manufacturer's recommended protocol. At the time of collection the tissue was removed from the plant and quickly placed into labeled foil packets, frozen in liquid nitrogen and stored at -80°C. Frozen tissue was ground in a pre-chilled mortar and pestle and approximately 0.2 g of powdered tissue was placed into a 2-ml microcentrifuge tube containing 1 ml of Tri-reagent. After incubation at room temperature for 10 min., 100µl of 1-bromo-3-chloropropane was added and tubes inverted for 15 seconds, and further inverted every two minutes for 10 minutes. Samples were centrifuged for 15 minutes at 4°C at 12000 x g. The aqueous phase was transferred to a 1.5 ml microcentrifuge tube and 250 µl of isopropanol and 250 µl of high salt solution of 1.2 M NaCl and 0.8 M Na-citrate, were added. After inversion and incubation at room temperature for 7 minutes, samples were centrifuged for 8 minutes at 4°C at 12000 x g. The supernatant was discarded and the RNA pellet was washed with 1 ml 75% ethanol in DEPC water. Samples were centrifuged for 5 minutes at 4°C at 7500 x g and the ethanol was removed and the pellet allowed to dry. The RNA pellet was resuspended with 30 µl DEPC-treated water, centrifuged for 30 seconds and placed on ice for 10 minutes. The sample was heated to 65°C for 5 minutes and RNA resuspended by pipetting up and down. The RNA was quantified using a Biospec spectrophotometer with sterile dd$H_2O$ as the blank.

The RNA was converted into cDNA with the BioRad Iscript kit; containing the reaction mix, reverse transcriptase and nuclease-free water. Total RNA was diluted to 1 µg in a total reaction volume of 20 µl. The cDNA synthesis reaction was incubated 5 minutes at 25°C, 30 minutes at 42°C, and 5 minutes at 85°C, followed by storage at -20 °C

For PCR amplification of plant samples, reactions were setup using Genescript 10x Taq buffer and Genescript Taq polymerase. Thermocycler conditions were 2 min at 95° for the first

denaturing step, followed by 25 cycles of 30 sec at 95°, 30 sec at 55° and 30 sec at 72°, followed by a single cycle of 5 min at 72°. Samples were held at 4° at the completion of the reaction. The products were analyzed on a 1% agarose gel, stained with Gel Green, run at 50V for 10 min followed by 25 min at 85V. The PCR products were visualized with a FluoroChem gel documentation system. The primers used to amplify plant gene transcripts via RT-PCR are listed in table 2.

## Results

### Selection of experimental plant lines

Four *M. truncatula* accessions were chosen based on their reported total triterpene saponin profiles in both foliar and root tissues (L. Sumner, personal communication; table 2). To utilize extreme differences in saponin amounts to test for impacts on pests, accessions A17, PRT178 and GRC43 were selected because each has relatively high saponin levels in leaves, and accession ESP105 was chosen because it had very low saponin levels in leaves (table 3 and figure 2). Demonstrating the wide variation in tissue-specific accumulation of saponins, GRC43 contains low root-saponin amounts whereas accession ESP105 roots are very high in saponins. Accession PRT178 and A17 have intermediate and high root-saponin concentrations, respectively.

To confirm that the experimental plant material matched the reported saponin profiles, individual leaf extracts for each accession were analyzed for total saponin levels. Saponin variants were grouped by the aglycone structure, and relative amounts quantified based on peak area. The first two hemolytic saponins produced in the pathway, hederagenin and bayogenin, were found at low levels in all four accessions (figure 2). Medicaginic acid and zanhic acid are higher in accessions A17, PRT178 and GRC43 and are lowest in ESP105 (figure 2). Two accessions, ESP105 and PRT178 have relatively low levels of soyasaponins and A17 and accession GRC43 have higher levels of soyasaponins (figure 2). The overall levels of each type of saponin in all accessions and tissues used in the experiments described here were in strong agreement with the previously reported values.

**Fungal disease on *M. truncatula* accessions**

Saponins have been reported to be antifungal in nature. To determine if saponins from *M. truncatula* possibly have this effect on a necrotrophic fungus, accessions that vary in saponin levels were challenged with *P. medicaginis* to observe the host-fungal interaction. Two inoculation assays were used to determine the susceptibility of the four accessions of *M. truncatula* to the fungal pathogen *P. medicaginis*.

Whole-plant inoculations were evaluated on the 13$^{th}$ day following application of spores. Accession A17 had an average rating of 3.0 and based on the rating scale is susceptible (figure 3). Accession ESP105 was also rated as susceptible, with an average rating of 3.3 on the rating scale (figure 3). Accession PRT178 had an average rating of 1.3 and GRC43 had an average rating of 1.2 and based on the rating scale are resistant (figure 3). Disease ratings on A17 and accession ESP105 were not statistically different, $p > 0.05$, though they differed from the other two accessions ($p < 0.10$). Disease ratings on PRT178 and GRC43 accessions had no statistical difference from each other, $p > 0.05$, as neither of these accessions showed any severe symptoms.

To evaluate the differences in disease progression on the four accessions at an earlier stage, multiple trifoliates were first wounded and then spray-inoculated with a spore solution of *P. medicaginis*. Wounding was performed with a tracing wheel, which creates a linear series of punctures across the leaf. This proved to be a rapid and reproducible method to assess susceptibility among the accessions of *M. truncatula*. The experiment was repeated three times with similar results each time. At three days post-inoculation, accessions ESP105 and A17 each had large, distinct brown lesions around wound sites (figure 4). The mean percent area of disease

was calculated and at three days post inoculation A17 and ESP105 had 7.74% and 14.39% leaf area diseased, respectively. Accessions GRC43 and PRT178, which were resistant in the whole-plant assays, showed only minor discoloration immediately around the wound sites and small chlorotic regions were occasionally observed (figure 4).

By five days post-inoculation A17 and ESP105 plants displayed chlorosis and spreading necrotic regions, and the brown lesions around wound sites had expanded and coalesced. At five days post-inoculation the mean percent areas of disease for A17 and ESP105 were 22.00% and 22.94%, respectively (figure 5). Lesions around wound sites on both PRT178 and GRC43 plants also increased in size by five days post-inoculation, but necrotic regions spreading from wound sites remained relatively small (figure 4). The mean percent areas of disease at five days post-inoculation for PRT178 and GRC43 were 2.14% and 2.19%, respectively (figure 5). Diseased leaf areas measured on accessions A17 and ESP105 at five days post-inoculation were not significantly different from each other. However, symptomatic areas on these lines were significantly greater than in either PRT178 or GRC43 (figure 4). Overall, the results from this assay were consistent with the whole-plant assay in terms of susceptibility of plant accessions.

**Aphid survival and fecundity on *M. truncatula* accessions**

Saponins have been reported to be insecticidal against species of aphids including pea aphid (Golawska, 2007). The potential effect of *M. truncatula* saponins on pea aphids was analyzed by observing population change over time on the accessions that vary in saponin levels. Aphids were removed and quantified for each replication 12 days post-infestation. Pea aphid populations after 12 days on accessions A17, ESP105 and PRT178 were not significantly

different, $p > 0.05$ (figure 6). The pea aphid population on GRC43 was statistically smaller from the other accessions, $p<0.01$, after 12 days (figure 6).

The size and leaf shape of A17, ESP105 and PRT178 are very similar, however early in its development accession GRC43 has small trifoliates and a more compact stature; it tends to s pread much more horizontally, compared to the other three accessions that have large trifoliates and robust vertical growth. To verify that the differences in aphid populations observed on GRC43 were not affected by the amount of foliar tissue available from the four accessions, the fresh weight of the above-ground biomass from each experimental unit was recorded after the aphids were removed. Pea aphids were found on aerial tissue, such as the petioles, leaves, and stems. There was no strong correlation between the tissue weight and the number of aphids present ($r^2 = 0.5411$), suggesting that aphid reproduction on GRC43 was not limited by availability of plant tissue.

**Root-lesion nematode populations on accessions of *M. truncatula***

The population sizes of *P. penetrans* on the four accessions of *M. truncatula* that differ in saponin levels in root tissue were measured. The population of nematodes present in the soil 50 days after inoculation was not significantly different among the four accessions ($p = 0.6205$; Figure 7). The number of nematodes that were extracted from the roots was minimal and indicated that the nematodes that had been present in roots moved out prior to extraction (data not shown).

**Trifoliate inoculations with GFP-*P. medicaginis* on accessions of *M. truncatula***

To observe infection of *P. medicaginis* microscopically, the fungus was transformed with a gene encoding GFP. The transformed fungus enables the pathogen to be visualized early in the infection process. Trifoliates were removed from each of the four accessions and were spot inoculated with GFP-fungus spore solution. The GFP-expressing isolates that were used, Pm_G5 and Pm_G7, have growth and visible characteristics in culture similar to the wildtype (WT) strain of *P. medicaginis* (figure 8). The inoculation site was observed under 100x magnification at four days post-inoculation and over the entire site of inoculation. Eight inoculation sites were observed on each accession and the experiment was repeated three times. The fungus can be observed colonizing the damaged cells surrounding the wound sites on all plants. There were apparent differences in hyphal growth among the accessions (figure 9). Specifically, A17 and ESP105, the two susceptible accessions, consistently appeared to support greater hyphae development than the two resistant accessions GRC43 and PRT178. Of all accessions, GRC43 appeared to support the lowest levels of fungal growth in this assay. At six days post-inoculation the puncture sites for A17 and ESP105 had more hyphae development; PRT178 had increased slightly and GRC43 had little change in development from four to six days post-inoculation (figure 9). Total GFP fluorescence at the inoculation site was measured at four days post-inoculation and A17 and ESP105 were not significantly different in percent fluorescence (figure 10). Accessions PRT178 and GRC43 were not significantly different in percent fluorescence (figure 10). Inoculated trifoliates of the accessions of *M. truncatula* at six days post-inoculation were significantly different in GFP-derived fluorescence levels. Accessions A17 and ESP105 were not significantly different in percent fluorescence at six days, but both display significantly different fluorescence levels from those observed on the other two accessions. Trifoliates were

inoculated with both the GFP-isolates and the WT of *P. medicaginis* and infection progressed at the same rate in all, indicating that virulence was not impaired by the transformation event (data not shown).

**Transcript accumulation for enzymes involved in triterpene biosynthesis**

To determine if *P. medicaginis* infection affects transcripts encoding putative saponin biosynthetic enzymes, plant tissue was collected from leaf tissue 13 days after infection with *P. medicaginis* and RNA was extracted for analysis.

Squalene synthase, squalene epoxidase and β-amyrin synthase catalyze the final steps in the synthesis of the triterpene precursor of saponins, β-amyrin. The enzymes thought to be responsible for converting β-amyrin into oleanolic acid and downstream triterpenes are classified as cytochrome P450s. One such enzyme, CYP71A612, has been characterized in *M. truncatula* and shown to catalyze synthesis of oleanolic acid (Carelli et al., 2011). Two additional sequences were selected for analysis from an *M. truncatula* expressed sequence tag (EST) database, based on their annotation as encoding cytochrome P450 enzymes and their high relative abundance in insect-damaged EST libraries. All target sequences were derived from A17.

In response to fungal infection, there were no large scale changes in transcript accumulation for any of the genes that were measured. Transcripts encoding enzymes in known and putative steps in triterpene synthesis were not observed to be altered in accessions ESP105, PRT178, or GRC43. In general, transcript sequence amplification for genes encoding triterpene biosynthetic enzymes was lower in these accessions for all genes, compared to levels in A17. In

A17, there appears to be a reduction in the transcripts tested in response to *Phoma* infection, with the exception of the transcript for CYP72A67 (figure 11).

When plants are challenged with insects, there can also be changes in transcript accumulation both locally and systemically. To determine if pea aphid feeding leads to changes in transcript encoding putative saponin biosynthetic enzymes, tissue was collected from leaf material 12 days post-infestation with pea aphids, and RNA was extracted.

For all transcript sequences measured, there was some constitutive expression in each accession, with the exception of CYP716A12 in GRC43 where there was no amplification (figure 12). Expression of the gene encoding the first enzymatic step in triterpene synthesis, squalene synthase, was not impacted by aphid feeding. The overall trend in this experiment was that transcripts for all subsequent steps were suppressed by aphid feeding. The most apparent example of this strong aphid-mediated suppression of transcripts is observed in accession ESP105, where there was little to no amplification for any transcripts in the latter steps of triterpene synthesis (figure 12).

The gene encoding β-amyrin synthase has previously been shown to be strongly induced by chewing insects, such as Diamondback moth larvae (*Plutella xylostella*), in *Barbarea vulgaris* (Wei et al., 2013). To test this response in the accessions tested here, and to contrast this response with the results after extensive feeding from pea aphid, transcripts were measured in leaves damaged by beet armyworm, *S. exigua*. Accessions damaged by *S. exigua* have strongly increased expression levels of β-amyrin synthase compared to non-damaged control plants (figure 13).

To determine if *P. penetrans* alters accumulation of transcripts encoding saponin synthesizing enzymes, nematode-damaged roots were collected 25 days post-infestation and RNA was extracted. Transcripts for each of the enzymes in accession A17 had strong nematode-induced response in the root tissue (figure 14). There were no apparent changes in transcript levels for the same enzymes when *P. penetrans* was present in PRT178 root tissue (figure 14). No amplification of transcript sequences was achieved for any sequences tested, in either ESP105 or GRC43 roots (data not shown).

**Discussion**

Four independent accessions of *M. truncatula*, representing a wide spectrum of foliar and root saponin concentrations, were used to test the hypothesis that high-saponin plants would display enhanced resistance to biotic pests.

Basal levels for triterpene saponins were measured among the *M. truncatula* accessions. As expected, saponins with aglycones oleanolic acid, hederagenin, bayogenin, medicaginic acid, and zanhic acid were detected. These same groups are found also in alfalfa, but generally at lower overall levels (Huhman and Sumner, 2002). As expected, leaves from accession ESP105 have low levels of medicaginic acid and zanhic acid, whereas A17, PRT178 and GRC43 have very high levels of these saponins. In general, the aglycone groups believed to be synthesized last in the pathway, *i.e*., medicagenic and zanhic acids, accumulate to the highest levels suggesting that the early precursors in the pathway are quickly converted to the final products. This finding is in strong agreement with previous reports (Huhman et al., 2005). This dataset confirmed that the plant tissue used in all subsequent experiments contained relative saponin amounts as expected.

Triterpene saponins have been reported to have anti-fungal properties and in this study *M. truncatula* saponins were tested against a necrotrophic fungal pathogen. A well-known example of triterpene saponins acting in disease resistance is the avenacins in oat (*Avena* spp.), which are active against *Gaumannomyces graminis* var. *tritici,* the causal agent of take-all disease. To prove that avenacins contribute to resistance to take-all, oat *sad* mutants, lines that lack the ability to synthesize avenacins, were inoculated with *G. graminis* var. *tritici* and *Fusarium* spp. When wild type oats were inoculated with both *G. graminis* and *Fusarium* pathogens, mild symptoms were present for *Fusarium* and no disease symptoms were seen for *G. graminis,* compared to the *sad* mutants. When oat *sad* mutants were inoculated with both pathogens,

disease incidence increased and the mutants were rated as susceptible to both pathogens. This work is evidence that triterpene saponins are effective against plant pathogenic fungi (Papadopoulou et al., 1999).

To determine whether *M. truncatula* accessions that have high foliar saponins are more resistant to a fungal pathogen, two assays were used. To rate the accessions as susceptible or resistant, Salter and Leath's protocol was adapted and the four accessions were inoculated with spores of *P. medicaginis* and evaluated (Salter and Leath, 1991). Thirteen days post-inoculation the accessions that showed few symptoms and rated as resistant were PTR178 and GRC43. Both of these accessions have high saponin levels in the foliar tissue. The most susceptible accessions were ESP105, which had low levels of foliar saponins and A17, which has high saponin levels in the foliar tissue. There is a trend of accessions having high saponin levels in the foliar tissue being more resistant to the fungal pathogen, *P. medicaginis*, with the exception of A17. The reaction of A17 to the fungal pathogen is unknown at this time but out of the four accessions chosen, it was the only genotype derived from a commercialized breeding line. The A17 line has previously been assessed for reaction to three Australian isolates of *P. medicaginis*, and was found to be susceptible to two isolates and moderately resistant to the third (Ellwood et al., 2006; Kamphuis et al. 2008). Only one isolate was tested in the experiments described here, but it is likely that differential responses could be found using other isolates.

The cultivar Jemalong was the parent line of A17, and has been used as forage for grazing animals. Forage crops are selected in breeding programs to have high amino acid content, to increase feeding quality. Because A17 has been a product of selective breeding, it could be that natural resistance has been decreased or lost due to lack of selection for the trait, and this might

partially account for why A17 reacts differently to *P. medicaginis* than the other three accessions.

The infection process for *P. medicaginis* takes several days and symptom development was sometimes sporadic, so a protocol was adapted from He and Dixon (2000) to observe the colonization of the fungus at an earlier time and to identify distinct differences in the infection process among the four accessions. A tracing wheel was used to create uniform puncture sites on the trifoliates that were then inoculated with spores of *P. medicaginis.* The wound sites increased the rate of colonization, possibly by damaging cells and thereby creating a suitable localized environment of dead cells for the necrotrophic fungus. In addition it is likely that the wound site provides a more accessible route of entry into the host.

At three days post-inoculation with *P. medicaginis* there were differences in symptoms among the accessions. Both ESP105 and A17 had lesions developing around the puncture sites with chlorosis beginning to show around the wound sites in ESP105 trifoliates. The other two accessions, PRT178 and GRC43, lacked symptoms around the wound sites. At five days after inoculation the symptoms had further progressed on A17 and ESP105 with necrotic lesions around the wound sites that had coalesced into larger areas. At five days there were lesions beginning to develop on accessions PRT178, and GRC43 had necrotic lesions surrounding the wound sites but they did not coalesce and did not progress any further on the leaf surface for these two accessions. The results are in agreement with the previous experiment with whole-plant disease ratings, in that accessions A17 and ESP105 are susceptible and accessions PRT178 and GRC43 are resistant. With these two experiments both showing A17 and ESP105 as susceptible and PRT178 and GRC43 as resistant to infection from *P. medicaginis* it further supports the trend that accessions with high saponin levels in the foliar tissue are more resistant

to fungal infection, with the exception of A17. There are other modes of resistance in legumes such as increased medicarpin production in alfalfa which contributed to resistance of alfalfa to *P. medicaginis*. There is also evidence that an isoflavonoid compound is involved in disease resistance to fungal pathogens (He and Dixon, 2000). The level and role of other defense compounds were not determined in this study but could affect disease reactions of the genotypes. Forage crop breeding programs often focus on protein content and forage quality as the primary traits of interest, whereas triterpene saponin content, isoflavonoid content, or disease resistance are often later considerations. Brefeldin A is a toxin that *P. medicaginis* produces both in pure culture and in infected plant tissue. This toxin is produced by the fungus as an effective component of defense against competitors (Weber et al., 2004). Levels of brefeldin A were not measured in this study, but its production could contribute to successful infection on A17 and ESP105. Other necrotrophic pathogens have adapted ways to combat saponins in host tissue. *Botrytis cinerea,* the causal agent of grey mold, has the ability to detoxify four steroidal and triterpene saponins by producing saponinases, xylosidase and glucosidases; saponinases impact the host specificity of the pathogen (Quidde et al., 1998).

Likewise, a study of accessions of *M. truncatula* and their resistance to powdery mildew (*Erysiphe pisi*) found that resistance can relate to how quickly an HR is induced. By inducing cell death it halts the progression of hyphal development from *E. pisi* and therefore stops disease progression (Prats et al., 2007). Interestingly, A17 was found to be resistant to *Colletotrichum trifolii* (race 1 and 2) and *Erysiphe pisi* (isolates Ep-p and Ep-a) (Ameline-Torregrosa et al., 2008).

Transforming organisms with a gene that encodes GFP and the use of a confocal microscope is a useful tool for monitoring pathogens over a length of time on host tissue. *Clavibacter*

*michiganesis* subsp. *michiganis* was transformed with enhanced green fluorescent protein (EGFP) and monitored for several days while it colonized and infected tomato seedlings. The ability to observe the bacterium and how it colonized tomato, and its adherence to the plant, confirmed the theory of how this bacterium colonizes tissue (Chalupowicz et al., 2012). Germinating conidia of GFP-*P. medicaginis* have previously been observed in the epidermal cells on *M. truncatula* leaves (Dhulipala, 2007), allowing visualization of epidermal cell and stomatal penetration by individual conidial germ tubes. To observe the necrotrophic fungus, *P. medicaginis*, colonization on trifoliates of A17 and accessions ESP105, PRT178 and GRC43, the fungus was transformed to express GFP. The fungus colonizes wounded cells and then produces hyphae to extend out on the susceptible A17 and ESP105, with minimal hyphae extensions from resistant accessions PRT178 and GRC43. This supports the whole-plant fungal assay using disease ratings. Saponins are sequestered in the cytoplasm of cells and as cell integrity is disrupted, by either the pipet tip or fungal hyphae, the contents in the cytoplasm are released and can potentially alter the response of the fungus. The saponin-rich cytoplasm of GRC43 and PRT178 could explain the lack of fungal spread in the reactions of both the wild-type *P. medicaginis* and the GFP-*P. medicaginis*.

Triterpene saponins have a negative impact on various aphid species. Both steroidal and triterpene saponins isolated from the soap bark tree, *Quillaja saponaria*, confer strong aphicidal and aphid deterrent activities. The presence of soap bark tree saponins repelled aphids from feeding on artificial diets. The midguts of aphids that consumed saponins had serious damage in cell membranes, which were broken and the contents of those cells were spilled into the gut lumen (De Geyter et al., 2012). De Geyter et al. (2012) observed both repellent and toxic activities of saponins, which destroyed the midgut of the aphid leading to starvation and death.

There were two reference genotypes of *M. truncatula,* A17 and A20, which were used to look at resistance to pea aphid and bluegreen aphid. Of the two genotypes A17 was more resistant to the two aphid species compared to A20. Bluegreen aphid feeding initiated a HR that is mediated by the semi-dominant gene AIN. Though A17 is more resistant than A20, A1N is not responsible for pea aphid resistance in A17 (Klingler et al., 2009). To follow up on A17's resistance to pea aphid, it was challenged along with three other accessions of *M. truncatula* that all differed in triterpene saponin levels to observe pea aphid behavior. At 12 days post-infestation there was a large population of pea aphid supported on accessions A17, ESP105 and PRT178. Of all accessions tested, only ESP105 has low levels of saponins in the foliar tissue. Interestingly, GRC43 has very high reported levels of foliar triterpene saponins and was the only accession that supported a very low population of pea aphid. Two near-isogenic *M. truncatula* lines, Jester and A17, were characterized by their reactions to pea aphids and bluegreen aphids (Gao et al., 2008). Jester was previously categorized as resistant to both pea aphid and bluegreen aphid (Hill, 2000), while A17 was susceptible to both species. Phloem ingestion differed between the two-isogenic lines, providing evidence that resistance is found in the phloem and that pea aphids preferred A17 to Jester (Gao et al., 2008). Although Gao et al. (2008) did not measure saponin levels, it is possible that differences in phloem saponin content might contribute to aphid preference.

Several studies have shown the aphicidal activity of alfalfa foliar saponins at relatively high concentrations. Therefore, Szczepaniak et al. (2001) set out to determine if combining alfalfa root and shoot saponins would be more effective at lower concentrations. They found that saponins from the shoots of alfalfa at 0.5% dose caused 100% mortality at six days, compared to root saponins at 0.5% concentration which at eight days lead to 100% mortality.

Cultivars of alfalfa with high or low saponin levels were compared for effects on pea aphids. Aphid fecundity and reduced performance was observed on a high-saponin line compared to low-saponin lines (Golawska et al., 2006). Aphid behavior was also impacted by saponins. Pea aphids that fed on high-saponin lines had reduced ingestion of phloem sap and prolonged penetration of the cells where saponins are potentially located. The conclusion Golawska et al. (2006) made was that *Medicago* spp. saponins have a strong negative effect on pea aphid reproduction and feeding. Alfalfa cv. Radius is high in saponins medacagenic acid, zanhic acid and soyasaponin I compared to a low-saponin cultivar of alfalfa with comparable levels of soyasaponin I, but low levels of medicaginic acid and no zanhic acid (Golawska et al., 2006). Three alfalfa saponins, zanhic acid and two medicaginic acid, were tested for altering pea aphid feeding behavior using electrical penetration graphs (epg). At high concentrations of saponins (100 ppm) the aphids ingested less phloem and xylem sap compared to low concentrations (50 ppm), where aphids increased phloem ingestion and probing (Golawska, 2007). The low-saponin cultivar lacked zanhic acid and its susceptibility to pea aphids agrees with the bioassay data presented in this study, that the accession with the highest zanhic acid levels also had the lowest population of aphids after 12 days.

The elimination of methyl-bromide as an agricultural nematicide has increased the demand for alternative products in the field. The most effective method to control nematodes is to use resistant plant cultivars, where resistance functions as the result of direct gene-mediated mechanisms or plant metabolites that impair feeding or reproduction. A group of promising plant metabolites with nematicidal activities is the triterpene saponins because of their chemical structure and their ability to disrupt cell membranes. The root-lesion nematode, *P. penetrans,* was chosen because it causes economic losses to many crops and because it is migratory and

endoparasitic. The feeding style of this nematode was desirable because it would travel more in the root system than a sedentary feeder would, and so *P. penetrans* will likely have an increased exposure to root saponins. Also, Elsen et al. (2006) concluded that both *M. truncatula* and *Lotus japonicas* were suitable hosts for several migratory endoparasitic nematodes, like *P. penetrans.* Saponins from alfalfa were tested both *in vitro* at various concentrations and as soil amendments on three nematode species, *Xiphinema index, Meloidogyne incognita* and *Globodera rostochiensis*. At eight hours after addition, the foliar saponins of alfalfa caused 100% mortality *in vitro* and at 24 hours the saponins from roots caused 100% mortality to nematodes. Leaf and root amendments at all doses reduced root and soil populations of nematode species. The most effective soil amendment dose was 4% of either root or foliar tissue added to the potting media to reduce the population of nematodes (D'Addabbo et al., 2010). In an *in vitro* experiment the specific saponin hederagenin had the highest activity of specific saponins, with 38% mortality after 1 hour at 125 µg $ml^{-1}$ against *X. index* (Argentieri et al., 2008). The four accessions used in this study were previously screened for root-knot nematode (*Meloidogyne* spp.) resistance. Dhandaydham et al. (2008) found that accession A17 and GRC43 were both resistant to *M. incognita* and *M. arenaria.* Accessions ESP105 and PRT178 were both resistant to *M. arenaria* but their susceptibility to *M. incognita* was not determined (Dhandaydham et al., 2008). In this study when A17 and the three accessions of *M. truncatula* were infested with *P. penetrans* for 50 days there was no significant difference in populations found in the soil.

Overall, there were differences observed among the accessions of *M. truncatula* when challenged with the three biotic pests tested here, but there was no strict correlation with high saponins and negative effects on the pests.

When *M. truncatula* accessions were challenged with *P. medicaginis,* transcripts encoding enzymes in known and putative steps in triterpene synthesis were measured, there were minimal differences observed in accessions ESP105, PRT178 and GRC43. Squalene synthase did not differ among the accessions. Squalene synthase is important because it is the starting substrate for the biosynthesis of the triterpenes in the genus *Medicago* (Tava et al., 2011). In accession A17 there is an observed suppression in response to fungal infection in all transcripts tested, with exception to CYP72A67.

Previous work shows that insects which cause wounds when feeding, such as beet armyworm, induce the production of β-amyrin synthase. This is important because wound-inducing insects induce plant defense responses quickly and the plant can sometimes deter the pest. Aphid feeding does not cause a wound and therefore does not induce the same immediate defense responses in the plant. This study showed that aphid feeding down-regulates the production of β-amyrin synthase and CYP716A12 in all accessions of *M. truncatula*.

Transcripts for enzymes involved in the biosynthesis of triterpene saponins were analyzed in roots of the different accessions when challenged with *P. penetrans.* In wheat there is a resistance gene, RInn1 that is effective against *Pratylenchus* spp. (Williams et al., 2002). Alfalfa was transformed to express oryzacystatins that when expressed at high levels increased the resistance to the root-lesion nematode, *P. penetrans* (Samac and Smigocki, 2003). There was a complication with RNA extraction for ESP105 and GRC43 accessions. The control transcript, actin, was not amplified and there was no amplification for other transcripts measured, therefore the results for accessions ESP105 and GRC43 were not shown. For A17 and PRT178 roots there were differences in transcript levels between them, with no change in transcript level for PRT178 and A17 having up-regulation of all transcripts tested. To our knowledge enzymes involved in

triterpene saponin synthesis and their induction by nematode feeding have not been studied in the accessions of *M. truncatula* reported here.

Though triterpene saponins from several plant species have been proven effective against biotic pests, the saponins from *M. truncatula* accessions did not show a strong correlation with effective control. In this study, protocols were established for aphid, fungal and nematode bioassays, that make testing the different accessions more efficient. The accessions chosen have different genotypic backgrounds, which add to the difficulty in determining the direct effects of triterpene saponins in this study. Future studies could benefit from crossing the high saponin accession GRC43 or A17 with the low saponin accession ESP105 to develop isogenic lines that differ only in triterpene saponin content.

Table 1. Properties associated with triterpene saponins in various hosts and against various pests

| **Property** | **Saponin, Host and Organism** | **Citation** |
|---|---|---|
| Fungicidal | Avenacins in Oat spp. against *Gaeumannomyces graminis* var *tritici* | Papadopoulou et al., 1999 |
| Insecticidal | *Quillaja saponaria* saponins against aphids | De Geyter et al., 2012 |
| | *Medicago sative* saponins against Colorado potato beetle | Szczepaniak et al., 2001 |
| | *Barbarea vulgaris* against diamondback moth | Shinoda et al., 2002 |
| Nematicidal | *Medicago spp.* saponins against *Xiphinema index* | Argentieri et al., 2008 |
| Antibacterial | *Medicago* spp. saponins against *Bacillus cereus, B. subtilis, Staphylococcus aureus and Enterococcus faecalis* | Avato et al., 2006 |
| Allelopathic | *Medicago sativa* against wheat and barley | Moyer et al., 2003 |
| Cytotoxic activities | Oleanolic acid as cancer treatment | Pollier and Goossens, 2012 |
| Bloat | *Medicago sativa* saponins in cultivars against sheep and cattle | Francis et al., 2002 |

Table 2. Oligonucleotide primers used to amplify transcripts for enzymes in the saponin biosynthetic pathway, and the internal control target actin, in *M. truncatula*.

| **Primer name** | **GenBank accession for target** | **Primer Sequence** |
|---|---|---|
| Squalene Synthase F* | XM_003606992 | 5'GCTTATCTCTCTGCCAATCG'3 |
| Squalene Synthase R* | | 5'AGGTTTGCTCACGATGCCTC'3 |
| Squalene Epoxidase F | XM_003608274 | 5'TCGGAGGTGTTTGCTCAGAT'3 |
| Squalene Epoxidase R | | 5'AAAAGGTCGTTCACACGGTC'3 |
| β- Amyrin synthase F | XM_003604074 | 5'ACAACACAGAGAGAGGATGG'3 |
| β- Amyrin synthase R | | 5'CAACCTTCTTCCGCTAACCG'3 |
| CYP72A67 F | AB558149 | 5'TCCACCAGCATTTGGTGTTA'3 |
| CYP72A67 R | | 5'ACCTCCCCCAGGATCTCAAA'3 |
| CYP72A68 F | DQ335782.1 | 5'GGTACCCTGATTGGCAAGAA'3 |
| CYP72A68 R | | 5'TAAAGGTTTCGTTGTTTGCC'3 |
| CYP716A12 F | FN995113 | 5'TTTGATCGGAGGACATGACA'3 |
| CYP716A12 R | | 5'CAAAAGGGTACGGTCTCTTT'3 |
| MtActin F | XM_003593074.1 | 5'GCTTGATTCTGGTGATGGTG'3 |
| MtActin R | | 5'TCACGGACAATTTCCTTCTCAG'3 |

*F and R designate forward and reverse oligonucleotide primers, respectively, for a target transcript.

Table 3. Reported basal levels of triterpene saponins among accessions of *M. truncatula*[a]

| | Tissue/Location | |
|---|---|---|
| Accession | Foliar | Root |
| A17 | High | High |
| ESP105 | Low | Very High |
| PRT178 | High | Intermediate |
| GRC43 | Very High | Low |

[a] Values based on unpublished metabolomic profiles (Lloyd Sumner and David Huhman, Samuel Roberts Noble Foundation, personal communication).

Table 3. Relative metabolic saponin levels * extracted from *M. truncatula* leaves

| | Accessions | | | |
|---|---|---|---|---|
| Saponin compound | A17 | ESP105 | PRT178 | GRC43 |
| Hederagenin | 1 | 1.55 | 1.1 | 2 |
| Bayogenin | 1 | 1.42 | 1.29 | 1.63 |
| Medicagenic acid | 1 | 0.05 | 0.99 | 0.81 |
| Zanhic acid | 1 | 0.09 | 0.87 | 1.37 |
| Soyasaponin | 1 | 0.5 | 0.47 | 1.09 |
| Soyasapogenol B | 1 | 0.46 | 0.4 | 1.22 |
| Soyasapogenol E | 1 | 0.11 | 0.49 | 0.18 |

*Purified saponin extracts from the leaves were analyzed by Lloyd Sumner and David Huhman, Samuel Roberts Noble Foundation, using reverse-phase high-performance liquid chromatography – mass spectrometry (HPLC/MS) and normalized to A17.

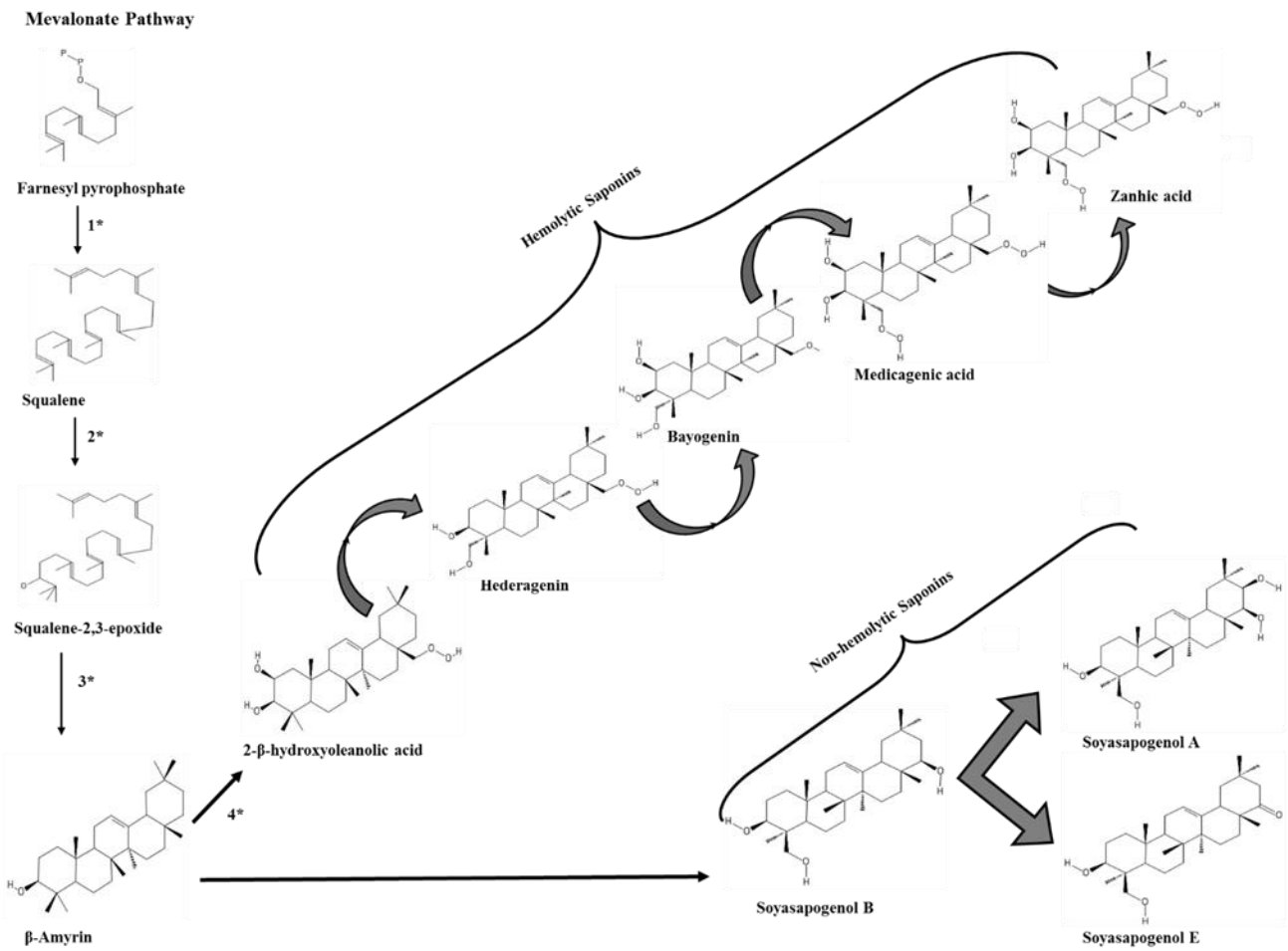


Figure 1. Proposed triterpene sapogenin biosynthetic pathway in *Medicago* spp.. β-amyrin is produced and subsequently converted to the triterpene sapogenin aglycones. Following biosynthesis of the structures shown here, the aglycones are modified with multiple sugar moieties to form saponins.Arrows with asterisks indicate enzymes involved in saponin synthesis, 1* is squalene synthase, 2* is squalene epoxidase, 3* is β- amyrin synthase and 4* is CYP716A12. The triterpene saponins are classified as either hemolytic or non-hemolytic (adapted from Fukishuma et al. 2011, Confalonieri et al., 2009).

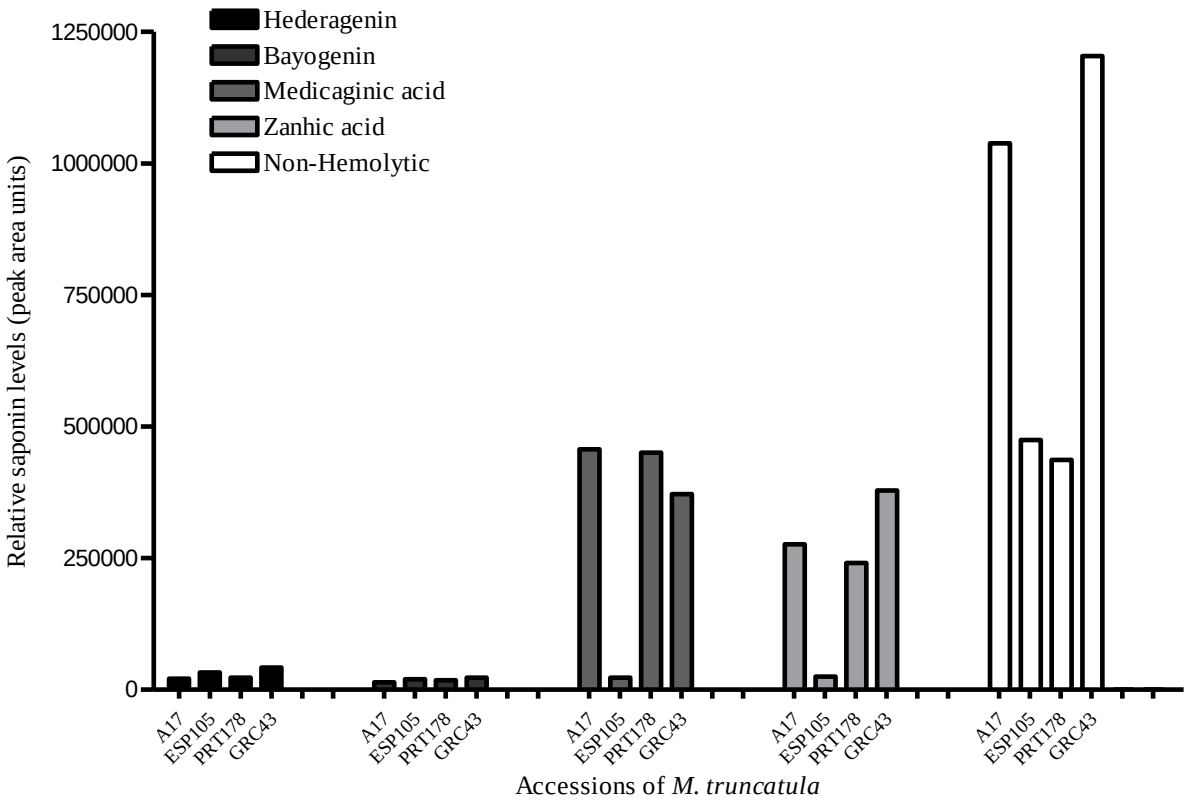


**Figure 2. Relative saponin levels of accessions of *M. truncatula.***
Bars indicate total levels of sapogenin aglycone classes, as determined via HPLC-MS single preparative extracts, from *M. truncatula* leaves. In all four accessions, products containing hederagenin and bayogenin were found at very low levels compared to downstream sapogenins. Hemolytic saponins in ESP105 are considerably lower than in the other accessions. Non-hemolytic compounds include soyasaponin, and soyosapogenols B and E.

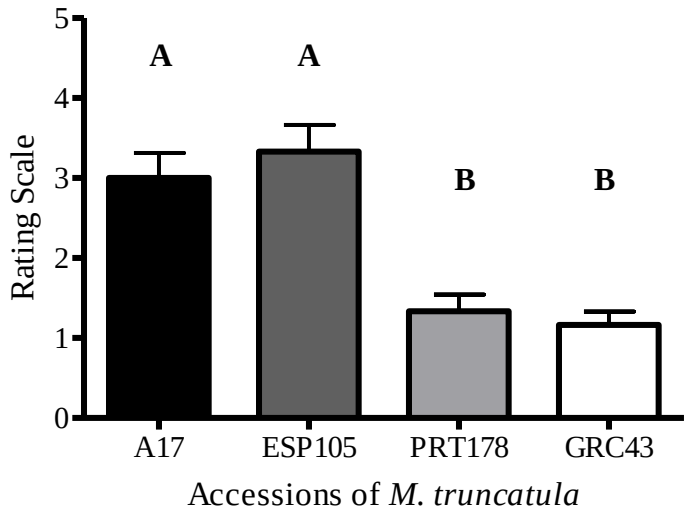


**Figure 3. Foliar disease symptoms caused by *P. medicaginis* on *M. truncatula*.**
Disease ratings are shown for each of four accessions inoculated with *P. medicaginis*. Both A17 and ESP105 are more susceptible to the pathogen than PRT178 and GRC43. The rating scale is adapted from Salter and Leath (1991) and described in the Methods section. A nonparametric Kruskal-Wallis ANOVA test was used to analyze data (n = 6); bars on the graph with same letters show no statistical difference ($p < 0.10$).

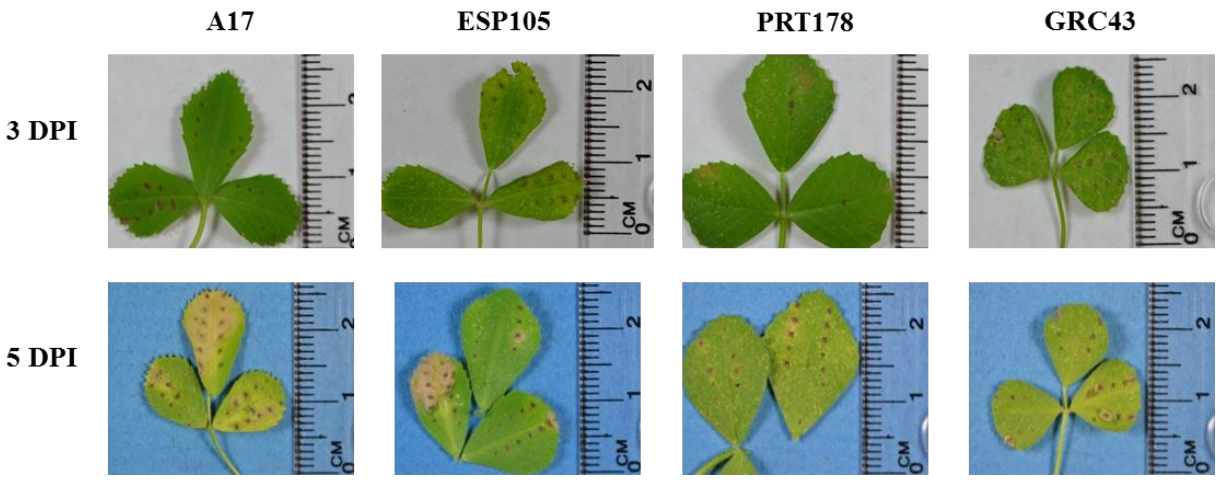


**Figure 4. Wounded trifoliate images of accessions inoculated with *P. medicaginis*.** Individual trifoliates, wounded and inoculated, observed post-inoculation with *P. medicaginis.* At three days post-inoculation lesions developed on accessions A17 and ESP105 surronding the wound site. Five days after wounding and inoculation A17 and ESP105 show chlorosis, large lesion diameters and coalescing necrotic areas while PRT178 and GRC43 have discrete lesions around the wound site.

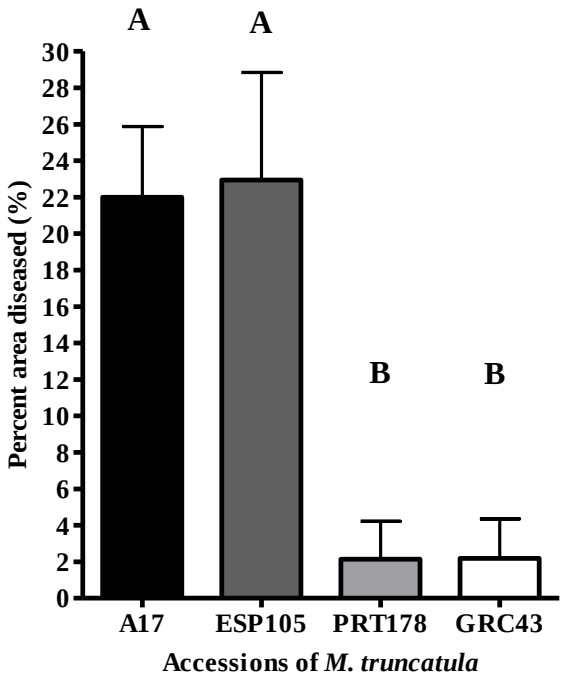


**Figure 5. Percent area of disease on wounded trifoliates of *M. truncatula* accessions five dpi.** Bars on the graph represent the percent area of disease. Trifoliates were photographed five days post-inoculation and images were analyzed using Image J©. An ANOVA and Tukey's multiple comparison were used to analyze data (n = 4); bars on the graph with same letters show no statistical difference at $p > 0.05$.

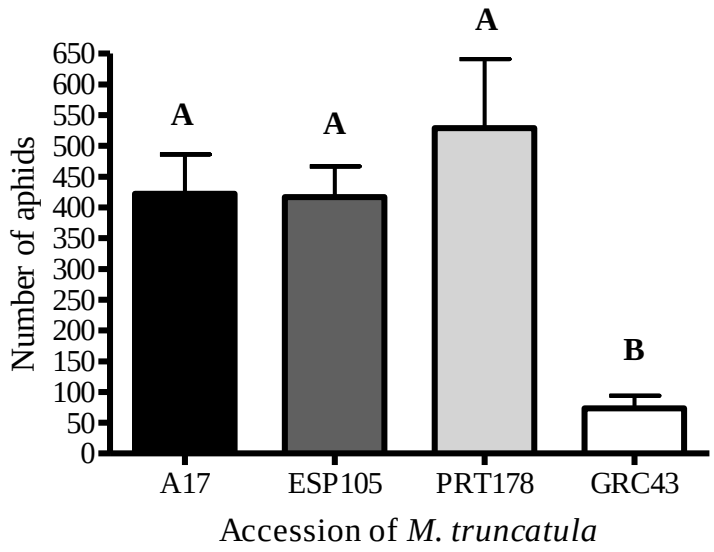


**Figure 6. Population of pea aphids after 12 days on four accessions of *M. truncatula*.** Means of aphid populations are shown and indicate reproduction on each of the four accessions. Population is the number of aphids counted after 12 days per whole plant. An ANOVA and Tukey's multiple comparison were used to analyze data ($n = 8$); bars on the graph with same letters show no statistical difference at $p > 0.05$. The accession GRC43 supports significantly less aphid reproduction, to a level of $p < 0.01$.

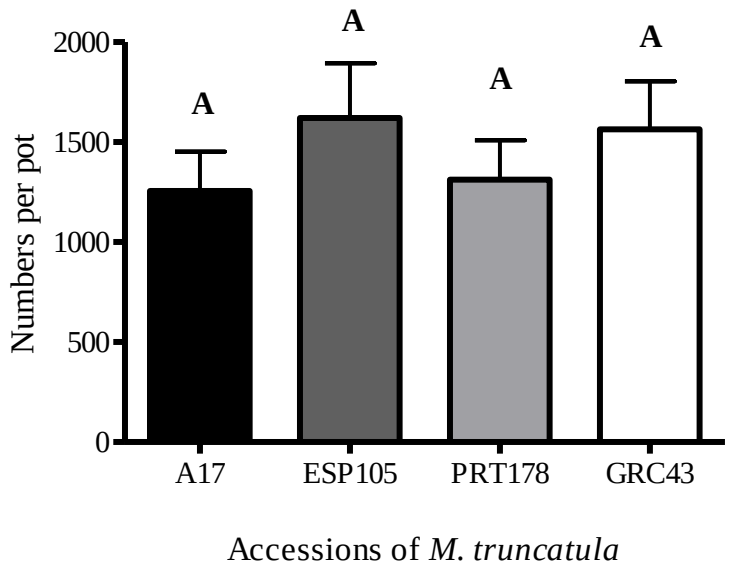


**Figure 7. Population of *P. penetrans* recovered from the soil 50 dpi on four accessions of *M. truncatula.***

Means of nematode populations are shown and indicate reproduction on roots from each of the four accessions. Nematodes were collected from soil used to grow potted plants, and manually counted. An ANOVA was used to analyze data (n = 8); same letters on the graph show no statistical difference at p = 0.62.

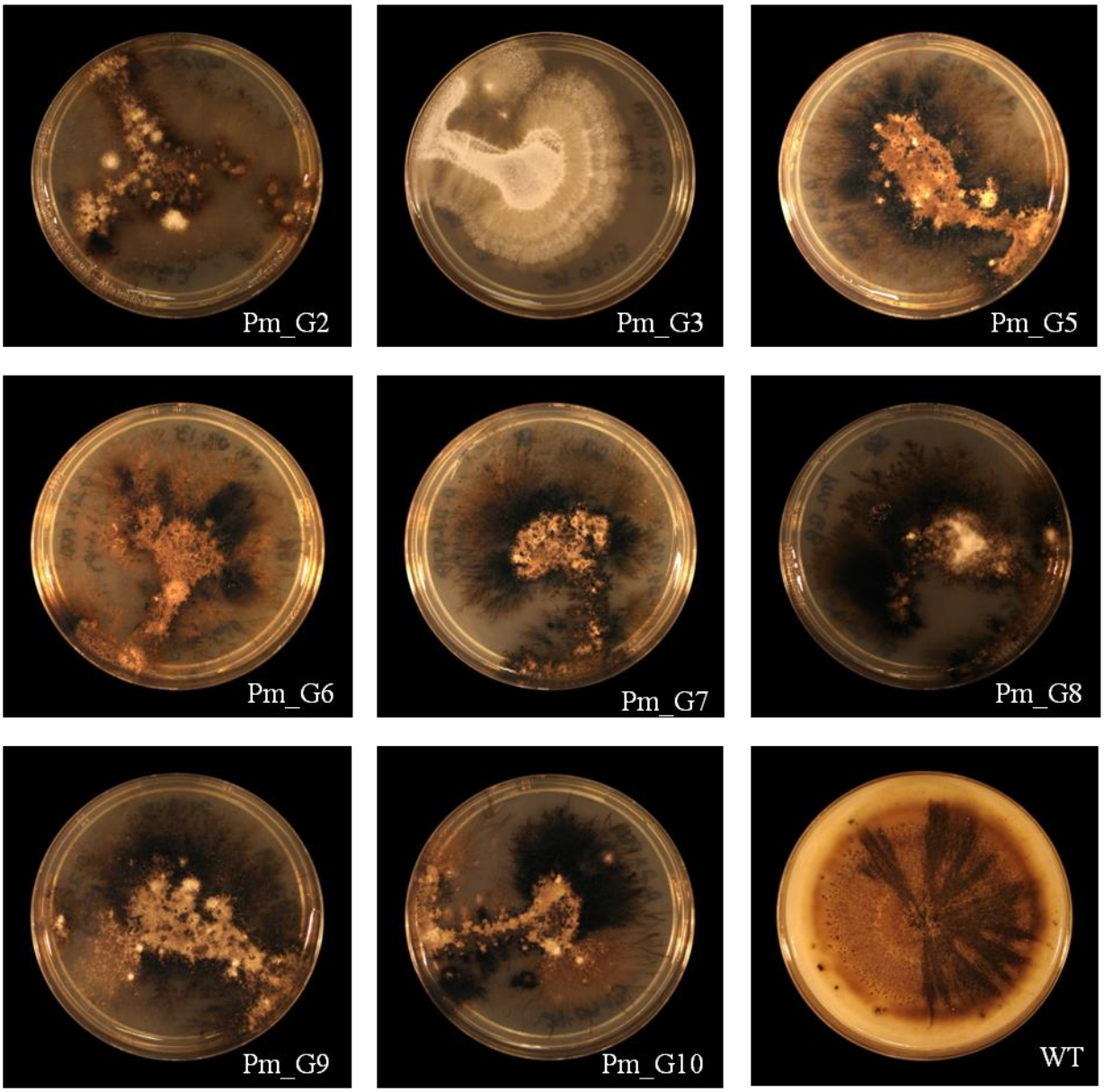


**Figure 8. *Phoma medicaginis* growth in culture.**
Eight transgenic fungal cultures were developed via *Agrobacterium* transformation that introduced the binary construct pBHt2_sGFP. Two isolates, Pm_G5 and Pm_G7, were selected for these studies, based upon their strong GFP expression and *in vitro* appearance and growth patterns similar to wildtype (WT) *P. medicaginis*.

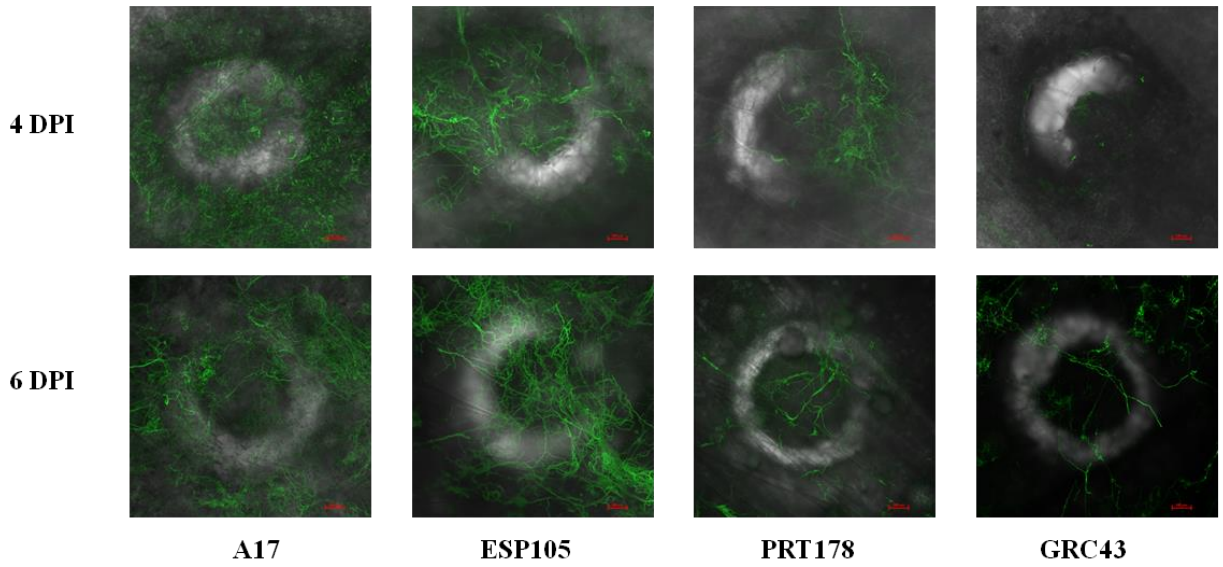


**Figure 9. Confocal images of GFP-*P. medicaginis* on four accessions of *M. truncatula*.** Spores from *P. medicaginis* cultures expressing GFP were dispersed on mature *M. truncatula* leaves wounded at the same time with a micropipettor tip. The circular wound is visible at the center of each image at 20x magnification. At four days post-inoculation (DPI) accessions A17 and ESP105 appear to support more hyphal growth whereas accessions PRT178 and GRC43 have less. The same trends are observed six DPI, and overall hyphal growth expands to cover more surface area of the leaf.

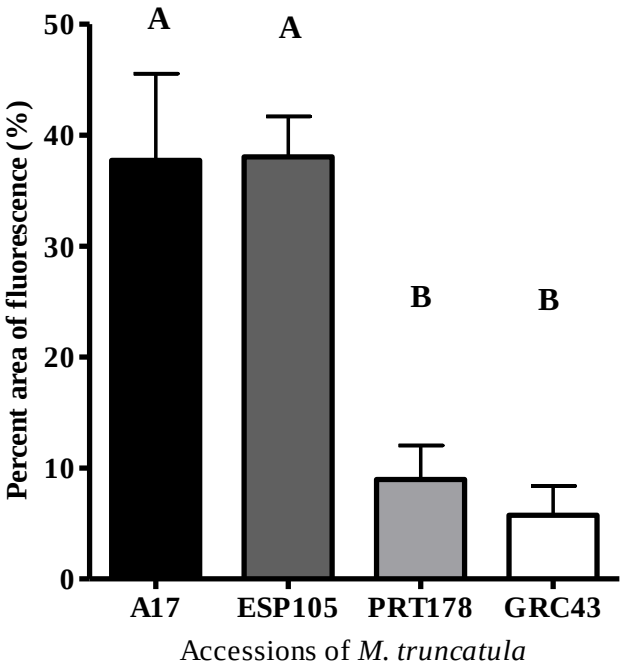


**Figure 10. Analysis of GFP images of *P. medicaginis* on *M. truncatula* accessions four dpi.** Bars on the graph are the means of the percent area of fluorescence. Trifoliates were scanned four days post-inoculation and were analyzed using Image J©. An ANOVA and Tukey's multiple comparison were used to analyze data (n = 3); bars on the graph with same letters show no statistical difference at $p > 0.05$.

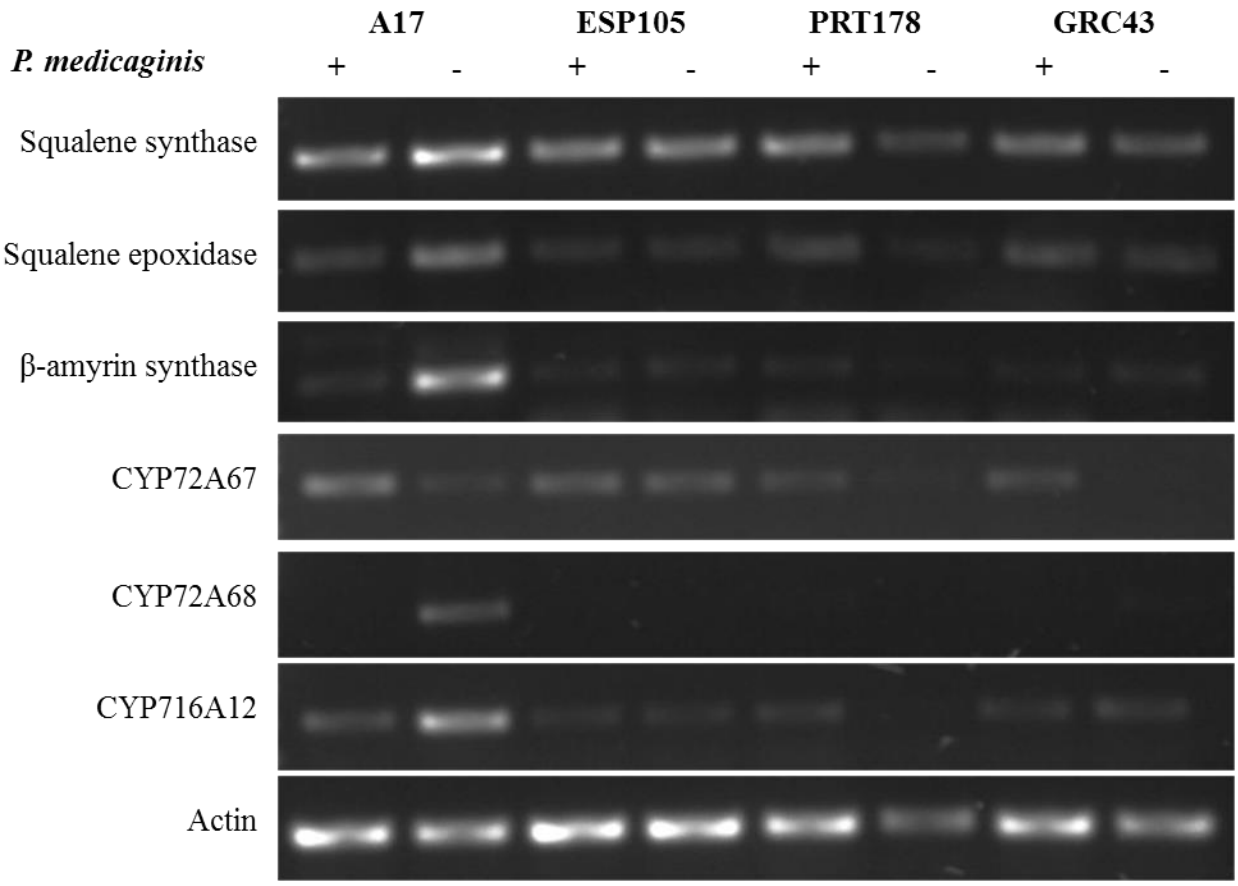


**Figure 11. Transcript levels of genes potentially encoding enzymes involved in triterpene saponin biosynthesis in response to infection with *P. medicaginis.*** Relative transcript accumulation was measured using RT-PCR in leaves of four *M. truncatula* accessions inoculated (+) or not inoculated (-) with *P. medicaginis*. Substantial reductions in transcript levels for several genes (*e.g.*, β-amyrin synthase) occurs in response to *P. medicaginis* inoculation in some accessions. Transcripts for the actin gene were amplified and run on the gel as an internal control.

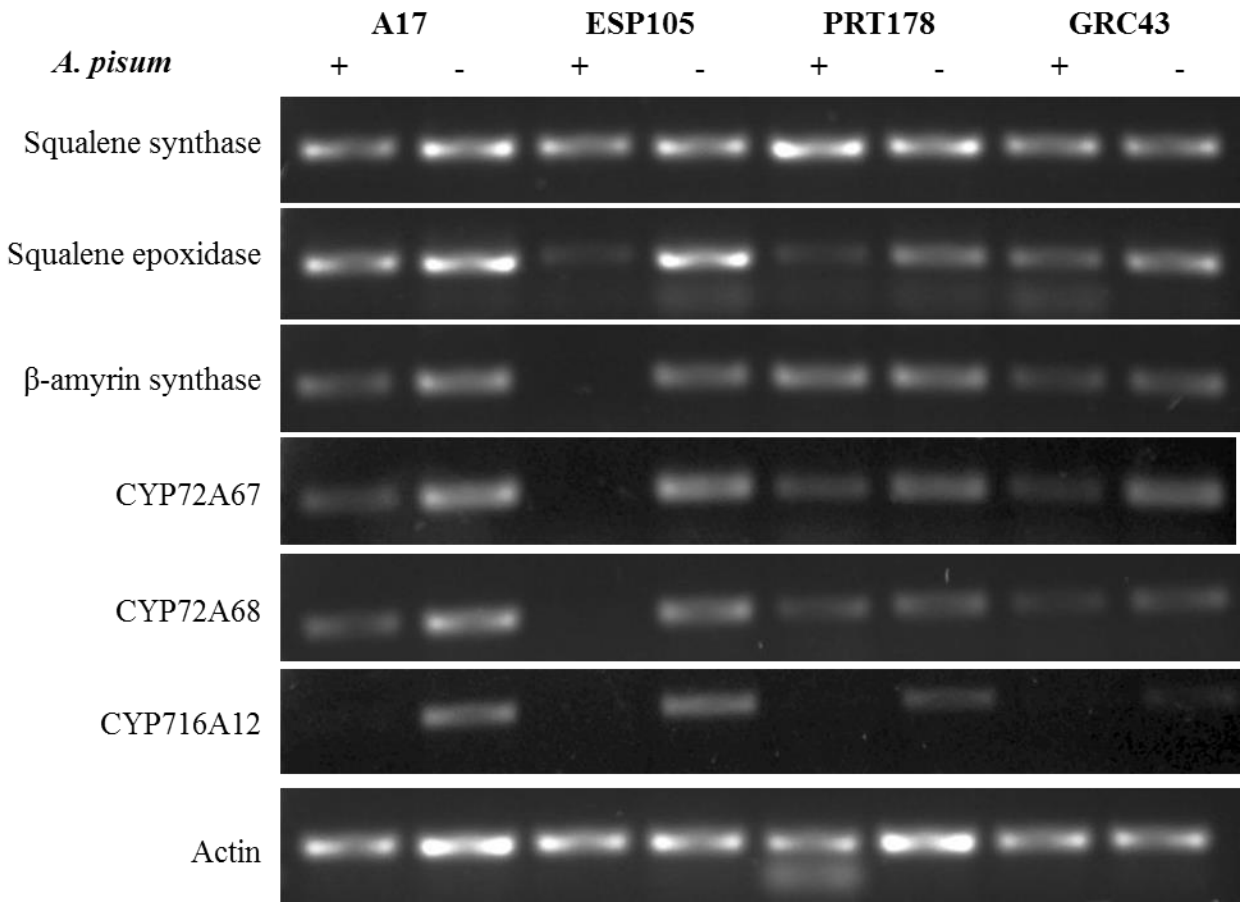


**Figure 12. Transcript levels of genes potentially encoding enzymes involved in triterpene saponin biosynthesis in response to pea aphid feeding.** Relative transcript accumulation was measured using RT-PCR in leaves of four *M. truncatula* accessions infested (+) or not infested (-) with pea aphids. Substantial reductions in transcript levels for several genes (*e.g.*, CYP716A12) occur in response to pea aphid feeding, and responses can vary by accession (e.g., β-amyrin synthase, CYP72A68). Transcripts for the actin gene were amplified and run on the gel as an internal control.

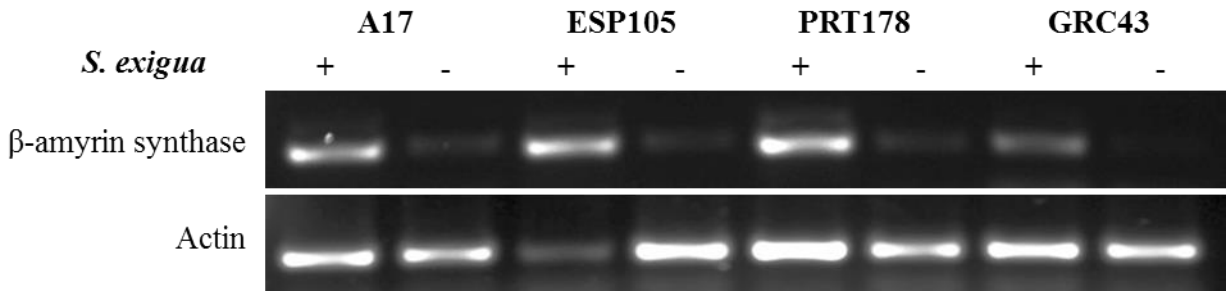


**Figure 13. Transcript levels for β-amyrin synthase in response to beet armyworm feeding.** Transcripts encoding β-amyrin synthase are strongly induced in leaves in response to *S. exigua* feeding in each of the four accessions. Transcripts for the actin gene were amplified and analyzed as an internal control.

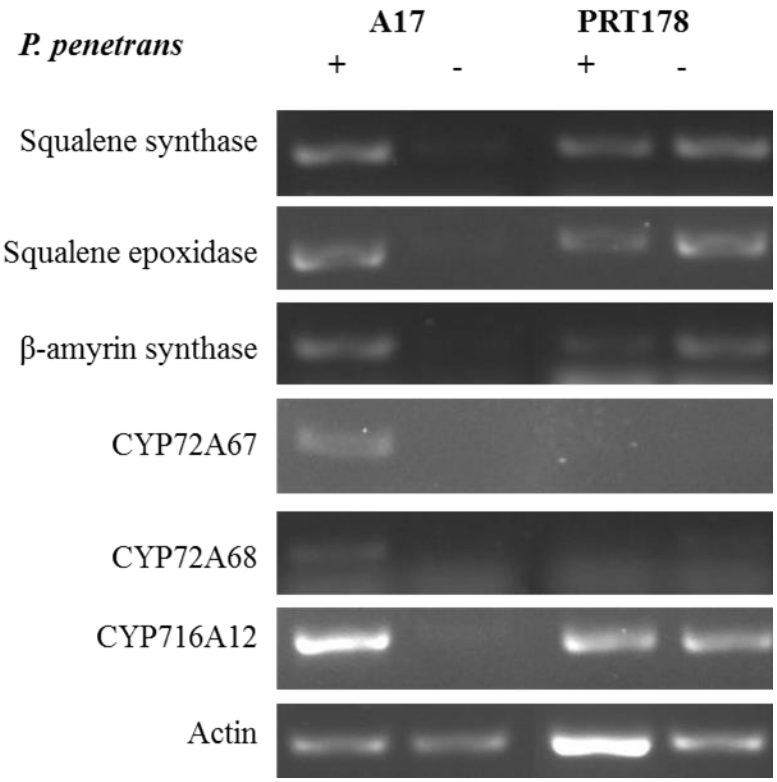


**Figure 14. Transcript levels of genes potentially encoding enzymes involved in triterpene saponin biosynthesis in response to inoculation with *P. penetrans*.** Transcripts for each of the genes tested showed strong nematode-induced increase in roots of *M. truncatula* A17. The same genes tested in PRT178 roots showed very little change in response to nematodes. None of the genes, including actin, was amplified from roots of ESP105 or GRC43 (data not shown). Transcripts for the actin gene were amplified and run on the gel as an internal control.

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