Inoculation of Almond Rootstock with Symbiotic Arbuscular Mycorrhizal Fungi

Project No.: **09-HORT9-Hua**

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Objectives:

1. Determine if there is value in adding AM fungi inoculum, particularly at planting of bare root (field grown) and the potted-plant nursery stock.
2. Determine if pre-plant fumigation impacts the extent and nature of mycorrhizal populations in the soil and is this of consequence?
3. Characterize the mycorrhizal fungi populations present on field grown nursery stock vs. potted plants the first season after planting as well as resulting tree performance
4. Determine if soil phosphate levels are too high to see beneficial effects of arbuscular mycorrhizal colonization

Interpretive Summary:

Increased pressure for food production has, in recent years, led to the development of intensive agricultural systems that use significant quantities of inorganic fertilizers and pesticides. However, there is now substantial evidence for the environmental costs of this high-input strategy. The estimated environmental and health care costs of the recommended use of pesticides in the U.S. are about $10 billion per year (Pimentel et al, 2005). Integrated pest and nutrient management systems and certified organic agriculture can reduce reliance on agrichemical inputs, and they are environmentally and economically sound. Arbuscular mycorrhizal fungi (AMF) form symbiotic associations with the majority of land plants, including many important agricultural crops. These fungi facilitate plant nutrient uptake, promote soil aggregation (Dodd, 2000; Gosling et al, 2006; Helgason and Fitter, 2009; Johnson et al, 2010). They are essential to the sustainability of agricultural ecosystems. AMF are particularly important in organic and/or sustainable farming systems that rely on biological processes rather than agrochemicals to control plant diseases. The symbiosis confers bioprotection to

plants against many soil-borne pathogens such as species of *Fusarium, Phytophthora, Rhizoctonia, Sclerotinium, Verticillium* as the outcome of complex interactions between plants, pathogens and AMF. The use of molecular tools in the study of these multifaceted interactions may aid the optimization of bioprotective responses and their utility within sustainable farming systems (Harrier and Watson, 2004). A recent publication reports that AM fungi increase biocontrol potential of *Pseudomonas fluorescens* (Siasou et al. 2009). AMF may help mitigate aflatoxin contamination of almond by improving water and temperature stress of almond trees before harvest (Ruiz-Lozano et al, 1995; Augé, 2001; Porcel and Ruiz-Lozano, 2004; Wu et al, 2006; Aroca et al, 2008; Bunn et al, 2009).

Yet the status of AM fungal population in almond orchard is not well understood. The purpose of this study is to determine if specific practices associated with planting almonds (e.g., pre-plant fumigation, inoculation with AM fungus, or other factors like choice of field grown vs. potted nursery stock) have an impact on AM fungal populations to the extent subsequent tree performance is affected.

The study of arbuscular mycorrhizal (AM) fungi has fundamental and practical importance. First because in most environments "root biology" is actually "mycorrhizal biology", and second because of the practical importance of AM in fields as diverse as sustainable agriculture, horticulture, reforestation, and ecosystem management In the last few decades, interest in AM fungi has increased. The symbiosis has the potential for sustainable production of important crops and reducing the use of chemical fertilizers and pesticides. For successful application of AM fungi with economically profitable returns, the soil conditions must be suitable for AMF colonization. Plant genotypes also influence the symbiosis. Almond growers are encouraged to participate in field experiments for optimizing the beneficial effect of AMF on almond production.

A field trial was initiated in early 2008 to examine the effects of Arbuscular Mycorrhizal (AM) fungi on almond tree growth. Trees were planted on 7 February 2008 at the San Joaquin Valley Agricultural Sciences Center. Trees used in the study were either traditional bare root (1/2” caliper) Nonpareil/Nemaguard or 3/8” caliper ‘potted’ Nonpareil/Nemaguard trees. Three AM treatments were imposed on the bare root trees (control, field cultured AM and commercial cultured AM) and potted trees were utilized as either controls, or field cultured AM (five total tree treatments). Tree growth, soil nutrient status, arbuscular mycorrhizal population and root colonization will be monitored for several years. The results of this study will provide scientific information to growers on utilization of AMF in almond orchards.

Materials and Methods:

Almond rootstock field trial

The trial site had been previously (September 2007) strip fumigated (chloropicrin) to provide ten single blocks (five fumigated, five non-fumigated), randomly arranged in two 5-block rows. Each block was of sufficient length to accommodate 12 trees planted at 12 ft intervals. Pairs of trees for each treatment were planted in a randomized order for each block, with a single Monterey/Nemaguard tree at the ends of each block. In addition to being guard or border trees, the Monterey/Nemaguard trees were planted to provide adequate pollination of the trial

trees in future harvests. The arrangement of trees in the plot was described in 2007-2008 final report and also shown here in **Table 3**.

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As planned, tree performance data to be collected will include:

a) Trunk circumference: initial and final yearly
b) Annual pruning weights
c) Nutrient status: Characterize nutritional deficiencies if and when symptoms arise
d) At end of trial: Whole tree top weight, trunk diameter, etc.

Soil extractable phosphorus –using Mehlich 2 extractant

This method estimates the relative bioavailability of inorganic ortho-phosphate (PO4-P) in soils using a dilute acid solution of acetic acid and hydrochloric acid containing ammonium fluoride. The orthophosphate ion reacts with ammonium molybdate and antimony potassium tartrate under acidic conditions to form a complex. This complex is reduced with ascorbic acid to form a blue color, which absorbs light at 880 nm. The method is shown to be well correlated to crop response on most soil types. The absorbance is proportional to the concentration of orthophosphate in the sample. The method has a detection limit of approximately 0.5 mg kg^{-1} (soils basis) and is generally reproducible within 8%.

Sudangrass trap cultures to detect AM fungi in newly planted almond rootstock

Thirty samples were collected from fumigated trees and the other from non-fumigated with or without AM inoculum on December, 2008 Roots and surrounding soil were added with a potting mixture (sand: soil at 1: 1 ratio, autoclaved before use). Ten Sudangrass seeds were planted in each 8 inch long planting cone on February 2009. The plants were maintained in greenhouse at Albany, CA. Quarter strength Hoagland solution with 1/8 strength phosphate was used to water the plants for the first four weeks and then phosphate was withdraw from the nutrient until May 26 2009. Plants were left dry to boost spore production. The tops of dried Sudan grass will be cut off; roots and soil will be collected in a pan and analyze for AM fungal spores. Work is in progress.

Molecular taxonomy to identify AM fungal
DNA extraction

AM fungal population diversity and species identification will be based on 18S small subunit ribosomal gene sequence (Redecker, 2000; Schwarzott et al, 2001). Colonized roots or spores (from soil of almond rootstocks) were used for DNA extraction by CTAB method. (Doyle JJ, Doyle JL. *Phytochem Bull.* 1987. 19:11–15). DNA extraction from the root\soil samples

using PowerSoil™ DNA Isolation Kit (Mo Bio Inc. Carlsbad, CA) were tested and adapted for routine use.

Cloning of ribosomal fragment for DNA sequence

A pair of primers specific to AM fungi was used to generate PCR fragments for TOPO® cloning (Invitrogen, Carsbad, CA). Fresh PCR product was cloned into the vector pCR®4-TOPO®. The cloned DNAs were transformed into E. coli using Gene Pulser Xcell electroporation system (BioRad, Hercules, CA). Each transformed E. coli clone harbors a fragment of partial DNA sequence unique to an individual AM fungal strain. DNA from each clone will be isolated and subjected to DNA sequencing using BigDye® Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems, Foster City, CA). The sequences of each DNA fragment were determined using an Applied Biosystems ABI 3700 genetic analyzer. These sequences were used for identification of AMF phylotypes by submitting to the BLAST query tool (Altschul et al, 1997) and database *www.ncbi.nih.gov/blast/* for an initial similarity analysis. Sequence alignment was performed manually. A phylogenetic determination was carried out by distance analysis using the neighbor-joining method in MEGA 4 (Tamura et al, 2007).

Colonization of Almond root stocks planted on 7 February 2008 at the San Joaquin Valley Agricultural Sciences Center.

Root samples were collected from soil 2 ft away from the tree trunk and I ft deep on July 22, 2010. Trees which were sampled are shown in **Table 3**. Soil and root samples were brought back to Albany lab. Fine roots were picked from the soil and stored in tubes at 4^0C. Experiments are planned to determine mycorrhizal colonization and mycorrhizal species identification. Soil phosphate and nitrate levels will also be determined as well.

Results and Discussion:

Almond trees planted for this project are now in their third year of growth in fumigated and non-fumigated soils. Trees bloomed for a first time during this growth season and bloom strength was evaluated at full bloom. Bloom progressed similarly throughout the various treatments, but bloom strength differed. Bloom was more intense among trees planted in fumigated plots, regardless of whether trees were bare root/potted or with/without arbuscular mycorrhiza treatment. To date in the third year, trunk caliper measurements have been taken twice (752 and 832 days after planting on 1 March 2010 and 20 May 2010, respectively). While no trees have died in the test, several trees in non-fumigated plots appear weak and unhealthy.

Growth trends that began during the first year after planting have continued throughout the third growing season. Our most recent growth measurements at 832 days after planting revealed highly significant differences ($p < 0.001$) are present between average trunk caliper of trees in fumigated plots (97.4 mm) and non-fumigated plots (58.5 mm). Comparing bare root versus potted trees at 832 days after planting, significant differences ($p=0.014$) were also noted. Trunk caliper of bare root trees averaged 80.6 mm, whereas potted trees had average trunk calipers of 75.3 mm. No significant differences were identified in growth comparisons of arbuscular mycorrhiza (AM) treated trees (average trunk caliper = 76.9 mm) versus non-AM treated trees (average trunk caliper = 79.0 mm) at 832 days after planting. ANOVA *p* values

obtained at the various sampling dates are presented in **Table 1**. Growth progression of trees since project inception in these main effect comparisons are presented in **Figure 1**.

Soil samples were randomly selected between almond young trees in November 2008. Soil phosphate concentration levels were determined. The values are expressed in ppm for the following samples: sample 3 (31 ppm), sample 6 (21 ppm), sample 7 (20 ppm), sample 18 (30 ppm), sample 24 (25 ppm) and sample 26 (26 ppm). The result indicates that there is sufficient phosphate in the soil to support young almond tree growth. The phosphate level is high and may prevent good mycorrhizal colonization. In some cases, mycorrhizal colonization can occur but it will not show stimulation in plant growth. Other beneficial effect of symbiosis may still function such as improvement of water stress and soil structures.

Roots of Sudangrass from trap cultures were analyzed for AM fungal colonization in fumigated and non-fumigated soils collected in 2007 at Firebaugh. Sudangrass plants were grown under limiting phosphate nutrient conditions for boosting colonization and inoculum production. Molecular techniques for identification of AM mycorrhizal fungal species were developed and performed at Albany lab. The procedure includes cloning and DNA sequencing. Several hundred clones have been sequenced and Blasted for ID using NCBI data base. The AMF species in Firebaugh soil are summarized in **Table 2**. Species abundance from fumigated (F) and non-fumigated (NF) soil is shown in **Figure 2**. Phylogenetic relationship of almond AM fungi to other species of AMF is illustrated in **Figure 3**.

We have sequenced more than 150 clones from root and soil samples collected from Parlier in December 2008. Results will be summarized. Almond soil and root samples collected from Parlier on July 22, 2010 will be analyzed for root colorization and AMF species. In addition soil phosphate and nitrate levels will also be determined.

Soil borne arbuscular mycorrhizal (AM) fungus forms a symbiotic (mutualistic) relationship with most plants. The fungus colonizes the root and grows out into the soil. Hyphae net work, the part of the fungus that's in the soil acts as an extension of the root system. The AM symbiosis improves plant phosphorus, nitrogen and mineral nutrition. Evidence also suggests the symbiosis provides protection of the plant against pathogens and improves plant water relations. In addition to facilitating nutrient uptake, some mycorrhizae secrete a gluey substance, called glomalin, which helps develop soil structure and soil aggregation favorable for plant growth. The beneficial effect of AMF on plant water stress should be investigated in almond production (Ruiz-Lozano et al, 1995; Augé, 2001; Porcel and Ruiz-Lozano, 2004; Wu et al, 2006; Aroca et al, 2008).

Research Effort Recent Publications:

Hua, S. S. T., Ledbetter, C., Browne, G. T., and Hernlem, B. 2010. Arbuscular mycorrhizal fungi associated with almond trees. Abstract. 110 General Meeting of American Society for Microbiology, San Diego, CA. May 22-27, 2010.

Hua, S. S. T. 2009. Saprophytic yeast, *Pichia anomala*. US 7,579,183 BI (patent).

References Related to this Research:

Altschul, S.F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein search programs. Nucl. Acids Res. 25:3389-3402.

Aroca, R., Vernieri, P., and Ruiz-Lozano, J. M. 2008. Mycorrhizal and non-mycorrhizal *Lactuca sativa* plants exhibit contrasting responses to exogenous ABA during drought stress and recovery. J. Exp. Bot. 59: 2029-2041.

Augé, R. M. 2001. Water relations, drought and vesicular-arbuscular mycorrhizal symbiosis Mycorrhiza. 11:3–42

Bunn, R., Lekberg, Y., Zabinski, C. 2009. Arbuscular mycorrhizal fungi ameliorate temperature stress in thermophilic plants. Ecology 90:1378-1388.

Dodd J.C. 2000. The role of arbuscular mycorrhizal fungi in agro- and natural ecosystems. Outlook in Agriculture, 29: 63-70.

Gosling, P., Hodge, A., Goodlass, G., and Bending, G. D. 2006. Arbuscular mycorrhizal fungi and organic farming. Agriculture, Ecosystems and Environment 113:17–35.

Harrier, L. A., and Watson, C. A. 2004. The potential role of arbuscular mycorrhizal (AM) fungi in the bioprotection of plants against soil-borne pathogens in organic and/or other sustainable farming systems. Pest Manag Sci 60:149–157.

Helgason, T, and Fitter, A. H. 2009. Natural selection and the evolutionary ecology of the arbuscular mycorrhizal fungi (phylum Glomeromycota). J Exp Bot 60:2465–2480.

Johnson, N. C., Wilson, G. W. T., Bowkera, M. A., Wilson, J. A., and Millerd, R. M. 2010. Resource limitation is a driver of local adaptation in mycorrhizal symbioses. Proc. Natl. Acd. Sci. 107:2093-2098.

Pimentel, D., Hepperly, P., Hanson, J. Seidel, R., and Douds, D. 2005. Organic and Conventional Farming Systems: Environmental and Economic Issues. http://www2.grist.org/gristmill/images/user/2988/pimentel_report_05_1.pdf

Porcel, R., and Ruiz-Lozano, J. M. 2004. Arbuscular mycorrhizal influence on leaf water potential, solute accumulation, and oxidative stress in soybean plants subjected to drought stress. J. Exp. Bot. 55: 1743-1750.

Redecker D (2000) Specific PCR primers to identify arbuscular mycorrhizal fungi within colonized roots. Mycorrhiza 10:73–80.

Ruiz-Lozano,J. M., 1 R. Azcon, I. R., and Gomez, M. 1995. Effects of arbuscular-mycorrhizal *Glomus* species on drought tolerance: physiological and nutritional plant responses. Appl. Environ. Microbiol. 61: 456-460.

Schwarzott, D., Walker, C., Schüßler, A. 2001. Glomus, the largest genus of the arbuscular mycorrhizal fungi (Glomales) is nonmonophyletic. Mol Phylogenet Evol 21:190–197.

Siasou, E., Standing D., Killham, K., Johnson, D. 2009. Mycorrhizal fungi increase biocontrol potential of *Pseudomonas fluorescens*. Soil Biol. Biochem. 41: 1341-1342.

Tamura, K., Dudley, J., Nei, M., and Kumar, S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24:1596-1599.

Wu, Q. S., Zou,Y. N., Xia, R. X. 2006. Effects of water stress and arbuscular mycorrhizal fungi on reactive oxygen metabolism and antioxidant production by citrus (Citrus tangerine) roots. Eur.. J. Soil Biol. 42: 166-172.

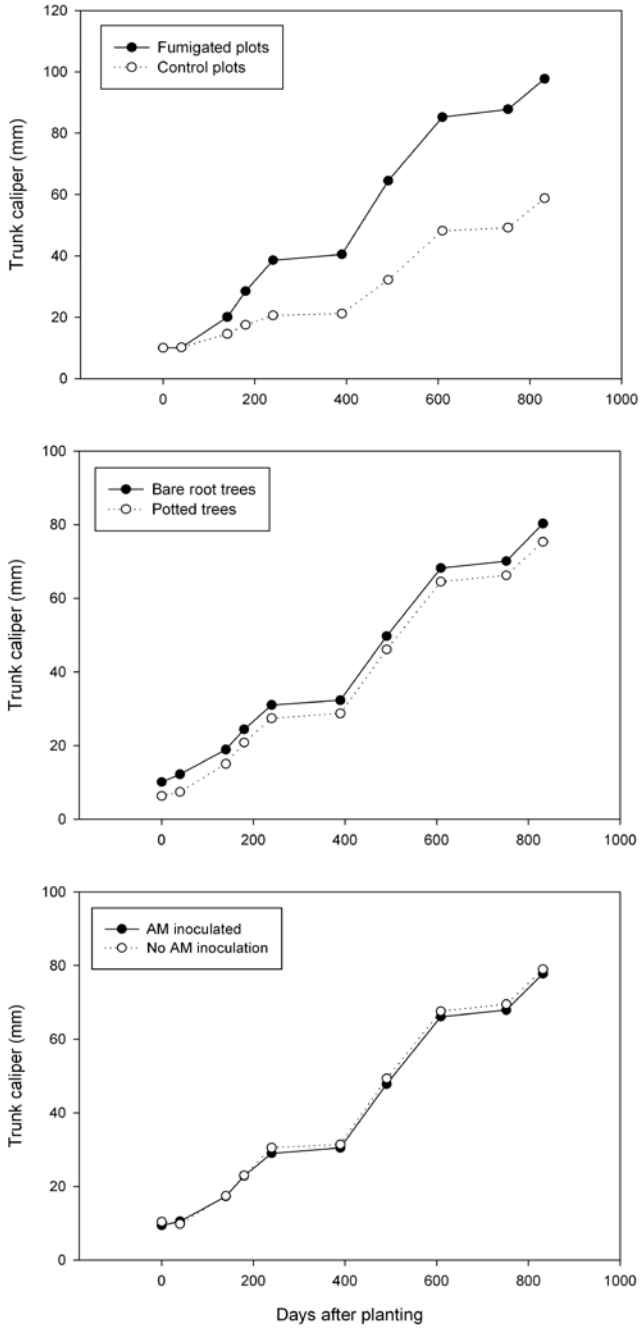

Figure 1. Trunk caliper increases for Nonpareil/Nemaguard trees planted at the San Joaquin Valley Agricultural Sciences Center as influenced by pre-plant fumigation, tree type and arbuscular mycorrhiza inoculation. Trees were planted in February 2008.

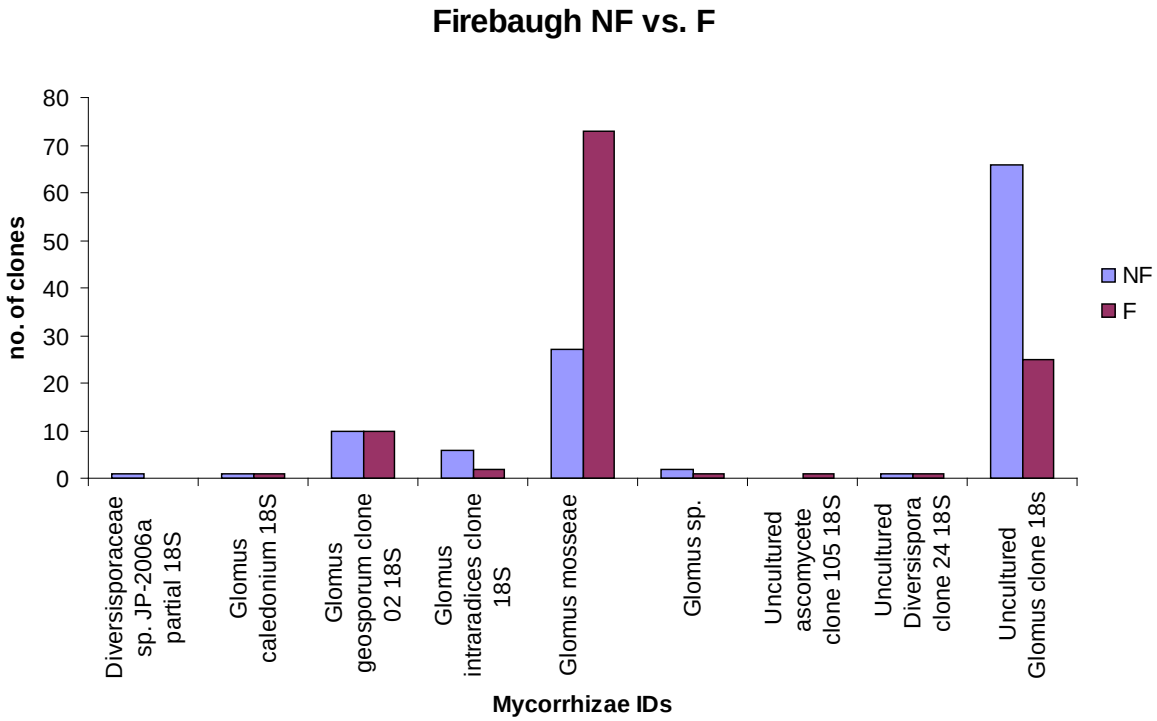

Figure 2. Arbuscular mycorrhizal fungal species from fumigated (F) and non-fumigated (NF) almond root samples

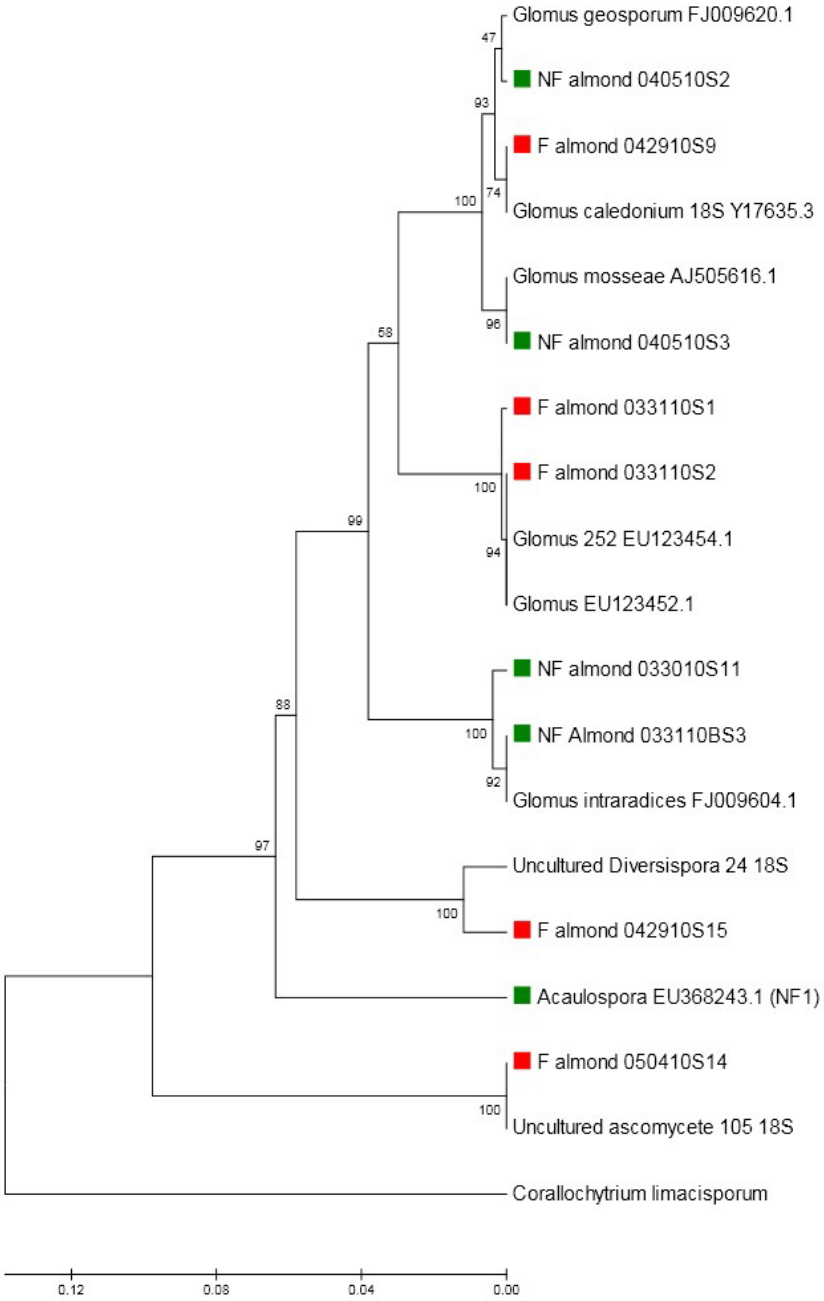

Figure 3. Phylogenetic relationship of almond arbuscular mycorrhizal fungal clones to other species of AMF using MEGA 4 software. Fumigated (F, in red), non-fumigated (NF, in green).

Table 1. Summary of ANOVA *p* values for main effects of AM field trial on Nonpareil / Nemaguard trees planted at the San Joaquin Valley Agricultural Sciences Center, February 2008.

	Effect		
Days after planting	Fumigation	Tree Type	AM treatment
491	<0.001	0.028	0.20
609	<0.001	0.048	0.27
752	<0.001	0.039	0.27
832	<0.001	0.014	0.32

Table 2. Arbuscular mycorrhizal fungal species identified based on 18S small subunit ribosomal gene sequence.

Summary of Arbuscular Mycorrhizal Species

ID	Firebaugh, NF*	Firebaugh, F*
Diversisporaceae sp. JP-2006a partial 18S	1	
Glomus caledonium 18S	1	1
Glomus geosporum clone 02 18S	10	10
Glomus intraradices clone 18S	6	2
Glomus mosseae	27	73
Glomus sp.	2	1
Uncultured ascomycete clone 105 18S		1
Uncultured Diversispora clone 24 18S	1	1
Uncultured Glomus clone 18s	66	25
Total clones	114	114

Table 3. Almond root samples collected in San Joaquin Valley Agricultural Sciences Center, for mycorrhizal colonization studies.

Parlier Plot - Samples collected on 07/22/10				West Side	
		Treatment		**Treatment**	
		Guard		Guard	
	Bag#26	5		2	
		5		2	Bag#25
		2		4	Bag#24
	Bag#27	2		4	
		1		1	Bag#23
	Bag#28	1		1	
		3		3	
	Bag#29	3		3	Bag#22
		4		5	
	Bag#30	4		5	Bag#21
		Guard		Guard	
		Guard	P	Guard	
		4	E	5	
	Bag#31	4	A	5	Bag#20
		1	C	1	
	Bag#32	1	H	1	Bag#19
		3		2	
	Bag#33	3	T	2	Bag#18
		5	R	4	Bag#17
	Bag#34	5	E	4	
		2	E	3	
	Bag#35	2	S	3	Bag#16
		Guard		Guard	
		Guard	I	Guard	
		1	N	4	Bag#15
	Bag#36	1		4	
		3	B	1	
South	Bag#37	3	E	1	Bag#14
Side		5	T	2	Bag#13
	Bag#38	5	W	2	
	Bag#39	4	E	3	Bag#12
		4	E	3	
	Bag#40	2	N	5	
		2		5	Bag#11
		Guard		Guard	
		Guard		Guard	
		4		1	
	Bag#41	4		1	Bag#10
		2		3	
	Bag#42	2		3	Bag#9
	Bag#43	3		5	Bag#8
		3		5	

	Bag#44	1		2	Bag#7
		1		2	
	Bag#45	5		4	
		5		4	Bag#6
		Guard		Guard	
		Guard		Guard	
		5		1	
	Bag#46	5		1	Bag#5
		4		4	Bag#4
	Bag#47	4		4	
		3		5	Bag#3
	Bag#48	3		5	
	Bag#49	2		2	Bag#2
		2		2	
	Bag#50	1		3	Bag#1
	Bag#51	1		3	
		Guard		Guard	
			East Side (Road)		

Footnotes: Yellow color indicates plot was fumigated. The number, 1, 2, 3, 4, or 5 indicate the type of mucorrhizal treatment applied to the rootstock at the time of planting (Feb. 2008).

Fumigated
Non-Fumigated

Trees plated on 02/07/08

1/2" Bare root Nonpareil/Nemaguard
1/2" Monterey/Nemaguard (Guard Trees)
1/4"-3/8" Potted Nonpareil/Nemaguard

Treatments

Trt 1 - Bare root control
Trt 2 - Bare root w/greenhouse inoc.
Trt 3 - Bare root w/commerical inoc.
Trt 4 - Potted control
Trt 5 - Potted w/greenhouse inoc.