Inoculation of Almond Rootstock with Symbiotic Arbuscular Mycorrhizal Fungi

Project No.: **08-HORT9-Hua**

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Objectives:

1. Determine if there is value in adding arbuscular mycorrhizal 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:

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 AM fungus cannot

multiply by itself. *Therefore inoculum production of AM is a challenge for the utilization of this fungus.* Recent literature review suggests the multifunctional nature of AM fungi includes better nutrient uptake, mineralization of organic nutrients, improving host plant's resistance to drought, seedling establishment, pathogen resistance, increased herbivore tolerance, increased pollination, heavy metal tolerance and increased soil stability. Research shows that AMF functioning may be more complex than previously thought. A diversified population of the symbiotic AM fungi in the soil in association with roots is important factor contributing to these beneficial effects.

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.

A field trial was initiated in early 2008 to examine the effects of AM fungi on almond tree growth. The trial was planted on 7 February 2008 at the USDA-ARS San Joaquin Valley Agricultural Sciences Center, Parlier. 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 arbuscular mycorrzal symbiosis 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.

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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

To determine if soil phosphate levels are too high to see beneficial effects of arbuscular mycorrhizal colonization, the Mehlich 2 extractant method is being used. 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%.

Molecular taxonomy to identify AM fungal

DNA extraction

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).

Cloning of ribosomal fragment for DNA sequence

Primers were selected for the ribosomal region of AM mycorrhizal fungi (Fig. 4). PCR fragments were cloned using Invitrogen™ TOPO TA Cloning® Kit for Sequencing. Fresh PCR product was cloned into the vector pCR®4-TOPO®. The cloned fragments were transformed into One Shot® Electrocompetent *E. coli*. Transformants were placed on LB (Lauria Broth) agar plate containing kanamycin (50µg ml^{-1}). Colonies were randomly picked for DNA sequencing. The nucleotide sequences of the insert were determined using the ABI Prism BigDye™ Terminator v3.1 Cycle Sequencing Kit. The labeled samples were analyzed using the ABI Prism 3100 Genetic Analyzer. Nucleotide sequences were viewed and edited using CodoneCode Aligner v.1.3.1. BLAST (basic local alignment search tool) was applied to compare the mycorrhizal sequences to NCBI data base (National Center for Biotechnology Information)

Phylogenetic tree of AMF_seq_image (including bootstrap)

This tree represents one of 4 most parsimonious trees found in heuristic searches using 10 replicates with random taxon addition, 1 tree held during tree–bisection–reconnection (TBR) branch swapping for each addition. Bootstrap analyses were conducted using 1000 bootstrap replicates with simple taxon addition, TBR branch swapping. Analyses were run with PAUP v. 4.0d81.

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. 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 2008. 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. 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.

Results and Discussion:

Trunk caliper was measured four times at 40, 140, 180, 240 days after planting (DAP) during the 2008 growing season to provide an indication of relative tree growth. No tree deaths were noted in the trial during the first growing season. There were however, significant differences in tree growth depending on fumigation status of the soil, and other experimental factors.

The most visually evident differences in tree growth were between fumigated and non-fumigated plots. Pre-plant soil fumigation did not affect tree caliper significantly at 40 DAP, but was a highly significant main effect from 140 through 240 DAP. Tree type (bare root vs. potted) affected trunk caliper throughout the 2008 growing season, being a highly significant main effect from 40 days after planting (DAP) onward. No significant differences in trunk caliper were noted between AM treated and control trees at 40, 140 or 180 DAP. However, trunk caliper differed significantly between AM treated and control trees at 240 DAP.

Almond trees planted for this project are now in their second year of growth in fumigated and non-fumigated soils. To date in the second year, trunk caliper measurements have been taken twice (390 and 491 days after planting). While no trees have died in the test, several trees in non-fumigated plots now appear weak and unhealthy.

With regard to tree growth at 491 days after planting, highly significant differences ($p<$ 0.001) are present between average trunk caliper of trees in fumigated plots (64.5 mm) and non-fumigated plots (32.2 mm). Comparing bare root versus potted trees at 491 days after planting, significant differences (p=0.028) were also noted. Trunk caliper of bare root trees averaged 49.7 mm, whereas potted trees had average trunk calipers of

46.1 mm. No significant differences were identified in growth comparisons of arbuscular mycorrhiza (AM) treated trees (average trunk caliper = 49.3 mm) versus non-AM treated trees (average trunk caliper = 47.8 mm) at 491 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**.

Performance evaluation will be continued for several years. Colonization by AM fungi causes a decrease of plant growth initially according to some published literature. AM fungi derive most of their carbon from the host plant. Estimates vary, but plants have been shown to direct 4% to 20% more photoassimilate to mycorrhizal root systems. Therefore mycorrhizal colonization may lead to carbon enrichment in the root zone and increase in soil microbial population and diversity. Further research is needed to investigate these effects.

Soil samples were randomly selected between almond young trees in November 2008 and 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. Results from PCR and gel electrophoresis analysis indicate that *Glomus Mosseae*, *Glomus 3*, *Gigasproa rosea*, *Glomus intraradices* were present in the soil and in colonized roots of Sudamgrass.

Molecular techniques for identification of AM mycorrhizal fungal species were developed and performed at the USDA-ARS Albany lab. The procedure includes cloning and DNA sequencing. Several hundred clones have been obtained for DNA sequence analysis. The current data were used to establish phylogenetic relationship of almond AM fungi to other species of AM mycorrhizal fungi. Two almond AM fungal strains can be clearly identified as *Glomaus intraradices.* Another almond AM fungal strain is closely related to *Glomus clarum.* Population analysis of AM fungal species in almond orchards is in progress. The results are summarized in **Figure 2 and 3**.

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 AM colonization. Plant genotypes also influence the symbiosis.

Recent Publications:

Ledbetter, C.A., Sisterson, M.S. 2007. Advanced Generation Peach-Almond Hybrids as Seedling Rootstocks for Almond: First Year Growth and Potential Pollenizers for Hybrid Seed Production. Euphytica. 160(2): 259-266.

Fennimore, S.A., Duniway, J.M., Browne, G.T., Martin, F.N., Ajwa, H.A., Westerdahl, B.B., Goodhue, R.E., Haar, M., Winterbottom, C.Q. 2008. Methyl bromide alternatives for california strawberry nurseries. California Agriculture. April-June 2008:62-67

Ledbetter, C.A. 2008. Register of New Fruit and Nut Varieties List 44. HortScience 43(5):1321-1343.

Sisterson, M.S., Chen, J., Civerolo, E.L., Ledbetter, C.A., Groves, R.L. 2008. Effects of Almond Leaf Scorch Disease on Almond Yield and Implications for Management. Plant Disease 92:409-414.

Hua, S. S. T. 2009. Saprophytic Yeast, *Pichia anomala*. US patent, allowed

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
40	0.79	<0.001	0.51
140	<0.001	<0.001	0.17
180	<0.001	<0.001	0.29
240	<0.001	<0.001	0.04
390	<0.001	<0.001	0.15
491	<0.001	0.028	0.20

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.

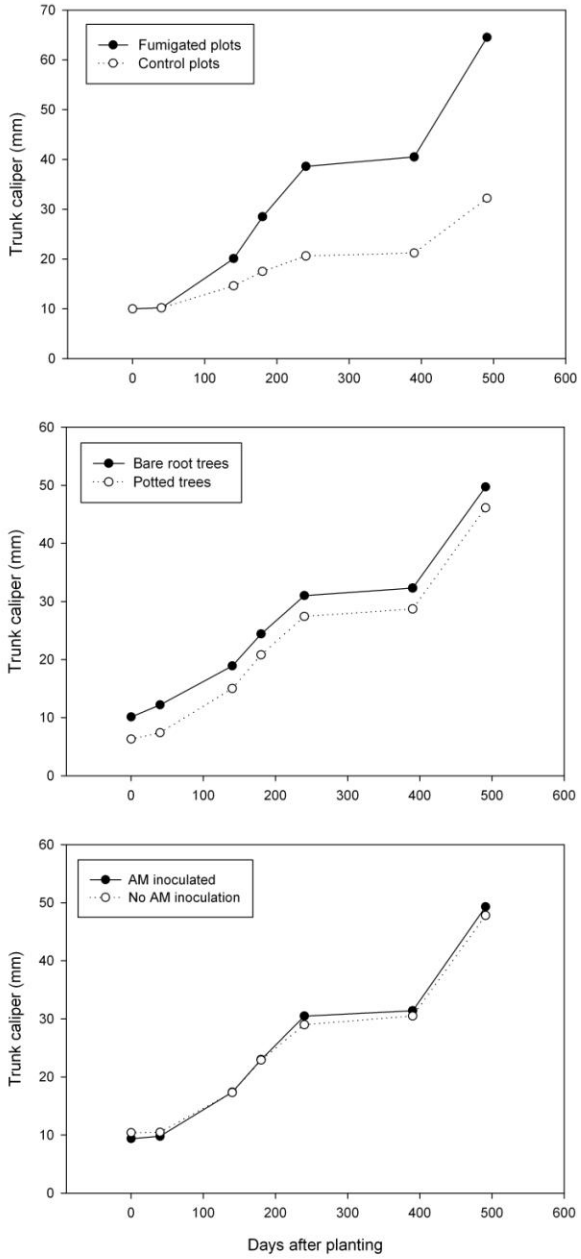

Figure 2. Identification of almond AM fungal species and phylogenetic relationship to other known AM fungal species.

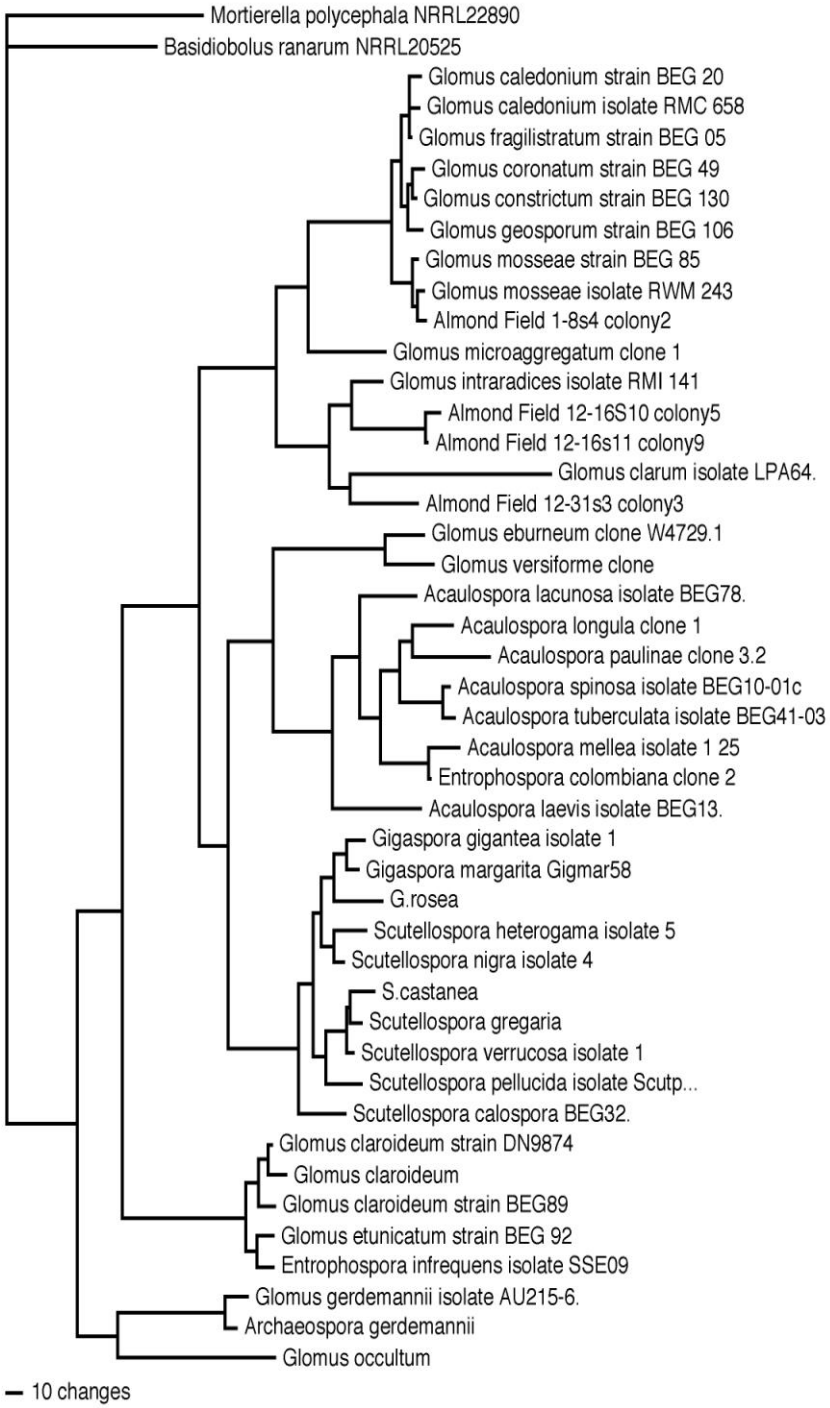

Figure 3. Bootstrap analyses of Almond AM fungi and other know AM fungal species using 1000 bootstrap replicates with simple taxon addition, TBR branch swapping.

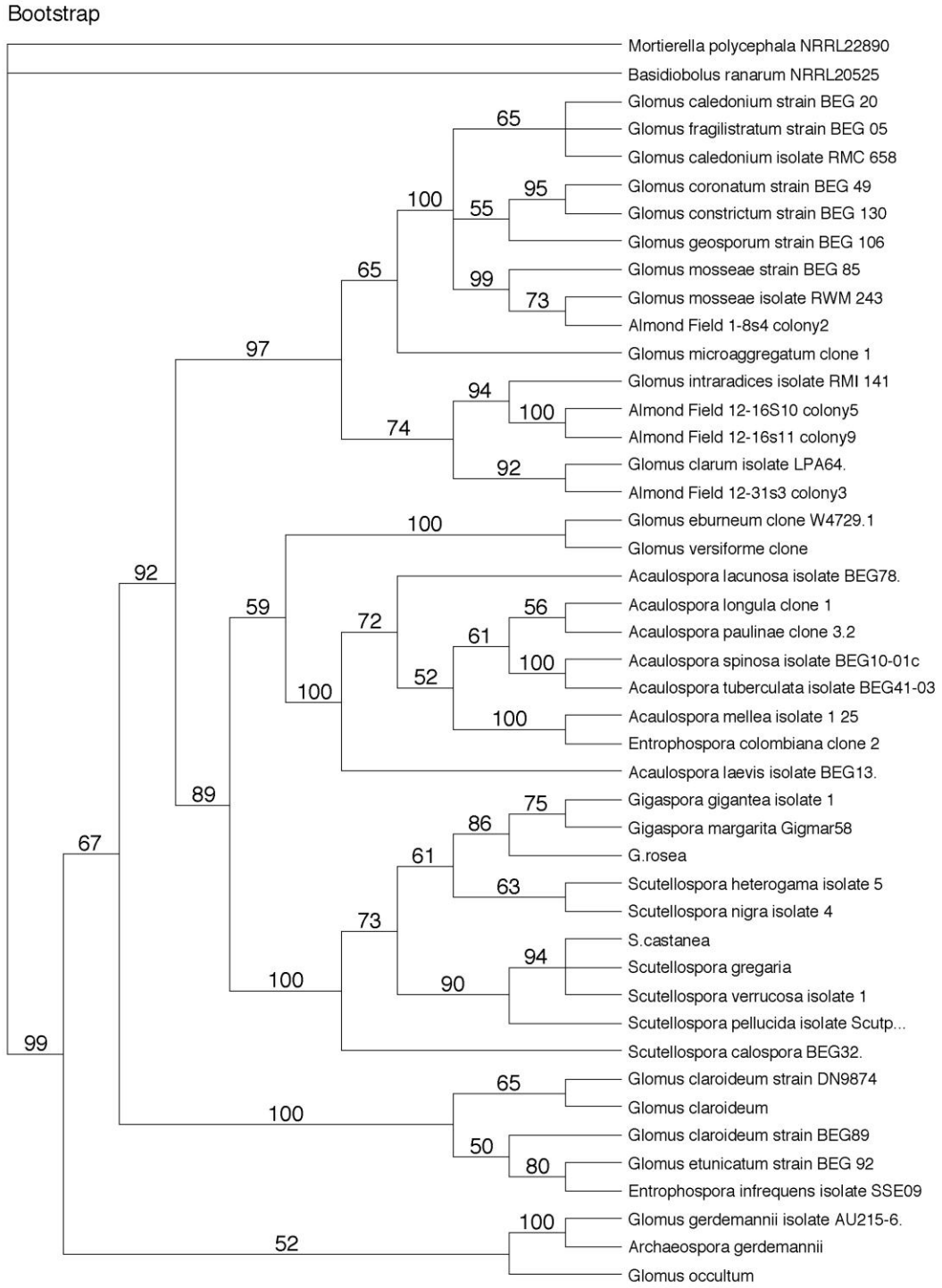

Figure 4. PCR primers for Arbuscular Mycorrhizal fungal identification and their position on partial map of ribosomal structural genes