Inoculation of Almond Rootstock with Symbiotic Arbuscular Mycorrhizal Fungi

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Interpretive Summary:

Soil borne arbuscular mycorrhizal (AM) fungi form a symbiotic (mutualistic) relationship with most plants. The fungui colonize the root and grow out into the soil. The hyphae network, the part of the fungi that is 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 purpose of this study is to determine if specific practices associated with planting almonds (e.g., pre-plant fumigation, inoculation with AM fungi, 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.

In August 2007 root samples were collected from an existing fumigation plot (Firebaugh, CA) in which trees were planted January 2007. These samples were used to develop AM inoculum. This was done by using trap cultures of Sudan to multiply the residual AM fungi. A special nutrient medium was applied to irrigate Sudangrass for boosting AM inoculum production. AM spores were produced in January 2008 and used to inoculate almond rootstocks planted in February 2008.

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 at the time of planting and during the first season after planting as well as resulting tree performance**.**

Materials and Methods:

Sudangrass trap cultures and inoculum production

Five samples were collected from fumigated trees and the other from non-fumigated. Roots and sourounding soil were added with a potting mixture (sand: soil at 1: 1 ratio, autoclaved before use). Fifty Sudangrass seeds were planted in each 8 inch pot on September 7, 2007. 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 withdrawn from the nutrient until December 10, 2007 (David Douds, USDA-ARS-ERRC, Philadelphia, PA and Kendra Baumgartner, USDA-ARS, Dept. of Plant Pathology, Davis, CA, personal communication). Plants were left dry until harvest in January, 2008.

The tops of dried Sudan grass were cut off; roots and soil were collected in a pan. Roots were then cut into 1-cm pieces and mixed with the center portion of the soils containing the AM spores. Two hundred grams of soil-root mixtures were used as inoculum for each almond rootstock.

Almond rootstock field trial

As planned Nonpareil almond trees on Nemaguard rootstock were planted in spring 2008 at USDA Parlier. In essence there is a split plot design contrasting fumigation in fall (October) 2007 vs. no fumigation and this is being done under the supervision of Greg Browne. There are 5 fumigated and 5 control strips and within these treatments are:

Fumigated (chloropicrin 400 #/ac)

1. No inoculation with AM – Field grown nursery stock
2. Inoculation with commercial AM inoculum – Field grown nursery stock
3. Inoculation with trap culture AM inoculum developed by Sylvia Hua – Field grown nursery stock
4. Inoculation with trap culture AM inoculum – Potted nursery stock
5. No inoculation with AM – Potted nursery stock

Not Fumigated

Same treatments as fumigated

The trial was 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).

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. Table 1 shows the arrangement of trees in the plot.

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.

AM fungal identification, cvolonization and molecular taxonomy

Spores were extracted from soils and roots by wet sieving. Sudangrass roots cut in 1-cm segments were stained with trypan blue to detect AM colonization. Spores and root colonization were viewed under a Zeiss stero-microscope.

DNA was extracted from Spores and colonized roots by CTAB method (Doyle JJ, Doyle JL. *Phytochem Bull.* 1987. 19:11–15). Primers from different region of AM fungal ribosomal genes were designed for identification AM fungal species. A list of primers is summarized in Table 2.

Extracted DNA was used a template in conjuction with a pair of primers for PCR amplication using “HotStarTaq Plus Master Mix kit” (QIAGEN, Valencia, CA, USA). PCR was carried out in a Bio-Rad DNA Engine. Amplified PCR products were subjected to agarose gel eletrophoresis, stained with ethidium bromide, viewed and recorded using a Bio-Rad gel documentation system (Bio-Rad, Hercules, CA, USA). The GeneRuler DNA Ladder Mix was used as the molecular weight marker (Fermantas, Glen Burnie, MD, USA) as well as Bio-Rad EZ Load 100bp molecular ruler. The sizes of DNA fragments and banding profiles were analyzed by using the Bio-Rad software Quantity One 4.6.3.

Results and Discussion:

Forty days after planting, tree caliper was obtained approximately 30 cm above the graft union. Trees were just beginning to push vegetative buds at this date. Without exception, all trees were beginning to grow. Trunk caliper was measured again at 140 days after planting (DAP), with no tree deaths being noted at that time (mid-June). However, visual differences in tree growth were then quite evident as related to fumigation treatment.

Initial tree size differences between bare root and potted trees complicate growth rate analyses; however, large differences in tree growth (as measured by percentage increases in trunk caliper) for both fumigation and tree AM treatments were noted at 140 DAP. Averaged across both fumigation and AM treatments, percentage increases in tree caliper were 54.9% and 102.7% for bare root and potted trees, respectively. Bare root trees planted in fumigated blocks had the largest tree calipers at 140 DAP (22.2 mm averaged across AM treatments), but potted trees planted in fumigated blocks had the highest percentage growth increase between 40 and 140 DAP (139.5% averaged across AM treatments).

Performance evalution will be continued for at least two years. *Colonization by AM fungi causes a decrease of plant growth initially according to some published literature*.

Roots of Sudan grass from trap cultures were analyzed for AM fungal colonization in fumigated and non-fumigated soils. Sudan grass plants were grown under limiting phosphate nutrient conditions for boosting colonization and inoculum production. Five hundred and fifty root fragments (I cm long) were stained with trypan blue and scored for colonization. 60% of the roots from *non-fumigated* soil were colonized. About 40% of the roots from fumigated soil were colonized. The data indicate that fumigation did reduce residual soil AM population. Root samples from planted almond tress in Parlier will be collected for evaluating root colonization later this summer.

Preliminary 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. More detailed analysis is in progress.

Recent Publications:

Hua, S.-S. T. Fungal media and methods for continuous propagation of vesicular arbuscular mycorrhizal (VAM) fungi in root organ culture. US patent No. 6,576,457 BI. 2003. (Patent)

Hua, S. S. T., Taurin, A. S., Pandey, S. N., Chang, L., and Chang, P. K. Characterization of *AFLAV*, a *Tf1/Sushi* Retrotransposon from *Aspergillus flavus.* Mycopathologia, 16: 97-104. 2007.

Hua, S. S. T. Environmental Adaptation of *Pichia anomala* WRL-076 as an effective biocontrol agent for pre-harvest application. IOBC Bulletin 30 (6) 241-244. 2007.

Hua, S. S. T., Brandl, M., and Eng, J. G. Fluorescent microscopic studies in the interactions of *Pichia anomala* and *Aspergillus flavus.* IOBC Bulletin 30 (6) 165-169. 2007.

Isakeit, T., Betran, F. J., Odvody, G., and **Hua, S. S. T**. Efficacy of *Pichia anomala* WRL-076 to control aflatoxin on corn in Texas, 2005. Plant Disease Management Reports 1: FC021. 2007.

Hua, S. S. T. Saprophytic Yeast, *Pichia anomala.* US patent, pending. 2006.

Chang, P. K., and **Hua, S. S. T**. 2006. Nonaflatoxigenic *Aspergillus flavus* TX9-8 competitively prevents aflatoxin accumulation by *A. flavus* isolates of large and small sclerotial morphotypes. Int. J. Food Microbiol. 108: 172-177. 2007.

Hua, S. S. T., Parfitt, D. E., and Holtz, B. A. Evaluation of a biopesticide, *Pichia anomala* WRL-076 to control *Aspergillus flavus* in a commercial orchard. Proceedings of the California Conference of Biological Control V, pp. 152-155. 2006.

Hua, S. S. T., McAlpin, C. E., and Ly, S. B. Population of *Aspergillus flavus* on pistachio buds and flowers. In: A. Mendez-Vilas (ed.). Recent Advances in multidisciplinary Applied Microbiology, Wiley-VCH Verlag GmbH&Co. KGaA, Weinheim, Germany, pp. 440-445. 2006. (Proceedings)

Hua, S. S. T. Progress in prevention of aflatoxin contamination in food by preharvest application of *Pichia anomala* WRL-076. In: A. Mendez-Vilas (ed.). Recent Advances in multidisciplinary Applied Microbiology, Wiley-VCH Verlag GmbH&Co. KGaA, Weinheim, Germany, pp. 322-326. 2006.

Table 1. Plot randomization plan

South Row	North Row
Guard	**Guard**
1	3
1	3
2	2
2	2
3	5
3	5
4	4
4	4
5	1
5	1
Guard	**Guard**
Guard	**Guard**
5	4
5	4
1	2
1	2
3	5
3	5
2	3
2	3
4	1
4	1
Guard	**Guard**
Guard	**Guard**
2	5
2	5
4	3
4	3
5	2
5	2
3	1
3	1
1	4
1	4
Guard	**Guard**
Guard	**Guard**
2	3
2	3
5	4
5	4
3	2
3	2
1	1
1	1
4	5
4	5
Guard	**Guard**

Planted on 7 February 2008

1/2" Bare root Nonpareil/Nemaguard
1/2" Monterey/NG (guard trees)
1/4" - 3/8" potted NonP/NG

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

Guard	Guard
4	5
4	5
3	3
3	3
1	1
1	1
2	4
2	4
5	2
5	2
Guard	Guard

.

Table 2. List of primers for Arbuscular Mycorrhizal fungal identification and map of ribosomal structural genes

Mycorrhizae Primers

Forward Primers
Reverse Primers

Forward	Forward Sequence	Reverse	Reverse Sequence	PCR/Seq	Species or Region
ITS1*	TCCGTAGGTGAACCTGCGG	ITS4	TCCTCCGCTTATTGATATGC	PCR2	Internal transcribed spacer (ITS) Region
ITS1F*	CTTGGTCATTTAGAGGAAGTAA	GIGA5.8R	ACTGACCCTCAAGCAKGTG	PCR2	Gigasporaceae (ITS and 5.8S Region)
ITS1F*	CTTGGTCATTTAGAGGAAGTAA	GLOM5.8R	TCCGTTGTTGAAAGTGATC	PCR2	Glomus mosseae/intraradices group (ITS and 5.8S)
ACAU1660*	TGAGACTCTCGGATCGGG	ITS4	TCCTCCGCTTATTGATATGC	PCR2	*Acaulosporaceae sensu stricto* (part of 18S(SSU), ITS, 5.8S)
ARCH1311*	TGCTAAATAGCTAGGCTGY	ITS4	TCCTCCGCTTATTGATATGC	PCR2	A. gerdemannii/A. trappei group/ G. occultum/G. brasilianum group (part of 18S(SSU), ITS, 5.8S)
LETC1670*	GATCGGCGATCGGTGAGT	ITS4	TCCTCCGCTTATTGATATGC	PCR2	Glomus etunicatum/claroideum group (part of 18S(SSU), ITS, 5.8S)
GLOM1310*	AGCTAGGCTTAACATTGTTA	ITS4	TCCTCCGCTTATTGATATGC	PCR2	Glomus mosseae/intraradices group (part of 18S(SSU), ITS, 5.8S)
G. Mosseae***	AAAGCCTTCGGATTCGCGG	FLR4	TACGTCAACATCCTTAACGAA	PCR2	*Glomus Mosseae* (LSU)
Glomus 2***	CATGAGGAGGAAACCCTCG	FLR4	TACGTCAACATCCTTAACGAA	PCR2	*Glomus 2* (LSU)
Glomus 3***	GAGCGTGAGGAGTTAAACGC	FLR4	TACGTCAACATCCTTAACGAA	PCR2	*Glomus 3* (LSU)
Glomus 4***	TCCTTATTTGCAAATTTGTATTC	FLR4	TACGTCAACATCCTTAACGAA	PCR2	*Glomus 4* (LSU)
Glomus 5***	GCCTTCGTTGCTTGCGTTA	FLR4	TACGTCAACATCCTTAACGAA	PCR2	*Glomus 5* (LSU)
A.spor. 1***	CAACATGAGGGTTCGCTTTC	FLR4	TACGTCAACATCCTTAACGAA	PCR2	*Acaulosporaceae 1* (LSU)
A.spor. 2***	TGTTCCCCCGGGAGCGATCT	FLR4	TACGTCAACATCCTTAACGAA	PCR2	*Acaulospora 2* (LSU)
A.spor. 3***	TTCGCTCGCGTACTTTCCGG	FLR4	TACGTCAACATCCTTAACGAA	PCR2	*Acaulospora 3* (LSU)
S.spor. 1***	GAACCTAACCTTGAAGTGCAC	FLR4	TACGTCAACATCCTTAACGAA	PCR2	*Scutellospora 1* (LSU)
S.spor. 2***	AGGGGAAACTCTGAGTGCA	FLR4	TACGTCAACATCCTTAACGAA	PCR2	*Scutellospora 2* (LSU)
NS5	AACTTAAAGGAATTGAGGGAAG	ITS4	TCCTCCGCTTATTGATATGC	PCR1	Universal Primers (part of 18S (SSU), ITS, 5.8S)

M13F	GTAAAACGACGGCCAG	M13R	CAGGAAACAGCTATGAC		After TOPO Cloning for Sequencing
ITS1	TCCGTAGGTGAACCTGCGG	NDL22	TGGTCCGTGTTTCAAGACG	PCR1	Universal Primers - First amplification (roots) (ITS, 5.8S and LSU)
LR1**	GCATATCAATAAGCGGAGGA	NDL22	TGGTCCGTGTTTCAAGACG	PCR2/Cl	2nd ampl. for cloning to sequence (LSU)
LR1	GCATATCAATAAGCGGAGGA	FLR2	GTCGTTTAAAGCCATTACGTC	PCR1	Universal Primers - First amplification (roots) (5' end of LSU) Used as 1st & 2nd ampl.
FLR3***	TTGAAAGGGAAACGATTGAAGT	FLR4	TACGTCAACATCCTTAACGAA	PCR2/Cl	2nd ampl. for cloning/seq. for primer design (FLR3 is between D1 and D2 of LSU, FLR4 is in the D2 domain)
5.23***	GTACGGTTAGTCAACATCG	FLR2	GTCGTTTAAAGCCATTACGTC	PCR2	*G. mosseae* (D2 & D3 of LSU)
8.23***	GTTCGGTTGATCAGATCCGCT	FLR2	GTCGTTTAAAGCCATTACGTC	PCR2	*G. intraradices* (D1,D2,D3 of LSU)
LR1***	GCATATCAATAAGCGGAGGA	23.46	GCTATCCGTAATCCAATACTG	PCR2	*Gig. Rosea* (D1&D2 of LSU)
ITS3	GCATCGATGAAGAACGCAGC	NDL22	TGGTCCGTGTTTCAAGACG	PCR1	*Universal Primer* (5.8S to LSU)
f6***	TAAATCTCCGAGGTTTCCTTGGC	r1	TCATCTTTCCCTCACGGTACTTG	PCR2	*A. paulinae* (D1 of LSU)
f4***	TAAATCTACCTGGTTCCCAGGTC	r2	TGAACCCAAAACCCACCAAACTG	PCR2	*Glomus sp.* (D1 and part D2 of LSU)
5.21***	CCTTTTGAGCTCGGTCTCGTG	NDL22	TGGTCCGTGTTTCAAGACG	PCR2	*Glomus mosseae (367bp)* (D2 of LSU)
LR1***	GCATATCAATAAGCGGAGGA	8.22	AACTCCTCACGCTCCACAGA	PCR2	*G. intraradices (455bp)* (D1 and part D2 of LSU)
LR1***	GCATATCAATAAGCGGAGGA	23.22	GAATCACAGTCAGCATGCTA	PCR2	*Gigaspora rosea (630bp)* *(D1 and part D2 of LSU)*
LR1***	GCATATCAATAAGCGGAGGA	4.24	TGTCCATAACCCAACTTCGT	PCR2	*Scutellospora castanea (615bp)* (D1 and part D2 of LSU)
5.25Forward ***	ATCAACCTTTTGAGCTCG	FLR2	GTCGTTTAAAGCCATTACGTC	PCR2	*Glomus mosseae (part of LSU)*
LeChs2-rtF	TTCGGTTAAGCGGCTCATGA	LeChs2-rtR	CTCGAGCACCCTTGTTGTTCTC	RT	*Lycopersicon esculentum* cv.Micro-Tom Chalcone synthase 2 GenBank # : X55195
Mtchit1-rtF	GGTGATGGTTGTCGAGTCAATG	Mtchit1-rtR	CTGTCGTCACGGTGCTTGAG	RT	*Medicago truncarula R108* Chitinase 1 GenBank # : Y10373

GiAM-rtF	GCTCTGGTGCCGAAAGCTT	GiAM-rtR	TAACCCGTTCTAACCTATTGACCAT	RT	*Glomus intraradices* 28S rDNA subunit GenBank # : AF396797
ITS1	TCCGTAGGTGAACCTGCGG	NL4	GGTCCGTGTTTCAAGACGG	PCR1	ITS and 26S rRNA
NL1	GCATATCAATAAGCGGAGGAAAAG	NL4	GGTCCGTGTTTCAAGACGG	PCR1/seq	D1/D2 region of LSU
LSU0061	AGCATATCAATAAGCGGAGGA			PCR1/Seq	*Glomus* sp. LSU rDNA , roots
LSU3f	AGTTGTTTGGGATTGCAGC			Seq.	*Glomus* sp. LSU rDNA , roots
LSU4f	GGGAGGTAAATTTCTCCTAAGGC			PCR2	*Glomus* sp. LSU rDNA , roots
LSU6f	AAATTGTTGAAAGGGAAACG			Seq.	*Glomus* sp. LSU rDNA , roots
LSU9f	ATTCGTTAAGGATGTTGACG			Seq.	*Glomus* sp. LSU rDNA , roots
LSU5r	CCCTTTCAACAATTTCACG			Seq.	*Glomus* sp. LSU rDNA , roots
LSU7r	ATCGAAGCTACATTCCTCC			PCR2	*Glomus* sp. LSU rDNA , roots
LSU8r	GGGTATCCGTTGCAATCCTC			Seq.	*Glomus* sp. LSU rDNA , roots
LSU0599	TGGTCCGTGTTTCAAGACG			PCR1	*Glomus* sp. LSU rDNA , roots
LSU0805	CATAGTTCACCATCTTTCGG			PCR1/seq	*Glomus* sp. LSU rDNA , roots

*Nested Primers : Use NS5 and ITS4 first PCR

**Nested primers : Use ITS1 and NDL22 first PCR