Progress and Final Report for
Micropropagation of Curry Tree for Nursery Sales and Production of Natural Enemies of the Asian Citrus Psyllid
California Citrus Nursery Board - GOD-13
Time Period: January 1, 2013 – December 31, 2013

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Executive Summary:

This project was funded for one year, so research outlined under objective 1 was conducted. The other 2 objectives (consumer acceptance and outreach) were to be performed during year 2 of the project and were not funded. However, we are looking for funding for the outreach component of this project, and the plants generated will be used for Asian citrus psyllid (ACP) rearing by the California Dept. of Food and Agriculture (CDFA). This report summarizes the research accomplished for objective 1, and all studies were conducted by the UC-Davis Plant Transformation Facility. Methods for micropropagation of curry trees were developed using several types of media for both seedlings from seed and nodal explants from mature trees. Establishment of *in vitro* shoot cultures and multiplication of shoots *in vitro* has been achieved and improvements in shoot quality were observed by altering the basal salt formulations. Subculturing of the shoots was most successful when dividing basal clumps of shoots, rather than using individual shoots. Problems encountered with shoot elongation were addressed through the incorporation of GA_3 into the medium. *In vitro* rooting of shoots has been demonstrated and plants acclimated to soil, but further increases in the efficiency of rooting would be beneficial. Basic methods for micropropagation of curry trees have been developed in this research. Although some improvements are still needed in shoot multiplication rates and rooting efficiency, these methods will allow the production of multiple curry trees from one seed.

Objective 1. Develop micropropagation methods for curry tree and establish a germplasm of *in vitro* plants that serve as nurse cultures.

Task 1a. Develop micropropagation methods from curry tree seeds and establish a germplasm bank of *in vitro* plants that serve as nurse cultures.

The UC-Davis Plant Transformation Facility obtained curry tree seeds from David Morgan on February 1, 2013. Seeds were surface sterilized for three minutes in 70 percent ethanol followed by 30 minutes in a 10 % Clorox solution containing 5ul Tween 20 and rinsed three times in sterile distilled water. Seeds were plated onto agar solidified Murashige and Skoog

(1962) minimal organics medium (MSO) supplemented with various plant growth regulator combinations including 5 mg/l benzylaminopurine (BAP) and 0.4 mg/l gibberillic acid (GA_3), or Lloyd and McCown's Woody Plant Medium (WPM) supplemented with 1.0 mg/l BAP and 1.0 mg/l GA_3. Seed germination was somewhat erratic possibly due to seed vigor. However, once germinated, multiple shoots developed from the germinating seedling (Figure 1). Shoot counts were recorded after 4-8 weeks in culture.

Both formulas of growth media produced multiple shoots from germinated seedling (Tables 1 and 2). The supplemented MSO media produced an average of 5.1 shoots per seedling (± 1.06) and the supplemented WPM media produced an average of 7 shoots per seedling (± 3.61). Clearly, either medium resulted in multiple shoots that could be used to start new plants and obtain multiple plants from a single seed, an improvement over using seeds to start individual plants

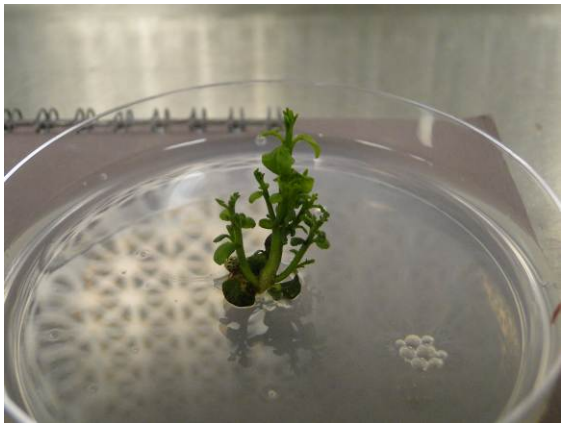

Figure 1. Multiple shoot development from an *in vitro* curry tree seed germinated on Murashige and Skoog (1962) minimal organics medium (MSO) supplemented with 5 mg/l benzylaminopurine (BAP) and 0.4 mg/l gibberillic acid (GA_3)

Table 1 Shoot multiplication response of seeds (i.e., number of shoots per seed) germinated on Murashige and Skoog (1962) minimal organics medium (MSO) supplemented with 5 mg/l benzylaminopurine (BAP) and 0.4 mg/l gibberillic acid (GA_3) after 4 weeks in culture.

Seed pedigree number	No. of shoots per seed
13047-01	4
13047-02	3
13047-03	5
13047-04	3

Seed pedigree number	No. of shoots per seed
13047-05	10
13047-06	8
13047-08	3
Mean (± std. error) shoots per seed	5.1 (± 1.06)

Table 2. Shoot multiplication response of seeds (i.e., the number of shoots produced) germinated on Lloyd and McCown's Woody Plant Medium (WPM) supplemented with 1 mg/l benzylaminopurine (BAP) and 1.0 mg/l gibberillic acid (GA_3) after 8 weeks in culture.

Seed pedigree number	No. of shoots per seed
13047-05m	10
13047-06m	8
13047-08m	3
Mean (± std. error) shoots per seed	7.0 (± 3.61)

Task 1b. If micropropagation from intact seedlings does not provide satisfactory results or seeds become too difficult to obtain, then micropropagation will be attempted from nodal explants or immature seeds.

A seed generated tree was obtained from Abhaya Dandekar on March 27, 2013, and maintained in the greenhouse and 4-inch shoot tips were removed from branches and from suckers that arose from the base of the tree. The leaves were removed and the shoot explants were rinsed for three minutes in 70% ethanol and transferred to 50 ml conical centrifuge tubes containing 20 % Clorox plus 5ul Tween 20 and agitated on a gyratory shaker at 250 rpms for 10 minutes followed by three rinses in sterile distilled water. The bottom 5 mm of the terminal ends of each cutting were damaged by this disinfestation procedure and therefore were removed and discarded prior to plating. The surface sterilized cutting was sectioned into pieces containing two lateral nodes and plated onto Murashige and Skoog (1962) minimal organics medium (MSO) supplemented with various plant growth regulator combinations which included 5 mg/l benzylaminopurine (BAP) and 0.4 mg/l GA_3, 1.0 mg/l BAP, 0.1 mg/l GA_3, and 0.1 mg/l indole-butyric acid (IBA) (Tricoli et al., 1985), WPM supplemented 1.0 mg/l BAP and 1.0 mg/l kinetin or WPM supplemented 1.0 mg/l BAP and 1.0 mg/l GA_3.

Seed and lateral bud explants produced shoots on all of the media (Tables 3 and 4; Figures 2-4). The best shoot multiplication rate was seen with WPM supplemented 1.0 mg/l BAP and 1.0 mg/l GA_3. Mean multiplication rates (± std. error) are given in Tables 3 -5.

Table 3. Shoot multiplication (number of shoots produced per lateral bud) 4 weeks after removal of lateral buds from tree and plating on MSO medium supplemented with 5.0 mg/l BAP and 0.4 mg/l GA_3.

Pedigree number	No. of shoots per lateral bud
13109-01	3
13109-02	3
13109-03	1
13109-04	0
13109-05	0
13109-06	0
Mean (± std. error) shoots per lateral bud	1.2 (±0.6)

Figure2. Shoot development from *in vitro* curry tree lateral bud explants grown on Murashige and Skoog (1962) minimal organics medium (MSO) supplemented with 1mg/l benzylaminopurine (BAP) 0.1 mg/l IBA and 0.1 mg/l gibberillic acid (GA_3)

Table 4 Shoot multiplication 4 weeks after lateral buds were removed from tree and plated on MSO medium supplemented with 1.0 mg/l BAP, 0.1 mg/l GA3 and 0.1 mg/l IBA.

Pedigree number	No. of shoots per lateral bud
13109-07	1
13109-08	2
13109-09	3
13109-10	5

Pedigree number	No. of shoots per lateral bud
13109-11	2
13109-12	3
13109-13	2
Mean (± std. error) of shoots per lateral bud	2.6 (±0.48)

Table 5. Shoot multiplication from lateral buds removed from a mature greenhouse grown tree and cultured on MSO medium supplemented with 1.0 mg/l BAP, 0.1 mg/l GA_3 and 0.1 mg/l IBA, MSO medium supplemented with 5.0 mg/l BAP and 0.4 mg/l GA_3 or WPM supplemented with 1.0 mg/l BAP and 1.0 mg/l GA_3 after 15 weeks in culture.

Media Formulation	Reps	Shoot increase	Ave shoot increase
MSO 1.0 mg/l BAP, 0.1 mg/l GA3 and 0.1 mg/l IBA	3	19	6.3
MSO 5.0 mg/l BAP, and 0.4 mg/l GA3	8	26	3.3
WPM,1.0BA and 1.0GA	17	166	9.7

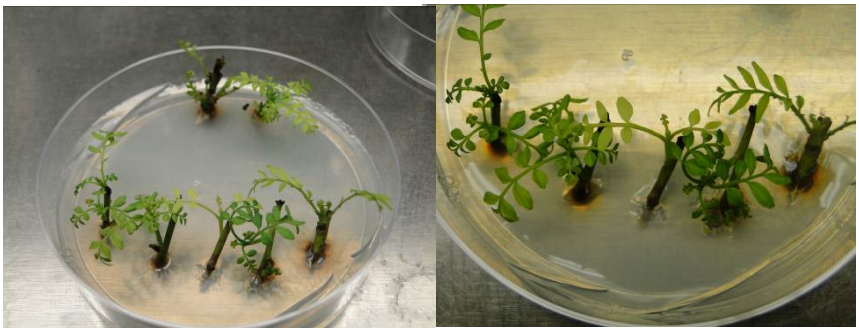

Figure 3. Shoot development from *in vitro* curry tree lateral bud explants grown on Lloyd and McCown woody plant medium (WPM) supplemented 1.0 mg/l BAP and 1.0 mg/l kinetin.

Figure 4. Multiple shoot development from in vitro curry tree lateral bud explants grown on Lloyd and McCown woody plant medium (WPM) supplemented 1.0 mg/l BAP and 1.0 mg/l GA_3.

In vitro shoot cultures produced a significant amount of phenolic compounds as can be seen in Figures 2 and 3. Phenolic production can be detrimental to continued multiplication of shoots in cultures. Phenolic compounds can reach concentrations where they become toxic to the cultures. We are evaluating various medium addendums to try to reduce phenolic production including ascorbic acid, cysteine and reduced glutathione.

Shoot production on WPM based medium resulted in adequate shoot multiplication, but the shoots were slightly chlorotic suggesting that modifying the salt formulation or iron content of the medium could improve shoot quality. To this end, we compared WPM medium to Driver and Kuniyuki plant medium (DKW) and have found that DKW medium produces healthier, darker green shoots (Figure 5). Based on these results we now routinely culture all our curry shoots on DKW medium. To further improve shoot quality we are investigating the effect of additional iron in the form of FeSO4, increased calcium supplied in the form of calcium gluconate into DKW medium, as well as the use of alternate carbon sources in the medium.

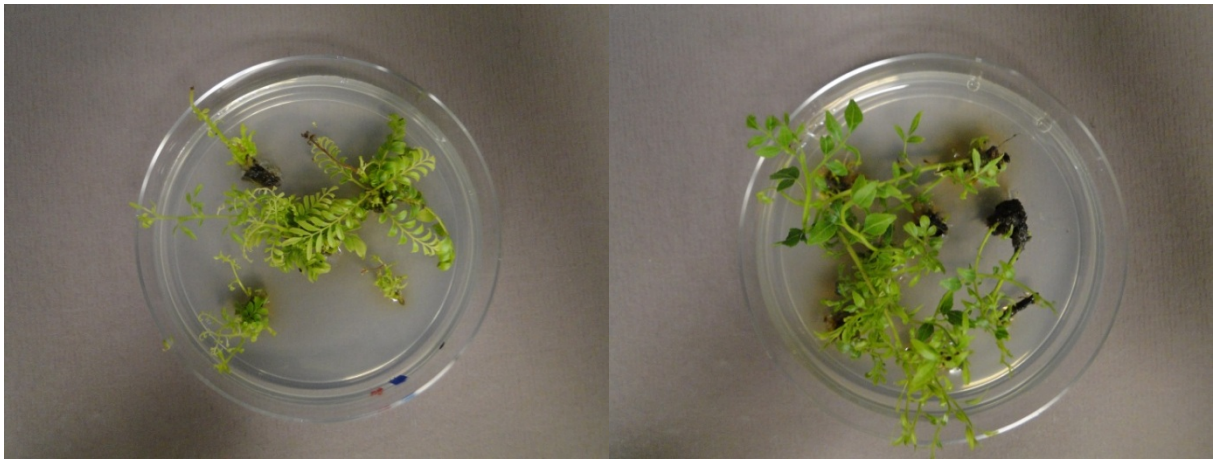

Figure 5. Multiple shoot development from *in vitro* curry tree lateral bud explants grown on Lloyd and McCown woody plant medium (WPM) supplemented 1.0 mg/l BAP and 1.0 mg/l GA_3 (left) compared to multiple shoot development from in vitro curry tree lateral bud explants grown on Driver and Kuniyuki plant medium (DKW) supplemented 1.0 mg/l BAP and 1.0 mg/l GA_3 (right)

Individual shoots which developed from seeds or from terminal or lateral buds were collected and either subcultured back onto shoot multiplication medium or transferred to onto one half strength MSO (½ x MSO) supplemented with 1.0 mg/l indole-3-butyric acid (IBA) for rooting as described by Bhuyan et al. 1997. When subcultured back onto shoot multiplication medium, some individual shoots developed additional multiple shoots but others fail to thrive. In addition, if shoots were removed too close to the basal region of the cluster often no further shoot development was seen. Therefore rather than singulating shoots by removing them from the shoot cluster and returning the base of the cluster back to multiplication medium, we divided large basal clusters of shoots into smaller clusters and subcultured them back onto multiplication medium (Figure 6).

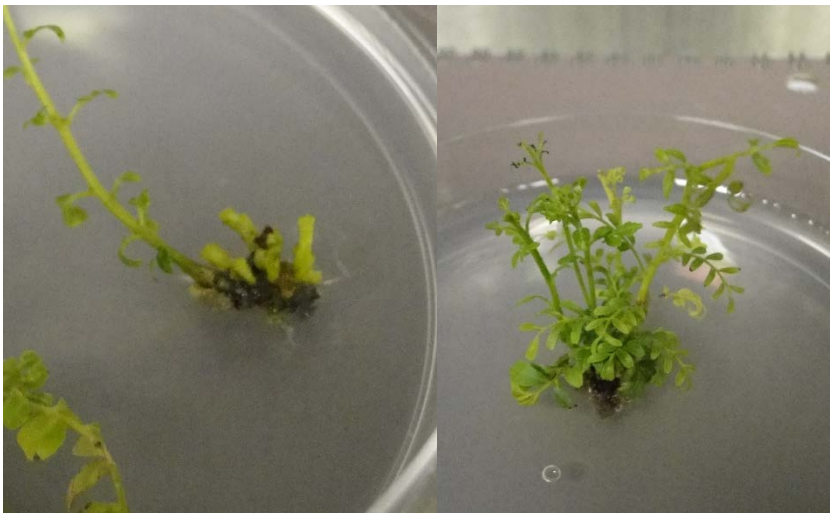

Figure 6. Delayed development of new axillary buds arising from the base of a singulated shoot (left) compared with multiple shoot development from a divided shoot cluster (right).

In our studies, very few of the isolated shoots transferred onto medium containing ½ x MSO supplemented with 1.0 mg/l IBA develop roots. Therefore we evaluated the effect of a short auxin pulse on root formation. Shoots were collected and transferred onto agar solidified one-half strength MS medium supplemented with 15 g/l sucrose and 5 mg/l NAA for five days and then transferred onto agar solidified one-half strength MS medium supplemented with 15 g/l sucrose without hormones. After 14 days only 15% of the shoots developed roots. The size of the shoots transferred to rooting medium maybe one of the factors negatively impacting rooting since larger shoots appear to root better than shorter shoots. However most of the shoots which were used in this rooting study were not well elongated which can hamper root formation. Increasing GA_3 levels may produce larger shoots which may in turn increase the rooting percentages. Preliminary results indicate that the addition of 1.0 mg/l GA_3 is beneficial to shoot elongation but when increased to 10 mg/l $GA_{3,}$ shoots exhibit excess elongation resulting in spindly shoots and strap like leaves. These shoots are more susceptible to damage when subcultured (Figure 7)

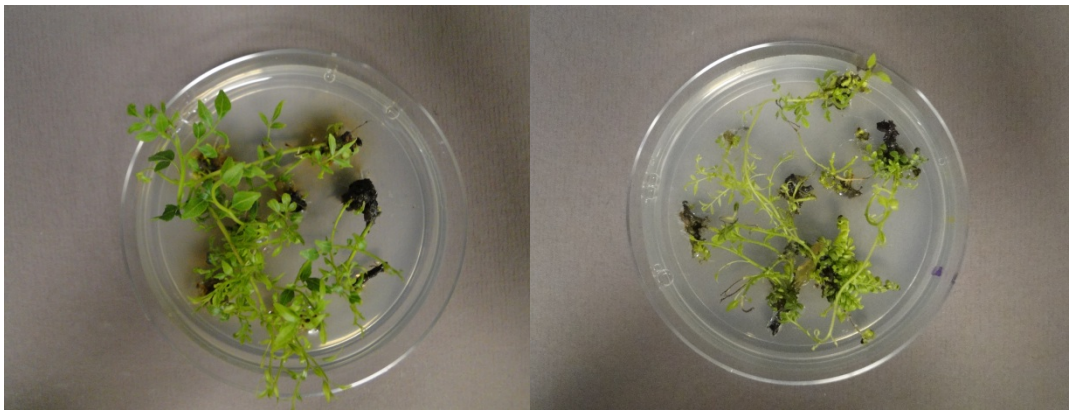

Figure 7. Curry shoot micropropagated on agar-solidified DKW medium supplemented with 1.0 mg/l BA and 1.0 mg/l GA_3 (left) or 10.0 mg/l GA_3 (right)

We have begun acclimating rooted shoots to soil by transferring them to Sunshine Mix #1 and maintaining them in a high humid environment for 3-5 days and then slowly reducing the humidity over the course of 7 to 10 days. Rooted plantlets have been establish in soil and although our sample size in small 83% (5/6) of the plants transferred to soil survived (Figure 8).

Figure 8. Tissue culture-generated curry trees after acclimatization to soil

Temporary immersion systems have been developed and utilized in numerous plant species for large-scale micropropagation. Temporary immersion systems have been used successfully to micropropagate banana, coffee, date palms, grapes, pineapple, potato and serviceberry. The system was designed to reduce manual labor associated with micropropagation systems. In a conventional plant tissue culture system, plants cloned in vitro expend the nutritional resources and growth factors in the media within 3-6 weeks. Therefore, plant material has historically been manually transferred to fresh culture vessel containing agar-solidified media every 3-4 weeks. The labor involved in this activity significantly increases costs of the production of in vitro plants. In fact, much of the overall cost associated with the micropropagation of plants is reflected in the cost of labor. Temporary immersion systems significantly reduce labor costs associated with micropropagation since rather than moving individual tissue pieces, the tissue remains in place and the medium is replenished. Temporary immersion systems also avoid continuous immersion of tissues in liquid, while allowing for efficient mixing and replenishing of nutrients in the medium. Shoot multiplication rates have been reported to be significantly higher using temporary immersion systems than on semi-solid medium. Shoots produced using temporary immersion are reported to be longer, of better quality, exhibit less hyperhydricity, and acclimate more efficiently to soil than shoots produce on semisolid medium. The system also allows plant cultures to be subjected to temporary desiccation in order to pre-condition plants prior to transfer to soil thereby improving acclimatization to soil.

We have begun to explore multiplication of curry trees in two different commercially available temporary immersion systems; the RITA system and the SETIS system (see Figures 9 and 10). Shoots were placed in these two temporary immersion systems and submersed in liquid WPM supplemented with1.0 mg/l BAP and 1.0 mg/l kinetin twice a day for duration of two minutes per immersion. Although shoot multiplication has been observed in these systems, the plants are chlorotic and exhibit some vitrification indicating either a modification of the nutrient formulation or change in the immersion time is required.

Figure 9. Multiple shoot development of in vitro curry tree on Lloyd and McCown woody plant medium (WPM) supplemented 1.0 mg/l BAP and 1.0 mg/l GA3 in a RITA temporary immersion bioreactor

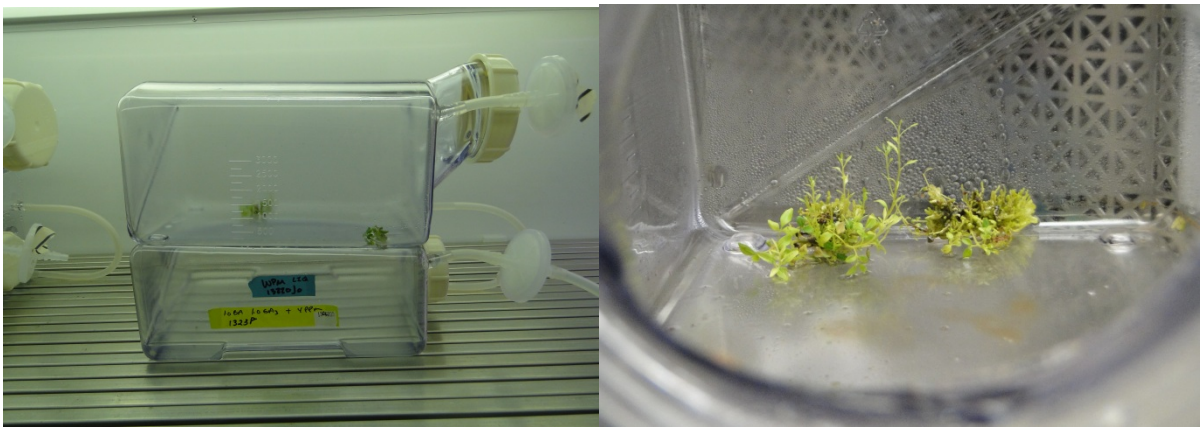

Figure 10. Inoculation of SETIS temporary immersion bioreactor with *in vitro* curry tree cultures on Lloyd and McCown woody plant medium (WPM) supplemented 1.0 mg/l BAP and 1.0 mg/l GA3-side view (left) or DKW supplemented 1.0 mg/l BAP and 1.0 mg/l GA3 view through opening (right) .

Task 2. Transfer technology to California citrus nurseries.

This task was scheduled to occur at the end of the second year of funding. However, the grant was only funded for one year. We are looking into the possibility of conducting the technology transfer using other funding sources.

Objective 2. Determine the suitability of the micropropagated curry tree for sale to and use by consumers.

Task 3. Conduct a survey of consumers as to their preferences for micropropagated or seed propagated curry trees.

The studies under this task were scheduled to occur in the second year of the project.

Objective 3. Determine the suitability of the micropropagated curry tree for rearing ACP.

Task 4. Conduct comparative studies on life history parameters of ACP on micropropagated and seed generated plants.

The studies under this task were scheduled to occur in the second year of the project. However, the plants generated by the UC-Davis Plant Transformation Facility will be made available to David Morgan at CDFA for use in rearing ACP and its parasitoid, *Tamarixia radiata*.

Conclusions and Application to a Citrus Nursery Situation:

This research has provided several basic methods to micropropagate curry trees from either seedlings (from seed) or from nodal explants. These methods allow propagation of multiple curry trees from a single seed, improving the efficiency of plant production over traditional methods (e.g., one seed, one plant). The plants are initially produced under aseptic conditions, so pest problems are minimized. These methods may be adapted by a nurseryman to produce sufficient curry tree plants to meet market needs, and possibly provide researchers with a method to produce plants for use in research on ACP.

Literature Cited:

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