Development of Disease Resistant Hybrid Rootstocks Through Cell Culture

Project No.: **HORT29.Dandekar**

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A. Summary

Almond (*Prunus dulcis*) is the #2 among California's top 20 agricultural commodities, grown on ~870,000 acres with an annual production in 2015 of close to 2 billion lbs valued at $5.9 billion (farm gate value). One hundred percent of U.S. almond production occurs in California, and CA almonds are a major export crop. The production of almonds in CA is targeted by a number of diseases and pests that limit their profitability including crown gall, nematodes and *Phytophthora* to name a few. Profits are reduced in the form of unsalable nursery stock, lowered productivity from infected trees, and increased susceptibility of infected plants to other pathogens and adverse environmental conditions. Many of these diseases can be reduced or eliminated by the selection of resistant rootstocks (Dandekar et al., 2012, Escobar et al., 2001; 2002; Walawage et al., 2013). The increased adoption of peach-almond hybrid rootstocks like Hansen has significantly improved almond yields for the industry but has also increased susceptibility to infectious diseases and other rootstock ailments. Therefore creating hybrid rootstocks resistant to these ailments is a high priority of the almond industry.

The focus of this work is to develop cell culture systems from hybrid almond rootstock tissues and to develop the ability to regenerate plants from these tissues. The development totipotent cell cultures will provide new opportunities to address genetically many of the disease and pest problems facing the almond industry. *Prunus* hybrid seed were created using parental materials that contain resistance traits of interest. A key objective of our study is to isolate and culture plant stem cells from these hybrid tissues and to develop cell and tissue culture systems to propagate individual hybrid seed sources, leading to the development of novel hybrid rootstocks for almond orchards in California.

B. Objectives

Goal: Develop embryogenic cultures of peach-almond hybrid rootstocks that can be regenerated into plants and that capture disease resistance present in one of the parental genotypes.

Objective 1: Develop embryogenic cultures of peach-almond hybrid rootstocks that capture existing disease/pest resistance traits leading to successful propagation of hybrid clonal rootstocks.[Years 1 and 2]

Activity 1: Culture immature embryos from hybrid almond seed. [Years 1 and 2]
Activity 2: Culture root tissues from germinating hybrid seed. [Years 1 and 2]

Objective 2: Micro propagation and validating disease/pest resistance [2 years; years 2 and 3]

Activity 3: Develop micro propagation system for new genotypes. [Years 2 and 3]
Activity 4: Validating disease resistance in the lab and greenhouse. [Years 2 and 3]

C. Results

Objective 1: Develop embryogenic cultures of peach-almond hybrid rootstocks that capture existing disease/pest resistance traits leading to successful propagation of hybrid clonal rootstocks.

Activity 1: Culture immature embryos from hybrid seed: Embryogenic cultures of peach-almond and almond-peach hybrid rootstocks were made to achieve this goal. With the help of Tom Gradziel, Greg Browne and others who have knowledge and developed crossing strategies we made several controlled crosses. Even though peach-almond cultures were not successful in regenerating into somatic embryos, almond-peach crosses were successfully generated independent somatic embryo lines. Multiple crosses were made using a peach selection with root knot nematode and possible ring nematode resistance in early springs of 2018 and 2019, to create hybrid seed for this study. Mission was used as the almond seed parent. Mission almond flowers were bulk pollinated with pollen of the different peach parents. This would allow greater genetic diversity in the pollen donor and so greater probability of fertilization and subsequent regeneration success. We will be using molecular markers to identify the specific parents of any regenerated plant. Several weeks after pollination embryos were rescued from hybrid seed at early stages of development to propagate embryonic stem cells. Multiple experimental conditions were evaluated, including age of embryo, culture media, growth factors and incubation conditions, to induce the embryos to proliferate in culture to generate somatic embryos (Figures 1A and 1B). In 2018 out of 50 immature nuts 10 embryogenic cultures were initiated and 4 independent hybrid somatic embryo lines were initiated (Table 1). All these cultures were validated for producing somatic embryos which continued to proliferate when exposed to embryo proliferation media. Desired embryo lines were desiccated to initiate germination and shoots were obtained (Figure 1C). Propagated plantlets look healthy and similar to most of almond x peach hybrids. More crosses were made in 2019 because the cooler weather prolonged bloom. In 2019 out of 50 immature nuts 20 cultures were initiated and 14 independent somatic embryo lines were obtained (Table 1). Germination systems were developed for the somatic embryos to enable the further testing of their genetic attributes in the laboratory and greenhouse.

Table1: Independent embryo lines obtained in years 2018 and 2019 from almond-peach crosses

2018				2019			
Nut harvesting date	Number of embryos rescued	Number of embryos established in the culture	Number of Independent somatic embryo lines established	Nuts harvesting date	Number of embryos rescued	Number of embryos established in the culture	Number of Independent somatic embryo lines established
5-3-18	10	0	0	5-17-19	10	0	0
5-7-18	10	0	0	5-21-19	20	9	6
5-9-18	15	6	2	5-22-19	20	11	8
5-13-18	15	4	2				

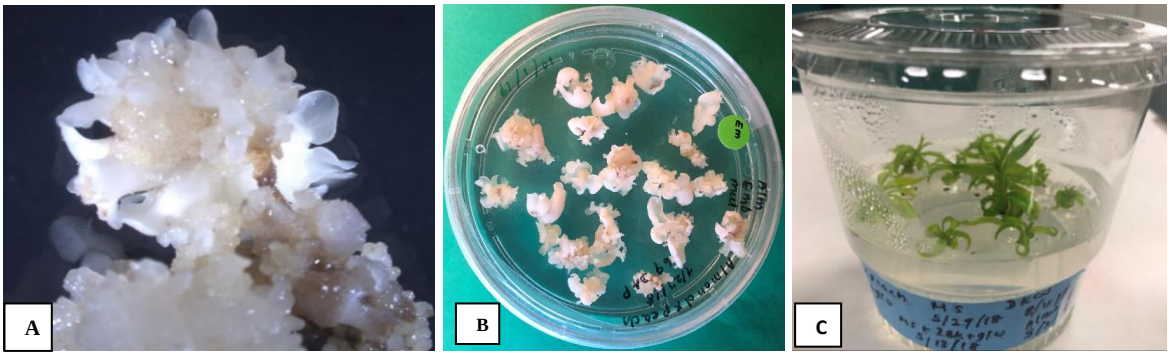

Figure 1: Somatic embryogenesis and micro-propagation of almond-peach crosses. A. Somatic embryos generated from almond-peach crosses, B. Proliferating embryos,
C. Plantlets regenerated from germinating hybrid somatic embryos

Activity 2: Culture root tissues from germinating hybrid seed: Hybrid almond seed will be obtained from different sources, surface sterilized and then allowed to germinate into sterile plantlets under various conditions. Different parts of the seedlings will be dissected and introduced into culture to determine which part of the seedling has embryogenic stem cells that could be propagated to develop embryogenic cultures. Multiple treatment combinations will be evaluated to define seedling age, tissue explant, culture media, and growth conditions that will allow the propagation of embryo stem cells and their development into somatic embryos. Once the cell lines can be established embryogenesis will be induced and plants regenerated by germinating the embryos. The plants obtained can then be evaluated in the greenhouse and the field for their horticultural characteristics the most pertinent of which is the maintenance of hybrid vigor. We have experience developing such systems for grapevine and walnut and (Dandekar *et al.*, 2012; Walawage *et al.*, 2013). Expected period for this activity: 0 - 36 months depending on the availability of good quality hybrid seed.

Objective 2: Micro propagation and validating disease/pest resistance.

Activity 3: Develop micro propagation system for new genotypes: Somatic embryos obtained from the activity 1, were germinated and shoots were obtained. They shoots were multiplied using modified shoot multiplication media (Figure 2D). The multiplied shoots were rooted to make plants, typical micro-propagation protocols (Figure 2E). At present shoots are in

multiplication and rooting stage to obtain required number of plants to test in the lab and green house for different pathogens.

Figure 2. Shoot proliferation and root induction under in vitro conditions. D. Shoot proliferation in shoot induction media, E. Root proliferation in root induction media

Activity 4: Validating disease resistance in the lab and greenhouse. Disease resistance will be validated in the lab and greenhouse. Propagated plants or shoots can be exposed to disease causing organisms and pests in culture and efficacy documented by a disease phenotype assay. We have done this successfully using bacterial pathogens that cause crown gall disease and Pierce's disease (Escobar et al., 2001, 2002; Aguero et al., 2005; Dandekar et al., 2012). We have successfully tested for resistance to nematodes (Walawage et al., 2013). Micro-propagated plants from tissue culture will be acclimatized transferred to the greenhouse to validate disease resistance phenotype as we have described earlier (Dandekar et al., 2012; Nascimento et al., 2016 and Gouran et al., 20160 (Expected period for this activity: 12 - 48 months depending on plants become available for testing for disease and pest resistance.

D. Discussion and Conclusions

The goal of this study is to culture plant stem cells from *Prunus*-almond hybrid seed tissues and to develop cell- and tissue-based propagation systems leading to the development of novel hybrid rootstocks for almond orchards in California. This inter-specific rootstocks can be tolerant, moderately resistant or resistant to pest and disease problems can significantly improve almond yields for the industry. When selecting the parents for crosses our main focus was to select parents with already has some resistance to certain pests and pathogens like phytophthora, crown gall or nematodes. By developing somatic embryo lines from these crosses we are able to produce plantlets in larger quantities with the same genetic backgrounds with shorter period of time than the conventional breeding programs.

Embryogenic cultures of peach-almond hybrid rootstocks and almond-peach were made to achieve this goal. Several controlled crosses were carried out during the spring of 2018 and 2019. In 2018 both peach-almond and almond-peach crosses were carried out while in 2019 we were only focused onto almond-peach crosses. Almond-peach crosses were successfully generated independent somatic embryo lines in the *in vitro* cultures while peach-almond cultures were not generated any somatic embryos. The reason could be the harvesting time of the immature nuts and embryo development stage at the time of harvesting nuts. Nut harvesting window and embryo development stages are very the critical factor when generating somatic embryos from this inter-specific hybrids. For almond-peach cultures harvesting window and embryo development stage was determined previous year and that helped when generating independent embryo lines from them. In almond-peach multiple crosses were made using a peach selection with root knot nematode and possible ring nematode resistance. Mission was used as the almond seed parent. Mission almond flowers

were bulk pollinated with pollen of the different peach parents. Molecular markers will be used to identify the specific parents of any regenerated plant. All the almond-peach cultures were validated for producing somatic embryos which continued to proliferate when exposed to embryo proliferation media. Desired embryo lines were desiccated to initiate germination and shoots were obtained. The roots were induced in those shoots. Propagated plantlets look healthy and similar to most of almond-peach hybrids. Germination systems were developed for the somatic embryos to enable the further testing of their genetic attributes in the laboratory and greenhouse.

Disease resistance will be validated in the lab and greenhouse. Propagated plants or shoots can be exposed to disease causing organisms and pests in culture and efficacy documented by a disease phenotype assay. We have done this successfully using bacterial pathogens that cause crown gall disease and Pierce's disease (Escobar et al., 2001, 2002; Aguero et al., 2005; Dandekar et al., 2012). We have successfully tested for resistance to nematodes (Walawage et al., 2013). Micro-propagated plants from tissue culture will be acclimatized transferred to the greenhouse to validate disease resistance phenotype as we have described earlier (Dandekar et al., 2012; Nascimento et al., 2016 and Gouran et al., 20160 (Expected period for this activity: 12 - 48 months depending on plants become available for testing for disease and pest resistance.

E. Materials and Methods

Immature fruits of controlled pollinated almond-peach were collected beginning 2 month after pollination and continuing through two weeks. Fruits were surface sterilized and dissected immediately or were stored at 2--4°C for later use. Sterilization consisted of a 10-s rinse in 70% (v/v) ethanol followed by a 20-min immersion in 10% (v/v) sodium hypochlorite (with 0.1 ml of detergent added per liter), and three washes in sterile distilled water. The fruits were then opened carefully and embryos were rescued using aseptic procedures. Twenty to twenty five fruits were dissected for each collection date. These explants were placed in petri plates on a conditioning medium of Driver and Kuniyuki which contained several plant hormones added to the basal medium. Gelzan (Sigma Co.) at 0.22% (w/v) was used for solid media. The pH was adjusted to 5.7 with KOH. The media were sterilized for 20 min at 121°C.

Explants were placed on the conditioning medium for 2--4 weeks, after which they were transferred to basal medium for repeated 2--4 week transfers. Cultures were grown in the dark at ambient room temperature or in a growth chamber set at 22°C with a 16-h photoperiod under standard cool white fluorescent lamps. When somatic embryos were emerging in this media they were moved to no hormone Driver and Kuniyuki media basal media to multiply them. The well-developed, healthy, white embryos were dried in a desiccator for a few days until they were ready to germinate and then cultured them on shoot induction media to obtain hybrid plantlets. Then these plantlets were transferred into a root induction media. Plants were grown under a cool white fluorescent lamps in a growth chamber with a 16-h photoperiod.

Media and culture conditions were optimized for shoots and root proliferation to use for validating disease resistance assays in the lab and greenhouse. These proliferated shoots with well-developed root system will be used for different pest and disease assays. Propagated plants or shoots will be exposed to disease causing organisms and pests in culture and

efficacy documented by a disease phenotype assay. Disease resistance will be evaluated in the lab and greenhouse under controlled conditions. Elite lines will be selected and further propagated.

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F. Publications that emerged from this work

No publications to report.