Unified Grant Management for Viticulture and Enology
INTERIM PROGRESS REPORT FOR 2021-2022 FUNDING CYCLE

CALIFORNIA GRAPE ROOTSTOCK IMPROVEMENT COMMISSION (CGRIC)
CALIFORNIA GRAPE ROOTSTOCK RESEARCH FOUNDATION (CGRRF)

1. **Summary**:

Project Title: Stacking disease and pest resistance in grapevine rootstocks
Principle Investigator: Abhaya M. Dandekar, Plant Sciences Department, UC Davis

The goal of this project is to develop grapevine rootstocks that combine their existing resistance to pests like Phylloxera and/or to nematodes with RNAi-mediated resistance to bacterial crown gall disease. The project was initiated on March 31, 2020 with input from members of the commission during their meeting on Feb. 4, 2020. Based on this input we will target in addition to the proposed rootstock GRN1 the following additional pest resistant rootstocks: 1103P, 101-14 Mgt, 110R and Freedom. We were successful in developing somatic embryo lines for 4 of the five target rootstocks except for Freedom which took a bit longer and was recently established (Dec 2021). This allowed us to accomplish objective 1. Using these somatic embryo cultures, we initiated the transformation process of introducing the RNAi-mediated resistance into the first 4 including Thompson Seedless (TS) as a control initiating Objective 2. Transformation of Freedom embryos will occur this year (2022). We have already started to obtain transgenic plants from the TS, GRN-1 and 1103P embryo lines. Plants from 101-14 Mgt and 110R will be received in a few weeks. In objective 2 of this proposal, we will propagate these plants to test their resistance to crown gall. We have successfully developed a rapid method to test crown gall resistance in young plantlets. This rapid method was validated on all 5 rootstock genotypes and showed that the wild type plants were all very sensitive to crown gall formation. These initial results will be compared to plantlets obtained from embryo lines expressing the RNAi-based resistance.

2. **Interim Progress Report**: Oct. 1, 2021 to Sept. 30, 2022 for 2021-2022 funding cycle.

3. **Project Title and UGMVE proposal number**: Stacking disease and pest resistance in grapevine rootstocks – 2021-2529.

4. **Principal Investigator**: Abhaya M. Dandekar, Department of Plant Sciences, University of California, Davis; 1 Shields Ave; Davis CA 95616.

Cooperator(s): Andrew M. Walker - Louise Rossi Endowed Chair, Department of Viticulture and Enology; University of California, Davis; 1 Shields Ave Davis CA 95616.

David M. Tricoli - Plant Transformation Facility; 192 Robbins Hall, University of California, Davis; 1 Shields Ave; Davis CA 95616.

5. **Objective(s) and Experiments Conducted to Meet Stated Objective(s)**:

Goal: To develop grapevine rootstocks that combine existing resistance to pests with RNAi-mediated resistance to disease. We propose to accomplish this goal via the following two objectives in a period of three years.

Objective 1: Develop and introduce crown gall resistance into grapevine rootstocks resistant to *Phylloxera* and nematodes

In our proposal we had selected a single rootstock GRN-1 (*V. rupestris* x *M. rotundifolia*) based on its high resistance to *Phylloxera* and nematodes. However, after our discussion with the board on Feb 4, 2020 we have included in this study rootstocks of their recommendation as they are of interest to California growers. These other rootstocks have a similar profile as they are resistant to different pests and environmental stress but are sensitive to crown gall. These include 1103P (*V. berlandieri* x *V. rupestris*), 101-14 Mgt. (*V. riparia* x *V. rupestris*), Freedom 1613 (*V. solonis* x. *Othello*) x Dog Ridge, 110R (*V. berlandieri* x *V. rupestris*) x Dog Ridge. Thompson Seedless somatic embryos will be used as the control. In the spring 2020, we initiated embryogenic callus from anther filaments of all of the above genotypes using methods that we have previously described (Aguero et al., 2006). The embryogenic callus was induced to make embryos (Fig. 1A) that can be clonally multiplied via repetitive embryogenesis where single embryos produce clones of themselves. We have begun to germinate these embryos to make sure that they are able to be germinated into plantlets as shown (Fig. 1B). This is where we currently are confirming the ability of all the embryogenic cultures to germinate.

The next step was to transform these embryos with the binary vector pDE00.0201 (Fig. 2A) to successfully express the RNAi-mediated resistance to crown gall as we have previously

described (Escobar et al., 2001, 2002, 2003). We transformed 4 embryo lines, GRN-1, 1103P, 101-14 Mgt, 110R and Thompson Seedless as a control. Freedom will be transformed in 2022. Freedom transformation and recovery work will take 6 to 9 months to complete. We have already started obtaining transgenic tissue culture plants from the TS, GRN-1 and 1103P embryo lines (Fig.1C and D). They are currently multiplying in the lab and greenhouse. Transgenic plants from other genotypes will be obtained in a few weeks.

Wild type plants of all the six genotypes that were obtained from Foundation Plant Services (FPS) at UC Davis are being maintained in the greenhouse (Fig. 1E). Some of the transgenic plants from genotypes TS, GRN-1 and 1103P are being maintained in the greenhouse as well. As transgenic plants of all the desired genotypes become available, they will be propagated and evaluated for crown gall resistance by challenging with different *Agrobacterium* strains.

Objective 2: Evaluate the efficacy of the combined resistance to disease and pests.

Here we have initiated two activities; the first is to develop an efficient micropropagation system to multiply plantlets obtained from individual embryo lines that will be used for testing the RNAi-mediated resistance to bacterial crown gall delivered by the binary vector pDE00.0201 shown in Fig 2A. The second activity that we have initiated is to develop efficient root systems that can be used for testing not only crown gall but also to confirm the pest resistance status of the combined resistance present in the individual rootstocks. To successfully accomplish this activity, we have cloned the two-root inducing (RolB and RolC) genes from *Agrobacterium rhizogenes* strain A4 (ArA4) (Britton et al., 2008). Using the known DNA sequence of this strain we were able to design appropriate primers to successfully PCR amplify these two genes on a single fragment of DNA. This fragment of DNA also contains the natural regulatory regions that are competent to express these two genes simultaneously in plant tissues. This piece of DNA that contains these two genes was incorporated to create two binary vectors shown in Fig 2B and C. The first (Fig 2B) also contains a gene that gives a red florescence and will be useful to identify the induced roots. The second binary (Fig 2C) contains a gene that improves the resilience of the root system. We plan to initially test these on wild type plants germinated from the different embryo lines that we now have in culture to validate their sensitivity to crown gall.

To evaluate the degree of resistance to crown gall, we developed a protocol using wild type tissue culture plants that were obtained from the somatic embryos and their leaf petioles. The assay we developed is very sensitive and quick, giving results within 3 weeks after challenging with *Agrobacterium* strains. For this experiment we used three different *Agrobacterium* strains; C58, 20W-5A and A281. Liquid *Agrobacterium* cultures were prepared with a cell density of 10^8 cells/mL by culturing bacteria from a fresh plate. Eight plants or leaf petioles were inoculated from each genotype. When inoculating the shoots, a scalpel was dipped in bacteria solution and

stabbed in three places on the stem starting from the bottom. In the leaf petiole assay, petioles were dipped in *Agrobacterium* solution for 20 minutes. Then the inoculated leaves were transferred to co-cultivation media for 48 hours and transferred to a media with timentin an antibiotic to control the overgrowth of the infecting bacteria. Tumor formation was observed over three weeks after inoculation. For this analysis the size of the gall was considered and a scale of 1-4 was used. No galls = 0, 25% =1, 50%=2, 75%=3 and 100%=4. Average score from eight replicates were considered when deciding the degree of resistance (Table 1). Galls were observed after 2 ½ to 3 weeks in inoculated shoots (Fig 3, A-D) and leaf petioles (Fig. 4, A-C). Protocols that were developed using stems and leaf petioles will be used to evaluate the degree of resistance to crown gall, in transgenic tissue culture plants that are available in the lab. Parallel experiments will be conducted with wild type plants from all the genotypes. This will help to evaluate the crown gall resistant genotypes compared to wild type.

We are in the process of developing a quick assay for greenhouse grown grape plants to evaluate resistance to crown gall. Cuttings from the initial plants can be rooted using an aeroponic system (mist bed) in the greenhouse (Fig 5A). Plants developing enough healthy roots (Fig 5B) can be inoculated with *Agrobacterium strain* A281. Roots were immersed in the bacterial solution for one hour and transferred back to the mist bed. Mist bed contained ½ Murashige and Skoog medium (Fig 5C), with pH adjusted to 5.2 to favor the growth of *Agrobacterium* strain A281. After three weeks of inoculation gall formation was observed (Fig 5D). We are still optimizing this protocol.

6. **Summary of Major Research Accomplishments and Results by Objective**

The current roster of grapevine rootstocks used in California are typically resistant to an individual disease or pest. Therefore, combining resistance traits in a single rootstock could make for a more sustainable and durable solution. The objective 1 of this project is to stack resistance traits in grapevine rootstocks and develop a single rootstock that has resistance to multiple pathogens. To achieve this objective, first we selected the rootstocks of interest to CA growers that are naturally resistant to *Phylloxera* and nematodes. In spring 2020, we initiated embryogenic callus from anther filaments of the genotypes of interest. The embryogenic callus was induced to make embryos that can be clonally multiplied via repetitive embryogenesis where single embryos produce clones of themselves. For accomplishments with Objective 2 we have begun germinating these embryos to develop plantlets that will be multiplied by micropropagation. Thompson Seedless somatic embryos and plants were generated to serve as the control. Table 2 summarizes all the events taken place from obtaining somatic embryos to transformation. We have also successfully cloned the root-inducing RolB and RolC genes from *Agrobacterium rhizogenes* A4 and build two binary vectors (Fig 2B, C) that will be used to

develop root systems for testing the RNAi-mediated resistance to crown gall more efficiently.

Wildtype grape shoots and leaf petioles of genotypes GRN-1, 1103P, 01-14 Mgt. and Thompson Seedless were challenged with *Agrobacterium* strains C58, 20W-5A and A281 to find out the degree of basal resistance to crown gall disease (Table 1). Wild type plants of all the genotypes showed susceptibility to crown gall when challenged with above *Agrobacterium* strains. Currently we have TS, GRN-1 and 1103P transgenic plants already being propagated. Transgenic plants of other three genotypes will be received in early 2022. Those shoots will be multiplied and plantlets challenged with *Agrobacterium* strains. Results will be compared with the wild type plants to determine the degree of resistance to crown gall disease.

7. **Outside Presentations of Research**:

Given the current COVID-19 situation there has been no opportunity to present this work. We look forward to communicating our research results to end-users and stakeholders given an appropriate opportunity.

8. **Research Success Statements**:

This project in 3 years will establish a pathway to stack disease and pest resistance in grapevine rootstocks. We are currently at 2 years into this 3-year project. The project output will be elite rootstock lines that are resistant to both crown gall and *Phylloxera*. The elite rootstocks developed by this project will be candidates for commercialization after field-testing to evaluate horticultural attributes and further validation of disease and pest resistance under field conditions. The validation of the stacking strategy developed by this project can be used to stack additional sources of resistance using RNAi against other pathogens.

9. **Funds Status**: Include a general summary of how funds were spent.

We have expended 50% of the funds the remaining 50% will be expended by Sept. 30, 2022. We have a continuing proposal that was submitted on Jan 31, 2022 and that will fund the work through to its conclusion in 2023.

Table 1: *In vitro* rapid leaf petiole assay to determine sensitivity of wild type grapevine rootstocks crown gall disease using different tumor forming *Agrobacterium* strains

Genotype	C58	20W-5A	A281
Thompson Seedless	3.3	2.8	1.9
GRN-1	1.6	1.8	3.7
101-14 Mgt.	2.6	2.3	3.1

Control	0	0	0

Scale of 1-4. No galls = 0, 25% =1, 50%=2, 75%=3 and 100%=4

Table 2: Status of somatic embryos and regenerated plants in the lab and greenhouse

Rootstock	Somatic embryos generated	Germinated to obtained wildtype plants	Embryo transformed to stack crown gall resistance	Transgenic plants obtained and in tissue culture	Wildtype stock plants available in the green house	Transgenic plants available in the green house
Thompson Seedless	Yes	Yes	Yes	Yes	Yes	Yes
GRN-1	Yes	Yes	Yes	Yes	Yes	Yes
1103 P	Yes	Yes	Yes	Yes	Yes	Yes
101-14 Mgt	Yes	Yes	Yes	In progress	Yes	No
110 R	Yes	Yes	Yes	In Progress	Yes	No
Freedom	Yes	No	No	No	Yes	No

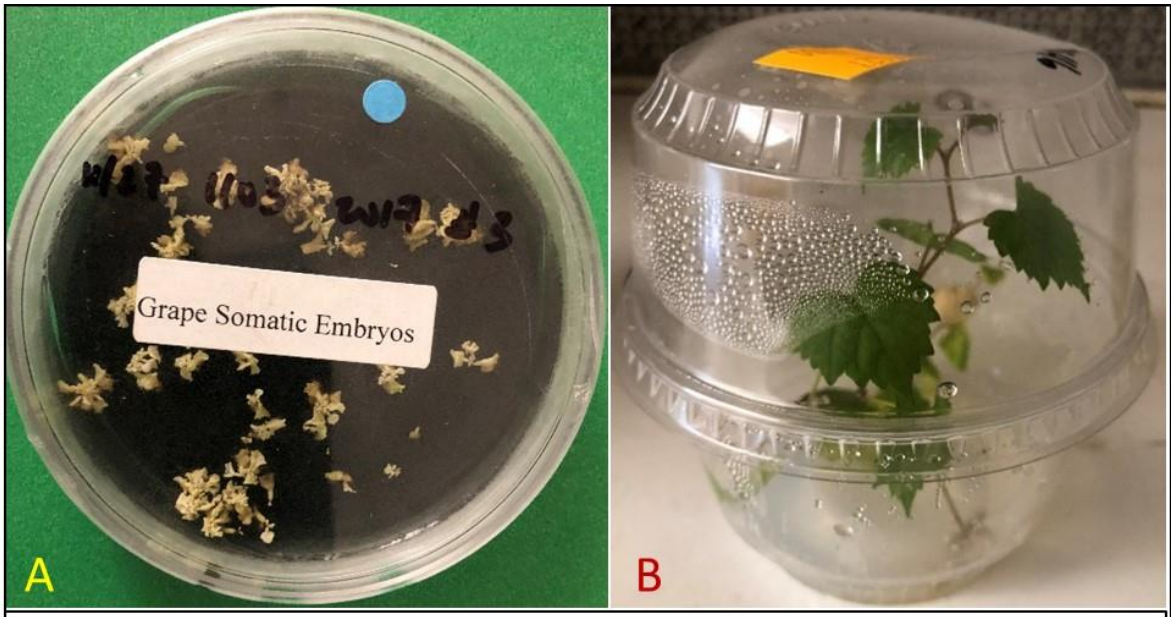

Figure 1: (A) Somatic embryo cultures developed from grapevine rootstocks for introduction of RNAi-mediated resistance crown gall. B) Grapevine rootstock seedling germinated from a somatic embryo culture ready for propagation and testing.

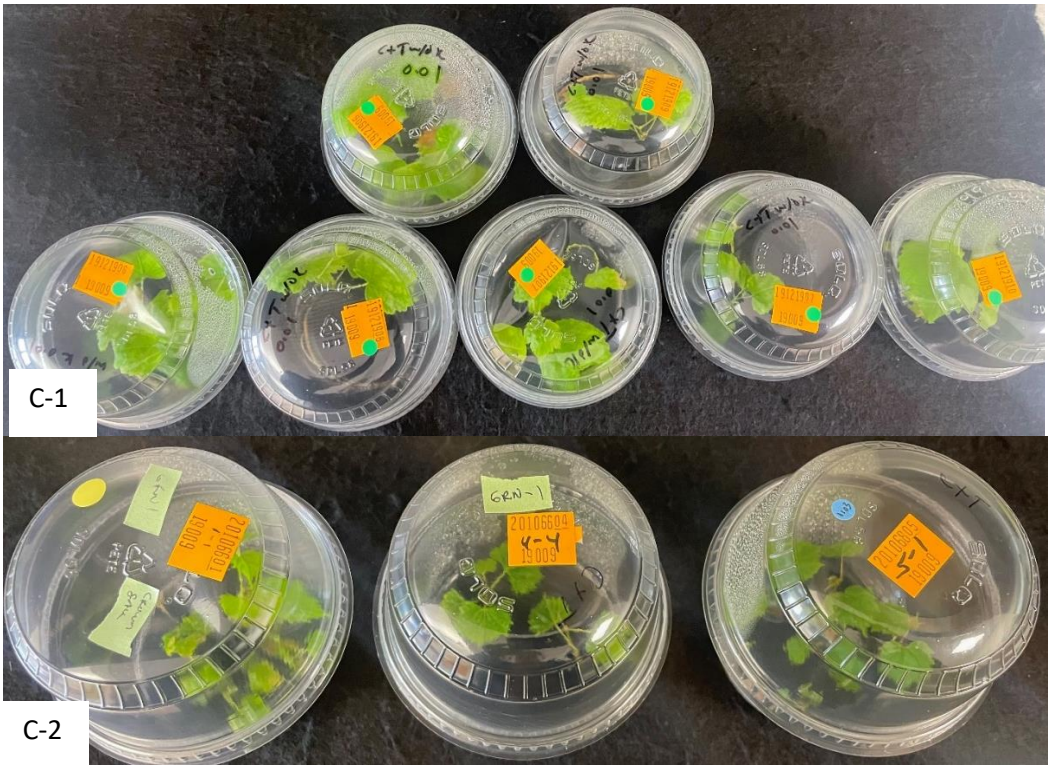

Figure 1(C): Transgenic grape tissue culture plants. (C-1)-TS, (C-2) GRN-1 and 1103P.

D

Figure 1(D): Transgenic TS, GRN-1 and 1103P plants in the greenhouse.

E

Figure1(E): Wildtype plants of GRN-1, 1103P, 01-14 Mgt., 110R, Freedom and Thompson Seedless plants in the greenhouse.

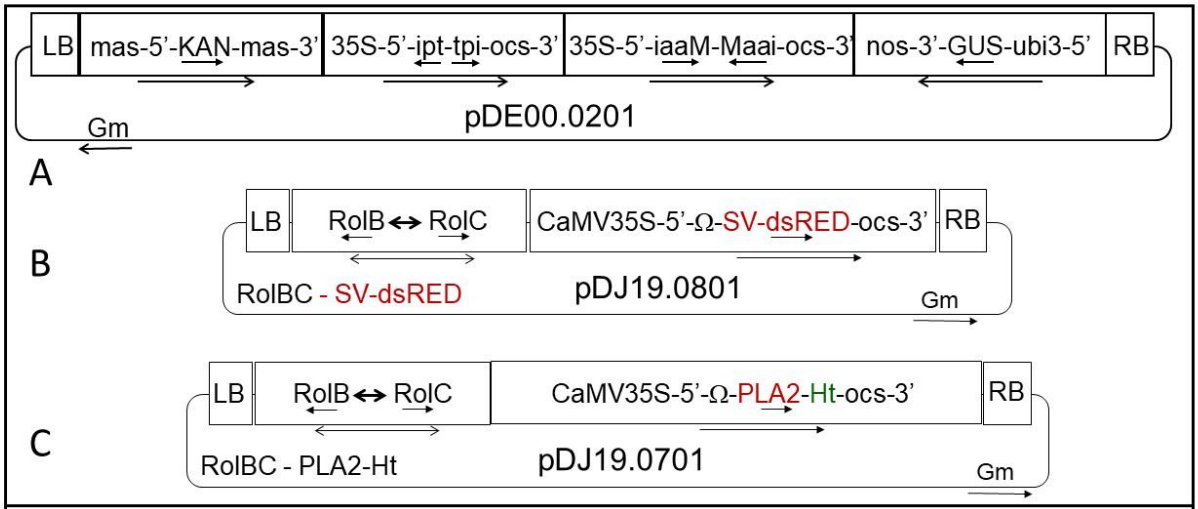

Figure 2: Binary vectors constructed to confer and to test for resistance to crown gall. A) Binary vector pDE00.0201 will be used to develop RNAi-mediated resistance to crown gall. Binary vector pDJ19.0801 (B) and pDJ19.0701 will be used to induce test for efficacy of RNAi-mediated resistance to crown gall.

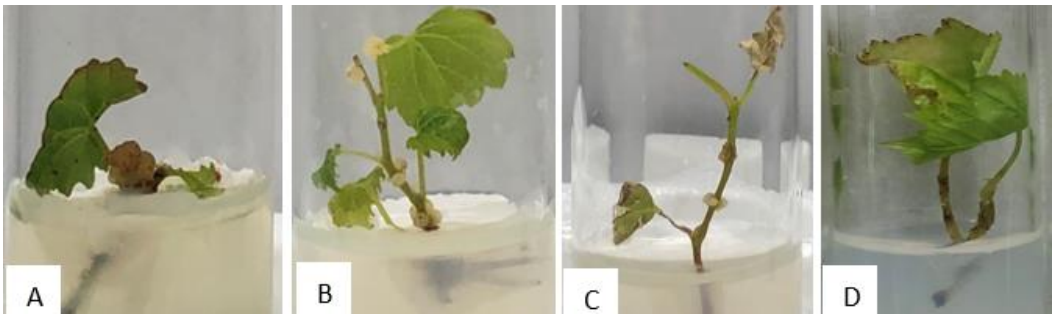

Fig 3 (A-D): *In vitro* shoots forming galls three weeks after inoculation with *Agrobacterium* strain A281 (A) GRN-1 (B) 1103P (C) 101-14 Mgt (D) TS.

Fig 4 (A-C): Leaf petioles forming galls three weeks after inoculation with *Agrobacterium* strain A281. (A) GRN-1 (B) 101-14 Mgt (C) TS.

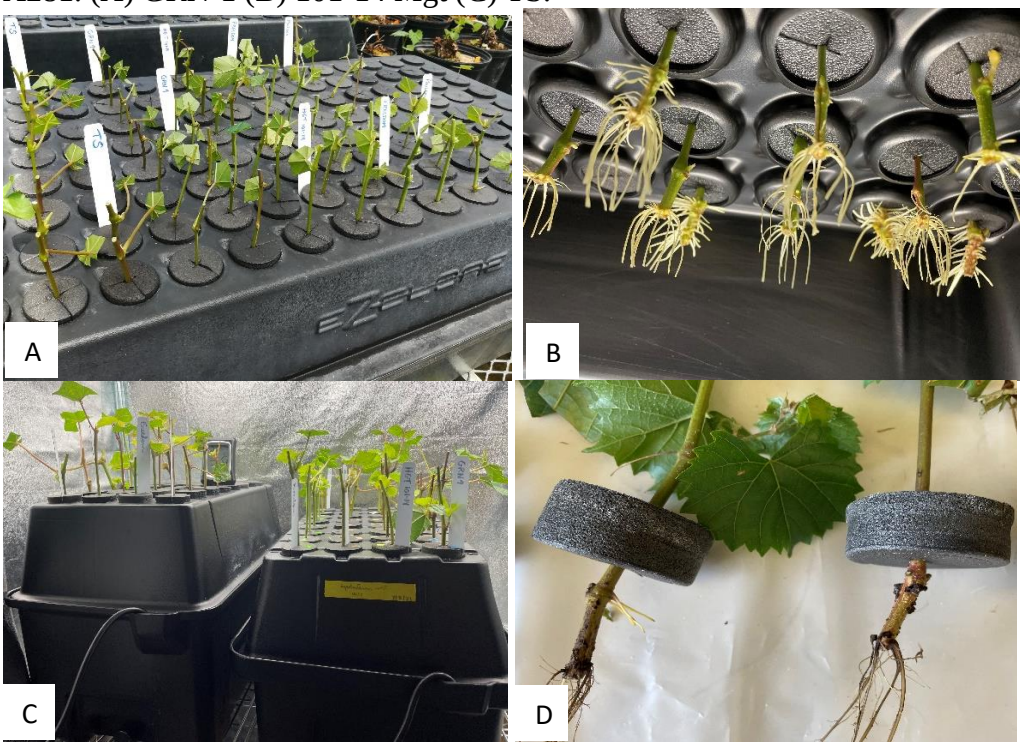

Fig 5 (A-D): Challenging wildtype plants with *Agrobacterium* strain A281.

(A). Inducing roots in the mist bed. (B). Roots trimmed and prepared for inoculation (C). Inoculated plants (D). gall forming in GRN-1 after three weeks.

10. **Literature Cited**:

Aguero, C.B., C.P. Meredith and A.M. Dandekar. 2006. Genetic transformation of *Vitis vinifera* L. cvs. 'Thompson Seedless' and 'Chardonnay' with the pear PGIP and GFP encoding genes. Vitis 45(1): 1-8.

Britton, M.T., M.A Escobar and A.M. Dandekar. 2008. The oncogenes of Agrobacterium tumefaciens and Agrobacterium rhizogenes. In: Agrobacterium: From Biology to Biotechnology. Eds Tzfira, T and Citovsky, V. Springer, New York. pp. 523-563.

Escobar, M.A., E.L. Civerolo, K.R. Summerfelt and A.M. Dandekar. 2001. RNAi-mediated oncogene silencing confers resistance to crown gall tumorigenesis. Proc. Nat. Acad. Sci. U.S.A. 98: 13437-13442.

Escobar, M.A., C.A. Leslie, G.H. McGranahan and A.M. Dandekar. 2002. Silencing crown gall disease in walnut (*Juglans regia* L.). Plant Sci. 163(3): 591-597.

Escobar, M.A., E.L. Civerolo, V.S. Polito, K.A. Pinney and A.M. Dandekar. 2003. Characterization of oncogene-silenced transgenic plants: Implications for Agrobacterium biology and post-transcriptional gene silencing. Molecular Plant Pathology 4(1): 57-65.