Monitoring pathogens and preventative control programs at a nursery producing container-grown plants©

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INTRODUCTION

Like any living organism, plants are susceptible to infection by environmental pathogens at all stages of development. The environmental conditions that nurseries must maintain to achieve plant growth coincide with the conditions necessary for pathogen growth and development. Although the chemical treatment of plants after a pathogen has infected its tissues is appropriate, the prevention of that initial pathogen/host interaction is more important to the long-term health and production of nursery plants. Thus, it is essential that nurseries have, in place, a system of prophylactic measures and monitoring that is designed to minimize this interaction. Such a system must be multi-layered and adaptable. By employing multiple prophylactic measures followed by close monitoring and laboratory testing of plants for potential pathogens, a high degree of success can be achieved.

ENSURING THE CLEANLINESS OF NEW TISSUE CULTURE INTRODUCTIONS

At Duarte nursery, our clean-plant protocol begins with every new plant species or clone we wish to introduce into propagation. These new introductions are put through a three-step virus and phytoplasma elimination protocol. This process begins with the harvest of apical meristems from new plants that are then cultured over a 1-week period. These meristem cultures then undergo thermotherapy, during which they are exposed to the highest temperature that the plant cells can tolerate and still grow. The high temperature thermotherapy lasts for 6 weeks. This will destroy the heat labile viruses only. However, it will also cause most other viruses to stop replicating and restrict the spread of viruses to the newly developed meristematic cells. The new meristematic tissue is then harvested and put through cryotherapy. During this process, meristematic cells are first dehydrated to reduce ice nucleation within the cells. They are then quick frozen in liquid nitrogen to kill any remaining viruses or phytoplasmas. The cells are then rehydrated to preserve the cells and then the surviving meristematic tissue is cultured to produce new plantlets.

PRODUCTION OF PATHOGEN-FREE MOTHER BLOCKS

The origin of plant source material and its previous exposure to pathogens is usually difficult to ascertain with certainty. However, it is not impossible. All properties utilized for our mother blocks were purchased as either uncultivated rangeland or farmland that was not growing the species we intended on planting. In theory, this limits the new plants exposure to a species specific pathogen. All plants designated to be used in our mother blocks start as micropropagated, “pathogen-free”, clones. These micropropagated clones are tested and are only used if they are free of pathogens at the time of planting. This helps reduce the potential pathogen load on a mother block, but does not prohibit the influx of pathogens in the future. For that reason, all scion and rootstock material is screened before the time of harvest and quality tested when it arrives at the nursery.

UTILIZATION OF TISSUE CULTURE TECHNOLOGY FOR PLANT PROPAGATION

At Duarte Nursery, tissue culture technology is used, not only to produce clonal mother stock, but is also used for propagating our fruit and nut trees. The process we use for establishing plants in vitro is multifaceted. First, the cuttings are excised from mother stock

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or other known pathogen-free sources and surface-sterilized with a dilute bleach solution and 70% alcohol to kill any pathogens that may be on the cuttings. These cuttings are then introduced to the proper medium and placed into culture. When suitable growth has occurred, sterile cuttings are made from the cultured mother stock and are then placed in the right nutrient medium. Plants produced from sterile cuttings are grown on sterile artificial media, under sterile growing conditions and controlled environments to multiply the number of stock plants and establish the line in vitro. The nutrient medium the plants are grown on contains sufficient nutrients to support the plant for about 4-6 weeks. At the end of this time the plants undergo the multiplication phase of our tissue culture process. During the first and each successive multiplication step, enough cuttings are taken from each plant to double or triple the amount of plant material in culture. We continue this multiplication until a critical mass of between 20,000-50,000 plants is achieved. This number of plants allows us to take the plants into the commercial production and rooting phase. At this point the extra cuttings from each successive multiplication are siphoned off and placed on an auxin-containing rooting medium to become a production run. Finally, plants cultured for production are grown and callused in the laboratory, then extracted from the rooting medium and planted in plug trays to develop roots. When sufficiently rooted and acclimated to the greenhouse environment, the rooted plugs are transplanted into pots containing a soilless medium. Four separate plant lines are created from each new introduction. Production run lots are kept segregated according to these plant lines throughout the production cycle. This ensures that if any somaclonal variation occurs, the line exhibiting an off-type can be omitted from the production run in the future and replaced by another genetically superior line. Additionally, all original lines are re-introduced every 4-5 yr to reduce the line's exposure to epigenetic effects.

TREATMENT AND SANITATION OF ROOTSTOCK AND BUDWOOD FROM THE FIELD

Grape rootstock and scion cuttings harvested from our mother blocks are all hot water treated to kill overwintering grape vine mealy bugs, if present. We have adapted this hot water treatment system to also clean the wood of any epiphytic fungal spores and bacteria. We accomplish this by filtering and recycling the water in the three hot water baths. We originally trialed commercial pool filters in 2013 and found them to be lacking (Figure 1). Fungal and bacterial contaminates were continually present in the water being recirculated into the bath after filtering. In 2014, we upgraded to industrial sand filters, filled with small pore size glass filtering media and added industrial UV filters to the water line. Testing indicated that the entire microorganism load on the wood was actively filtered out by the upgraded filtration system (Figure 2).

Figure 1. (A) Old pool filtering apparatus used to filter contaminates from the sanitation tanks used to clean rootstock and budwood from the field; (B) Culture plate from tank water containing fungal and bacterial contaminates prior to filtration with the old apparatus; and (C) Culture plate containing tank water contaminates after an 8-h day of filtration utilizing the old apparatus.

Figure 2. (A) New industrial filtering apparatus used to clean tank water; (B) Culture plate containing fungal and bacterial tank water contaminates prior to filtration with the new apparatus; and (C) Culture plate containing tank water contaminates after an 8-h day of filtration utilizing the new apparatus.

SEASONING ROOM SANITATION WITH OZONE

The simple movement of plant material in the external environment can expose that material to potential air-borne pathogens. Therefore, we continue to treat rootstock and scion budwood both during and after processing to inhibit pathogen inoculation. Grape wood that is retrieved from cold storage for seasoning is treated prophylactically in the seasoning room with ozone. Ozone is produced by a corona-discharge ozone generator and pumped into the room by a series of manifolds and jet fans to produce an even dispersal throughout the room. The efficacy of the treatment is periodically tested and has been found to be efficient in the prevention of bacterial and fungal development on the seasoning wood (Figure 3). In addition to the nightly treatment of the room with ozone, the entire room is sterilized every week with bleach.

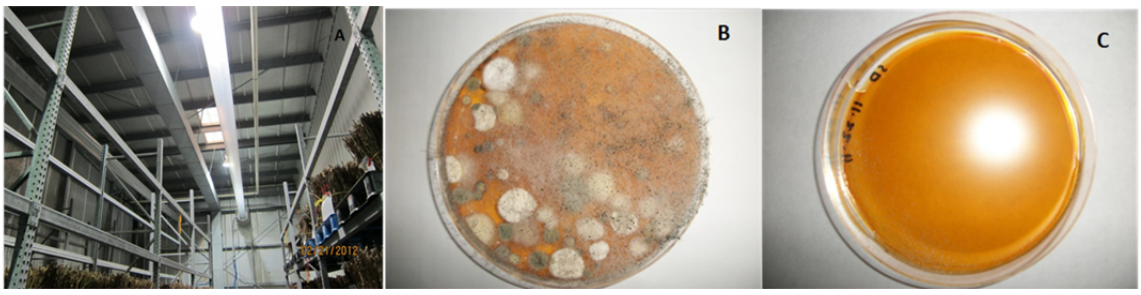


Figure 3. (A) Seasoning room with jet fan for distributing ozone. (B) A culture plate left open in the room for 15 h, without ozone. (C) The same test was done while ozone was actively being pumped through the air into the room.

GRAFTING SANITATION

The cuts made to the rootstock and budwood during grafting provide a direct route for pathogen inoculation into the plant. The sanitation procedures used during grafting are aimed at preventing possible infections. The procedure includes the flame sterilization of both clippers and grafting blades every 30 min during the grafting cycle, disinfecting the entire room with chlorine foam and changing out the polyethylene covering the grafting tables every 2 weeks. Additionally, the pallets and flats used to transport the wood are sterilized with bleach at the start of each day.

CALLUSING WATER FILTRATION AND ULTRAVIOLET AIR SANITATION

After grafting, our grape vines are placed in hydroponic callusing baths. The water used to fill the baths is filtered by the same industrial designed filters and in-line UV water sanitation used in the hot water treatment area (Figure 2A). In addition, the air ducting that supplies outside air to the room is equipped with UV radiation emitting lights to clean the air

coming into the callusing area. Testing of the callusing water filtering system has proven that it is capable of removing fungal spores and bacteria from the water supply feeding the baths (Figure 4). Additionally, ultraviolet irradiation of the incoming air is able to kill any air borne fungal spores (Figure 5). These mechanisms aid in protecting our newly grafted grape vines from potential bacterial and fungal pathogens.

Figure 4. (A) Water inflow to callusing filtration system showing high levels of bacterial contamination; (B) Water outflow from the filtering system to the callusing baths with no biologicals present.

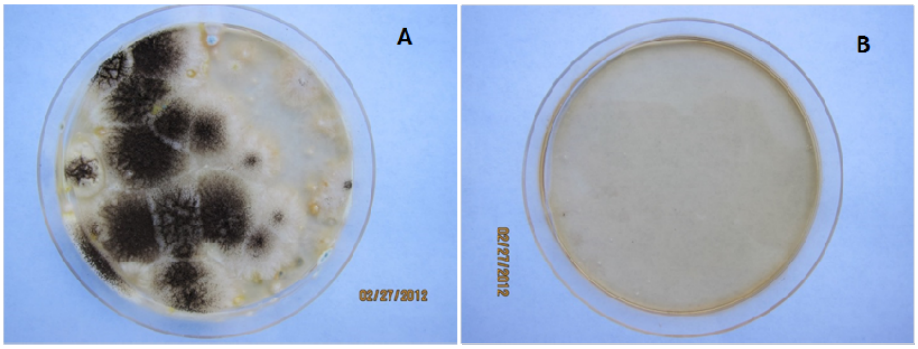


Figure 5. (A) Culture plate exposed to air inflow to the callusing room for 5 h without ultra-violet radiation; (B) Analogous culture plate exposed to ultra violet radiated air inflow for 5 h.

CONTAINERIZED PLANT PRODUCTION IN THE GREENHOUSE

Every plant produced at Duarte Nursery is grown in a container from propagation, through greenhouse growth and until the point of delivery to our customer. The containerization of our plants gives them several benefits with regard to their potential exposure to pathogens. Containerized plants never interface with the soil until they are planted in a customer's field. Since the plant has never been planted in the earth, this minimizes the plants exposure to soil-borne pathogens and nematodes. By being grown in a container, the product is planted with a complete intact root system (Figure 6). This reduces transplant stress, encourages fast establishment and reduces root-wound exposure to crown gall. Using the containerized plant platform also allows us the ability to screen our soilless potting mix periodically for pathogens and add beneficial organisms like mycorrhizae to the mix to increase pathogen resistance to root and soil pathogens.

Figure 6. Fully developed root system on a containerized grafted grape vine.

FACILITY RETROFITS TO IMPROVE SANITATION

The facilities that are used to produce our plants also play a role in the overall sanitation of our nursery. Facilities can either aid or hinder sanitation and pathogen prevention efforts. For example, wooden benches and dirt floors, provide good environments for both bacterial and fungal pathogens to colonize. These older structures, once infected, are very difficult to sanitize. To that end, Duarte Nursery has embarked on a multi-year capital expansion program in which older wood-constructed greenhouses have been removed and 18 acres of new steel-constructed, concrete-floored greenhouses have been built (Figure 7). In addition to the new indoor areas, 35 acres of new outdoor growing space has been added with steel bench construction over concrete pads. At the end of each production cycle excess soil and plant material is collected and discarded and benches and floors are washed thoroughly. After cleaning, benches and floors are treated with oxidizing chlorine foam to kill any pathogens before the next crop occupies that space (Figure 8).

Figure 7. (A) Old type greenhouse with wooden benches over dirt floor; (B) Modernized greenhouse; and (C) modernized outdoor growing area, both constructed with steel benches and concrete floors to minimize pathogen exposure to the plants.

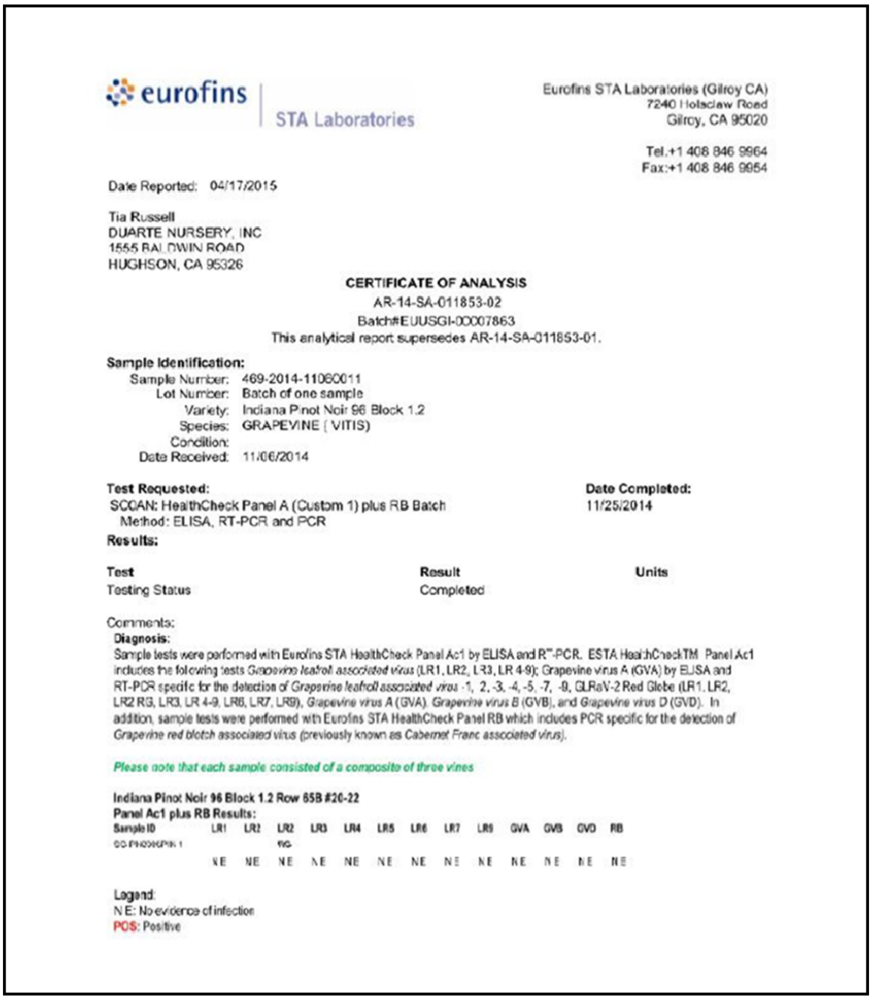
Figure 8. Using chloride foam to sanitize greenhouse and outdoor surfaces used for plant production.

CONTINUED TESTING AND SCREENING OF PLANT PRODUCTS

The prophylactic measures employed by any nursery, no matter how precise or controlled, only limit the ability of pathogens to infect plants. Therefore, programs designed to monitor, test and screen plant material coming into the nursery, plants growing at the nursery and plants ready for sale is essential. Duarte Nursery rootstock and budwood mother blocks are routinely tested every year by state employees through the voluntary CDFA Certification Program. In addition, Duarte Nursery's Quality Assurance Department, samples and screens all Duarte-owned mother blocks and any non-Duarte budwood from outside sources through ELISA and PCR testing at private laboratories (Figure 9). While it is truly impossible to test each individual plant prior to sale, having a thorough screening protocol in place can minimize the number of plants that slip through at the point of sale. At Duarte, we go further than a simple visual screening just prior to shipment. Here, plants are periodically screened for bacterial and fungal pathogens at the tissue culture, acclimation and growth stages of development. This screening process entails plant sampling, sample processing, bacterial and fungal culture, and colorimetric identification of potential pathogens via a GEN III OmniLog Microbial Identification System (BIOLOG, Inc., Hayward, California) (Figure10). Finally, each plant is visually inspected by our shipping department before they are transported to our customers.

SUMMARY

Due to the environmental conditions nurseries must maintain to be productive, pathogen growth and development is inevitable. However, nurseries can minimize their plants' exposure to pathogens through the use of preventive measures implemented in a multi-layered, adaptable sanitation system. Any such system must be combined with a developed integrated pest management program and an appropriate plant monitoring, screening and testing program that ensures a high number of pathogen-free plants reach the customer.



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Date Reported: 04/17/2015

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CERTIFICATE OF ANALYSIS
AR-14-SA-011853-02
Batch#EUUSGI-00007863
This analytical report supersedes AR-14-SA-011853-01.

Sample Identification:
Sample Number: 469-2014-11060011
Lot Number: Batch of one sample
Variety: Indiana Pinot Noir 96 Block 1.2
Species: GRAPEVINE (VITIS)
Condition:
Date Received: 11/06/2014

Test Requested:
SCCAN: HealthCheck Panel A (Custom 1) plus RB Batch
Method: ELISA, RT-PCR and PCR

Date Completed:
11/25/2014

Results:

Test	Result	Units
Testing Status	Completed	

Comments:
Diagnosis:
Sample tests were performed with Eurofins STA HealthCheck Panel Ac1 by ELISA and RT-PCR. ESTA HealthCheckTM Panel Ac1 includes the following tests *Grapevine leafroll associated virus* (LR1, LR2, LR3, LR 4-9); Grapevine virus A (GVA) by ELISA and RT-PCR specific for the detection of *Grapevine leafroll associated virus* -1, 2, -3, -4, -5, -7, -9, GLRaV-2 Red Globe (LR1, LR2, LR2 RG, LR3, LR 4-9, LR6, LR7, LR9), *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), and *Grapevine virus D* (GVD). In addition, sample tests were performed with Eurofins STA HealthCheck Panel RB which includes PCR specific for the detection of *Grapevine red blotch associated virus (previously known as Cabernet Franc associated virus)*.

Please note that each sample consisted of a composite of three vines

Indiana Pinot Noir 96 Block 1.2 Row 65B #20-22
Panel Ac1 plus RB Results:

Sample ID	LR1	LR2	LR2 RG	LR3	LR4	LR5	LR6	LR7	LR9	GVA	GVB	GVD	RB
[illegible]	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE

Legend:
NE: No evidence of infection
POS: Positive

Figure 9. Example of test results from ELISA and PCR analysis of grapevine budwood.

Figure 10. (A)Plated samples ready for preparation, (B) sample preparation for colorimetric assay, (C) GEN III OmniLog system, and (D) identification via colorimetric assay showing different color patterns for four different bacteria.