## **Exploring Potential Induction of Grapevine (*Vitis* spp.) Root Phenolic Compounds by Ring Nematodes, *Mesocriconema xenoplax***

## **In-depth Materials and Methods**

In May 2018 a total of 16 ‘Cabernet Sauvignon’ grapevine scions established on either ‘Saint George’ or ‘Schwartzman’ rootstocks were planted in a 2:1 (volume/volume) mixture of sand:autoclaved field soil (collected from Parlier, CA, USA) in 5-gallon (~20L) pots. After a week, eight plants for each rootstock were left healthy or were inoculated with an estimated 1,000 ring nematodes applied as a 1mL suspension of 250 nematodes/mL pipetted into four different holes in the sand/soil mix. Similarly, in April 2019 a total of 16 ‘Cabernet Sauvignon’ grapevine scions established either on its own ‘Cabernet Sauvignon’ rootstock or on ‘O39-16’ rootstocks in the same 2:1 (volume/volume) sand:soil mix. One month later, eight plants each were left healthy or inoculated with ring nematodes in a similar manner as in 2018.

For 2018, plants were harvested and the soil was sampled for nematodes three months after inoculation in August, whereas for 2019 plants were harvested and the soil was sampled for nematodes four months after inoculation in September. The difference was due to uncontrollable logistical reasons (personnel and equipment availability). For nematode counts, a total of 50 mL of soil was collected into 50 mL centrifuge tubes from the soil mix in each pot. These were then delivered to commercial sources for nematode counts via the sugar flotation-sieve technique [1-2].

Roots were randomly sampled to assess phenolics by collecting roughly 10 g from at least five different spots of the root ball and placing into 50 mL centrifuge tube and then flash-freezing in liquid nitrogen. Samples were kept frozen at -20°C until further processing.

Phenolics were assessed using the Wallis et al. [3] and Wallis and Chen [4], with most reagents and solvents from Thermo-Fisher Scientific (Waltham, MA, USA) unless specified. Frozen root samples were pulverized with a mortar and pestle in liquid nitrogen and had a 0.10 g aliquot weighed out into 1.5 mL centrifuge tubes and then extracted overnight at 4°C in 0.5 mL methanol. Pellets remaining after centrifugation the following morning were re-extracted in 0.5 mL of methanol as well, with this second extract combined with the first to yield a total of 1.0 mL methanol extract.

High-performance liquid chromatography (HPLC) was conducted using a Shimadzu (Columbia, MD, USA) LC-20AD pump based liquid chromatograph equipped with Supelco Ascentis RP-18 (Sigma-Aldrich, St. Louis, MO, USA) column and a Shimadzu PDA-20 photodiode array detector. A total of 50 µL of the methanol extract was injected into the HPLC for each sample. Standards were obtained by Sigma-Aldrich when available, and other compounds were putatively identified via liquid chromatography-mass spectrometry using a Shimadzu LCMS2020 system running similar conditions to the HPLC. Peak areas were converted to mg/g fresh weight amounts by using standard curves made with commercially available standard compounds from the same phenolic subclass, i.e. procyanidin B2 for proanthocyanins, catechin for flava-3-ols, quercetin glucoside for flavonoid glycosides, and resveratrol for stilbenoids [4].

In addition to the plant experiments, a microplate bioassay also was conducted to assess the potential of stilbenoids to affect ring nematode survival. For this, 200 µL solutions were applied to each well of one of two 96 well plates. Eight wells consisted of water as controls. Additionally, wells contained 2.5 ppm, 5 ppm, 10 ppm, or 20 ppm of stilbenoid compounds, either five wells for each concentration of the monomer piceid (resveratrol glucoside) (from Sigma), five wells per concentration for the dimer ε-viniferin (from Sigma), six wells per concentration for the resveratrol trimer (putatively miyabenol C, from fraction collection), or six wells per concentration for the resveratrol tetramer (putatively a mixture of vitisin B and hopeaphenol, from fraction collection). Fraction collection was performed with the same HPLC conditions as the analysis study, using extra root methanol extractions to collect a 0.5 min window around the putative compound peaks. Compound purity was confirmed by running fractions on LC-MS, with purity of stilbenoid trimers or tetramers over 95%. Each 96-well plate was dried completely using a vacufuge.

To perform the ring nematode assays, a suspension of about 10 nematodes per 200 µL was prepared and then added to each well of the two 96-well plates to cover all treatments. Each well was then immediately examined using a microscope to count the number of live and dead ring nematodes. One day later, the number of live and dead nematodes per each well was reassessed as well. Percent mortality per well after one day was then calculated.

For statistics, IBM (Armonk, NY, USA) SPSS statistics version 24 was used with α = 0.05. Pearson’s correlations were utilized to find associations between total phenolics and the subclasses of phenolics (flavonoids and stilbenoids). Analyses of variance were used to compare total phenolics (the sum total of individual phenolics) with year, rootstock cultivar, ring nematode treatment, and all interactions as independent variables. Because of the great differences due to year, ANOVAs were performed again for 2018 and 2019 separately. Multivariate analyses or variance (MANOVA) was run including all individual phenolics, with each year assessed separately and rootstock, ring nematode treatment, and the interaction as independent variables. Follow-up ANOVAs were performed when appropriate. ANOVAs also were utilized to observed differences in ring nematode mortality in the bioassays.

**References**

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