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Final Report

Mealybug species composition and management in Virginia vineyards

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Final report for the 2020-2021 project involving mealybugs on grapevines, and exploration on role of ants in mealybug distribution:

Introduction

Mealybugs are minute, white, soft-bodied insects belonging to the family Pseudococcidae (Hemiptera). These insects use their piercing and sucking mouthparts to feed directly on the phloem sap. Phloem sap is a source of nitrogen, sugars, and minerals; sugar is present in a relatively higher proportion than other essential nutrients needed by these tiny insects. Hence excess sugars contribute to sticky, sugary fluid (honeydew) excreted by these insects. Honeydew produced by these insects is often deposited on the surface of grapevines, which supports the growth of sooty mold and attracts ant populations towards the grapevines. Healthy plants can tolerate low populations without significant damage while the high populations reduce the plant vigor, yield and fruit quality. Sometimes, the insects would not be detected on the plant until they appear on fruit clusters, reducing their value. Some mealybug species have been observed to transmit the grapevine leafroll virus in California. The mitigation of damage due to arboviruses transmitted by mealybug is largely dependent on the control of these vectors. It is important to study the species composition and their management in Virginia vineyards. Species differ in vector competency, and number of generations. The vine mealybug is an invasive species that has been introduced into California. It is a more problematic virus vector and its presence or absence must be determined here.

Distribution

Mealybugs include different species that are not limited to greenhouses and nurseries but also infest wide varieties of annual plants, perennial plants, grasses, and conifers. The primary vineyard-infesting mealybugs fall under the subfamily Pseudococcinae. Some of the important vineyard infesting mealybugs include grape mealybug, *Pseudococcus maritimus* (Ehrhorn), obscure mealybug, *Pseudococcus viburni* (Signoret), longtailed mealybug, *Pseudococcus longispinus* (Targioni- Tozzeti), citrophilus mealybug, *Pseudococcus calceolariae* (Maskell), vine mealybug, *Planococcus ficus* (Signoret), citrus mealybug, *Planococcus citri* (Risso), pink hibiscus mealybug, *Maconellicoccus hirsutus* (Green) and Gill's mealybug, *Ferrisia gilli* (Gullan).

Grape mealybug has been the predominant pest of mealybug in the past (Pfeiffer 2008). In an earlier survey of mealybugs in Virginia (part of a larger study on grapevine viruses),

Jones (2016) identified 100 mealybugs, composed of 67 grape mealybugs, 31 Gill's mealybugs, and 2 obscure mealybugs. Vine mealybug has been found in all the grape growing regions of California; however, it was not reported in Virginia in the previous study. Longtailed mealybugs appear to be cosmopolitan in a tropical and subtropical environment, while they are present in greenhouses and homes in temperate regions (Tenbrink and Hara 2007). Citrus mealybug is an important pest in vineyards in Spain and Brazil (Cid et al. 2010). It is a polyphagous pest, which prefers citrus plant. It is a common pest of citrus plants primarily in greenhouses and on several ornamental plants in nurseries. Pink hibiscus mealybug arrived in Hawaii in 1984 and was discovered in southern California in 1999 and Broward County in Florida in 2002 and has currently spread north into southern Georgia (Roltsch et al. 2006, Hoy et al., 2003). Gill's mealybug is a newly described species of mealybug found in pistachio growing regions of California and found infesting almonds, grapes, persimmons, and stone fruits as well as mulberry (Gullan, Downie, and Steffan, 2003). It has also been reported from Virginia by Jones in 2016. Grape mealybug and obscure mealybugs are easily confused with each other and both have been found in Virginia (Jones and Nita, 2019).

In an earlier survey of mealybugs in Virginia (part of a larger study on grapevine viruses), Jones (2016) identified 100 mealybugs, composed of 67 grape mealybugs, 31 Gill's mealybugs, and 2 obscure mealybugs; vine mealybug was not found. It would be useful to survey mealybugs in root infestations, especially in outbreak conditions.

Description

Identification of the mealybugs and their common name is based on adult females. Adult females are distinctly segmented and thinly or thickly covered with mealy or cottony wax secretion, which is often extended out along the sides of the body in a series of shorter filaments, while longer ones are present towards the caudal regions of the body. The mouthparts are often threadlike and longer than the body itself, which is used to pierce through the leaf or bark of plants to suck out phloem sap. Males, rarely seen, are delicate, winged (infrequently wingless), and gnat-like, possessing long caudal wax filaments. Their sole function is reproduction and have abortive mouthparts and hence do not feed. Mealybugs vary in size from 0.5 mm long (minute young ones) to up to 5 mm long (adult females). Many mealybug species can reproduce asexually, without mating (McKenzie, 1967).

Life Cycle

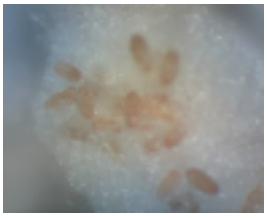
The life cycle of mealybugs varies within different species. Although these species look strikingly similar, these species have slight variations in geographic ranges, host plant preferences, economic injuries, and management strategies. Generally, females have three nymphal instars, while the males have four. The males and females are similar during an immature stage but differ completely as an adult. Males undergo complete metamorphosis after the third instar, developing wings. Males are short-lived, lasting 1-2 days only to reproduce. Females undergo incomplete metamorphosis, resembling the immature stage but larger in size and retain their legs. Females then slowly move within the vines, occasionally transferred within the vines by plant materials, farm equipment, or wind

current.

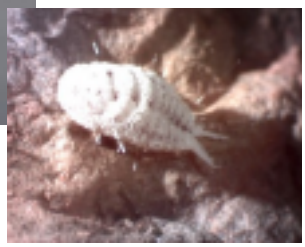
Mealybugs are present on different parts of the vines depending on the season and different species. Grape mealybug overwinters as egg or the first instars called crawlers underneath the bark of cordons, vine trunks, or spurs. Crawler is the dispersal stage which often moves to find the feeding spot. With the onset of favorable conditions during spring, crawlers move up to feed on exposed canes and leaves. Those that do not move will remain on the trunk, feeding on the phloem sap and lay eggs there when mature. Adult females after mating oviposit within ovisacs and deposit these ovisacs underneath loose barks on trunks, cordons, and spurs. Females lay several hundred eggs in cottony ovisacs. The first instar crawlers hatche out of the eggs and disperse into the remaining part of the vines. Depending on the climate, grape mealybugs and obscure mealybugs have two to three generations per year, while vine mealybugs can have three to nine generations per year. Grape mealybug and obscure mealybug look remarkably alike, obed. Grape mealybug releases reddish orange defensive fluid releases clear defensive fluid.

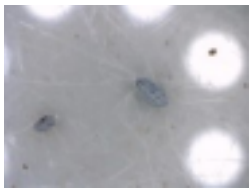
Male prepupae

Life-cycle



Second Instar





Male pupae Second and third Instar

Introducon

Egg mass

First Instar First Instar

Figure 1. Life cycle of mealybug

Economic Impact

One of the primary effects of the presence of mealybug as mentioned earlier is the production of honeydew, that supports the growth of sooty mold and attracts ant populations towards the grapevines. Sometimes the mealybug infestation will not be evident on the vineyard until harvest, when the appearance of mealybugs on the clusters forces the grape growers to drop the clusters. Infestations in low populations are often tolerated by the grapevines, while in high populations, plants often lose vigor, yield, and fruit quality. Thus, at high populations, vines may be induced to drop their clusters in late season because of stress associated with this feeding. The greatest economic impact resulting from mealybugs is its potential role as vectors of important vineyard viral diseases, notably grapevine leafroll-associated viruses (GLRaV). The most common mealybug in Virginia, grape mealybug, is a known vector of GLRaV-3 It is the most severe of the eight types of grapevine leafroll reported so far. Golino et al. (2002) reported that they were able to confirm that four species [mealybug] found in California – obscure (OMB), longtailed, citrus and grape mealybug have the potential to transmit GLRaV-3 isolates. This was the first experimental evidence of grapevine leafroll virus transmission by obscure and grape mealybug. In addition, it was also reported for the first time that GLRaV-5 could be transmitted by longtailed mealybug. Management of mealybugs will be critical to the management of GLRaV (Cooper et al. 2018).

Mealybug Habitat

Introduc&#65533;on

Figure 2 Habitat of mealybug

Association with ants

Ants have been observed in proximity with honeydew-producing insects including mealybugs. The interactions between ants and honeydew-producing hemipterans has been studied extensively in multiple ecosystems and this association has been found to be beneficial to both insects (Styrsky and Eubanks 2006, Brightwell and Silverman 2010, Wilder et al. 2011, Renault, Buffa and Delfno 2005). In the association, ants tend and protect the honeydew-producing hemipterans from predators and parasitoids, while hemipterans provide them an important food supply as honeydew. In a recent study of three ant species native to Mediterranean region, foraging on the vine canopies, were found to induce population increase of vine mealybugs. However, in the same study, only 16% of the total mealybug population on the site was tended by the ants (Beltrà et al. 2017).

Most mealybugs have the potential to form root colonies on grapes, though the tendency varies among species. Their movement to roots, and spreading in that area, is facilitated by ants (Daane et al. 2007). When we collected mealybugs from grape roots in Albemarle County, at least three species of ants were present, the most common being smaller yellow ant *(Acanthomyops claviger)*, and pavement ant *(Tetramorium caespitum)* and thief ant *(Solenopsis molesta)*. When smaller yellow ants were collected into a container that contained a root sample with mealybugs attached, a worker picked up a mealybug and ran around the container in an agitated fashion. An understanding of the role of ants may provide a clearer view of the epidemiology of grapevine leafroll disease. Grasswitz and James (2008) studied the movement of grape mealybug between vines, including self directed movement by walking, or movement aided by wind. Movement by either means was limited. However, an ant-assisted movement was not included. In a study of mealybugs and GLRaV, Jones and Nita (2016) found that movement of the disease was not affected by wind – this would be consistent with ant-assisted movement of the vector mealybugs.

The objectives of this project were to determine the species composition of grapevines, both on aerial portions and roots, examine role of ants in movement of mealybugs on grapevines, and evaluate chemical control agents.

Methods

1) **Part 1 – determine the species composition of mealybugs in Virginia vineyards, comparing aerial populations with those inhabiting roots**

Sampling Sites

We scouted five commercial vineyards with a history of mealybug infestation or Grape leafroll virus (GLRaV) infection (Horton (H), Saunders, Virginia Mountain Vineyard (VMV), Pearmund Cellars (P) and Grace Estate Winery) (GEW). The sites were monitored once a week from the end of April 2020 to October 2020. The traps were set up at H, GEW and S on April 17, 2020, on April 20, 2020 at VMV and on April 28, 2020 at Pearmund cellars. Aerial samples (mealybugs on cordons, shoots, canes and clusters) as well as the root samples were surveyed by visual examination of at least one row of vines per vineyard per day. During early season, when insects were not spotted in the field, some leaf/shoot samples were taken back in 70% alcohol to check for the presence of mealybug nymphs. An attempt was made to sample mealybugs in GLRaV-positive vines and those without known GLRaV. Mealybugs were photographed before being collected into 70% ethanol.

Relative Sampling

We used red plastic delta traps and sticky liners from Alpha Scents to monitor the male mealybug populations. The pheromone lures were ordered from Evergreen Growers Supply. Lures specific to grape mealybug, vine mealybug, citrus mealybug, obscure mealybug and longtailed mealybug were used, with one of each type per vine row per site. In addition to checking mealybug traps each week, we monitored the population of mealybugs by visual inspection of the vines for about five minutes per vine. Crawlers were counted separately from the rest of the life stages. During that time-period, we examined the aerial parts of the plants including spurs, leaves and trunk. For non-destructive sampling, we examined the plant parts visible. For destructive sampling, we removed some portions of the bark to check for the presence of mealybugs in the trunk. For the root
samples, we carried out nondestructive sampling by digging up the soil to check for the presence of mealybugs on the roots.

Genetic Analysis

The genetic analysis of mealybugs is based on a tool developed by Daane et al. (2011). DNA extraction was carried out using DNeasy Blood and Tissue kit. Due to the limitation in the reagents available, we pooled out the sample and carried out genetic analysis of 24 samples from three different sites (7 samples from GEW, 4 from VMV and 13 samples from Barboursville). Several genomic regions have been used for the identification of

mealybugs and other insects. One of these regions that has been used is mitochondrial cytochrome oxidase subunit I gene (COI). The species-specific primers designed for grape mealybug, scarlet mealybug (*Pseudococcus calceolariae* Maskell), long-tailed mealybug, vine mealybug, citrus mealybug, obscure mealybug and Gill's mealybug were used for the species identification (Table 1). PCR was carried out in BIO-RAD C1000 thermal cycler using multiplex PCR plus kit. An initial denaturation step at 95 ℃ for 5 min was followed by 30 cycles of 30s at 94 ℃, 90s at 53 ℃ and 90s at 72 ℃, with a final extension of 10 minutes at 72 ℃. All reactions used QIAGEN multiplex PCR master mix that includes $MgCl_2$ (3mM), buffer, dNTPs and *Taq* polymerase.

After amplification, 4µl of each PCR product was visualized by electrophoresis on a 2% agarose gel using GelRed. Each reading consists of a single mealybug. Our gel reading was divided into two replicates of each sample and two replicates of a no template control (no DNA). The positive control contains the DNA samples of grape mealybug and Gill's mealybug from previous research by Taylor Jones in 2012 from AREC lab, Winchester. The first replicate was loaded with forward primer for scarlet mealybug (*Pseudococcus calceolariae* Maskell (PCa), vine mealybug (PF), citrus mealybug (PC), and Gill's mealybug (FG) and the reverse primer. The second replica was loaded with forward primer for grape mealybug (PM), long-tailed mealybug (PL), and obscure mealybug (PV) and the reverse primer.

Size and Name of species-specific primers used for mealybugs

Amplicon lengths	Size	Primers used
Scarlet mealybug	650 bp	PCa / MB-R
Long-tailed mealybug	600 bp	PL / MB-R
Vine mealybug	450 bp	PF / MB-R
Grape mealybug	400 bp	PM / MB-R
Citrus Mealybug	350 bp	PC / MB-R
Obscure mealybug	250 bp	PV/ MB-R
Gill's mealybug	150 bp	FG/ MB-R

Primer sequences:

FG 5'-GAA TCA TTA ATT TCT AAA CGT TTA CTA A-3'

MB-R 5'-CAA TGC ATA TTA TTC TGC CAT ATT A-3'

PC 5’-TAA TCT ATT TTT ATC TAT CAA TTT AAC C-3’

PCa 5’-TGC AAC AAT AAT TAT TGC CAT C-3’

PF 5’-CTT TGT TGT AGC TCA CTT TCA C-3’

PL 5’-CCA TTT ATC TTT GAT CCA CAG-3’

PM 5’-CTG ATT TCC TTT ATT AAT TAA TTC AAC-3’

PV 5’-ATA TTT CTT CTA TTG GTT CAT TC-3’

2) Part 2 - Determine the most common ant species in association with root infestations

Ants in close association with mealybugs were collected in 70% ethanol for species identification. Identification will be provided by the Insect ID Lab in the Department of Entomology.

This year we tried to conduct an experiment on ant assisted movement on the field. Ant were provided with either only mealybugs or sugar solution and the mealybug to assess effectiveness of ants in moving mealybugs across the vines. The duration and distance moved by ants carrying mealybugs was recorded.

3) Part 3 - Carry out an insecticidal efficacy trial

An insecticide trial site will be selected after infestations are determined. Four single vine replicates of each treatment will be included. Insecticides planned for this trial include Assail (acetamiprid), Applaud (buprofezin), and Movento (spirotetramat). These represent three different modes of action (4A - Nicotinic acetylcholine receptor (nAChR) competitive modulator; 16 - Chitin biosynthesis inhibitor; 23 - Acetyl CoA carboxylase inhibitor, respectively). There will be no issue with crop destruct since all are currently registered on grape (Pfeiffer et al. 2019). Movento is of special interest since it is translocated to the roots and has the potential to control root infestations. Pesticide manufacturers will be contacted to determine if there is any new chemistry in development that can be incorporated. Data will be analyzed using analysis of variance, with mean separation using Tukey’s HSD.

The pesticides were not available for the 2020 field season because a key product was not delivered. The trial was initiated in spring 2021. Data collection and analysis are proceeding.

Results

Part 1 - Mealybug species present

Initial field days (17 April-23^{r} May 2020)

The third instars of Gill’s mealybugs were observed in the field as early as the first week of May in Virginia Mountain Vineyard, Grace Estate Winery and Horton Vineyard. Adult

Gill's mealybugs were observed as early as the third week of May (May 18 in Horton and Grace Estate and May 23 in VMV). Grape mealybug crawlers were found on 23 May at Pearmund along with ant mounds underneath the vine. Gill's mealybug late instars and adult females were observed feeding and resting on the grape spurs/buds present on the lower region of the trunk during early season, while grape mealybug instars and adults were mostly seen inside the trunk during early season. The grape spurs and buds taken back to the lab during the first field day (17 April 2020) shows the presence of crawlers. June to October.

Throughout the field season, Virginia Mountain Vineyard, and Horton Vineyard were recorded to have higher number of mealybugs than the last season. Grace Estate Winery had lower number of mealybugs than the year earlier. It was the first year of mealybug recording for Pearmund Cellars, and mealybug number varied with respect to the location of grapevines. One section was lightly infested, while the other was heavily infested despite having the same spray schedule. On high infestation, many adults along with several egg masses can be seen on the trunk (Figure 1).

Figure 1. Adult mealybugs on trunk

Male mealybugs were recorded on the traps as early as 28th of May on Horton, Grace estate, Saunders and Pearmund. Although the activity of male mealybugs was not that dominant in the traps, the study was able to record the activity of male mealybugs as late as the end of September inside the trunk, although their trap capture was continuously null during that time. The male captured on the trunk, although winged, was considerably sluggish and could be captured easily with forceps. Although their activity is rarely

noticed on the field, more male mealybugs were on plant itself than trap might point out to the fact that they are considerably sluggish in flying. The life cycle was also observed to have highly overlapping life stages.

Species identification of **grape, obscure and Gill's mealybug** for this season was based on morphology. Grape mealybug and obscure mealybug can be easily differentiated in the field, as when they are poked, grape mealybug releases reddish orange defensive fluid while obscure mealybug releases clear defensive fluid. Gill's mealybugs are distinct due to the presence of white wax with bare patches on the back that gives the impression of two white stripes down on the back. They also have long white glassy rods around their body. They have longer caudal filaments than grape mealybug.

Genetic analysis of the species

Amplification of the COI fragment by using multiplex PCR primers yielded species specific fragments that provided the direct diagnosis of twenty-four samples. The method accurately narrowed down the samples submitted to two species of mealybugs i.e., grape mealybug and Gill's mealybug. The band was also observed in the positive control that contained the sample from the same two species. The negative control failed to reveal any band. The samples from Barboursville and VMV revealed the presence of single species i.e., **grape mealybug**, while GEW had both **grape mealybug** and **Gill's mealybug** (Fig. 2 and 3). This data is based on samples from last year and current year's samples are still under analysis.

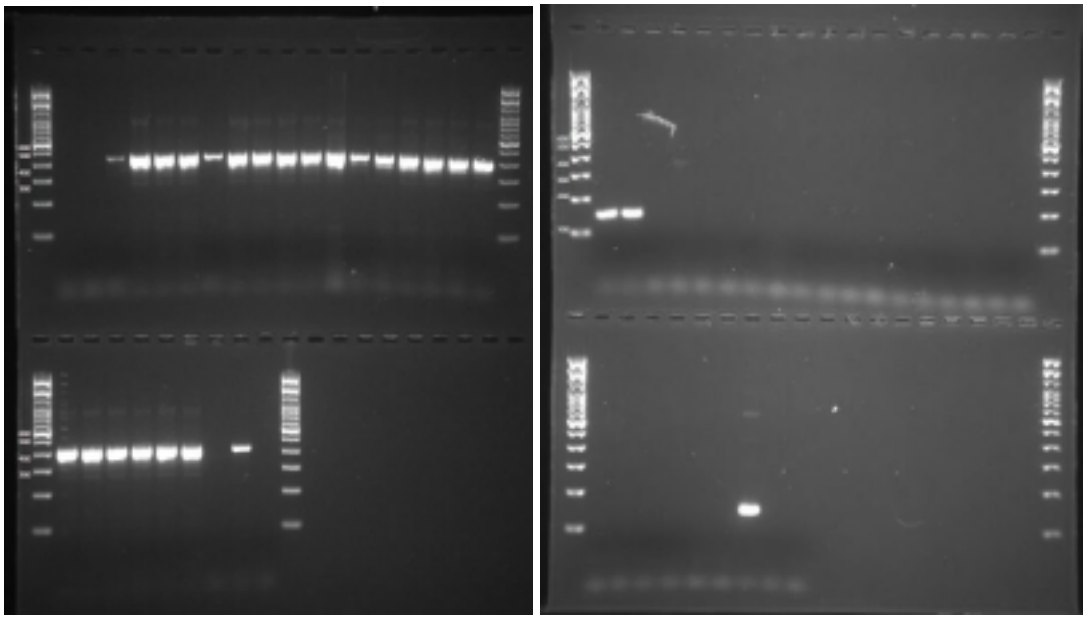


Figure 2 and 3. PCR reading on 2% agarose gel revealing 450bp band width on 22 samples from three different field sites and a single band of positive control

Part 2 - determine the most common ant species in association with root infestations

This year we tried to conduct an experiment on ant-assisted movement in the field. Ants were provided with either mealybugs or sugar solution and the mealybug to assess effectiveness of ants in moving mealybugs across the vines. The duration and distance moved by ants carrying mealybugs was recorded.

Ants were observed in close association with mealybugs at all the field sites. Ant nests were either present at the base of the trunks or near the vines. We had found ants tending the mealybugs in these sites. While carrying out the sampling of the mealybugs on these sites, we observed ants picking up the nymph when disturbed and transporting nymphs around. The **pavement ant**, *Tetramorium immigrans* and the **smaller yellow ant**, *Lasius (Acanthomyops) claviger*, were found in close association with ants this year, while **pavement ant** (Barboursville and Grace Estate Winery) and the **smaller yellow ant**, **odorous house ant** *(Tapinoma sessile)* and **cornfield ant**, *Lasius (Lasius) neoniger* (Virginia Mountain Vineyard) were recorded in 2019. The species identification of the ants was provided by the Insect ID Lab in the Department of Entomology.

The choice and no-choice test between ant-mealybug association with or without the presence of sugar solution were conducted in the field throughout the season. However, we were only able to calculate the duration and distance of self and ant assisted movement of mealybugs. Third instar or adult female movement varies from few inches to 36 inches looking for suitable location within the grapevine; while ant assisted movement from few inches (2 minutes) to 38 inches (in between two grapevines for up to 10 minutes). Ants were also observed to move different life stages of mealybugs from surface of trunk/ant nests to the more inner parts of the trunk/ant nests, when mealybugs were disturbed. Ant activity dropped to minimum by the first week of September.

Being social insects, it had been difficult to run choice and no-choice test in the field (Figure 4). In the 2021 field season, we continue work on sugar bait stations ant activity. This may lead to a control of ant nest near grapevines, that may lessen transport of mealybugs among vines.

Figure 4. Choice and no-choice test on the field

Part 3 - carry out an insecticidal efficacy trial

The trial was initiated in spring 2021, including multiple modes of action. This includes the product Movento, which will translocate to roots. We hope to demonstrate efficacy to root-infesting mealybugs. Data collection and analysis are proceeding.

Dissemination of results

The student supported by this project, Ms Pragya Chalise, presented some of her results to the summer technical meeting of the Virginia Vineyards Association on 28 July 2021.

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