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Mealybug species composition and management in Virginia vineyards

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1) determine the species composition of mealybugs in Virginia vineyards, comparing aerial populations with those inhabiting roots
2) determine the most common ant species in association with root infestations
3) carry out an insecticidal efficacy trial

Introduction

Mealybugs are minute, white, soft-bodied insects belonging to the family Pseudococcidae. These insects use their piercing and sucking mouthparts to feed directly on the phloem sap. Plant sap is rich in protein, sugars, and potassium and contains sugar in a relatively higher proportion than other essential nutrients needed by these tiny insects. Hence a larger proportion of sticky, sugary fluid is excreted by these insects, which is also known as honeydew. 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 making it unfit for sale. Some mealybug species have been observed to transmit the grapevine leafroll virus in California. The mitigation of damage due to arboviruses or due to the presence of mealybug is largely dependent on the control of its vector i.e. mealybug. It would be interesting to study the species composition and their management in Virginia vineyards.

Distribution

Mealybugs include different species that are not just limited to the greenhouses and the 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 has not been 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 up to 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 et al. 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 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 has vestigial mouthparts and hence do not feed. They 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 among 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 larval instars, while the males have four. The males and females are similar during an immature stage but differ completely adults. Males form a pupal stage 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 laying 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 often known as crawlers hatches 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 between three and nine generations per year. Grape mealybug and obscure mealybug look remarkably like each other, except when gently probed, grape mealybug releases reddish orange defensive fluid while obscure mealybug releases clear defensive fluid.

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, longtailed, citrus and grape mealybug have the potential to transmit GLRaV-3 isolates. This has been 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).

Association with ants

Ants have been observed in proximity with the 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 of the insects (Renault et al. 2005, Styrsky and Eubanks 2006, Brightwell and Silverman 2010, Wilder et al. 2011). In the association, ants tend and protect the honeydew producing hemipterans from predators and the parasitoids, while hemipterans provide them an important food supply as honeydew. In a recent study of vine mealybugs in table-grape vineyards in eastern Spain, three ant species native to Mediterranean 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 in 2018, at least three species of ants were present, the most common being smaller yellow ant *(Acanthomyops claviger)*, and also 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 mealybugs 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.

Objective of the study

Grape mealybug has been the predominant mealybug in Virginia vineyards in the past (Pfeiffer 2008). Grape mealybug, obscure mealybug and Gill's mealybug have been reported from Virginia in the latest study (Jones 2016). Some of the mealybug species are not known to occur in the east. Their introduction into Virginia would greatly complicate management. For example, vine mealybug is known only from California, where it has posed a disproportionate problem because of its greater number of generations, greater honeydew production, and increased tendency to occur on grape roots (Daane et al. 2012). The first objective of the study is to identify the mealybug species in Virginia Vineyards. We will also examine the aerial population and root population of the infested sites.

The second objective of our study is to investigate the potential role of ants in movement of mealybugs in the vineyard. Finally, the third objective of our study would be to run an insecticidal efficacy trial.

Material and Method

Sampling Sites:

We scouted five commercial vineyards with the previous record of mealybug infestation or Grapevine 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 July 2019 to September 2019. Barboursville Vineyard (B) was visited for destructive sampling of the mealybugs. 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. An attempt was made to sample mealybugs in GLRaV-positive vines and those without known GLRaV. With growers' assistance, vines along with the roots were removed from the ground and examined in one of the sites. Mealybugs were photographed before being collected into 70% ethanol.

Relative Sampling:

We used the red plastic delta trap and sticky liner from Alpha Scents to monitor male mealybug populations. The pheromone lures were ordered from Evergreen Growers Supply. Vine mealybug, grape mealybug and citrus mealybug lures were used, with each

one of its type on the central position and edge of the vine row. We had placed a total of six traps per site, three towards the edge and three towards the center. In addition to checking mealybug traps each week, we monitored the population of mealybugs by visual inspection of the vines for about five minutes. Crawlers were counted separately from the rest of the life stage. 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. The destructive sampling was carried out by uprooting the grape plant.

Genetic Analysis

The genetic analysis of mealybugs is based on a similar 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, citrophilus (or scarlet) mealybug, 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 citrophilus mealybug (PCa), vine mealybug (PF), citrus mealybug (PC), and Gill's mealybug (FG) and the reverse primer. The second replicate 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

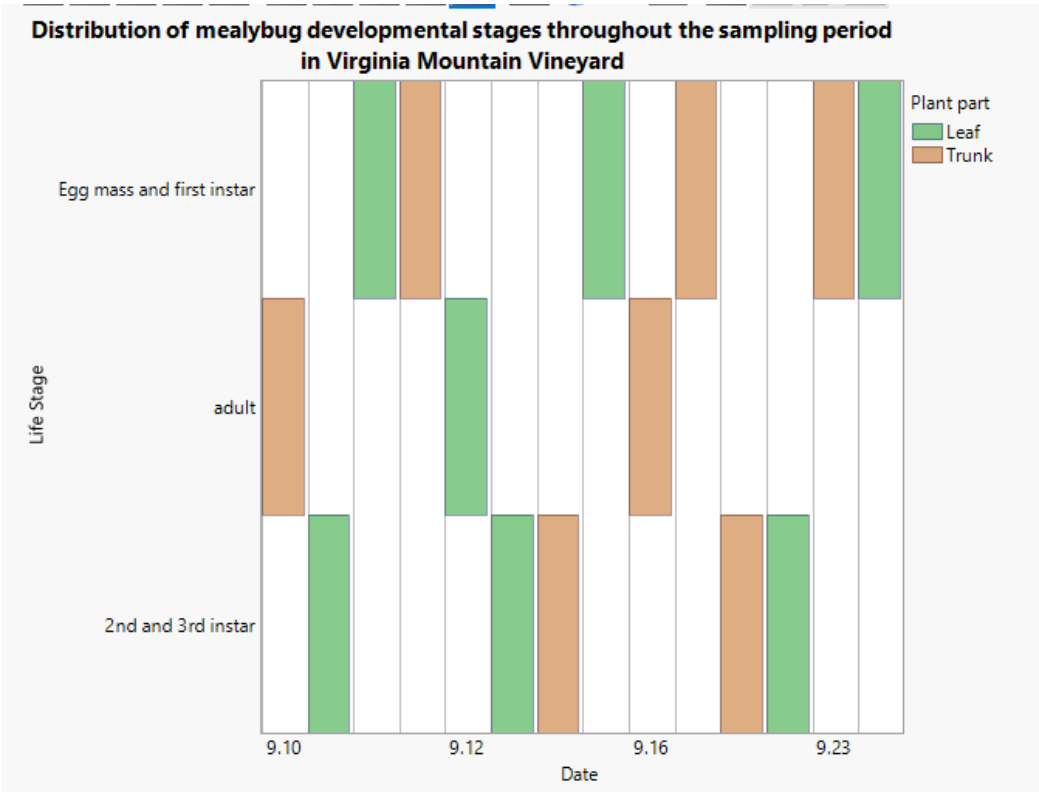
Primer sequences:
FG 5'-GAA TCA TTA ATT TCT AAA CGT TTA CTA A-3'
MB-R 5'-CAA TGC ATA TTA TTC TGC CAT ATT A-3'
PC 5'-TAA TCT ATT TTT ATC TAT CAA TTT AAC C-3'
PCa 5'-TGC AAC AAT AAT TAT TGC CAT C-3'
PF 5'-CTT TGT TGT AGC TCA CTT TCA C-3'
PL 5'-CCA TTT ATC TTT GAT CCA CAG-3'
PM 5'-CTG ATT TCC TTT ATT AAT TAA TTC AAC-3'
PV 5'-ATA TTT CTT CTA TTG GTT CAT TC-3'

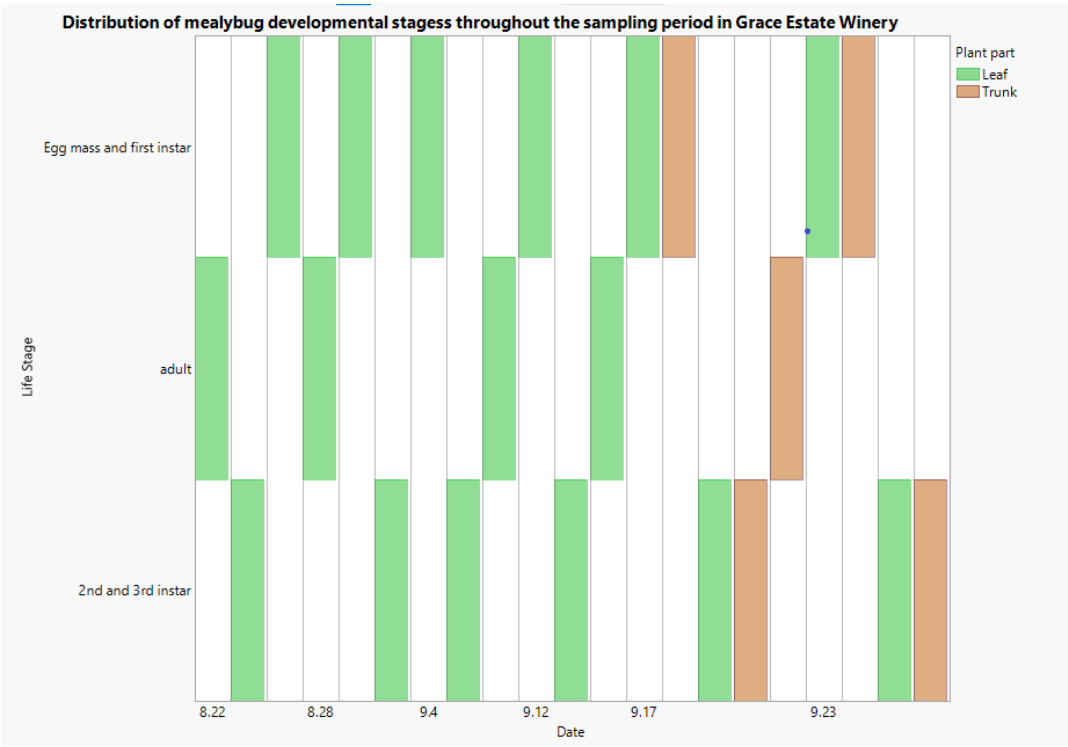
Result

Mealybug seasonal abundance and distribution

Five-minute relative sampling:

The study was carried out in five different vineyard sites in Virginia from mid-July to the end of September. We also collected several samples from Barboursville, the sixth site, which was not scouted but the vines were uprooted to check and sample for the mealybugs present on roots. Among the three sites scouted, the average number of mealybugs (instars and adult female) present in the vineyard peaks around the end of August and population declined towards the end of sampling period. In the second week of September, we only found mealybugs in VMV (Table 1, Figure 1, 2 and 3). The VMV population consisted of adult females or egg masses predominantly on the trunk. GEW and Horton had a higher number of mealybugs on the leaves than on the trunk. Throughout the sampling season, GEW had higher number of mealybugs than rest of the vineyards. Barboursville had relatively high infestation on the roots while none were found on the aerial parts. At the Saunders vineyard, only a few crawlers and one dead mealybug devoured recently by a spider were found on the leaf and trunk over the entire sampling period. Pearmund Cellars lacked any sign of mealybug throughout the entire three months of sampling period. Root infestation was found only at Barboursville vineyards. Trunks were full of egg masses towards the end of sampling period (Table 1, Figure 1,2 and 3).


Distribution of mealybug developmental stages throughout the sampling period in Virginia Mountain Vineyard
Plant part
Leaf
Trunk
Egg mass and first instar
adult
2nd and 3rd instar
Life Stage
9.10
9.12
9.16
9.23
Date


Distribution of mealybug developmental stagess throughout the sampling period in Grace Estate Winery
Plant part
Leaf
Trunk
Egg mass and first instar
adult
2nd and 3rd instar
Life Stage
8.22
8.28
9.4
9.12
9.17
9.23
Date

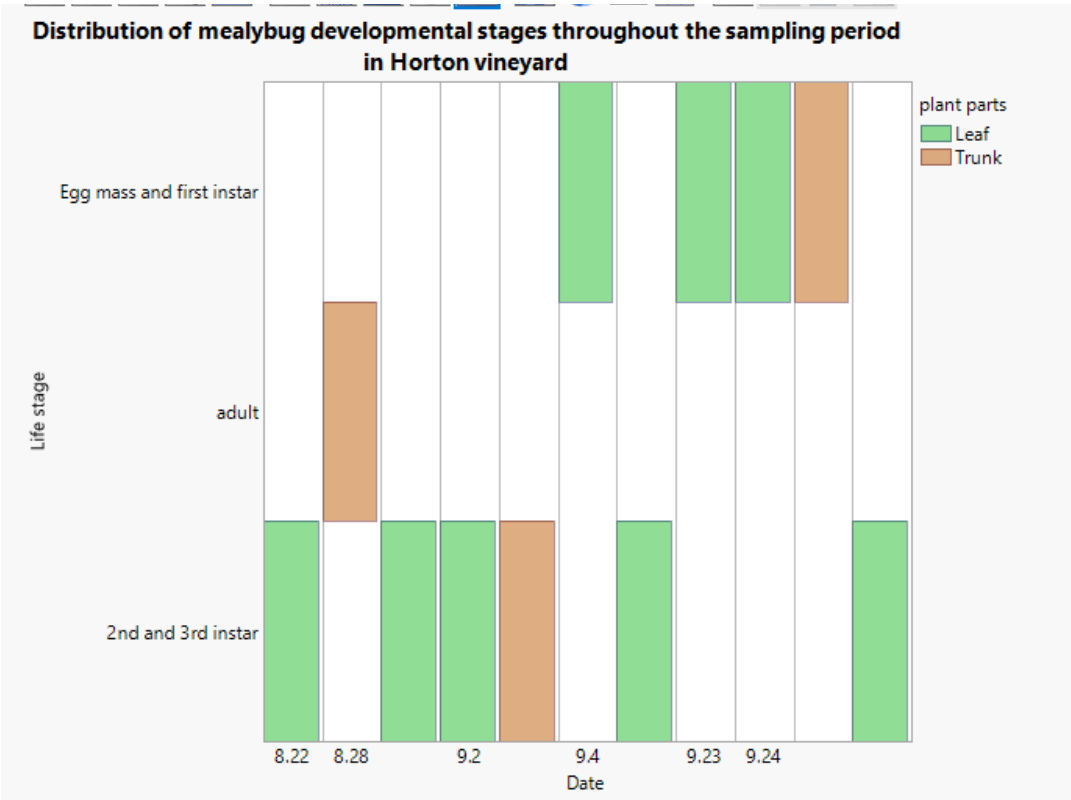


Figure 1, 2 and 3. Distribution of mealybug developmental stages throughout the sampling period in Virginia Mountain vineyard, Horton vineyard and Grace Estate winery (Date in month and date)

Table 1. Average number of mealybugs per site

	Horton Vineyard	Grace Estate Winery	Virginia Mountain vineyard
Date	Average number of mealybugs found	Average number of mealybugs found	Average number of mealybugs found
8/22	1	2.33	Na
8/28	6	4	Na
9/2	2.67	0	Na
9/4	1	2.23	Na
9/10	Not applicable (na)	Na	2.5
9/12	0	2	4.2

9/16	Na	Na	3.86
9/17	0	3	Na
9/23	1	2.33	1.2
9/24	2.5	0	Na

Trap capture data of male mealybug

The delta trap, set up for the male mealybugs that was placed in the GEW, peaked during midweek of August, and the trap capture decreased till the first week of September. We did not observe any activity on the trap after first week of September. Minimum trap capture (0-3) was recorded from Horton where we continued to observe the male mealybugs on the trap on the trap until the third week of September. We only recorded a trap capture during mid- August until first week of September in the Saunders vineyard. The highest trap capture was recorded from the Pearmund Cellars, which continued from midweek to the first week of September. We only observed a single trap capture during second week of August from VMV (Table 2, Figure 4).

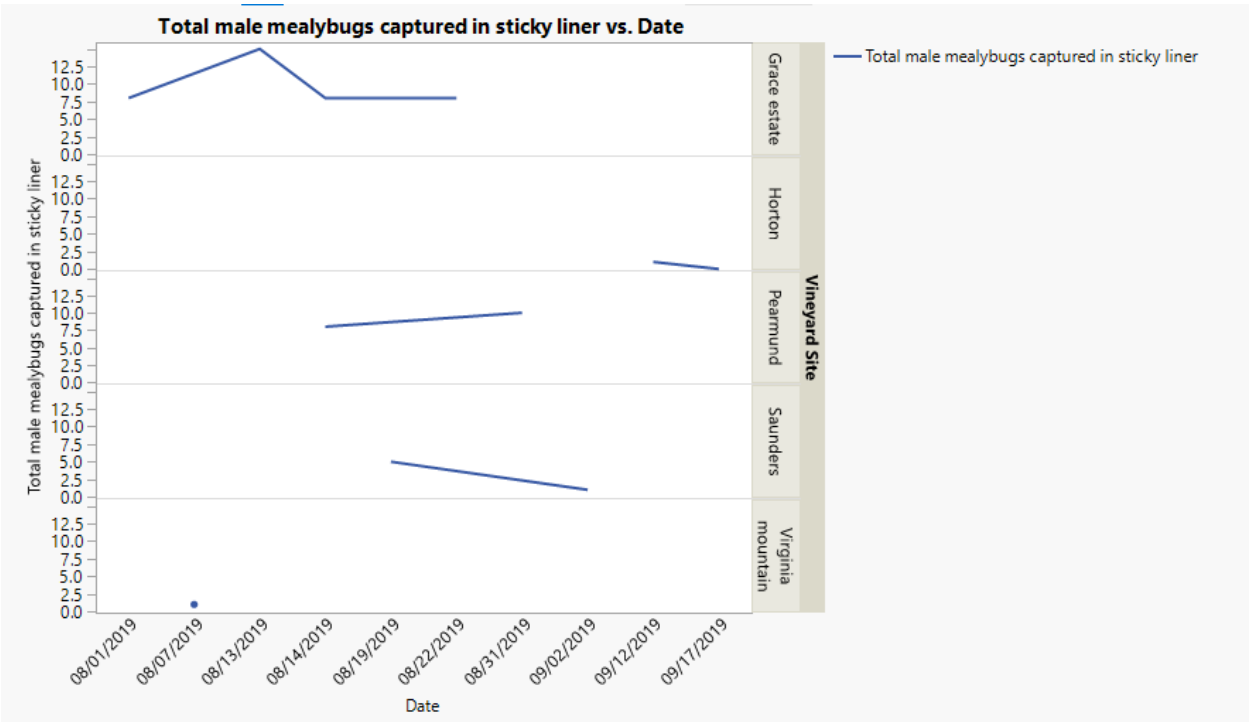


Figure 4, Male mealybugs captured in the Delta trap

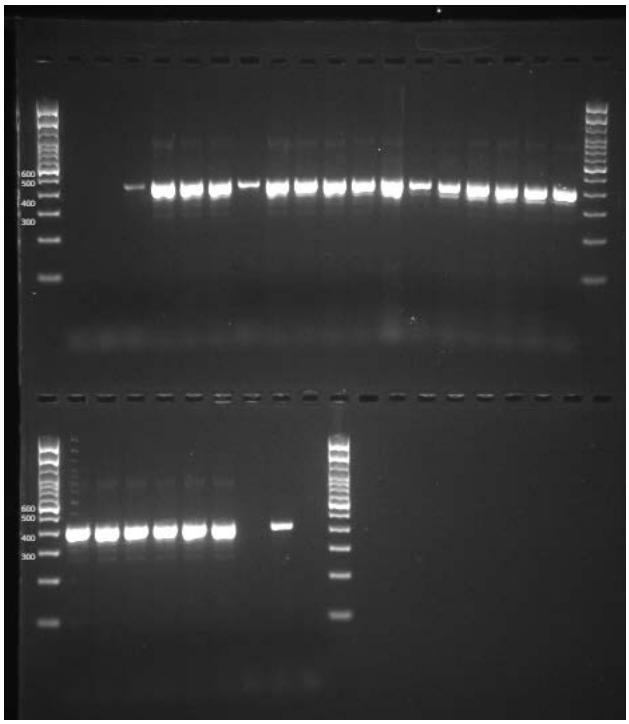
Table 2. Number of male mealybugs captured on the traps placed on edge vs center of vine row

Date	Name of vineyard	Total number of male mealybugs captured on the stick trap	Number of male mealybugs captured on the edge vs central position of vine row
7.30.19	Horton	0	0x0
8.01.19	Grace estate	8	6x2
	Horton	0	0x0
08-07-19	Grace estate	8	6x2
	Horton	3	2x1
08-07-19	Virginia mountain	1	0x1
08-14-19	Grace estate	8	6x2
	Saunders	2	0x2
	Pearmund	8	3x5
08-13-19	Grace estate	15	7x8
08-19-19	Saunders	5	2X3
	Horton	0	0X0
08-22-19	Grace estate	8	8X0
	Horton	1	1X0
	Pearmund	1	0X1
08-31-19	Pearmund	10	10X0
09-02-19	Saunders	1	1X0
	Virginia mountain	0	0X0
09-04-19	Horton, Saunders and Grace estate	0	0X0
09-12-19	Horton	1	1X0
09-17-19	Horton	0	0X0

	Grace estate	4	2X2

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. 5 and 6).



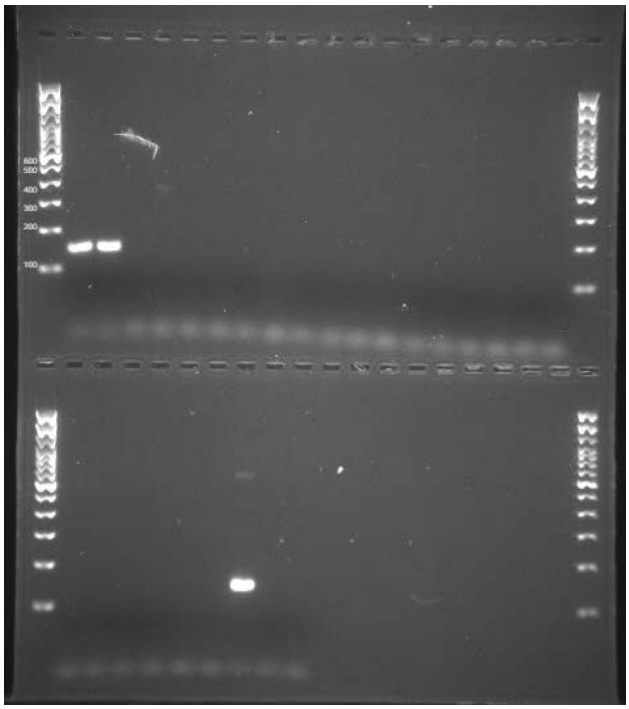


Figure 5 and 6. PCR reading on 2% agarose gel revealing 450bp band width on 22 samples from three different field sites and a single band of positive control

Role of ants:

Ants were observed in close association with mealybugs on VMV, Grace Estate, Horton and Barboursville. Ant mounds were present at the base of the trunks in Barboursville and VMV. We had found ants tending the mealybugs in VMV, Grace Estate, Horton and Barboursville. While carrying out the sampling of the roots in Barboursville, we observed ants picking up the nymphs when disturbed and transporting nymphs around. A single specimen of pavement ant, *Tetramorium immigrans* was present in close association with mealybug in Barboursville and GEW. In VMV, we found three species of ants, the smaller yellow ant, *Lasius (Acanthomyops) claviger*, odorous house ant *(Tapinoma sessile)* and cornfield ant, *Lasius (Lasius) neoniger*. The species identification of the ants was provided by the Insect ID Lab in the Department of Entomology.

Discussion

Mealybug seasonal abundance and distribution

The species level identification of mealybugs was solely based on genetic analysis. We still have male and female mealybug samples from last year, that needed identification. We aimed at finishing the genetic analysis of the samples this year. We scouted the vineyard from end of July to the end of September. We have been able to identify the species from three different vineyards in Virginia. In the future, we aim at collecting more samples and have a more detailed description of mealybugs from the rest of our sites as well. The mealybugs number recorded from the field may not represent actual number of mealybugs

present because some of the days we were unable to record the number of species on the field due to rain, difficulty in sampling near the trunk. and other factors. The growers dropped clusters on mealybug-infested vines because of low quality in GEW, while in Horton, though the number of mealybugs recorded from the field was not that high, mealybugs reached the clusters and injured the fruit causing the growers to drop some of the fruit. The insecticidal trial for control of mealybugs will be carried out this year if the situation is more favorable. We also aimed at establishing the potential role and control of ants on the field as well as carry out the morphological identification of the mealybugs.

Figure 7. Gill's mealybug from the field

Figure 8. Male mealybug captured on sticky trap

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