

‘Munger’ black raspberry: Insights into its role as a virus indicator for *Rubus* species

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ABSTRACT

'Munger' black raspberry (*Rubus occidentalis*) has been a preferred indicator for *Rubus* viruses. The working hypothesis behind the 'Munger' elevated susceptibility to viruses is its ability to sustain elevated virus titers; however, no research has yet explored this concept. To address this, we utilized an infectious clone of blackberry chlorotic ringspot virus to study the differences in virus expression between two *Rubus* species: *R. occidentalis* ('Munger') and *R. L. subgenus Rubus* Watson (blackberry, 'Natchez'). Our data demonstrate that virus accumulation in 'Munger' is over 47,300 times higher compared to 'Natchez,' enhancing our understanding of virus-host dynamics and providing valuable insights into why 'Munger' is a preferred indicator species for virus detection in *Rubus* spp.

Keywords: *Rubus*, diagnostics, virus accumulation, indicator.

1. INTRODUCTION

Symptom expression serves valuable diagnostic purposes. For this reason, scientists have identified susceptible hosts (indicators) that exhibit distinct symptomology when infected by an array of pathogens. A notable example is 'Munger' black raspberry

(*Rubus occidentalis*), which was released in the late 19th century (Hedrick, 1925) and is considered the best indicator for the majority of *Rubus* viruses (Martin et al., 2016; Martin & Tzanetakis, 2008) even though many of the ones discovered in the 21st century tend to be asymptomatic (Martin et al., 2013; Villamor et al., 2022). Still, the factors contributing to its greater vulnerability to viruses have not been studied.

The research presented within was initiated based on a simple inquiry: Why has 'Munger' been the preferred indicator for *Rubus* viruses for over a century? To explore this question, we formulated a straightforward hypothesis: 'Munger' can sustain high virus titers over other *Rubus* species and cultivars.

To test this hypothesis, we required two essential components: virus-free plant material and a pure, homogeneous virus inoculum. This inoculum needed to be free from the inherent quasi-species diversity, titer variability, and potentially varying biological properties typically associated with grafting or mechanical inoculations of field material. We obtained 'Munger' and 'Natchez' (*R.L. subgenus Rubus* Watson, blackberry) plants free of viruses based on HTS analysis (Druciarek et al., 2024). We selected the infectious clone of blackberry chlorotic ringspot virus (BCRV), a member of the genus *Ilarvirus* (Sierra-Mejia et al., 2025), as the model virus to compare virus expression between species over a six-month time period.

It will appear contradictory to use BCRV in the study given its asymptomatic nature in single infections across both host species (Poudel et al., 2011). The choice was deliberate and is grounded in the study's hypothesis – 'Munger' sustain high virus titers compared to other *Rubus* species and cultivars. This approach also eliminates the confounding influence of symptoms, which could inherently skew virus titer comparisons

between tissues (Quito-Avila and Martin, 2012; Ammara et al., 2017; Bin et al., 2022). The results of the study will help us deepen our understanding of virus-host interactions, essential for enhancing agricultural practices and improving plant health management.

2. MATERIALS AND METHODS

2.1 Plant maintenance

The plants selected for this study were confirmed to be singly infected with BCRV (Druciarek et al., 2024, Sierra-Mejia et al., 2025). BCRV-infected ‘Natchez’ plants were acquired using dodder to transfer the virus from *Nicotiana benthamiana* (Sierra-Mejia, 2024), whereas the BCRV-infected ‘Munger’ plants were obtained via direct agroinoculation. Infected plants were vegetatively propagated to increase their numbers. To establish a consistent baseline and minimize the impact of environmental variations and virus delivery, all plants were trimmed and subjected to chilling conditions by placing them in a cold room at 4°C in complete darkness for one month. The surviving plants—17 ‘Natchez’ and 8 ‘Munger’—were transferred to a growth chamber, where they were maintained under a 16/8-hour light/dark cycle at 22°C.

2.2 Detection

Sampling was conducted monthly over a six-month period. To minimize titer variation related to tissue age, we consistently sampled only newly expanded leaves. Total nucleic acid extractions and cDNA synthesis were performed as described by Poudel et al (2013). cDNA was diluted at a ratio of 1:4 and 1:4,000 for virus and 18S rRNA detection, respectively. All RT-qPCRs were conducted using a CFX96 Touch real-time

PCR detection system (Bio-Rad, Hercules, CA, USA). For virus detection the thermocycler conditions consisted of 50° C for 2 min, 95° C for 10 min, followed by 40 cycles of 95° C for 15 sec and 60° C for 1 min using the primers/probe of Poudel et al (2014) (Supplementary Table 1). For 18S rRNA detection was performed using primers and probes designed by Osman et al (2007). The probe's quencher and fluorophore were further modified to meet the specific requirements of the equipment (Supplementary Table 1). The final reaction volume was 20 µL and included 2 µL of cDNA, 10 µL of TaqMan™ Universal PCR Master Mix II with UNG (Thermo Fisher Scientific), 25 nM of each 18S rDNA-specific primer, and 12.5 nM of the corresponding probe. The thermocycler conditions were the same as those used for BCRV detection, and appropriate controls were included in each run. Each sample was analyzed in triplicate through qPCR, and the average CT value from the three independent runs was calculated (Table 1).

2.3 Statistical analysis

The resulting qPCR runs were compiled into a dataset, categorizing the data by month and species. Cycle threshold (CT) values were normalized for viral quantification using 18S rRNA as an internal control, and we included this value in our dataset. For normalization purposes, a CT value of 40 was assigned when the virus was not detectable. Normalization was calculated using the equation:

$$\Delta CT = CT_{\text{virus}} - CT_{18s}$$

Where, ΔCT represents the difference in CT values between virus and internal control (18S rRNA). The normalized value, reflecting relative virus expression, was derived from:

$$\text{Normalized Value} = \text{Relative Viral Expression (RVE)} = 2^{-\Delta CT}$$

The dataset was imported into R version 4.4.1 for analysis (R Core Team, 2023) which was used in all downstream analyses. We employed a linear mixed effects model, with the normalized values as the response variable. Species and month were included as fixed main effects, and an interaction effect between month and species was also included. Each individual plant was accounted for as a random factor to address the variability amongst plants. A Type III analysis of variance (ANOVA) using Satterthwaite's method was conducted to determine the significance of the fixed and interaction effects in a linear mixed-effects model. Following this, a post hoc analysis was conducted to address specific group differences in the mixed effects model. For this, marginal means were evaluated, and pairwise comparisons among these means were conducted using Tukey's Honest Significant Difference (HSD) method ($P < 0.05$).

3. RESULTS

BCRV was consistently detected in all 'Munger' plants at all sampling points. In 'Natchez' the virus was undetectable at certain sampling points in some plants, without any consistent patterns emerging (Table 1).

Overall, the RVE over the length of the study was 0.109 for 'Munger' and 2.31×10^{-6} for 'Natchez', yielding an RVE ratio of over 47,304. The ANOVA test showed that both species ($p=7.47 \times 10^{-10}$) and sampling time ($p=8.66 \times 10^{-10}$) had a significant effect on virus

RNA accumulation (Table 2). Additionally, the interaction between the two was also significant ($p = 8.64e^{-10}$), suggesting that the effect of species on the virus accumulation may vary depending on the month (Table 2). The post hoc analyses revealed significant differences in the estimated marginal means (emmeans) of virus accumulation between 'Munger' and 'Natchez' over the length of the study. For example, in the first month, the emmean for 'Munger' was 0.05, whereas for 'Natchez', it was $6.10e^{-7}$, indicating higher virus accumulation in the former. This trend continued in subsequent months, with 'Munger' consistently displaying higher emmean values (Figure 1). Furthermore, these analyses revealed significant differences in virus accumulation associated with the sampling time. For 'Munger', there was considerable variability, with a notable increase in virus expression during the second sampling time, whereas 'Natchez' exhibited less variability across sampling times.

4. DISCUSSION

This study advances our understanding of BCRV-host interactions by providing insights into the dynamics of virus accumulation over time in two distinct genotypes, 'Munger' and 'Natchez'. We monitored BCRV accumulation over six months, coinciding with the growing season, revealing that 'Munger' is favorable host of the virus, consistently supporting higher virus accumulation across all sampling points. In contrast, 'Natchez' did not support high virus accumulation, and detection was sporadic throughout the study. These results align with the fact that blackberries are generally more resistant to viral infections than black raspberries (Finn & Clark, 2012). Moreover, these results

shed light on why ‘Munger’ has been the standard indicator for virus detection for *Rubus* species.

Ilarviruses typically display uneven distribution and fluctuating titers within woody hosts throughout the year (Uyemoto & Scott, 1992; Zotto et al., 1999). This pattern was evident in 'Natchez,' but not in 'Munger,' where viral titer levels remained high, facilitating consistent detection. The intermittent detection of the virus in ‘Natchez’ corroborates the observations of Villamor et al (2022), where the authors noted that viruses in berry crops were not consistently detected at four sampling points. The authors hypothesized that this inconsistency could be due to temporal variations in virus accumulation and distribution within the host, as well as the influence of individual host genotypes on virus-host interactions (Villamor et al., 2022). Similar situations have been observed in other plant models, such as sugarcane, where virus detection varies with season and tissue type (Bulus et al., 2023; Malapi-Wight et al., 2021). In our study, we minimized the influence of seasonality and tissue-related titer variation by maintaining consistent temperature and light conditions and sampling newly expanded leaves from both hosts.

Despite the high virus accumulation in ‘Munger’ that was at least 47,300 times greater than in 'Natchez', the former remained asymptomatic throughout the study. This situation has also been documented with other viruses infecting *Rubus*, such as blackberry yellow vein associated virus and blackberry virus Y, where ‘Munger’ serves as a host without exhibiting symptoms (Susaimuthu et al., 2007; Martin et al., 2013). Ilarviruses, including BCRV, are known to cause latent infections. While BCRV remains asymptomatic during single infections in blackberry (Poudel et al., 2014) and raspberry,

it can transition to a symptomatic state in mixed infections, such as those causing blackberry yellow vein disease.

Such asymptomatic conditions are often associated with latent infections, where a host plant remains symptomless despite a virus infection, potentially due to virus tolerance or viral persistence. Virus tolerance is observed when high viral titers do not trigger a strong antiviral response. In contrast, virus persistence occurs when the virus continues to replicate at low levels over time without inducing disease symptoms (Takahashi et al., 2019). We hypothesize that 'Munger' exhibits tolerance to BCRV infection, as it shows no symptoms despite high virus accumulation.

A mixed-effects statistical analysis model was employed to account for repeated measures collected over different months and the variability in observation numbers across groups. This approach effectively addressed the unbalanced data, allowing us to assess the effects of species, month, and their interactions on virus titer accumulation while considering the random effects associated with each individual biological sample. The two-way ANOVA results indicated significant influences from these factors on virus accumulation. Post-hoc analysis revealed higher virus accumulation in 'Munger', with viral fluctuating titers across the months and a significant increase during the second sampling point. In contrast, 'Natchez' exhibited lower accumulation overall with less variability. The mechanisms underlying these observations remain to be explored.

Plants were subjected to low temperatures for a month before the start of this experiment. Virus replication is believed to slow in cooler conditions, with rising temperatures correlating with increased viral titers, a trend noted in studies linking seasonal changes to viral dynamics (Honjo et al., 2020). This increase is attributed to

early viral accumulation in young leaves, with the host's defense system activating RNA silencing genes as the viral load increases (Honjo et al., 2020). This could explain the significant increase in virus accumulation observed in 'Munger' during the second month, likely driven by the temperature shift from 4°C to 22°C. As viral titers peaked, an antiviral response may have been triggered, leading to a decline in virus expression in the following months.

Host genotype significantly influences virus expression, as shown by Smith and Campbell (2004), who studied how time and plant genotype affect the spread of poplar mosaic virus. They found that the virus' systemic spread varies by genotype, with different *Populus* genotypes exhibiting distinct levels of susceptibility and virus accumulation.

Despite having fewer observations for 'Munger' than for 'Natchez,' statistical analysis delivered a high level of confidence for several reasons: 1) BCRV was consistently detected at every sampling point in 'Munger' unlike 'Natchez'; 2) 'Munger' showed consistent and homogeneous observations, with CT values fluctuating by less than five cycles within each plant (Table 1); and 3) our statistical model accounted for unbalanced data.

Future research should focus on uncovering the host factors that either inhibit or facilitate BCRV's systemic spread, potentially enhancing our understanding of resistance traits among different *Rubus* genotypes and facilitating their incorporation into breeding programs.

This study offers a detailed analysis of the interaction between blackberry chlorotic ringspot virus (BCRV) and two *Rubus* genotypes, 'Munger' and 'Natchez',

highlighting key differences in virus expression and detection. We found that 'Munger' serves as a more permissive host, exhibiting higher and more consistent virus accumulation, making it a valuable indicator for monitoring viruses in *Rubus*. Although both genotypes remained asymptomatic, differences in virus accumulation suggest that further research into host mechanisms could deepen our understanding of virus tolerance and persistence.

DECLARATIONS

During the preparation of this work the authors used ChatGPT v.4 to assist in generating R code for statistical analysis and to improve the language. No content was directly generated by the tool; rather, it was used to help enhance the overall clarity of the text. After using this tool, the authors reviewed and edited the content and code as needed and take full responsibility for the content of the publication.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available from the corresponding author.

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Table 1. RT-qPCR results for detecting blackberry chlorotic ringspot virus in ‘Munger’ and ‘Natchez’ plants over a six-month period. A given value indicates that the virus was detected, the number corresponds to the average CT amongst three independent runs, (-) indicates that the virus was not detected, a CT value of 40 was given to allow for the statistical analyses, and (NA) signifies that the plant was lost and could not be sampled for further analysis.

Plant	Month					
	1	2	3	4	5	6
Ct Values- Munger						
MD1	21.86	21.38	23.90	22.72	21.14	24.67
MD2	21.85	18.41	NA	NA	NA	NA
MD3	20.38	20.28	21.67	23.41	20.57	22.92
MD4	21.38	18.07	22.86	19.82	21.74	23.10
MD5	19.39	17.16	19.65	20.72	20.43	23.14
MD6	24.32	20.81	21.00	20.41	22.29	24.85
MD7	22.79	19.93	20.59	22.79	20.71	24.13
MD8	22.45	21.95	21.39	21.47	21.06	23.88
Ct Values- Natchez						
ND1	- (40.00)	38.37	- (40.00)	- (40.00)	- (40.00)	- (40.00)
ND2	- (40.00)	- (40.00)	- (40.00)	39.25	- (40.00)	- (40.00)
ND3	34.53	- (40.00)	- (40.00)	- (40.00)	- (40.00)	- (40.00)
ND4	- (40.00)	- (40.00)	- (40.00)	- (40.00)	- (40.00)	- (40.00)
ND5	- (40.00)	38.73	- (40.00)	- (40.00)	- (40.00)	39.13
ND6	- (40.00)	- (40.00)	- (40.00)	38.90	- (40.00)	38.77
ND7	- (40.00)	- (40.00)	- (40.00)	- (40.00)	- (40.00)	39.48
ND8	- (40.00)	38.97	- (40.00)	- (40.00)	- (40.00)	38.91
ND9	36.12	39.68	- (40.00)	- (40.00)	- (40.00)	- (40.00)
ND10	- (40.00)	- (40.00)	- (40.00)	- (40.00)	- (40.00)	- (40.00)
ND11	- (40.00)	- (40.00)	- (40.00)	- (40.00)	- (40.00)	- (40.00)
ND12	- (40.00)	- (40.00)	- (40.00)	- (40.00)	- (40.00)	- (40.00)
ND13	38.52	37.55	- (40.00)	- (40.00)	- (40.00)	- (40.00)
ND14	33.14	- (40.00)	- (40.00)	- (40.00)	- (40.00)	- (40.00)
ND15	- (40.00)	37.27	- (40.00)	- (40.00)	- (40.00)	39.45
ND16	- (40.00)	- (40.00)	- (40.00)	- (40.00)	- (40.00)	39.23
ND17	- (40.00)	36.17	- (40.00)	- (40.00)	- (40.00)	- (40.00)

Table 2. Type III Analysis of Variance with Satterthwaite's Method. The table shows the results for the effects of species, month, and their interaction on the response variable. All factors (species, month, and species:month interaction) are statistically significant, with p-values less than 0.001.

Sum Sq(Sum of Squares), Mean Sq(Mean Square), NumDF (Numerator Degrees of Freedom), DenDF(Denominator Degrees of Freedom),F value, and Pr(>F) (p-value).

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Species	0.17005	0.170049	1	23.853	96.407	7.468e-10
Month	0.11198	0.022395	5	112.722	12.697	8.659e-10
Species:Month	0.11199	0.022397	5	112.722	12.698	8.644e-10

Distribution of Normalized Virus Titer by Species and Month

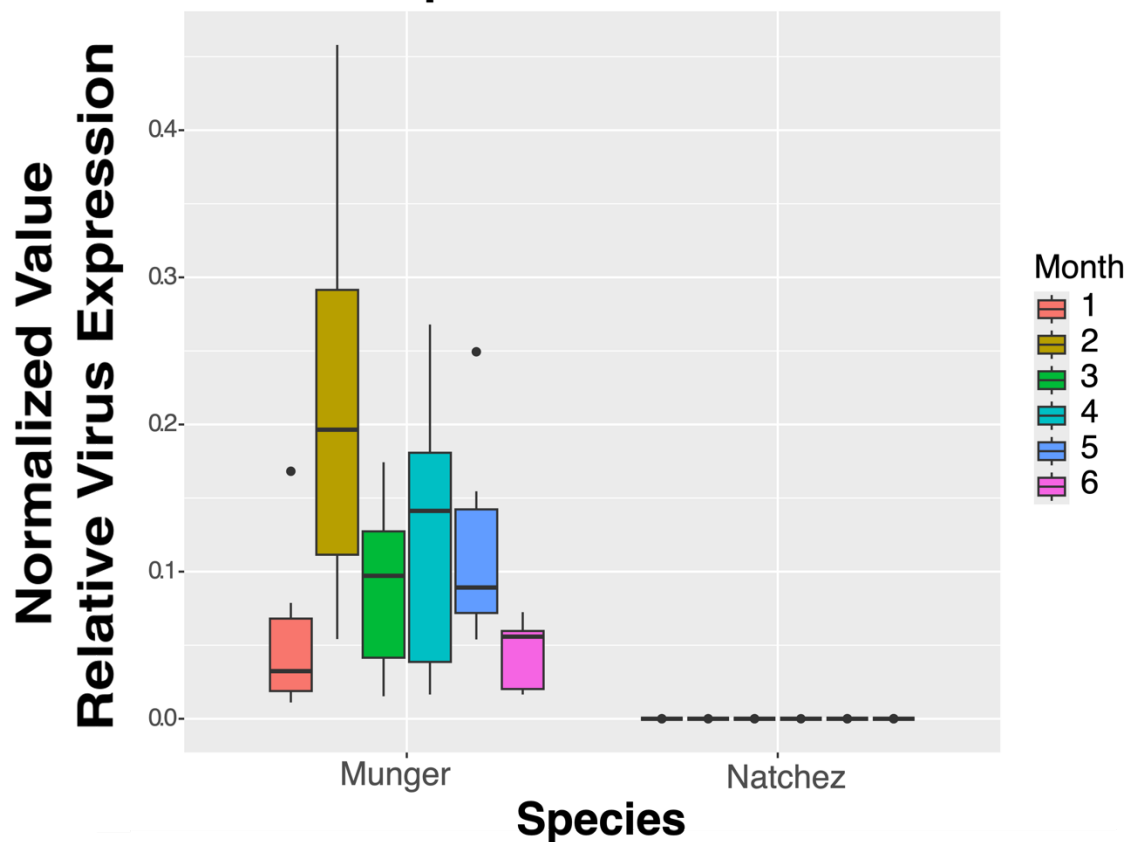


Figure 1. Box plot illustrating the distribution of normalized virus RNA accumulation for blackberry chlorotic ringspot virus (BCRV) in 'Munger' black raspberry and 'Natchez' blackberry, across a six-month period. Dots represent outliers. Compact letter display (CLD) results showing pairwise comparisons of species and month combinations, where groups sharing the same letter are not significantly different, and groups with different letters are significantly different based on Tukey's post-hoc test at the 5% level of significance.

Supplementary Table 1. Detection primers and TaqMan™ probes used in this study.

Primer Name	Nucleotide sequence 5' – 3'	Reference
qPCR BCRV3.298Fa ^a	AGGTTGAAATGGCTTTGACCC	Poudel et al. (2014)
qPCR BCRV3.298R ^a	AAGCAGCRCATCGCCTTATAC	
qPCR BCRV3.298 probe ^a	/56- FAM/TCTCGCTGG/ZEN/TTCGATGGAGCT/3IA BkFQ/	
18S rRNA 449f ^a	GTGACGGAGAATTAGGGTTCTGA	Osman et al (2007).
18S rRNA 498r ^a	CTGCCTTCCTTGGATGTGGTA	
18S rRNA 475p ^a	/5HEX/CCGGAGAGG/ZEN/GAGCCTGAGAAAC GG/3IABkFQ/	Osman et al (2007) modified for this study

^a Primers used for RT-qPCR detection

Supplementary Material

R Code Utilized to perform the statistical analysis in R studio. This code was generated with the aid of ChatGPT v.4

```
# Load necessary libraries
install.packages('emmeans')
install.packages('lmerTest')
install.packages('multcompView')
install.packages('multcomp')
install.packages("pbkrtest")
library(pbkrtest)
library(lme4) # For mixed-effects models
library(lmerTest) # For p-values in mixed-effects models
library(emmeans) # For post-hoc comparisons
library(ggplot2) # For plotting
library(multcompView)
library(multcomp)

# Load your dataset
# Set working directory and load your dataset
setwd('~\\Desktop\\') # Change this to your actual path
# Replace 'your_data.csv' with the actual path to your CSV file
data <- read.csv("Viraltiter6months9.csv")

# Normalize the CT values, ensuring NA handling
data$Normalized_Value <- 2 ^ (data$CT_Internal_Control - data$CT_Virus)

# Ensure proper factor levels for species and month
data$Species <- as.factor(data$Species)
data$Month <- as.factor(data$Month)
data$Plant_ID <- as.factor(data$Plant_ID)

# Fit a mixed-effects model, ignoring NA values in the analysis
##model <- lmer(Normalized_Value ~ Species * Month + (1 | Plant_ID), data = data)
model <- lmer(Normalized_Value ~ Species * Month + (1 | Plant_ID), data = data)

# Summarize the model
summary(model)

# Check for significant differences using ANOVA
anova(model)

# Post-hoc comparisons
emmeans_results <- emmeans(model, ~ Species * Month)

#emmeans_results <- emmeans(model, ~ Species * Month, mode = "asymptotic")
summary(emmeans_results)
post_hoc_results <- pairs(emmeans_results, adjust = "tukey")
summary(post_hoc_results)
```

```

425 plot(emmeans_results)
426 plot(post_hoc_results)
427
428 # Convert results to a data frame
429 post_hoc_df <- as.data.frame(post_hoc_results)
430 multcomp::cld(emmeans_results)
431 letters_df <- multcomp::cld(emmeans_results, Letters = letters)
432
433 # Get compact letter display
434 letters_df <- multcomp::cld(emmeans_results, adjust = "sidak", Letters = letters)
435
436 # Compute mean normalized values for each species
437 mean_munger <- mean(data$Normalized_Value[data$Species == "Munger"], na.rm = TRUE)
438 mean_natchez <- mean(data$Normalized_Value[data$Species == "Natchez"], na.rm = TRUE)
439
440 # Calculate how many times Munger is larger than Natchez
441 ratio <- mean_munger / mean_natchez
442 # Print the result
443 ratio
444 # Get the unique months from the data
445 unique_months <- unique(data$Month)
446
447 # Initialize a result dataframe to store the ratios for each month
448 results <- data.frame(Month = character(), Ratio = numeric(),
449                       Mean_Munger = numeric(), Mean_Natchez = numeric(), stringsAsFactors =
450 FALSE)
451
452 # Loop through each month and calculate the means and ratio
453 for (month in unique_months) {
454   # Compute mean normalized values for Munger in this month
455   mean_munger <- mean(data$Normalized_Value[data$Species == "Munger" & data$Month ==
456 month], na.rm = TRUE)
457
458   # Compute mean normalized values for Natchez in this month
459   mean_natchez <- mean(data$Normalized_Value[data$Species == "Natchez" & data$Month ==
460 month], na.rm = TRUE)
461
462   # Calculate the ratio (how many times Munger is larger than Natchez)
463   ratio <- ifelse(mean_natchez == 0, NA, mean_munger / mean_natchez) # Handle division by
464 zero
465
466   # Append results to the dataframe
467   results <- rbind(results, data.frame(Month = month, Ratio = ratio,
468                                         Mean_Munger = mean_munger, Mean_Natchez = mean_natchez))
469 }
470
471 # View the results
472 print(results)
473
474 # Visualization of normalized values over time, handling unbalanced data
475 ggplot(data, aes(x = Month, y = Normalized_Value, color = Species)) +

```

```

476   geom_point(position = position_jitter(width = 0.1), alpha = 0.7) + # Add jitter to points for
477   visibility
478   geom_line(aes(group = interaction(Species, Plant_ID)), alpha = 0.5) + # Connect points for
479   the same plant
480   labs(title = "Normalized Viral Load Over Time", x = "Month", y = "Normalized Viral Load") +
481   theme_minimal()
482
483   # Violin plot of normalized viral load by Species and Month
484   ggplot(data, aes(x = Month, y = Normalized_Value, fill = Species)) +
485   geom_violin(trim = FALSE, alpha = 0.7) + # Violin plot
486   geom_boxplot(width = 0.1, position = position_dodge(0.9), outlier.shape = NA, alpha = 0.5) +
487   # Boxplot on top
488   labs(title = "Violin Plot of Normalized Viral Load by Species and Month",
489        x = "Month",
490        y = "Normalized Viral Load") +
491   theme_minimal() +
492   theme(legend.title = element_blank())
493
494   # Bar Plot
495   ggplot(data, aes(x = Species, y = Normalized_Value, fill = Month)) +
496   geom_bar(stat = "summary", fun = "mean", position = "dodge") +
497   labs(title = "Normalized Virus Titer by Species and Month", y = "Normalized Value")
498
499   # Box Plot
500   ggplot(data, aes(x = Species, y = Normalized_Value, fill = Month)) +
501   geom_boxplot() +
502   labs(title = "Distribution of Normalized Virus Titer by Species and Month", y = "Normalized
503   Value")
504
505
506

```