Secondary Metabolite Analysis to Understand Tree Response to Spruce Beetles

2022-23 ESS SUPER Program

Skills for Undergraduate Participation in Ecological Research

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Research Summary

Bark beetles (*Dendroctonus rufipennis*) have long been a significant source of disturbance in the Rocky Mountain region, but the frequency and intensity of outbreaks have increased in recent years. This trend can be attributed to the warmer temperatures and drier conditions associated with climate change, which have created more favorable conditions for bark beetle infestations. In response to these stressors, trees have needed to modify their defense mechanisms in order to better defend themselves against pests, yet unfavorable climate conditions have made it difficult for trees to adapt to these conditions. This is because when trees experience drought, they often allocate more resources to survival functions like maintaining water balance and less to secondary functions like resin production. As a result, the production of resin may decrease, leaving trees more susceptible to damage from pests and pathogens. Additionally, drought stress can alter the composition of volatile organic compounds (VOCs) released through resin, reducing the resin's ability to repel pests and pathogens. To better understand how drought stress affects the production and composition of resin, we are exploring the differences in defense strategies by examining the varying components within resin compounds from a mesic (wet) site in Cameron Pass, Colorado and a xeric (dry) site in Alma, Colorado. This research aims to provide insights into how trees defend themselves under drought stress, as the global climate continues to warm, and insect herbivory increases.
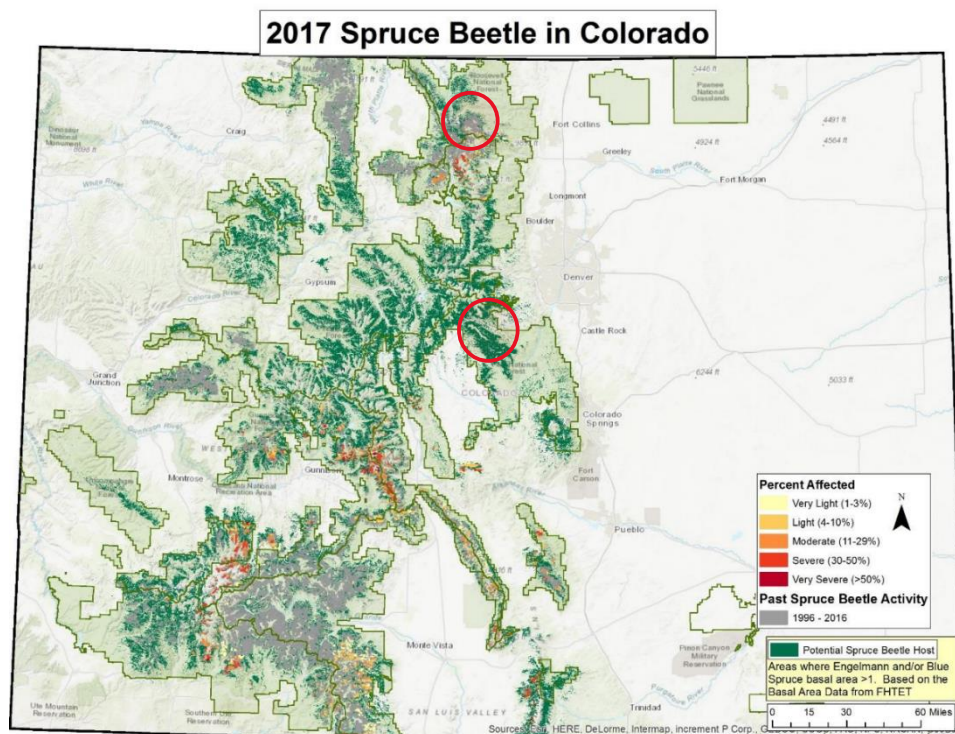
Introduction

Fig 1. *Potential for spruce beetle host (green) with past spruce beetle activity (grey), and 2017 severity categories (yellow to red) in Colorado.* The red circles indicate the areas of study for this project. (Image by: USDA Forest Service)

Background:

Bark beetles (*Dendrocton*us *rufipenniss*) have been affecting spruce trees in the Rocky Mountain region and Alaska since the 1700s, but recently the outbreaks of this pest have become larger and more frequent (Jenkins et al. 2014). The recent surge in bark beetle infestations in the last century has been attributed to environmental changes resulting from drought and climate change (Hart et al. 2013). This has weakened the trees' defenses, thus making them easier targets for the beetles by providing them with suitable food, habitat, and conditions for reproduction. In the past, these infestations did not have as significant of a negative environmental impact, as historical outbreaks have contributed to the health of vegetation dynamics in mountain ecosystems (Rodman et al. 2021). However, the recent surge in intensity is resulting in higher rates of tree mortality, which is exacerbating other environmental concerns such as increased severity of wildfires and reduced CO_2 sequestration (Katz et al. 2017). This increase in mortality is negatively altering the trajectory of the subalpine forest ecosystems and causing a series of cascading effects that is prompting concerns for the health of other plants and animals within the surrounding areas.

With this being said, Spruce trees are attempting to address the bark beetle infestations by adapting their defense mechanisms to counter the increased outbreaks. One of the main defense mechanisms that protects spruce trees against pest invasions is secondary metabolites found in resin (Fig. 2) (Franceschi et al. 2005). This strategy of defense involves expelling the invading organisms from the bark, sealing the wound with a hardened resin, and producing toxic compounds that discourage and mitigate the pest's activity. However, during times of high stress, such as droughts caused by climate change, the trees are unable to produce these secondary metabolites, which would otherwise provide protection (Huang et al. 2019). This is because, during these events, spruce trees resources are being allocated to growth and survival rather than protection.

Previous studies have investigated chemical defense mechanisms produced by trees, however, the research questions and hypotheses in this study differ from those in past research. The aim of this study is to explore the differences in resin compounds between two forest sites - one that is mesic (Cameron Pass) and another that is xeric (Alma). By studying these sites, we hope to gain a better understanding of the dynamics of spruce trees in response to environmental stress, herbivory, and their defense mechanisms.
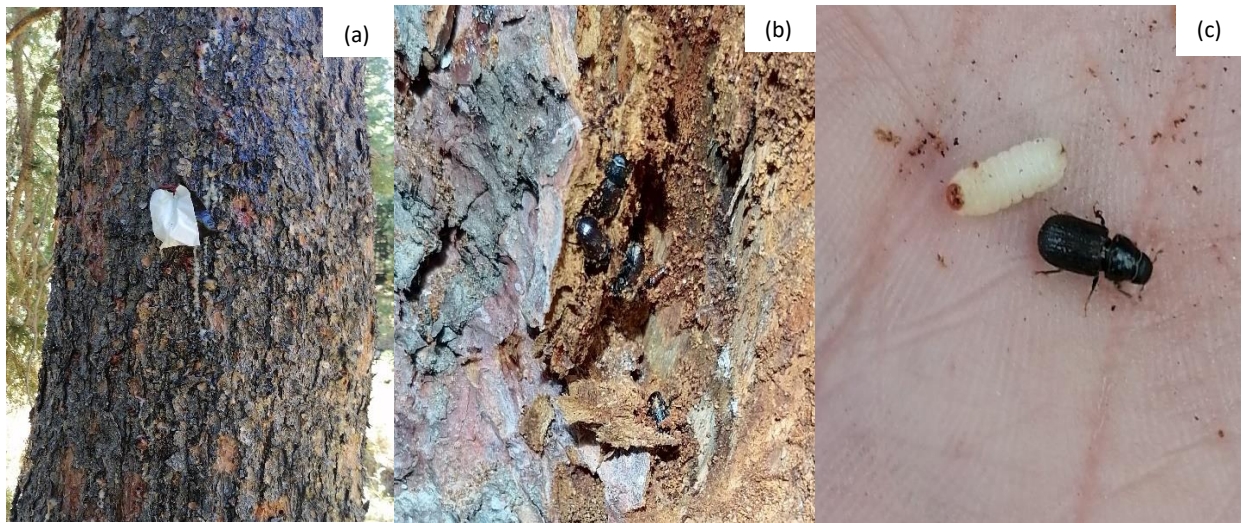
Fig 2. (a) Picture of a baited tree with resin from the research study (Photo by: Ehsan Khedive). (b) Bark beetles within the bark of an infested tree (Photo by: Ehsan Khedive). (c) Bark beetle adult and a bark beetle pupa (Photo by Ehsan Khedive).

Research Question

How will bark beetles (Dendroctonus rufipenniss) affect components of spruce resin in two forest sites (Cameron pass and Alma) after the beetle starts diapause in October?

Expected Outcome and/or Hypothesis

We hypothesize that components of resin will be different between control (wounded) and baited trees. We also hypothesize that site specific properties also affect host resin response to bark beetles. Hence, the two sites were selected so that one site is mesic (Cameron pass), and the other site is xeric (Alma).

Emergent Null Hypothesis

Although there are differences in specific site properties between the baited and non-baited trees and across both sites, the components in the resin will remain consistent.

Methods

Data Collection:

On October 15th and 16th, 2022, data collection was conducted in Alma and Cameron Pass (Fig 9). The sites featured a combination of trees with gallery wounds or intact bark and trees that were baited or non-baited. For the baited trees, the resins ranged from clear/crystallized to brown/yellow, whereas non-baited trees only had clear/crystallized resins. As a result, an average of 3-4 samples were collected per baited tree and 1-2 samples per non-baited tree at each site, resulting in around 80+ samples for both sites. Each sample was labeled with information indicating the site, tree number, whether it was baited/non-baited, and the color of the resin.

Data Processing/Entry:

Following collection, the samples were stored in a freezer at the forestry health lab on Colorado State Universities campus, and data processing began on October 31, 2022 (Fig 9). The resin was weighed, and 10-30mg of each sample was transferred to a new vial. Then, a solvent of Hexane/Dichloromethane was added to the sample, and the vial was vortexed for 30 seconds to completely dissolve the resin. After this, the vials were put into a centrifuge at 12000 rpm for 2 minutes so that the solid debris precipitate. Next, 200 µL of the diluted and dissolved resin sample was added to a new GC/MS vial containing 800 µL of Hexane. The GC/MS vial was subsequently inserted into the mass spectrometry autosampler, where the analysis began. All data collected for this project has been entered and stored on a computer located within the forestry health lab at Colorado State University.

Data Analysis:

Once the GC/MS analysis was complete, the results were imported into a program called "MassHunter" where the compounds peaks were able to be identified using mass libraries (NIST and Wiley) and exported into an CSV file (Fig 9). The results were filtered by only looking at the 10-C compounds that were identified by the results. After this, an aliquot of external standard monoterpenes with concentrations from 5 to 100 ppmv were provided using Hexane as a solvent. The aliquots were then injected into GC/MS using the same programming as the resin sample analysis. Finally, a concentration curve was fitted using linear regression in order to convert the chromatogram area to concentration as volumetric part per million (ppmv). This allowed us to better understand and interpret the concentration of each of the monoterpenes presents within the resins we were testing. Once this was finished, all of the Excel data was inputted into RStudio where the data was compiled and converted into graphs that will be displayed in the results.

Data Interpretation:

The findings were interpreted in order to determine if there was a discernible pattern of resin components between the baited and non-baited trees at both sites (Fig 9). If such a pattern is identified, additional research will be necessary to comprehensively investigate the compounds and their role in augmenting the trees' defense mechanisms against the bark beetle infestation in Colorado.

Results

The results of terpene measurements in different sites with varying treatments indicate that the range and average concentrations of monoterpene in collected resin samples are very similar to those from intact bark of baited trees. Cameron Pass (CBN) had the highest variation and median concentration, while the lowest concentration of monoterpenes was found in samples taken from beetle galleries on baited trees in Alma (ABG), but the difference between treatment groups was not statistically significant (Figure 3).
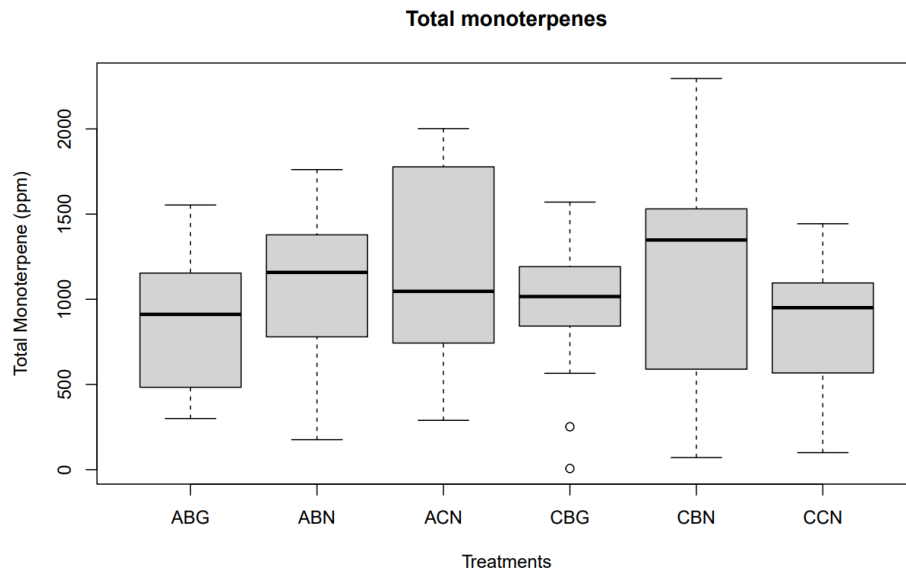

Fig 3. Boxplot of total monoterpene concentration in resin samples from different sites and treatments: ABG (Alma, Baited, Gallery wounds), ABN (Alma, Baited, Intact bark), ACN (Alma, Control, Intact bark), CBG (Cameron Pass, Baited, Gallery wounds), CBN (Cameron Pass, Baited, Intact bark), and CCN (Cameron Pass, Control, Intact bark).

The results of terpene measurements in the sites showed that Alma samples tend to have lower concentration of monoterpenes compared to Cameron pass. More specifically, there are higher concentration of 4(10)-Thujene, 3-Thujen, and cis-Ocimene observed in Alma and higher concentration of 4-Carene, alpha-Phellandrene, gamma-Terpinolene, 2-Thujen, beta-myrcene, Linalool, and Orthodene observed in Cameron pass samples (Figure 4).
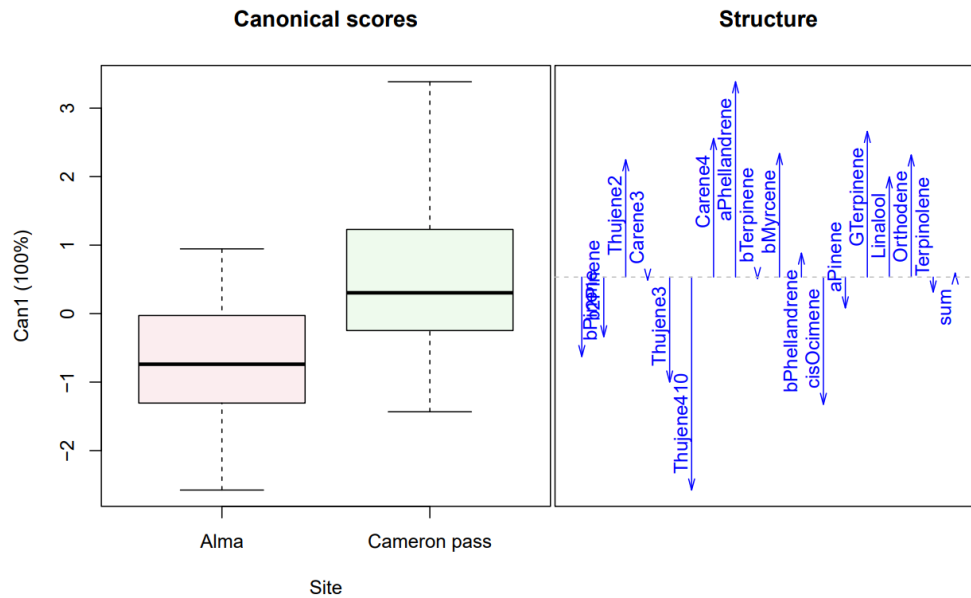
Fig 4. The boxplot shows the canonical scores (standardized results of all variables) for Alma on the right and Cameron Pass on the left. It also displays the most prevalent compounds at each site, with downward arrows indicating a higher concentration in Alma and upward arrows indicating a higher concentration in Cameron Pass samples. The size of the arrows reflects the magnitude of the difference in compound concentration between the two sites, with longer arrows indicating a greater concentration at the corresponding site, and shorter arrows indicating more similarity between the compounds present at each site.

The results of terpene measurements in the beetle and control trees showed that control trees tend to have lower concentration of monoterpenes compared to beetle trees. More specifically, there are higher concentration of 4(10)-Thujene, beta-Phellandrene, beta-myrcene, and 2bPinene observed in the control samples and higher concentration of 4-Carene, beta-Pinene, alpha-Phellandrene, beta-Terpinene, alpha-Pinene, gamma-Terpinolene, Linalool, and Orthodene observed in the beetle samples (Figure 5).
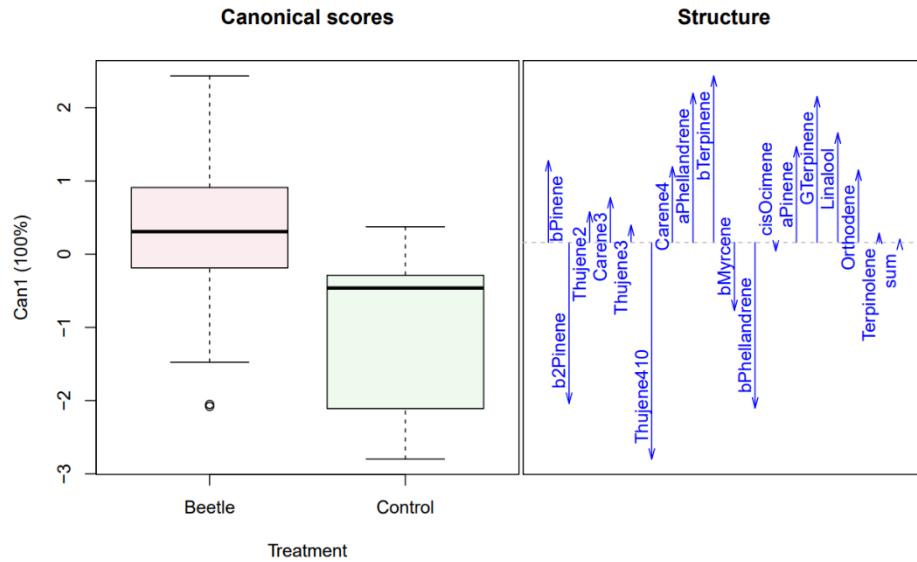
Fig 5. The boxplot shows the canonical scores (standardized results of all variables) for the beetle trees on the right and control trees on the left. It also displays the most prevalent compounds at each site, with downward arrows indicating a higher concentration in the control samples and upward arrows indicating a higher concentration in the beetle samples. The size of the arrows reflects the magnitude of the difference in compound concentration between the two treatments, with longer arrows indicating a greater concentration in the corresponding treatments, and shorter arrows indicating more similarity between the compounds present in each treatment.

The results of terpene measurements in the gallery wounds (Yes) and intact bark (No) show that gallery wounds tend to have lower concentration of monoterpenes compared to intact bark. More specifically, there are higher concentration of 4-Carene, alpha-Phellandrene, beta-Terpinene, alpha-Pinene, gamma-Terpinene, and Orthodene observed in the samples taken from the gallery wounds and a high concentration of 3-Carene, 3-Thujene, 4(10)Thujene, beta-Myrcene, beta-Phellandrene, cisOcimene, Linalool, and Terpinolene in the intact bark samples (Figure 6).
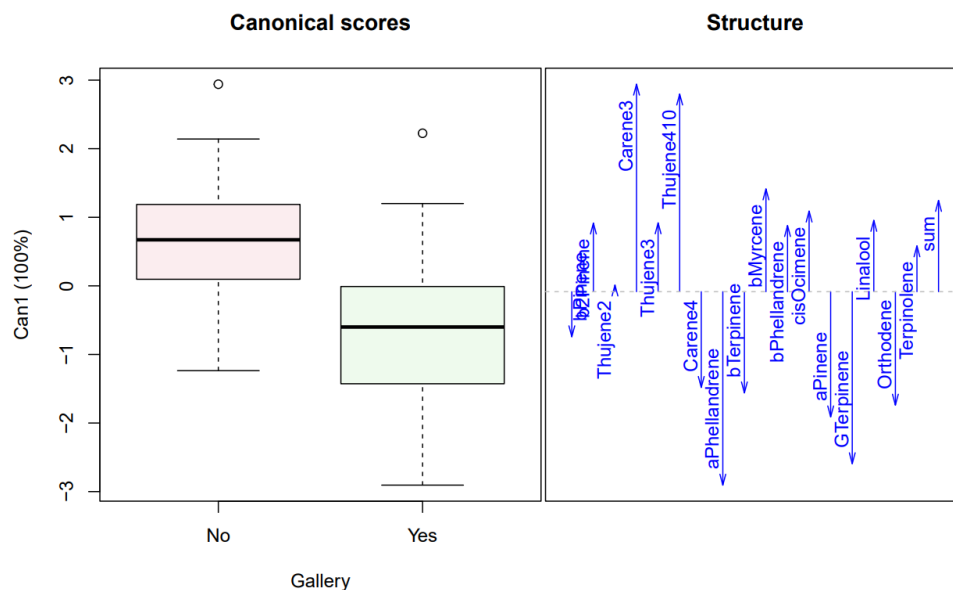
Fig 6. The boxplot shows the canonical scores (standardized results of all variables) for the gallery wounds (Yes) on the right and intact bark (No) on the left. It also displays the most prevalent compounds at each site, with downward arrows indicating a higher concentration in the gallery wound samples and upward arrows indicating a higher concentration in the intact bark samples. The size of the arrows reflects the magnitude of the difference in compound concentration between the two treatments, with longer arrows indicating a greater concentration in the corresponding treatments, and shorter arrows indicating more similarity between the compounds present in each treatment.

The results of terpene measurements in the resin colors at both of the sites show that crystalized and yellow have similar components made up of gamma-Terpene, alpha-Pinene, 4-Carene, beta-Terpinene, and 2-Thujene. The black colored resin only had components of beta-pinene monoterpenes. The clear resin has high concentrations of the sum, 4(10)Thujene, beta-Phellandrene, Orthodene, Linalool, Terpinolene, 3-Carene, 2bPinene, cisOcimene, 3-Thujene, and beta-Myrcene (Figure 7).
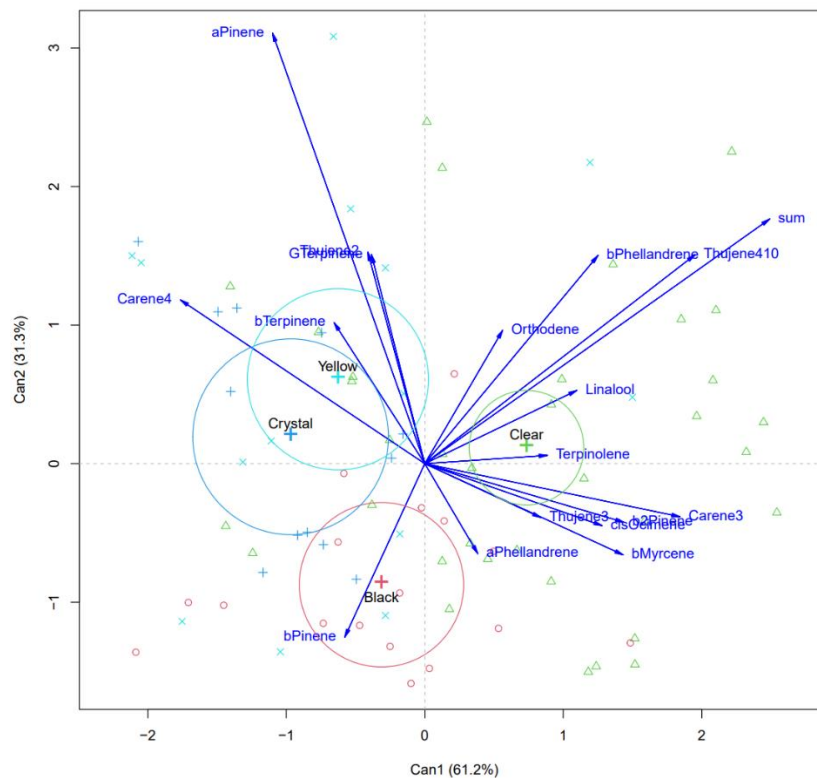

Fig 7. A graph that represents the compounds present within each resin type (clear, crystallized, yellow, and black) using two different canonical functions (Can1 and Can2). The size of the circles reflects the variations within the treatments, while the position of the circles indicates the similarity in components to each resin type. The size of the arrows indicates the magnitude of the difference in compound concentration between the treatments, with longer arrows indicating a greater concentration in the corresponding resin types, and shorter arrows indicating more similarity between the compounds present in each resin type.

The results of terpene measurements in the different treatments show that CCN exhibits high concentrations of beta-Phellandrene, while ABG and CBG show high concentrations of alpha-

Pinene, Orthodene, 4-Carene, gamma-Terpinene, 2-Thujene, beta-Terpinene, and alpha-Phellandrene. CBN has high concentrations of Linalool, 3-Carene, beta-Myrcene, beta-Pinene, Terpinolene, and their sum, whereas ABN has high concentrations of 3-Carene, cisOcimene, 3-Thujene, beta-Myrcene, beta-Pinene, and Terpinolene. Lastly, Treatment ACN demonstrates high concentrations of 4(10)Thujene, b2Pinene, and beta-Phellandrene (Figure 8).
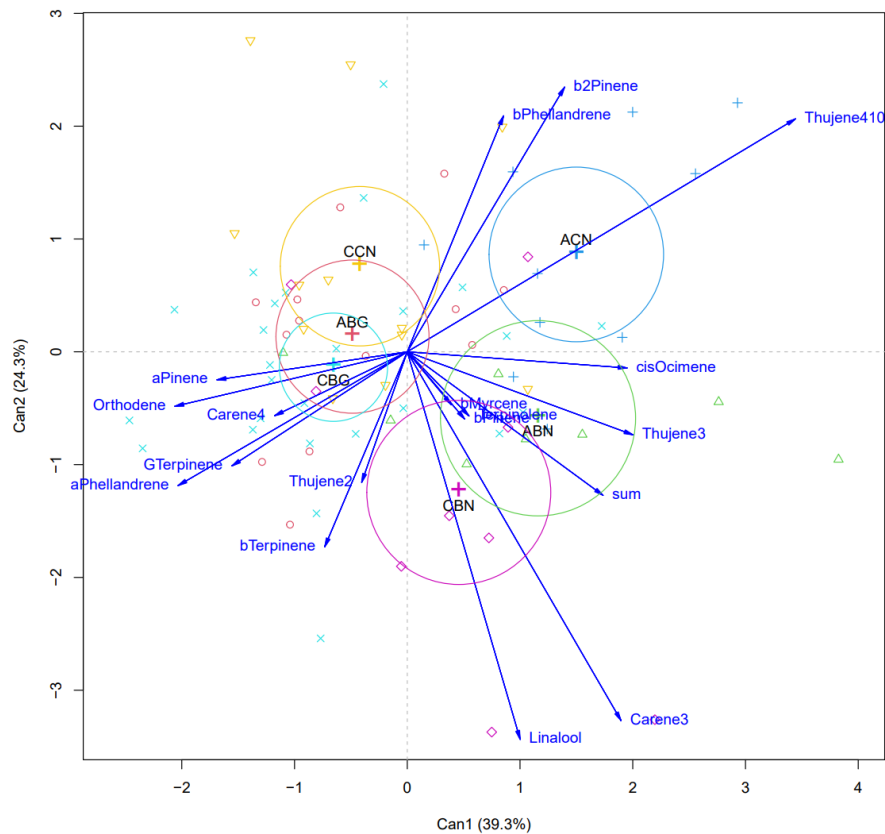

Fig 8. A figure that represents the compounds present within each treatment (ABG, ABN, ACN, CBG, CBN, and CCN) with the variables being represented by two different canonical functions (Can1 and Can2). The size of the circles reflects the variations within the treatments, while the position of the circles indicates the similarity in the components to each treatment type. The size of the arrows indicates the magnitude of the difference in compound concentration between the treatments, with longer arrows indicating a greater concentration in the corresponding treatment types, and shorter arrows indicating more similarity between the compounds present in each treatment type.

Discussion

The hypothesis of this study is supported by the data presented above, which indicates that there is not a difference in the total amount of terpenoids produced, but rather a difference in the type of terpenoids produced by the host trees to combat bark beetle infestations (Fig 1 and 8). Specifically, the findings suggest a significant difference in the concentration of

monoterpenes between the various site properties, indicating a potential variation in defense mechanisms among host trees at the different research sites.

Some of the differences may be attributed to the age of the trees, since we know that the trees in Alma are older and more susceptible to mortality after infestation, particularly when weakened by outside factors such as drought and disease (Gouvernement du Canada, 2020) (Fig 4). Additionally, the presence of galleries in the trees appears to play a role in their defense mechanisms, as indicated by positive correlations between the compound components of the galleries at both Alma and Cameron Pass (Fig 6 and 8). The differences observed between the beetle (baited) trees and control trees also provide support for our hypothesis, as they demonstrate that the trees are actively combatting infestations and producing distinct resin compounds compared to the control trees that are not directly infested (Fig 5). Additionally, the differences in resin compounds can be observed by the color of the resin, as the control trees only produced clear, crystallized, and yellow resin, while the beetle trees produced black resin (Fig 7). All of these findings indicate that the trees are generating unique compounds in response to infestation and actively working to mitigate the bark beetles.

These findings are consistent with a prior study conducted by Trowbridge et al. (2021), which concluded that drought could alter the allocation and concentration of defense chemicals in trees, further reinforcing our results. This is critical because it illustrates that the current and future increase in herbivory will continue to place pressure on trees in the Rocky Mountain region, but they have the capacity to adapt their resources to overcome it.

Conclusions

The findings suggest that trees can allocate resources to different defense mechanisms depending on the type of disturbance and insect infestation they are experiencing. However, further research is needed to confirm these findings, as only two research sites were studied and may not represent the entire Rocky Mountain region. Additionally, more research is required to investigate the specific compounds involved in the trees' defense mechanisms, which will provide a deeper understanding of how they mitigate beetle infestations.

Understanding these findings is crucial, as dead and dying trees caused by bark beetle infestations can increase the severity and extent of forest fires (Katz et al. 2017). Additionally, bark beetles have a significant impact on carbon sequestration because when trees die, they release stored carbon, thus contributing to climate change (Katz et al. 2017). Given these facts, it is crucial to acknowledge the implications of bark beetle infestations and take steps to mitigate their impact on forest ecosystems. This approach will not only help safeguard our ecosystems from the impacts of climate change but also ensure that we can continue to enjoy the Rocky Mountain region for many years to come.

References

Bark beetles. Forest Service National Website. (n.d.). Retrieved February 6, 2023, from

https://www.fs.usda.gov/detail/r3/forest-grasslandhealth/insects-diseases/?cid=STELPRD
B5228457

Brownell, J. (2020, August 12). *Aerial survey shows bark beetle damage still spreading in Colorado*. Colorado Public Radio. Retrieved February 20, 2023, from https://www.cpr.org/2019/01/17/aerial-survey-shows-bark-beetle-damage-still-spreading-in-colorado/

Franceschi, V. R., Krokene, P., Christiansen, E., & Krekling, T. (2005). Anatomical and chemical defenses of conifer bark against bark beetles and other pests. New Phytologist, 167(2), 353–376. https://doi.org/10.1111/j.1469-8137.2005.01436.x

Gouvernement du Canada. (2020, July 7). *Forest ecosystem impacts*. Natural Resources Canada. Retrieved March 29, 2023, from https://natural-resources.canada.ca/our-natural-resources/forests/wildland-fires-insects-disturbances/forest-pest-management/forest-ecosystem-impacts/13389

Harris J.L., R2 FHP staff, and States' Forest Health specialists 2018. 2017 Forest Health Conditions of the Rocky Mountain Region (R2). USDA Forest Service. https://www.fs.usda.gov/Internet/FSE_DOCUMENTS/fseprd586755.pdf

Hart, S. J., Veblen, T. T., Eisenhart, K. S., Jarvis, D., & Kulakowski, D. (2014). Drought induces spruce beetle (dendroctonus rufipennis) outbreaks across northwestern Colorado. Ecology, 95(4), 930–939. https://doi.org/10.1890/13-0230.1

Jenkins, M., Hebertson, E., & Munson, A. (2014). Spruce Beetle Biology, Ecology and Management in the Rocky Mountains: An Addendum to Spruce Beetle in the Rockies. Forests, 5(1), 21–71. https://doi.org/10.3390/f5010021

Katz , C., Wendle, J., Wendle, J., Pearce, F., Pearce, F., Langlois, J., & Langlois, J. (2017). *Small pests, big problems: The global spread of Bark Beetles*. Yale Environment 360. Retrieved April 4, 2023, from https://e360.yale.edu/features/small-pests-big-problems-the-global-spread-of-bark-beetles

Rodman, K. C., Andrus, R. A., Butkiewicz, C. L., Chapman, T. B., Gill, N. S., Harvey, B. J., Kulakowski, D., Tutland, N. J., Veblen, T. T., & Hart, S. J. (2021). Effects of Bark Beetle Outbreaks on Forest Landscape Pattern in the Southern Rocky Mountains, U.S.A. Remote Sensing, 13(6), 1089. https://doi.org/10.3390/rs13061089

Trowbridge, A. M., Adams, H. D., Collins, A., Dickman, L. T., Grossiord, C., Hofland, M., Malone, S., Weaver, D. K., Sevanto, S., Stoy, P. C., & McDowell, N. G. (2021). Hotter droughts alter resource allocation to chemical defenses in Piñon Pine. *Oecologia*, *197*(4), 921–938. https://doi.org/10.1007/s00442-021-05058-8

Acknowledgements

Appendix

Data Collection
- 80+ samples collected in Alma and Cameron Pass.
- Collected based on...
  - Color and consistency (Clear, brown, yellow, crystalized)
  - Baited trees (pheromone traps)
  - Non-baited trees (control)

Data Interpretation
- Determine a difference in the resin components based off the RStudio results.
- Look at the site specific properties between Alma and Cameron Pass and determine if this is a factor to bark beetle response.

Data Processing/Entry
- weighed, mixed, and diluted, then transferred the resin into new vials.
- Put vials into GC/MS machine to be analyzed
- Entered vial names/data into Masshunter.
- Entered resin weights into Excel.

Data Analysis
- Imported the results into MassHunter then converted it into a CVS file by Identifying the 10-C compounds.
- Built an aliquot of external standard monoterpenes.
- Injected aliquots into the GC/MS machine
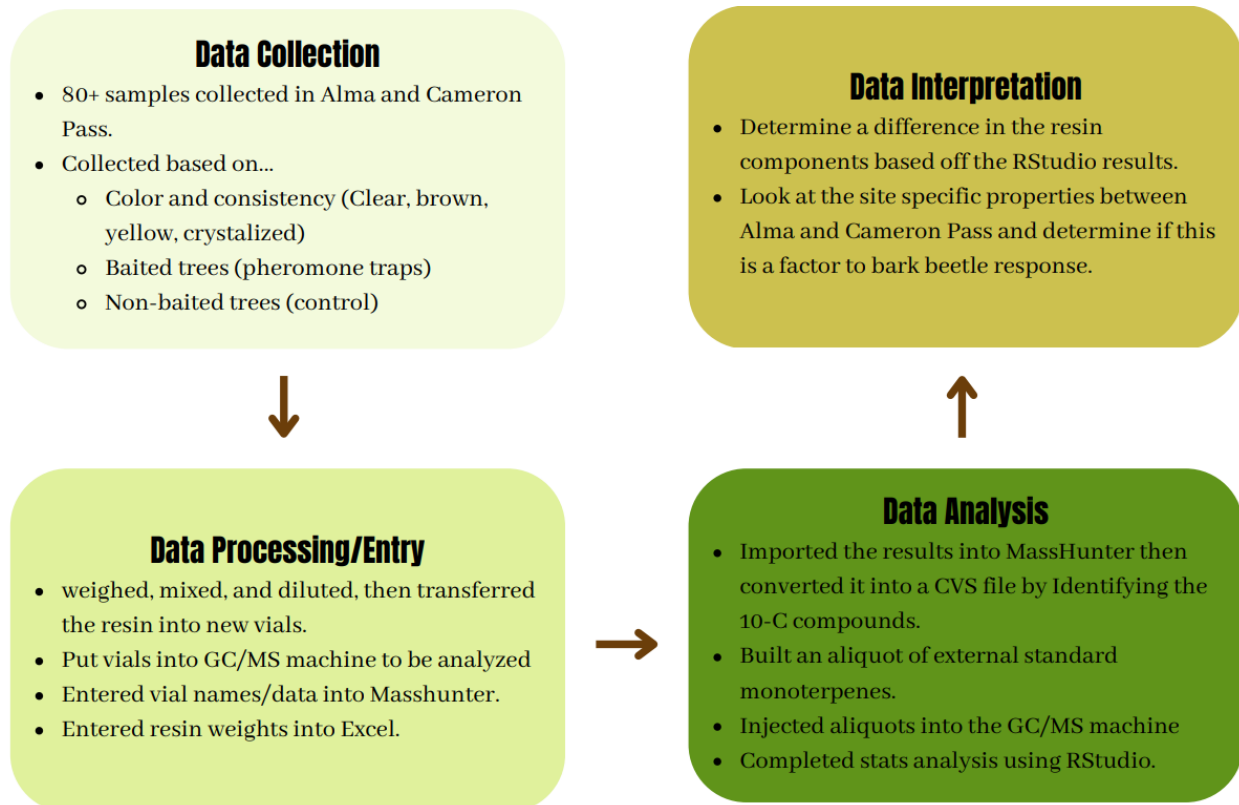- Completed stats analysis using RStudio.

Fig 9. Outline of the methodology used throughout this research project, Collection, processing, entry, analysis, and interpretation of the resin samples.