Allelopathic Effects of *Centaurea stoebe* on the Germination of Nearby Plant Species

Grace Justice

1. Introduction:

The spread of invasive plant species is one of the most serious threats to native ecosystems (Neubert and Parker 2004). The rapid spread of many species is a sign of highly destructive invasion in native ecosystems. Invasive plants use a wide range of strategies to establish themselves in new ecosystems, and each strategy can confer a different competitive advantage. One strategy commonly used by invasive species is allelopathy (Kalisz et al. 2021). Allelopathy is the ability of a plant to release chemical compounds that either directly or indirectly affect the surrounding plants in a negative way (Rice 2012). This release of allelopathic compounds better equips the plants to spread through a novel ecosystem. There are multiple different ways in which allelopathic compounds can have an effect on neighboring plants (Inderjit and Keating 1999). There can also be a difference in function and allelopathic composition based on where a compound originates on a single individual (Sodaeizadeh et al. 2009). In this study, they found that leaves had the highest level of toxicity, followed by stems and then roots. Allelopathic compounds can be classified as a wide array of chemical compounds within the broader subject of secondary metabolites. Secondary metabolites are a broad range of chemical compounds within plants that don't serve an obviously essential purpose for survival, but are still needed for the makeup and behaviors of the plant (Bennett and Wallsgrove 1994). There are many types of secondary metabolites, and they all have a wide range of functions, not just for defense and competition.

Allelopathic compounds make up a subset of secondary metabolites which are specialized for competition with nearby plants. Their classification under the broader subject of secondary metabolites doesn't mean they are a small subject, however. The subject of allelopathy refers to a wide range of known and unknown compounds with many different functions. There are many allelopathic compounds that have been the focus of projects with similar desired outcomes as this one, and some studies have successfully identified the exact compound responsible for an observed competitive mechanism. One example of a successful connection between compound and function is the compound juglone found in *Juglans nigra* (Black walnut) (Zubay et al. 2021, Rietveld 1983). The toxicity of Black Walnut is a trait that is well studied, and was better understood with the consideration of allelopathy as the reason for the toxicity. In this study, different species were tested to find the sensitivity of each species and the impact high levels of juglone had on germination.

Spotted knapweed (*Centaurea stoebe*) is not a new plant studied in allelopathy research. It is, however, a controversial subject that has contradicting findings and opinions connected to it (Duke et al. 2009). There have been studies done that have shown a positive correlation between

catechin in spotted knapweed and decreased plant growth, but these results have not been repeatable.

Plants can use secondary metabolites for a wide range of defensive processes (Teoh 2016). When a plant that contains certain secondary metabolites enters an ecosystem that it has never evolutionarily existed in, secondary metabolites can cause more damage than normal. When this happens, it can be described by the novel weapons hypothesis. The novel weapons hypothesis is the process where an introduced plant is able to dominate an area, because it contains compounds that the existing plants have never faced before, and are therefore defenseless at first (Blair et al. 2005).

The purpose of this study is to test the hypothesis that secondary metabolites from spotted knapweed inhibit germination rates of nearby plant species. A second hypothesis is also tested that there is a different level of germination inhibition for compounds exuded by roots and leaves of spotted knapweed. These are tested with treatments of different parts of spotted knapweed plants on easily germinated plants. The goal of this project is to investigate how spotted knapweed uses chemicals as a plant defense, releasing these chemicals in the soil to overtake nearby plant species. In the project, plant material was collected from about 10 individuals in a population for bulk extraction and separated by leaves and roots of each plant. Extract from each plant part and a control was pipetted into the soil where one of three species of lettuce seeds were placed in a greenhouse experiment. Germination success of the seeds was monitored over the span of 2 weeks, with data collected at day 10 and day 14.

2. Methods:

2.1 Study System

spotted knapweed (*Centaurea stoebe*) is a biennial or short lived perennial herbaceous plant native to Europe and Western Asia. (Knochel and Seastedt 2009). spotted knapweed has spread throughout North America very quickly, primarily affecting disturbed ecosystems (Innes 2021). This species is listed as highly invasive in the invasive plant species list by the Virginia Department of Conservation and Recreation (Heffernan 2024). spotted knapweed is commonly found in disturbed grasslands and fields. This species is a member of the Asteraceae family, and it has purple flowers that attract pollinators. Secondary metabolites can be released from spotted knapweed plants by means of root exudates, which are compounds being released directly from roots that spread through the ecosystem, or from leaf litter falling to the ground releasing these compounds through decomposition. Once these compounds are released, they may alter soil chemistry in a way meant to compete with surrounding plants.

The sample collection site was the VT Stream Lab, a field site owned by Virginia Tech for research. This collection site is located at (37.2122524, -80.4424668), which is right off Heth Farm Road in Blacksburg, VA. This site is a restoration site on Stoubles Creek that was

previously agricultural land, and is now a riparian buffer site (Barlow 2016). The site was chosen because of the previous disturbance to the area as well as the high volume of spotted knapweed dominating the area.

2.2 Plant Sample Collection

Plant material was collected from about 10 individuals of a population. The process of collection consisted of first choosing a plant. Plants in vegetative stage that were flowering and that had grown to an average height of 2 feet were chosen for collection. A small hand trowel was used to dig around the plant and carefully work through the soil in order to pull out as much root matter from each plant as possible. The roots were delicate, so root material wasn't successfully collected from all plants that leaf matter was collected from. For leaf matter, leaves were picked off the live stems by hand. At this time of year some plants had already started to die and some stems had dried up, so no material was collected from these plants. Plant samples were separated into roots and leaves for analysis of the material. These samples were held in plastic bags and stored in a -20o C freezer prior to extraction.

2.3 Bulk Extraction

Frozen samples were submerged in liquid nitrogen and ground to a powder using a mortar and pestle. Next, they were added to an equal volume ratio of methanol to plant material; 300 mL methanol was added to 56.31g of root material, and 500 mL methanol was added to 90.1g of leaf material. After being left on a stir plate overnight, the solid material was separated out of the extract using vacuum filtration. A Buchi R-300 Rotavapor was used to concentrate the solvents to equal volume (Nickols 2017). Separations were performed on a Shimadzu single quadrupole LC-MS system (LC-2050), which was equipped with an integrated liquid chromatography system (pumps, autosampler, column oven) and UV detector. The column was a Kinetex reversed-phase column (XB-C18, 2.6 um particles, 2.1 x 100 mm), maintained at 40 °C. Solvent A was water and Solvent B was methanol, both solvents containing 0.1% formic acid (v/v). The flow rate was 0.4 mL/min with the UV detector monitoring 278 nm and the mass spectrometer scanning between 100-1000 m/z. The gradient separation started at 5% Solvent B and at 2 minutes a linear gradient increased the concentration of B to 95% at 24 minutes where it was held for 2 minutes. The gradient was quickly ramped down to 5% B at 26.1 min and held there until 30 min, at which point the next injection occurred. The first minute of elution was put to waste to avoid salts on the mass spectrometer. Analyses were conducted in both positive and negative ion modes.

2.4 Seed Germination Experiment

Three species of lettuce seed were used for the germination experiment. These were Red Romaine, Green Ice, and Bibb. 3 trays were prepared with basic potting soil in each small well. 180 seeds were placed ⅛ inch into soil, and covered in a thin layer of vermiculite. Vermiculite was added to help seeds germinate and improve reliability of test (Reis et al. 2025). 100

microliters of each treatment (root, leaf, and control) were pipetted out into each replication. The control treatment consisted of only methanol to account for the methanol used in the extracts. Humidity hoods were placed on each tray, and the trays were kept in an empty fridge that had been turned off for this process. Supplemental lighting was applied for 6 hours each day in the fridge, and trays were watered and monitored every day for 2 weeks. Seeds were considered germinated when growth could be seen from the soil (Miransari and Smith 2014).

2.5 Data Analysis

Data were plotted in R to first visually assess seed germination in response to the treatment added. The effect of secondary metabolites within spotted knapweed plant material on germination was assessed using a linear regression model. The linear regression included plant species, treatment, and the interaction between treatment and plant species as predictor variables. The package ggplot2 was used to make a bar graph to visualize the data.

Results

Germination tests showed a significant effect of treatment (χ^2= 15.0500, P=0.0005394), a significant effect of species (χ^2= 43.5500, P=3.493e-10), and significant interactions between treatment and plant species (χ^2= 9.5001, P= 0.0497459). The control treated samples showed about 35% germination. Seeds treated with leaf extract only had about 14% germination. This means there was a 21 percent-point decrease in germination. Seeds treated with root extract didn't show any significant change compared to the control samples.

Using this code, the data was plotted with a bar graph showing germination proportion in response to the three treatments and three species. Red Romaine lettuce had the highest overall germination success. Within Red Romaine, the root treatment had the highest germination, which was slightly above control. The leaf treatment had lowest germination, and was substantially lower than the control. One replicate of Red Romaine with leaf treatment only had 20% successful germination, and the highest replicate had 75% successful germination, so more replication to ensure there was no experimental error could give different results. Green Ice lettuce had the smallest variability of germination across treatments. With Green Ice, the root treatment still had highest germination, and the control had the lowest germination success. The control was only slightly lower than the leaf treatment. Bibb lettuce had the highest variability across treatments, with control giving the highest germination success followed by root. With this species, the leaf treatment had much smaller germination success than the other two treatments.

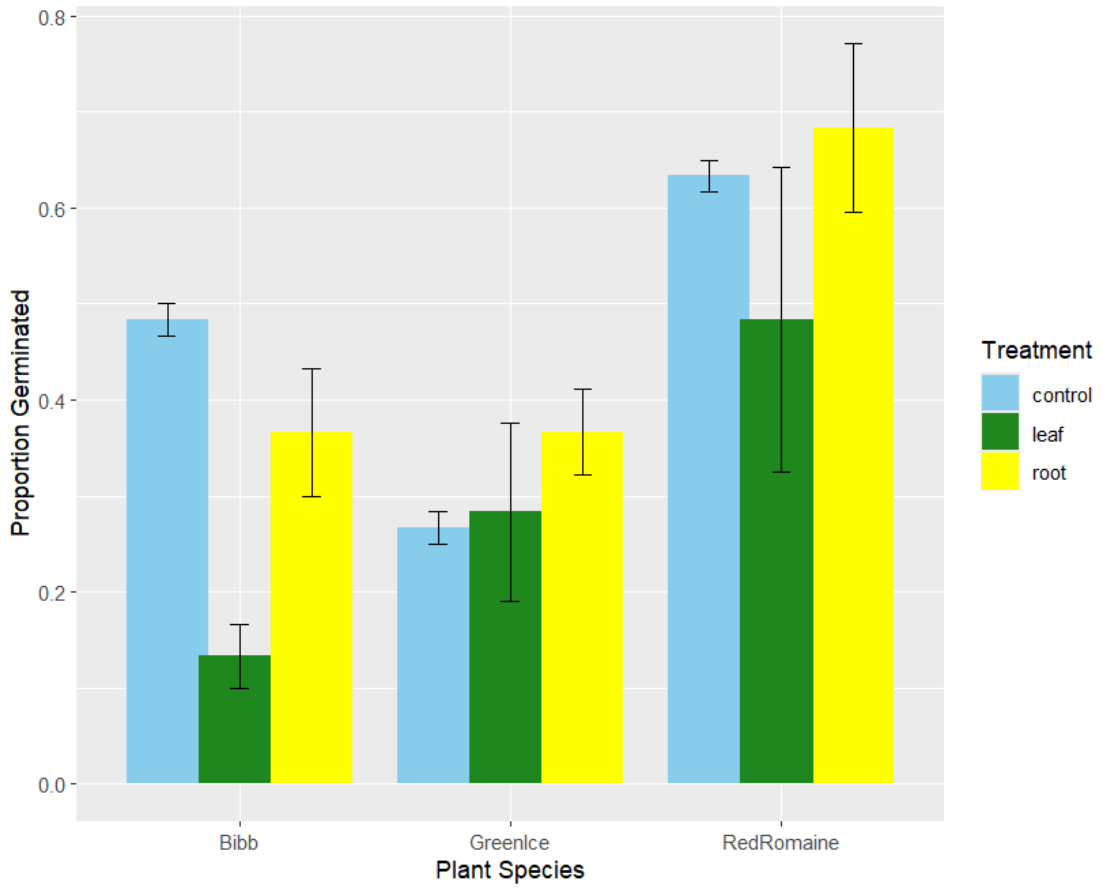

Figure 1: *Proportion of seed germination across 3 lettuce species when treated with root, leaf, and control extract.*

LCMS images showed the absorbance peaks of aliquots of the root and leaf extracts. Peaks that reached an absorbance over 20 were noted. Between 14 and 14.5 there is a peak reaching ~40 in the leaf extract, and only a small peak under 10 for root extract. At 16.5 minutes there is a peak to ~40 in the root extract, and there is no peak in leaf extract. At 17 minutes there is a peak to ~250 in the leaf extract and a peak to ~110 in the root extract. Between 23 and 23.5 there is a peak reaching ~140 in the root extract and no peak in leaf extract. This means that there are 2 main compounds present at high concentration.

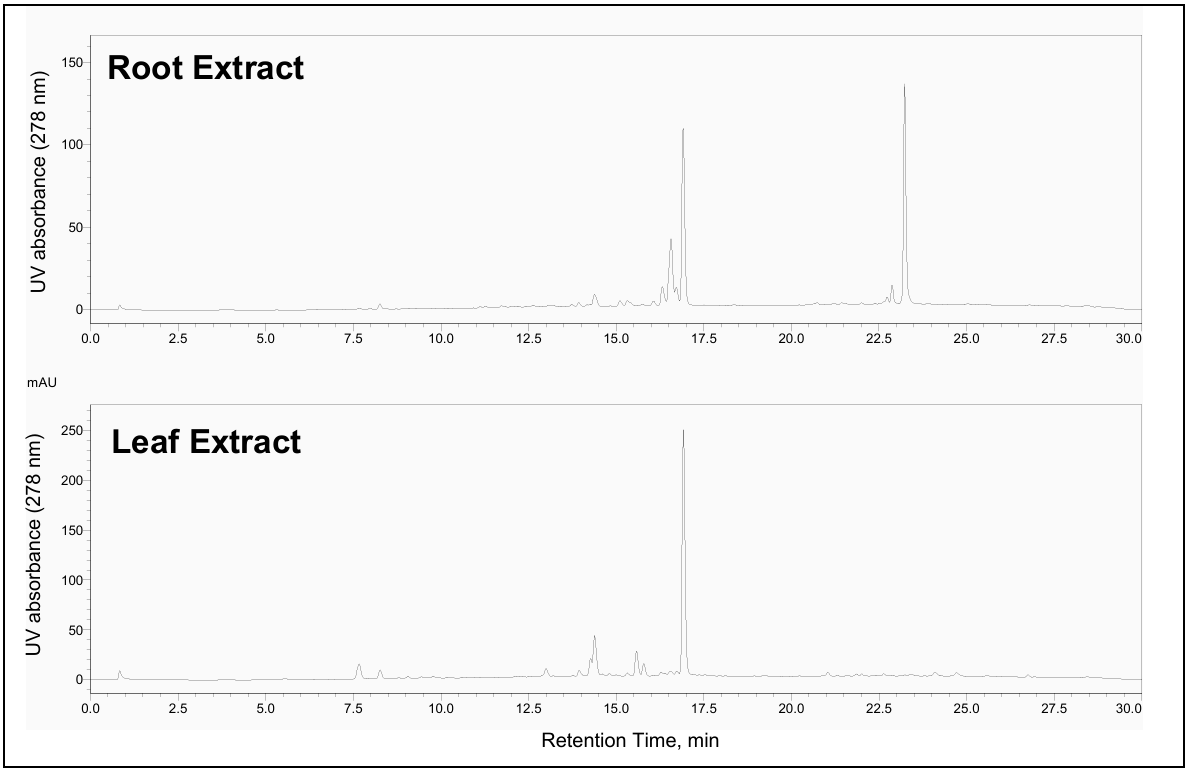

Figure 2: *Liquid Chromatography Mass Spectrometry (LCMS) images showing peaks of chemical compounds in leaf and root extract.*

The LCMS data show the same general idea that leaves and roots have different chemical composition. These results match up well with the germination inhibition data. The cluster of strong peaks between 15-18 minutes indicate that there are multiple allelochemicals present.

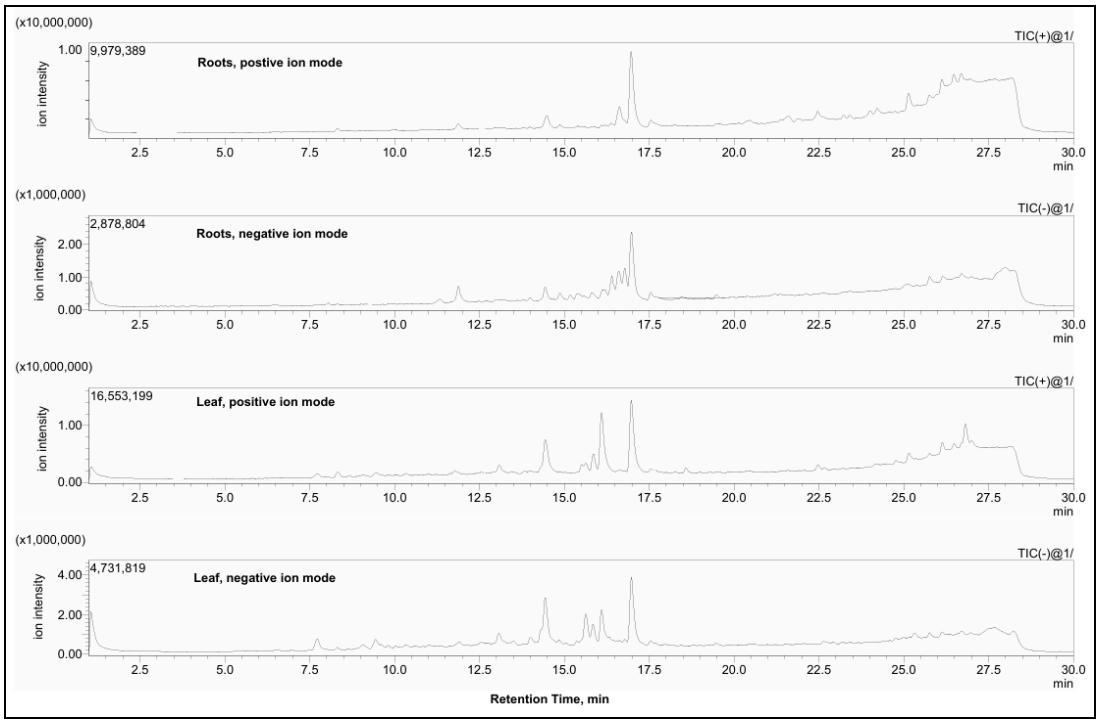

Figure 3: *LCMS images showing ion intensity of leaf and root extracts looked at in positive and negative ion modes*

Overall, leaves contain more abundant and more active compounds, and leaves have higher germination inhibition; roots contain less active compounds, show less germination inhibition, and even support germination.

Discussion

My findings that leaf litter inhibits seed germination and root litter slightly supports seed germination is notable and important for future understanding of plant interactions. Future research with these findings could be done with a similar approach, but could use native plants that are more accurate to this process in nature. There could also be a different result from this same process if completed in the Spring, since the spotted knapweed plants would be in a different stage of life at that time of the year.

The results from this experiment give some insight to what might be happening between spotted knapweed and its environment, but this is just a starting point. It can be seen from this project that during September-November at least, leaves contain some compound that directly inhibits seed germination. This does not mean that we can assume leaves always reduce germination, but this is one piece of the puzzle. This time of year is the time when a spotted knapweed plant is dying and the leaves may fall to the ground at a higher rate. It would make sense, then, that the plant would release compounds as a defense from these falling leaves as a way to take advantage of this step of their life cycle.

The data from the mass spectra and chromatograms together can be used to determine the identity of the unknown compounds. Even though these compounds are currently unknown, further investigation into the structure could allow for understanding of the elemental composition. The data showing multiple potential allelochemicals could mean there is not one major compound responsible for spotted knapweed dominance, but instead multiple compounds doing multiple different things creating favorable conditions for spotted knapweed.

Using all data collected, it can be concluded that leaf extract causes a decrease in seed germination and leaf extract also has higher peaks at 3 different locations. Root extract has a high peak that is not present in the leaf extract, and root treatment also caused slight increase in seed germination. Further investigation into what compounds these peaks represent would be able to prove if these findings are connected, and if there is a connection, replication could further demonstrate what exactly these compounds have the ability to do as a novel weapon.

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