DISSERTATION

# PLANT-MEDIATED INTERACTIONS BETWEEN HERBIVORY AND SOIL MICROBIAL COMMUNITIES IN BIOCONTROL PROGRAMS OF RUSSIAN KNAPWEED

Submitted by

Giovana Matos Franco

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Doctoral Committee:

Advisor: Paul Ode

Ian Pearse
Melinda Smith
Pankaj Trivedi

ABSTRACT

PLANT-MEDIATED INTERACTIONS BETWEEN HERBIVORY AND SOIL MICROBIAL COMMUNITIES IN BIOCONTROL PROGRAMS OF RUSSIAN KNAPWEED

Russian knapweed (*Rhaponticum repens*) is an invasive noxious weed present in the United States and two insect biocontrol agents have been released to assist with its management: the gall midge (*Jaapiella ivannikovi*) and the gall wasp (*Aulacidea acroptilonica*). Since their establishment, no concrete impacts of biocontrol agents onto Russian knapweed have been measured, neither their impacts on interactions between Russian knapweed and local microbiomes. To address this knowledge gap, observational and manipulative studies were conducted to investigate the effects of biocontrol agents on Russian knapweed fitness as well as its associated microbiomes. We found that Russian knapweed associates with a core microbiome that can assist with invasion in the introduced range as well as, in root samples collected from sites where gall wasp were present, lower microbiome diversity was observed, indicating potential negative effects on overall plant health. In garden conditions, water availability positively correlated with plant growth, negatively correlated with insect establishment, and shaped microbiomes in root associated tissues. Results of this dissertation highlights how introduction of biocontrol agents shifts pre-established relationships between invasive plants and microbiomes as well as how such relationships could be impacting the success of biocontrol programs.

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# CHAPTER 1: Introduction

## The good and the bad: how may interactions with other species shape the competitiveness of an invasive plant?

When the Hutchinsonian concept of niche was developed, competition between species was taken as the central factor determining the likelihood of coexistence (Hutchinson, 1957). However, in the 1990s, several authors emphasized the importance of positive interactions in niche modelling (Bertness and Callaway, 1994; Bruno et al., 2003; Maestre et al., 2009). Interactions between species can indeed pose negative effects through competition, parasitism, and predation. On the other hand, positive interactions can expand the realized niche through facilitation and habitat amelioration (Bruno et al., 2003; Maestre et al., 2009). Understanding facilitative interactions through the lens of invasive plants is especially important because it may help elucidate the roles of each group of organisms and predict how the management of invasive plants can be more efficient.

When considering the invasion "timeline" of an exotic plant (figure 1), different biotic and abiotic factors can affect the arrival of the primary inoculum (seed or vegetative propagules) and establishment, the ability to compete with other species, and the ability to withstand stressors (figure 1). Highly competitive plants are successful in each of these steps. However, which factors influence a plant's competitiveness and invasiveness will depend on the biotic and abiotic characteristics of an area (Dlugosch et al., 2015) and

potential interactions formed with local microbiomes.

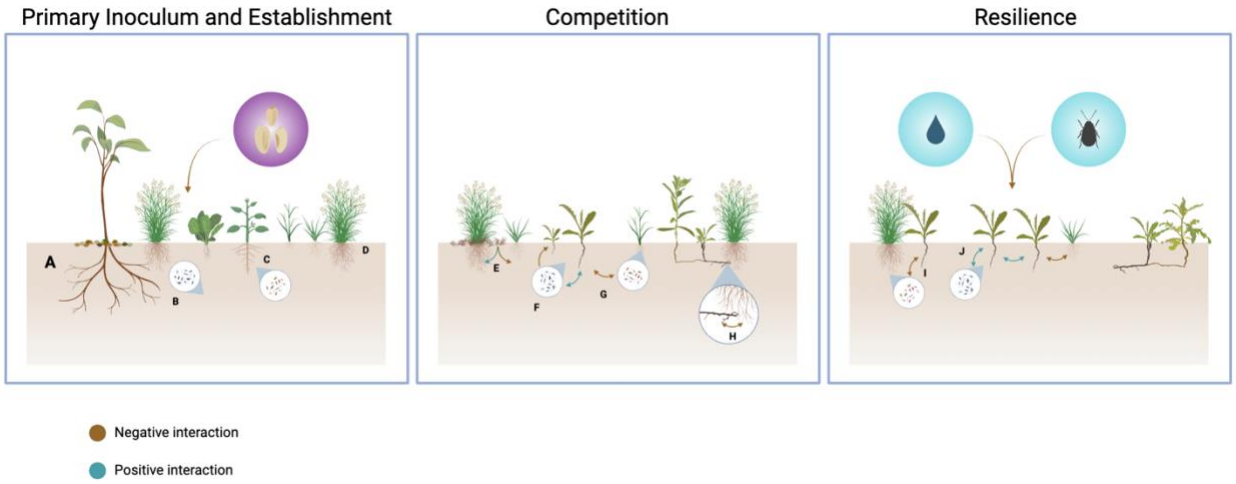



Figure 1: Timeline of invasion. Interactions considered in this review are marked by letters in each invasion phase. It is important to emphasize that all interactions and phases occur simultaneously, this is depicted in distinct steps to facilitate understanding. **Primary Inoculum and Establishment**: A – soil nutrient and chemicals released through litter decomposition. B – local microbiome and its potential negative, neutral, or positive interactions towards incoming inoculum (seeds or vegetative propagules) of exotic plants. C – plant-soil feedbacks in the invaded area affecting the establishment success of the exotic plant. D – 'biotic resistance' of local native plant communities to the establishment of exotic plants through selection of microbiomes, release of allelochemicals to the soil, and efficient use of resources. **Competition:** E – positive effects of native plant litter decomposition to conspecifics and negative effects on other plant species. F – negative plant-soil feedbacks on conspecifics and heterospecifics. G – negative effects of microbiomes selected by native plant species on the exotic species. H – negative interactions between root exudates of competing species. **Resilience:** I – negative soil feedback between microbiomes selected by different plant species while under biotic and abiotic stress. J – positive effects provided by microbiomes under biotic and abiotic stress.

## Competitiveness and other factors: single interactions

Biotic and abiotic factors can interact in complex ways within a community. To disentangle complex interactions and explain the details of each component, single interactions will be explained in this section (figure 2) and how organisms can further

interact will be covered in the next section (Competitiveness and other factors: multiple interactions).

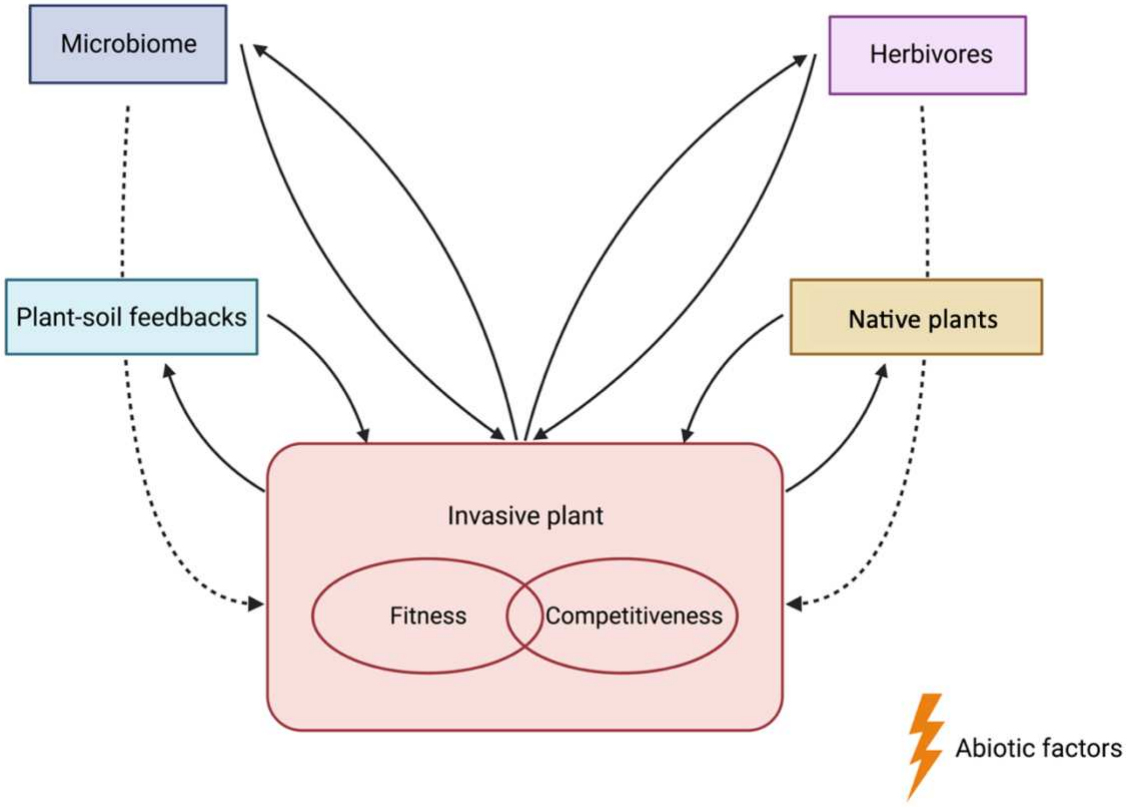



Figure 2: Simplified diagram showing interactions within a community invaded by an exotic plant. Solid lines represent direct interactions and dashed lines represent indirect interactions. Biotic effects in rectangles may impact and be impacted by invasive plants via competition. Each of the effects are further explained and exemplified in the following sections.

Plant-associated microbiomes and plant-soil feedbacks

Plant-associated microbiomes can lead to higher tolerance towards biotic and abiotic stresses, higher nutrient acquisition, and promotion of plant growth (Trivedi et al., 2020, Pieterse et al. 2014). Although microbiomes from introduced ranges are often

distinct from native ranges (Lu-Irving et al., 2019), invasive plants can form relationships with local microbiomes (Borda et al., 2022; Bunn et al., 2023; Yu et al., 2022). For instance, the ability of an invasive plant to form associations with arbuscular mycorrhizal fungi (AMF) can be much higher than native plants, possibly because of composition of root exudates (i.e. flavonoids in root exudates of woody species – Borda et al., 2022). However, how such interactions may contribute to the competitiveness of the invasive plant should be expanded. The invasive *Centaurea stoebe* forms associations with AMF, and in conditions of low water availability, it can enhance *C. stoebe*'s biomass and its competitive ability with the native *Bromus marginatus* compared to plants grown under conditions of high water availability (Bunn et al., 2023). Additionally, under conditions of high-water availability, native pathogenic microbes can preferentially attack *C. stoebe* and give *B. marginatus* a competitive advantage (Bunn et al., 2023). This finding helps understanding why *C. stoebe* commonly invades dryer areas and highlights the importance of water availability and soil microbiomes on invasion success.

The exudation of chemicals that are novel in the introduced range is thought to provide a competitive advantage to invasive plants (Callaway and Ridenour, 2004). Examples of direct interactions between chemicals released by invasive plants and microbiomes include attraction of beneficial microorganisms to invasive plants' rhizosphere (Yu et al., 2022) and disruption of pre-established relationships between native plants and beneficial microorganisms (Callaway and Aschehoug, 2000). Such changes in the local microbiome may provide negative, neutral, or positive feedbacks to invasive

plants (Inderjit & van der Putten, 2010), affecting the establishment and competitiveness of invasive plants.

Several species of invasive plants release chemicals to the soil (Jara-Servin et al., 2023) and plants that release such chemicals can attract more beneficial microorganisms than their competitors (Borda et al., 2022; Sun et al., 2021; Xu et al., 2012; Yu et al., 2022). *Ageratina adenophora* (crofton weed) invades several areas of Eastern Asia where it releases root exudates that have direct effects on the microbial community. Besides selecting for nitrogen fixing bacteria in the genus *Bacillus* (Y. Sun et al., 2021), root exudates from crofton weed select for bacteria in other genera such as *Pseudomonas*, *Rhizobium*, and *Duganella*, which are usually associated with phosphorus solubilization, as well as the accumulation of the plant-growth promoting hormone IAA (Fang et al., 2019). The increase in abundance and activity of non-symbiotic nitrogen-fixing bacteria increased the availability of nitrogen in invaded areas (Du et al., 2022), leading to higher competitiveness of *A. adenophora* against the native plant *Rabdosia amethystoides*. Areas that crofton weed invades typically have low fertility and nitrogen concentrations; therefore, interactions with microbes can provide habitat amelioration. Similar patterns are observed with the invasive buffelgrass (*Pennisetum ciliare*) in the Sonoran Desert of the southwestern US. Buffelgrass invades areas with low nutrient and water availability, and promotion of a core microbiome that can assist with nutrient availability and plant growth promoting bacteria allows this invasive plant to succeed in otherwise unfavorable habitats (Jara-Servin et al., 2023).

Chemicals released by invasive plants can also disrupt pre-established relationships between native plants and their microbiomes. For instance, garlic mustard (*Alliara petiolata* – Brassicaceae) is an invasive plant that releases root exudates (the glucosinolate sinigrin and its metabolite allyl isothiocyanate (AITC)) that disrupt associations between native plants and their AMF (Cantor et al., 2011). Sinigrin/AITC, even when released in low concentrations, can have a negative effect on naïve soil communities leading to a reduction of fungi in the soil (Cantor et al., 2011). Through the disruption of relationships between native plants and soil microbiomes, garlic mustard can have enhanced competitiveness over native species (Barto et al., 2011; Cantor et al., 2011; Lankau et al., 2014; McCary and Wise, 2019; Roberts and Anderson, 2001; Roche et al., 2021). However, removal of *A. petiolata* from field sites does not change the total number of spores of AMF (Burke et al., 2019, 2011), suggesting that there is selection for AMF that are tolerant to exudates released by garlic mustard (Barto et al., 2011). These studies show that the introduction of novel chemicals to an area may help during the establishment phase of an invasive plant (Figure 2 – "Competition"); however, communities may adapt to these chemicals and other factors need to be considered for understanding the persistent competitiveness of an invasive species (Figure 2 – "Resilience"). In the case of the garlic mustard system, another factor that may enhance plant competitiveness is changes in C:N ratios in the soil resulting from litter decomposition (Burke et al., 2019, 2011), where the presence of garlic mustard decreased C:N ratio and might have decreased C storage in roots (Burke et al., 2019).

Plant-soil feedbacks may lead to negative effects for native (Duchesneau et al., 2021) or invasive plants (Luo et al., 2021; Waller et al., 2016). Invasive plants may release root exudates that select for pathogens of native plants. This process highlights the importance of chemicals released by invasive plants during the establishment and competition phases. Meanwhile, native plants may also select for microbiomes that will have negative effects on invasive plants, reducing their fitness and competitiveness (Luo et al., 2021; Waller et al., 2016). For instance, the presence of AMF enhanced the competitiveness of native species, decreasing the fitness of the invasive *Plantago virginica* (Luo et al., 2021).

Herbivore-mediated interactions between invasive and native plants

Interactions between plants and herbivores impact the competitiveness of invasive plants in relation to other native plant species. Absence of herbivores during the establishment phase reduces biotic stress upon invasive plants and increases establishment success, as discussed by the enemy release hypothesis (Keane and Crawley, 2002a). As a result of reduced herbivory, there is a reduction of resource allocation to induced defense mechanisms, which may select for less well-defended plants leading to increased investment in reproductive effort (Blossey & Notzold, 1995; Maron et al., 2004), resulting in increased competitiveness against other plants during the competition phase (Figure 2). However, as time since initial invasion increases, native herbivores may start consuming the invasive plant (Crous et al., 2017; Verhoeven et al., 2009). Nevertheless, generalist herbivores in the introduced range are hypothesized to

prefer native over invasive plants (Keane and Crawley, 2002a), providing an opportunity for invasive plants to have higher competitiveness. Therefore, disentangling mechanisms of herbivore-mediated interactions are crucial for understanding the competitiveness of invasive plants.

Native plant communities shape herbivore composition and, depending on the phylogenetic distance between invasive and native plants, native specialist herbivores may consume invasive plants (Crous et al., 2017; Eckberg et al., 2014). For instance, when the invasive *Cirsium vulgare* (bull thistle) expanded its range to non-invaded areas containing the native *C. altissimum* (tall thistle), native herbivores fed on the invasive thistle, leading to lower population growth rates compared to areas where the insect was not present (Eckberg et al., 2014).

Plants can allocate resources towards anti-herbivore defenses that could be otherwise used for plant growth (Herms & Mattson, 1992); box 1); yet, how this trade-off will affect invasive plants is variable. Invasive plants often escape from their specialist herbivores from their home range and novel chemical defenses may not be efficiently metabolized by generalist herbivores in the introduced range (Sedio et al., 2020). Together, mismatches between invasive plant and native herbivores may provide resistance to native herbivores in the introduced range (Verhoeven et al., 2009). Meanwhile, new associations between native herbivores and invasive plants may be advantageous to herbivores in cases where their feeding mechanisms are not perceived by the invasive plant. This can lead to biotic resistance and establishment failure by exotic plants (Verhoeven et al., 2009). However, native herbivores need to recognize invasive plants as potential hosts (Pearse et

al., 2013), which may be challenging if the appropriate morphological and chemical cues are absent. Initial contact by native herbivores may be due to similarity with phylogenetically related species (Crous et al., 2017) or accidental consumption (Santamaría et al., 2022). Implications of the integration of invasive plants into native herbivore diets include the potential reduction of invasive plant competitiveness and cascading effects on higher trophic levels (Fortuna et al., 2012).

*Box 1: Herbivore effects on plant resistance and competitiveness*.

*Plants* resistance can be categorized as either*: constitutive or induced (Verhoeven et al., 2009). Constitutive resistance refers to mechanisms that are expressed in the plant at levels independent of attack by herbivores; while induced resistance are traits whose levels of expression can be triggered by herbivory. Both types of resistance are costly for plants, since the synthesis of secondary chemicals or enhancing structural barriers require reallocating resources* (Kruger et al., 2020; Paul-Victor et al., 2010). *The induction of defense mechanisms can use some of same precursors from basic plant functions, such as growth and cell differentiation, and since the amount of resources available for a plant are finite, plants experience trade-offs between growth and defense (*Herms & Mattson, 1992).

Competitiveness traits are particularly interesting in the context of biological control programs, since the introduction of specialist herbivores from the native range of the plant may stress the invasive plant and, potentially, reduce its competitiveness and fitness

relative to native plants. Life histories of biological control agents and how they impact their host species shape the success of biological control programs. For instance, the salvinia weevil, *Cyrtobagous singularis*, introduced to manage the invasive aquatic fern giant salvinia, *Salvinia molesta*, feeds on the rhizome, roots, and leaves of their host, which reduces reproduction, photosynthesis, and ability to float (Room et al., 1981). Without the ability to float the invasive giant salvinia cannot survive and compete with other plants, making this an excellent example of highly successful biological control program. Other biological control agents alter the physiology of their host plants, reducing competitiveness of species; for instance, the gall midge, *Jaapiella ivannikovii*, has been introduced to the USA to manage Russian knapweed, *Rhaponticum repens* (Djamankulova et al., 2008). The galls are formed by a series of physiological changes induced by the feeding of the insects resulting in stunted plant growth and reduced flowering. Although these changes negatively impact the fitness of Russian knapweed, the impact on its competitiveness with native plants remains uncertain.

Plant-plant interactions

Availability of resources and competitive ability of local plant community (native and invasive plants) shape plant-plant interactions and establishment of new invasive plants ("Primary Inoculum and Establishment" in Figure 1). Abiotic resources such as light, water, and nutrients limit the establishment of plants and dictate the nature of plant-plant interactions (Dlugosch et al., 2015; Wright et al., 2014). The ability to acquire such

resources, directly or through interactions with other taxa, while coping with competition are characteristics of high competitiveness of invasive plants.

Allelochemicals released by root exudation and decomposition of biomass can enhance competitiveness of invasive plants (Callaway and Aschehoug, 2000; Dayan et al., 2010; Loydi et al., 2015). Root exudation of allelochemicals is particularly relevant in agricultural settings, since competition between invasive plants and crops is cited as one of the biggest contributing factors for yield loss (Ma et al., 2023; Pimentel et al., 2005). Plant litter composition can change the nutrient balance of an area (Burke et al., 2011, 2019), or release chemicals with growth deterrent properties (Dai et al., 2016). Litter leachates of invasive plants delayed seed germination, reduced germination percentage and root growth of native plants (Loydi et al., 2015). These results show that allelochemicals present in invasive plants may increase their competitiveness by opening a niche in the introduced range and providing time for their offspring to germinate and establish.

Plants can also dominate areas through rapid growth, higher biomass, and larger number of seeds (Ni et al., 2020). In such scenarios, higher fitness is associated with higher competitiveness. Earlier germination can lead to higher fitness because plants will be in later developmental stages and be stronger competitors when other plant species germinate (Stevens and Fehmi, 2011). In contrast, some species germinate later, allowing them to take advantage of resources made available by the growth of earlier plants (Gioria et al., 2018).

### Competitiveness and other factors: multiple interactions

Even though the topics in the current paper were approached separately, plant-soil feedbacks and microbiomes, plant-plant interactions, and insect herbivores conjointly affect invasive plants during the establishment, competition, and resilience phases. For instance, microbiomes have been reported to enhance herbivore-resistance traits in invasive plants, shifting herbivore preference away from invasive plants (Kalske et al., 2022; Kempel et al., 2013; Luo et al., 2021); and reduction of competitiveness of invasive plants was correlated microbiome mediated interactions, leading to higher success of native plants (Li et al., 2019, 2017). Additional examples of multiple interactions are covered in the next sections.

### Microbiome-mediated effects between herbivores and plants

Studies on plant-mediated interactions between microbes and herbivores are commonly conducted by investigating single interactions with specific taxa and how it impacts plant responses to herbivory or herbivore preferences. For instance, when barley plants (*Hordeum vulgare*) were inoculated with beneficial bacterium *Acidovorax radicis* N35e, population sizes of the aphid *Sitobion avenae* were reduced (Sanchez-Mahecha et al., 2024). Furthermore, in other cultivated crop systems, plants inoculated with a blend of microbes had reduced damage by herbivores (Komatsu et al., 2023; Simmons-Elliott et al., 2023). This finding suggests the importance of understanding effects of microbiomes towards plant-insect interactions instead of single interactions.

Pineda et al., (2010) first highlighted the potential for plant-mediated effects between root-associated microbes and herbivores. Since then, several studies have confirmed their expectations that significant interactions are present. Fungal microbiomes conditioned by different plant groups (forbs, grass, or legumes) shifted the number of aphids (*Aphis jacobaeae*) per ragwort plant (*Jacobaea vulgaris*) (Kos et al., 2015). Such shifts may have occurred due to changes in nutrient acquisition and plant responses to herbivory. Microbial mutualists can also trigger induced systemic resistance (ISR) in plants but not cause pathogenicity (Pieterse et al., 2014). Once ISR is triggered, plants defenses are primed and have increased responses to subsequent attacks (Conrath et al., 2006). Therefore, if a plant is attacked by pathogens or herbivores, it can have faster and/or stronger responses against the attacker. For instance, ISR induced by *Bacillus subtillis* can slow the growth rate of the silverleaf whitefly *Bemisia tabaci* in tomato plants (*Solanum lycopersicum*, Valenzuela-Soto et al., 2010) and induce resistance in wheat (*Triticum aestivum*) towards the bird cherry – oat aphid *Rhopalosiphum padi* (Rumyantsev et al., 2023).

Herbivory can also have plant-mediated impacts on the rhizosphere microbiome, since changes in plant resource allocation occur during insect feeding. In normal conditions, nearly half of assimilated carbon goes towards root exudation (Bais et al., 2006). These exudates recruit and select for desired microorganisms and reduce abundance of potential pathogens (Pieterse et al., 2014, Bais et al., 2006). When plants are fed upon by insect herbivores, the identity of compounds stored in roots changes, increasing associations between roots and arbuscular mycorrhizal fungi (Xing et al., 2024).

Additionally, caterpillar saliva has been shown to play an important role in changes in root metabolites (Xing et al., 2024). Products of secondary metabolism can also change rhizosphere microbiome composition, and vice-versa. The rhizosphere microbiome can affect and be affected by the abundance of glucosinolate compounds in rhizosphere samples of *Brassica rapa*; however, no microbiome effects on herbivory were observed (DeWolf et al., 2023). Additionally, microbiome composition and colonization of plant tissues can depend on induced responses to external factors. One common response to herbivory is the accumulation of salicylic acid (SA), and in *Arabidopsis* mutants with enhanced and depleted levels of SA in plant tissues have been shown to negatively impact root colonization by bacteria (Lebeis et al., 2015), which can lead to negative effects on plants due to reduction of positive associations.

Most literature on plant-mediated impacts between microbiomes and herbivores has focused on model plants (e.g., *Arabidopsis thaliana*) and agricultural crops (Backer et al., 2018). However, such interactions also need to be further investigated on invasive plants, since plant-microbiome interactions have been shown to impact invasive plants' competitiveness and could potentially impact the efficacy of invasive species management and biological control programs.

## Conclusion

Interactions between introduced biological control agents and the communities into which they are introduced are important but understudied (Blossey et al., 2018). Here, I

also stress the need to test the effects of biological control agents on their new communities. I suggest thinking critically how biological control agents impact not only plant fitness, but also plant competitiveness, and how their introduction may shift pre-established interactions (figure 2).

Predicting relationships that have never previously existed can be complicated; however, understanding individual interactions can be useful to predict multiple interactions if direct measurement is not possible (Boardman et al., 2022). Modeling and other mathematical techniques have long been used in ecology to predict interactions that cannot be directly measured. Such methods have been used to estimate the spread of invasive species and establishment of biological control agents (Mukherjee et al., 2021; Muskett et al., 2020). I propose adding impacts of biological control agents towards competitiveness of invasive plants and estimating long term effects.

The success of invasive plants depends on a variety of interactions. Resource availability in new range defines which niches can be occupied by invasive plants. Biotic Interactions with components of the novel environment, such as the microbiome, herbivores, and the native plant community can expand (Bruno et al., 2003; Dukes et al., 2019; Keane and Crawley, 2002a) or reduce (Luo et al., 2021; Waller et al., 2016) the niche of the invasive species through positive and negative interactions, respectively. Additionally, the competitiveness of invasive plants can also impact on the area's invasibility via exudation of novel chemicals and higher fitness (Callaway & Ridenour, 2004). Learning how an invasive plant fits into new communities helps understand how to manage them. Biological control programs can be a great approach for invasive plant

management; however, their success rate and non-target effects are variable. Consideration of the competitiveness factors of invasive plants may enhance the success of biological control programs and reduce non-target effects.

### The Russian knapweed (*Rhaponticum repens*) study system

Russian knapweed is an invasive noxious weed commonly found in western US and Canada (EddMapS 2024). Seeds were likely introduced in the early 1900s through alfalfa seed importation from Turkestan and elsewhere in central Asia (Maddox et al., 1985; Watson, 1980; Zouhar 2001). As a successful invader, Russian knapweed can tolerate a wide variety of water availability and nutrient scarcity conditions (Watson, 1980). While overall seed germination is low, it is the main mechanism of long-distance dispersal (Watson, 1980). Once it establishes in a new location, Russian knapweed forms dense patches through vegetative growth that can suppress other plant species (Watson, 1980). In addition to competing with crops and leading to yield loss, Russian knapweed is toxic to horses, causing the untreatable nigropallidal encephalomalacia disorder (chewing disease) where movement of lips is impaired, continuous chewing is observed, and animals loose ability to drink or feed, leading to death by starvation or dehydration if not euthanized (Elliott and Mccowan, 2012).

Current management strategies rely heavily on pesticide applications; however, in natural areas such as wildlife refuges, or areas with low financial input (such as roadsides and railroads) application of herbicides is unfeasible. Mowing and sheep grazing can reduce Russian knapweed growth, but these approaches need to be consistently applied,

which is not practical in large areas. To help manage Russian knapweed, two biological control agents were selected in the native range and introduced in the US (classical biological control): a gall midge (*Jaapiella ivannikovi*) and a gall wasp (*Aulacidea acroptilonica*). Both insects stress plants through oviposition in/on plant tissues, which through oviposition fluids or feeding by immatures will lead to formation of galls, providing feeding sites for the immatures. Gall midges have been reported to have up to four generations per year (Sonya Daly *personal communication;* Djamankulova et al., 2008) while wasps are univoltine. Both biological control agents are frequently released together, but it is not clear whether the presence of one impacts the establishment of the other. Therefore, one of the goals of my dissertation is to investigate how both gall-forming insects interact. Additionally, it is not known if insects negatively impact Russian knapweed in the invaded range. It has been reported that in the native range, both insects successfully reduced the fitness of Russian knapweed (Djamankulova et al., 2008); however, impacts should be further investigated in the introduced range, where they have formed novel interactions and adapted to local biotic and abiotic conditions.

# CHAPTER 2: Connecting the dots: How microbiomes can help us understand plant-insect interactions

## Introduction

What conditions allow some exotic plants to become dominant within the communities they invade has been the subject of numerous hypotheses and countless studies over the past several decades. Prominent among these has been the enemy release hypothesis (ERH, Keane & Crawley, 2002), which posits that many exotic plants (and other organisms) become invasive in novel habitats because they have left their own natural enemies behind. Indeed, this has served as the justification for introduction (classical) biological control programs where natural enemies of the invasive plant are imported from the plant's region of origin to re-establish herbivore regulation of the plant population (Heimpel & Mills, 2017). Furthermore, invasive plants that have escaped control by herbivores are generally thought to be under relaxed selection pressure to invest in anti-herbivore defenses (Keane & Crawley, 2002). Such plants may be able to divert resources to invest in growth and reproduction, which would potentially explain their evolution of increased competitive abilities (EICA, Blossey & Notzold, 1995).

Another hypothesis to explain the success of exotic plants has been the novel weapons hypothesis in which invasive plants possess unique chemistries that enable them to suppress the growth of native plants (Callaway and Ridenour, 2004). Original iterations of this hypothesis focused on the direct effects of root exudates of the exotic plant that function as allelochemicals against native plants. More recent versions have begun to

explore how root exudates can either disrupt the beneficial associations between native plants and arbuscular mycorrhizal fungi (AMF, Cantor et al., 2011) or promote the formation of beneficial associations between the roots of the invasive plant and soil microbiomes that can increase the competitiveness of the invasive plant (Bunn et al., 2023; Trognitz et al., 2016; Yu et al., 2022). Plants can recruit beneficial microbes in the rhizosphere through the release of low and high molecular weight compounds (Bais et al., 2006). Such microorganisms colonize the soil layer next to roots and can ameliorate biotic (Pineda et al., 2010) and abiotic stresses (Pineda et al., 2013), which can enhance the competitiveness of invasive plants. For instance, AMF can assist the plant with nitrogen and phosphorus solubilization in exchange for carbon (Tedersoo et al., 2020). Additionally, endophytic colonization by AMF can prime plant response mechanisms and enhance and/or expedite subsequent responses to biotic stressors (Conrath et al., 2006). Other microorganisms, such as *Pseudomonas* spp., *Bacillus* spp., *Streptomyces* spp., and *Trichoderma* spp., can prime plant responses to herbivory (Pieterse et al., 2014). These microbes can trigger induced systemic resistance (ISR) and prime defense mechanisms. The interactions of microbiomes and plants have been widely studied in cultivated and model systems in recent years; however, how such interactions alter the competitiveness of invasive plants still needs to be further investigated.

In addition to invading and establishing in new areas, invasive plants must also adjust to damage by herbivores when classical biological control agents are introduced. Introduction of herbivores can change plant-associated microbiomes (Kong et al., 2016; Malacrinò et al., 2021) and microbiome composition may impact herbivory levels (Howard

et al., 2020). Yet, studies of how plant – microbial associations influence plant – insect herbivore interactions and their role as potential explanations for the success and/or failure of biological control attempts are still poorly known. These effects have received substantial recent attention in systems where plant pathogens are used as biocontrol agents. For example, the endophytic community associated with invasive plants can compromise the success of biological control programs using rust fungi (Currie et al., 2020; Kentjens et al., 2024); however, effects of plant-associated microbiomes on insect herbivores as biological control agents are largely unexplored. Understanding how invasive plant – biological control agents interactions shape microbiome compositions will help us understand the success of biological control programs.

As plants establish in new locations, they select for a core microbiome that can help with response to biotic and abiotic stressors (Toju et al., 2018). Species in core microbiome are the ones that shape microbial communities by being highly connected with other species and being consistently present across similar growing environments (Shade and Handelsman, 2012). In plant-associated microbiomes, core microbiomes can provide stress amelioration and promote growth promotion. For instance, the core root-associated microbiome of invasive buffelgrass (*Pennisetum ciliare*) contained bacterial taxa associated with nitrogen availability to plants, plant growth promotion, and plant protection (Jara-Servin et al., 2023). The association with such taxa can enhance the ability of buffelgrass to invade new arid and nutrient-poor habitats (Jara-Servin et al., 2023). Core microbiome selection in buffelgrass occurs through release of allelochemicals (Jara-Servin et al., 2023), a frequently observed trait among invasive plants (Bais et al., 2006).

Several species of knapweeds in the genus *Centaurea* (Asteraceae) have established populations in North America. The allelopathic effects of root exudates have been suggested to enhance the competitiveness of at least three species (Bais et al., 2006; Callaway and Aschehoug, 2000; Duke et al., 2009). Differences in native vs. exotic soils have been suggested to be responsible for the invasiveness of at least one species of *Centaurea*. The highly invasive yellow star thistle *C. solstitialis* experiences increased growth rates when grown in soils from the invaded range compared to soils from its native range in Spain (Montesinos and Callaway, 2020), arguably because the soil microbial community in the plant's native range suppresses plant growth and this microbial community is absent in North America. Additionally, interactions between microbiomes and water availability can affect invasion success. For instance, application of live microbes from invaded sites and high watering levels decreased the biomass of the invasive *C. stoebe* relative to that of its native competitor *Bromus marginatus*, possibly due to accumulation of pathogens targeting *C. stoebe* and the positive AMF associations benefitting *B. marginatus* (Bunn et al., 2023). This possibly explains why *C. stoebe* is commonly found in drier locations than the wet conditions used in this study. Two additional *Centaurea* species that are not considered to be invasive exhibited similar growth performances in soils from their native (Spain) and introduced (California) ranges (Montesinos and Callaway, 2020). Collectively, these studies suggest that differences in soil microbial communities may be responsible for whether a given species becomes invasive.

Here we examine the relationship between soil microbiomes, insect herbivory, and the invasive Russian knapweed *Rhaponticum* (formerly *Centaurea*) *repens*. Russian knapweed has been present in the United States since the early 1900s (Zouhar, 2001), and two biological control agents have been introduced in the 2010s to help manage this invasive plant: the tip gall wasp (*Aulacidea acroptilonica* – Cynipidae) and the stem gall midge (*Jaapiella ivannikovi* – Cecidomyiidae). Females of both insects lay eggs solely on Russian knapweed and induce formation of galls that provide specialized feeding sites for the development of immatures. The presence of galls reduces growth and seed production of Russian knapweed (Djamankulova et al., 2008). Since development of galls and immatures are dependent on the host plant, this system allows us to assess the impacts of microbiomes on plant responses to herbivory and how microbiome composition influences the success of biological control programs through plant-mediated interactions.

When established, Russian knapweed forms dense patches of mostly clonal growth (Gaskin and Littlefield, 2017) that persist in the same location for several years. Because of its growing patterns, we hypothesize that Russian knapweed can select for microbiome communities that also persist for several generations. These communities can have direct impacts on plant growth, as observed in other invasive plant systems (Jara-Servin et al., 2023; Trognitz et al., 2016). However, we do not know (1) if there is a core microbiome associated with Russian knapweed across different sites; (2) the predicted functions associated with core microbiomes; and (3) how biological control agents alter microbiome – Russian knapweed associations. To assess the relationships between soil microbiomes, biological control agents, and Russian knapweed, we analyzed the microbiome

composition of rhizosphere, root, and leaf tissue samples from sites with and without the presence of biological control agents. We initially hypothesized that sites would have different microbiomes due to site-specific abiotic factors; however, sites still may have common core microbiomes associated with Russian knapweed. Additionally, we hypothesized that microbiome composition would reflect the presence of biological control agents; more specifically, microbial taxa associated with plant responses to herbivory would be common across sites where biological control agents are present.

## Methods

Six Russian knapweed field sites (two where *J. ivannikovi* were released, two where *A. acroptilonica* were released, and two where no biological control agents were released) were selected throughout Colorado (Figure 3). Within each site, soil samples directly in contact with roots (rhizosphere), root samples (both, the inner portion of roots – endosphere – and the outer layer immediately outside of roots – phyllosphere), and aboveground tissue (buds) were collected from each of six plants (total of 18 samples per site), snap frozen in liquid nitrogen and transported in a Dewar flask to the lab, where they were kept in a -80˚C freezer until DNA extraction. Sample and library preparation followed the "Earth Microbiome Project" protocol (Thompson et al. 2017). Rhizosphere samples were extracted using MoBio PowerSoil kit (MoBio, Carlsbad, CA, USA). Plant samples (root and buds) were rinsed by placing samples into a sterilized 15mL falcon tube with DI water and shaken for 5 minutes, ground in liquid nitrogen using a mortar and pestle, and DNA was extracted using MoBio Plant Pro kit (MoBio, Carlsbad, CA, USA) following the

recommended protocol. Quantity and purity of DNA in extracted samples were measured using a spectrophotometer (NanoDrop Technologies LLC, Wilmington, DE, USA) and primers targeting the V3-V4 regions of 16S (bacterial) and ITS1-ITS2 (fungal) regions containing Illumina markers were used. Final concentrations of samples were measured using the Quant-iT PicoGreen dsDNA assay kit (Invitrogen, Grand Island, NY). Individual samples were pooled based on dsDNA concentration and cleaned using MoBio UltraClean PCR Clean-Up Kit (MoBio, Carlsbad, CA, USA). Samples were sequenced in an Illumina MiSeq series.

Bioinformatics:

Samples were de-multiplexed and pre-processed using Qiime2 (2022.2 – Boylen et al. 2019). Quality filtering, chimera removal, and merging of forward and reverse reads were conducted using DADA2 (Callahan et al., 2016). Only forward reads for ITS samples were used due to the low quality of reverse reads. Bacterial samples were matched with the Silva database (v138.1, Quast et al. 2013) with 99% confidence while fungal samples were matched with the UNITE (v8, Abarenkov et al. 2022) with 99% confidence using the function "qiime feature-classifier classify-sklearn" (qiime2, Bokulich et al. 2018).

Data analysis:

Amplicon sequence variant (ASV) and taxonomy tables were imported into RStudio (v.2023.06.2, Posit team 2023) and analyzed using the packages "vegan" (v.2.6-4, Oksanen et al. 2022), "mctoolsr" (v.0.1.1.9, Leff 2016), "BiodiversityR" (v.2.15-1, Kindt and Coe

2005), “metagenomeSeq” (v.1.42.0, Paulson and Talukder 2017), “microbiome” (v.1.23.1, Lahti et al. 2019), “phyloseq” (v.1.44.0, McMurdie and Holmes 2013), and “stats” (4.3.1, R Core Team 2023). Shannon index for alpha diversity and species richness were calculated and used as response variables to test if either insect establishment or site impacted community richness and diversity (Tukey HSD and Kruskal-Wallis tests). Samples were normalized using cumulative sum scaling (CSS). Bray-Curtis estimates for beta diversity were calculated, plotted into NMDS, and differences between groups were calculated through a perMANOVA. Abundance bar plots showing influential taxa were constructed using insect presence as a predictor for each sample type. Additionally, reads were grouped at the phylum or genus level and zero-inflated models (“fitZig” function from “metagenomeSeq” package) were built to calculate differential abundance of taxa and log-fold change in each taxonomic level. Results were compared between insect presence levels. Taxa that are commonly associated with ISR and plant responses to stress were selected based on the literature (Pieterse et al., 2014), taxa were selected from samples using the function “taxa_summary_by_sample_type” from the “mctoolsr” package and analyzed with a Kruskal-Wallis ANOVA. To calculate core microbiome composition across sites, we grouped reads at the genus level and further calculations were conducted using the function “plot_core” from the “microbiome” package. For a taxon to be considered part of the core microbiome, it had to be present in at least 50% of the samples at an abundance of 0.01.

Normalized taxonomy tables and core microbiome outputs were further analyzed to estimate functional groups and guilds for bacterial and fungal data using FAPROTAX (Louca

et al., 2016) and FUNGuild (Nguyen et al., 2016), respectively. Both systems match the input datasets with their functional database and provide an output that summarizes which functions are present in each sample. Due to the high variety of functions provided in FAPROTAX outputs for the whole normalized dataset, data were analyzed for differential abundance using the "DESeq2" package. Outputs from FUNGuild were organized in three different trophic modes (Nguyen et al., 2016): saprotrophs (break down dead cells), pathotrophs (harms host cells), and symbiotrophs (exchange nutrients with host). All classifications were kept regardless of confidence level. Differences in abundance of guild presence per sample type were analyzed as a response for count data models ("glm" function in "stats" package) with insect presence as only predictor.

Results:

We took rhizosphere, root, and bud samples from six populations of Russian knapweed under three levels of herbivory and analyzed the bacterial (16S) and fungal (ITS) communities.

Bacterial communities:

The survey of bacterial communities resulted in 5,796,397 reads. Filtering for non-chimeric reads and merging forward and reverse reads resulted in 4,833,724 reads. Even though PCR blockers for plant DNA (PNA) were added to prevent replication of plant-associated DNA, most of the reads obtained from bud tissue were from chloroplast and mitochondria, which prevented us from conducting further analyses. Mitochondria and chloroplast reads were filtered out of rhizosphere and root samples and samples

containing low reads (less than 30 reads) were removed. Species diversity and richness were higher on rhizosphere than root samples (Shannon diversity: Kruskal-Wallis $X^2$ = 62.235, df = 1, p-value < 0.001; richness: Kruskal-Wallis $X^2$ = 60.056, df = 1, p-value < 0.001; Figure 4). Within sample types, no differences on species diversity or richness were detected for rhizosphere samples between insect establishment levels (Shannon diversity: Kruskal-Wallis $X^2$ = 0.9074, df = 2, p-value = 0.6353, Figure 4A; species richness: Kruskal-Wallis $X^2$ = 2.0015, df = 2, p-value = 0.3676, Figure 4B). Root samples from sites where wasps were established had significantly lower diversity (Shannon diversity: Kruskal-Wallis $X^2$ = 7.489, df = 2, p-value = 0.0236, Figure 4C) and species richness(Kruskal-Wallis $X^2$ = 8.062, df = 2, p-value = 0.0178, Figure 4D) compared to sites where midges established. Samples from sites where no insects were released were intermediate in terms of both diversity (Figure 4C) and species richness (Figure 4D).

Within sample types in rhizosphere samples, insect treatment and site identity were associated with distinct microbial beta diversity [perMANOVA was significant when comparing dissimilarities between insect establishment levels (F = 4.0093; df = 2; R2 = 0.1490; *p* = 0.001) and sites (F = 3.5963; df = 3; R2 = 0.2005; *p* = 0.001) – Figure 5A, stress = 0.1955], as well as among root samples [perMANOVA was significant when comparing dissimilarities between insect establishment levels (F = 2.3557; df = 2; R2 = 0.0835; *p* = 0.001), and sites (F = 2.5574; df = 3; R2 = 0.1361; *p* = 0.001) – Figure 5B, stress = 0.2403].

For rhizosphere samples, the most abundant classes were Actinobacteria, Alphaproteobacteria, Nitrososphaeria, Gammaproteobacteria, Bacilli, Bacteroidia, Planctomycetes, Thermoleophilia, Verrucomicrobiae, and Vicinamibacteria (Figure 6A). At

the genus level, *Canditatus, Nitrocosmicus, Bacillus, Vicinamibacteraceae,* and *Nitrososphaeraceae* were most abundant (Figure 6B). Additionally, several unculturable genera were highly abundant (Figure 6B). For root samples, most common taxa were Actinobacteria, Alphaproteobacteria, Bacilli, Bacteroidia, Gammaproteobacteria, Nitrosophaeria, Planctomycetes, Polyangia, Verrucomicrobiae, and Vicinamibacteria (Figure 7A). At genus level, *Actinophytocola, Actinoplanes, Glycomyces, Nocardioides, Phylobacterium, Promicromonospora, Pseudomonas, Steroidobacter,* and *Streptomyces* (Figure 7B).

Core microbiome analysis:

Core microbiome analysis for bacterial samples resulted in a total of 203 genera in rhizosphere samples and 27 genera in root samples. Genera with a prevalence higher than 5% are shown in figure 8A. Core genera with highest relative abundance in rhizosphere samples were *Candidatus Nitrosocosmicus* (average relative abundance = 7.04%), an uncultured bacterium in the order *Vicinamibacterales* (average relative abundance = 4.85%), *Bacillus* (average relative abundance = 3.93%), *Vicinamibacteraceae* (average relative abundance = 3.45%), *Nitrososphaeraceae* (average relative abundance = 3.39%), and an unclassified bacterium in the order Solirubrobacterales (average relative abundance = 3.24%). For root samples, genera with prevalence higher than 5% are shown in figure 9A. Core genera with highest relative abundance were *Promicromonisora* (average relative abundance = 10.69%), *Actinophytocola* (average relative abundance = 9.41%), *Streptomyces* (average relative abundance = 9.10%), *Nocardiodes* (average relative

abundance = 6.30%), *Actinoplanes* (average relative abundance = 4.50%), *Glycomyces* (average relative abundance = 4.31%), *Phyllobacterium* (average relative abundance = 3.78%), *Steroidobacter* (average relative abundance = 3.40%), and the grouped genus Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium (family Rhizobiaceae, average relative abundance = 2.51%).

Functions associated with the core microbiome were mostly chemoheterotrophy and aerobic chemoheterotrophy (figures 8B and 9B). Two genera (*Nitrososphaeraceae* and *Candidatus Nitrocosmicus*) in the rhizosphere samples were associated with aerobic ammonia oxidation and nitrification. *Rubrobacter*, in addition to chemoheterotrophy and aerobic chemoheterotrophy, was associated with nitrate reduction.

Specific taxa:

Three genera were selected to be further investigated due to their association with plant responses to herbivory: *Streptomyces*, *Pseudomonas*, and *Bacillus* (Pieterse et al., 2014). Mean relative abundances of each genus were compared across insect presence treatments with a Kruskal-Wallis test. No statistical differences were observed in any of the investigated genera in rhizosphere samples (table 1). The relative abundance of *Pseudomonas* spp. in root samples from wasp sites was significantly lower than midge and no insect sites ($p$-value < 0.001), although, no difference was observed in rhizosphere samples ($p$-value = 0.32, table 1).

Fungal communities:

The survey of fungal communities led to 4,925,859 reads. Removing chimeric reads resulted in 2,236,842 reads. Species richness and diversity were highest on rhizosphere samples (Shannon diversity: Kruskal-Wallis $X^2 = 54.019$, df = 2, p-value < 0.001; richness: Kruskal-Wallis $X^2 = 77.418$, df = 2, p-value < 0.001; Figure 10). Neither species diversity (Shannon diversity: Kruskal-Wallis $X^2 = 2.991$, df = 2, p-value = 0.2242, Figure 10A) nor species richness (richness: Kruskal-Wallis $X^2 = 0.514$, df = 2, p-value = 0.773, Figure 10B) in rhizosphere samples differed as a function of insect treatment. However, root samples from midge sites were significantly more species diverse (Shannon diversity: Kruskal-Wallis $X^2 = 7.2654$, df = 2, p-value = 0.0264, Figure 10C) and species rich (species richness: Kruskal-Wallis $X^2 = 8.938$, df = 2, p-value = 0.0115, Figure 10D) than sites containing wasps; no insect sites were intermediate both in terms of species diversity (Figure 10C) and richness (Figure 10D). Species diversity from bud samples, on the other hand, were higher in wasp sites than in midge sites (Shannon diversity: Kruskal-Wallis $X^2 = 8.5903$, df = 2, p-value = 0.0136, Figure 10E). No differences in microbiome species richness was seen in bud samples across the three insect treatments (Kruskal-Wallis $X^2 = 3.376$, df = 2, p-value = 0.1849, Figure 10F).

Microbiome composition was correlated with insect treatment and site in root samples [insect treatment (perMANOVA F = 2.3132; df = 2; R2 = 0.0796; *p*-value = 0.001) and sites (F = 2.4824; df = 3; R2 = 0.1282; *p*-value = 0.001) – Figure 11B, stress = 0.2021] and bud samples [insect treatment (perMANOVA F = 1.8034; df = 2; R2 = 0.0935; p-value = 0.001) and sites (F = 1.9929; df = 2; R2 = 0.1549; p-value = 0.001) – Figure 11C, stress =

0.1423]. No differences were observed in rhizosphere samples when comparing insect treatment (perMANOVA: F = 1.0109; df = 2; R2 = 0.0504; p-value = 0.162) but differences were observed across sites (F = 1.0245; df = 2; R2 = 0.0766; p-value = 0.035 – Figure 11A, stress = 0.1415).

The Sordariomycetes and Dothideomycetes were the most abundant classes in rhizosphere samples with similar abundance across insect treatments (Figure 12). In root samples, the most abundant classes were the Sordariomycetes, Leotiomycetes, Agariocomycetes, and Dothideomycetes and their abundance varied greatly across insect treatments (Figure 12). The most abundant classes in bud samples were the Tremellomycetes and Dothideomycetes (Figure 12).

Core microbiome analysis:

Core microbiome analysis for fungal samples yielded a total of 52 genera from the rhizosphere samples, 5 genera from the root samples, and 4 genera from the bud samples. Genera with prevalence higher than 5% are shown in figures 15 and 16. Core genera with highest relative abundance in rhizosphere samples were *Fusarium* (average relative abundance = 40.95%), *Preussia* (average relative abundance = 9.82%), an uncertain genus in the order Hypocreales (average relative abundance = 6.50%), *Mortiella* (average relative abundance = 6.39%), *Gibberella* (average relative abundance = 4.74%), *Chaetomium* (average relative abundance =4.60), and *Acrostalagmus* (average relative abundance = 3.45%). For root samples, core genera with highest relative abundance were *Polyscytalum* (average relative abundance = 10.96%), *Paraphoma* (average relative abundance = 3.24%), *Alternaria* (average relative abundance = 2.24%), *Mortierella* (average relative abundance =

4.34%), and *Fusarium* (average relative abundance = 7.62%), and for bud samples, core genera with highest relative abundance were *Filobasidium* (average relative abundance = 13.44%), *Alternaria* (average relative abundance = 24.10%), *Udeniomyces* (average relative abundance = 4.33%), and *Cladosporium* (average relative abundance = 15.13%).

We also looked at trophic modes of core microbiome from rhizosphere (Figure 15B), root (Figure 16B), and bud samples (Figure 16D). Our results show that organisms that could be associated with all three trophic modes were abundant all sample types (rhizosphere = 15.4%; root = 40%; bud = 25%). Additionally, potentially pathogenic organisms were present in rhizosphere samples.

Discussion:

While associations between plants, microbiomes, and herbivores have been explored (Friman et al., 2021; Pangesti et al., 2013), the expansion of this knowledge to invasive plants and their introduced herbivores (biological control agents) is still in its infancy. This paper focused on investigating microbiome-invasive plant associations, and how these associations shift when biological control agents are introduced. Few studies integrating these three levels have been conducted. Microbiomes associated with invasive plants in their native and introduced range are known to differ (Lu-Irving et al., 2019); however, how patterns within invaded range change in relation to herbivory are understudied.

Looking at the core microbiome of an invasive plant may help us understand its mechanisms to colonize new areas. The invasive *Pennisetum ciliare* (buffelgrass) selects

for a core microbiome that is capable of metabolizing allelochemicals synthesized by this invasive plant as well as assist with nutrient acquisition (Jara-Servin et al., 2023). The root-associated microbiome of Russian knapweed is associated with nutrient cycles and their availability to plants (*Candidatus Nitrosocosmicus*, *Nitrososphaeraceae*, *Mortierella*). Russian knapweed frequently grows in disturbed areas; therefore, forming associations with microbes that help with nutrient availability or ameliorate abiotic stressors is advantageous. Additionally, pathogenic root-associated microbes were found (e.g., *Gibberella* and *Fusarium*), possibly because knapweed has been present in the sampled sites for several decades (Sonya Daly *personal communication* and NAIP imagery). Invasive plants may be less exposed to pathogenic microbes in the introduced range possibly enabling their establishment (Gioria et al., 2023; Inderjit and van der Putten, 2010; Klironomos, 2002). However, negative interactions may evolve as species persist in the same location (Hannula et al., 2021; Strauss et al., 2006; Van der Putten et al., 1993).

Taxa associated with plant response to biotic stressors (*Nocardioides, Bacillus*, *Streptomyces*) were also found in core microbiome samples of Russian knapweed. Presence of *Bacillus* spp. have been associated with induced systemic resistance (ISR - Pieterse et al., 2014), and *Bacillus subtillis* have been reported to form biofilm in roots of *Arabidopsis* due to plant responses to pathogens (Rudrappa et al., 2008). Our results show that *Bacillus* spp. were highly abundant across rhizosphere and root samples; however, how it could be impacting Russian knapweed's response to biological control agents remains unknown. We hypothesize that plant response to herbivory by biological control agents led to changes in root exudation and enhanced the recruitment of those taxa,

similarly to Rudrappa et al. (2008). Although not statistically different, the relative abundance of *Bacillus* spp. in root samples was at least two times higher in midge and wasp sites when compared to insect-free sites, despite relative abundance in rhizosphere samples of insect-free sites being at least twice as much as both sites with insects.

Sites and insect presence are associated with differences in species richness and diversity in fungal and bacterial communities (Eldridge et al., 2017). In the present study, microbiome composition and richness changed among sample types (rhizosphere, root, or bud samples), with rhizosphere samples being the most diverse and rich, as observed in other studies (Hamonts et al., 2018). Our results go against what was found by Kong et al. (2016), where evenness and diversity of bacteria in rhizosphere samples was impacted by introduction of insect herbivores. However, our results agree with Liu et al. (2017), where the authors found the application of jasmonic acid (JA) and its cascading effects on upregulating response mechanisms not having impacts on rhizosphere microbiome richness and evenness but do impacting root microbiome composition. We found that microbiome richness and diversity were distinct in root samples; more specifically, sites associated with wasps had lower richness and diversity of fungal and bacterial communities compared to midge-associated sites. This finding suggests that Russian knapweed root microbiome is shifted in response to herbivory and changes associated with wasps are greater than in midges. This finding goes against our hypothesis since we expected insect-associated sites to have similar microbial community due to top-down effects (presence of herbivory). However, it seems like nature of herbivory was a very

important factor leading to distinct levels of plant stress and, in consequence, microbiome associated to plants.

Plants' ability to respond to herbivory can impact root-associated microbiomes (Hamonts et al., 2018; Lebeis et al., 2015; Trivedi et al., 2012). In studies conducted by Lebeis and collaborators (2015), the authors concluded that the phytohormone salicylic acid (SA) has an important role in root microbiome colonization, where *Arabidopsis thaliana* mutants with enhanced or depleted synthesis of SA led to lower microbial diversity in roots. In addition, colonization of *A. thaliana* roots by *Pseudomonas*, *Escherichia coli*, *Streptomyces*, and *Chryseobacterium* sp. present in synthetic communities (SynComs) was reduced in SA enhanced and depleted mutants (Lebeis et al., 2015). Bacteria in the genus *Pseudomonas* were less abundant in root samples from wasp sites; although equally abundant in rhizosphere samples, suggesting that wasps may be shifting response hormones in plants and leading to reduced root colonization. Insect establishment did not reduce root colonization by microbes in the genus *Streptomyces*, in fact *Streptomyces* shown as a core microorganism for root samples.

In conclusion, the present study exemplifies how invasive plants could be using interactions with microbiomes to expand their range and increase their competitiveness against other plant species. Russian knapweed selects for a core microbiome that contains taxa that ameliorates nutrient deficiencies, pathogens, and beneficial organisms. Additionally, we see that establishment of wasps negatively impacts the root microbiome of Russian knapweed. Where root samples from wasp-associated sites had lower abundance of *Pseudomonas* spp., and richness and diversity of species suggesting that

plant responses to herbivory shifts root microbiomes. However, we cannot make inferences of whether microbiome composition negatively impacted the establishment of biological control agents, as we selected sites where insects were already present or not. Further studies should be conducted to test whether plants inoculated with distinct microbiome composition have varying levels of response to herbivory or if biological control agents have different preferences to plants associated with distinct microbiome composition, as conducted by Howard et al. (2020) while investigating the effects of microbial communities on tall golderod (*Solidago altissima*) and preference by its herbivores. To our knowledge, this is the first paper to study the associations between and invasive plant, its microbiome, and biological control agents. We believe that investigating such interactions will help understand biological control programs of invasive plants, more specifically, why its success is inconsistent (Kentjens et al., 2024).

Figures and tables:

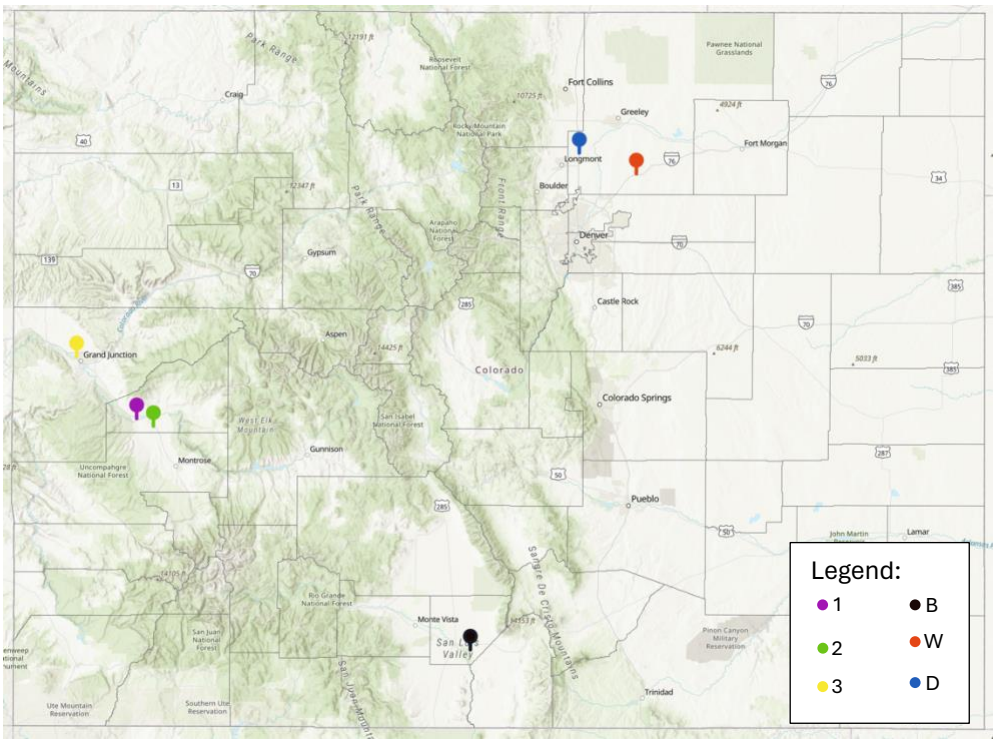
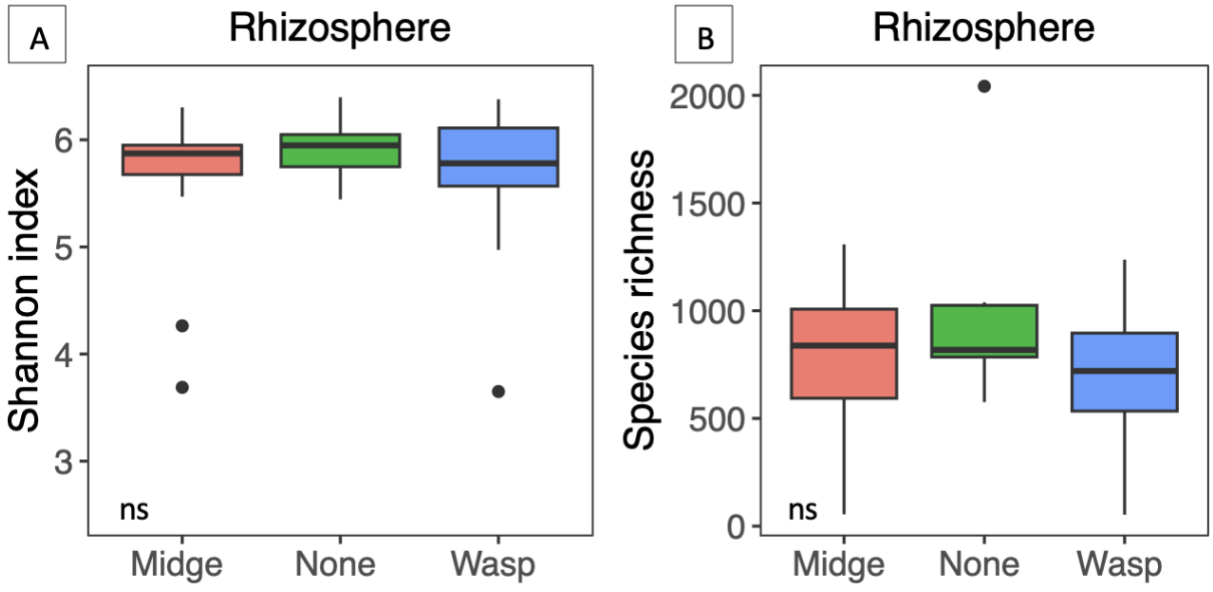



Figure 3: Distribution of knapweed sites throughout Colorado. Sites 1 and W have *A. acroptilonica*, 2 and D have *J. ivannikovi*, and B and 3 have no insects.

<u>Bacterial communities</u>:



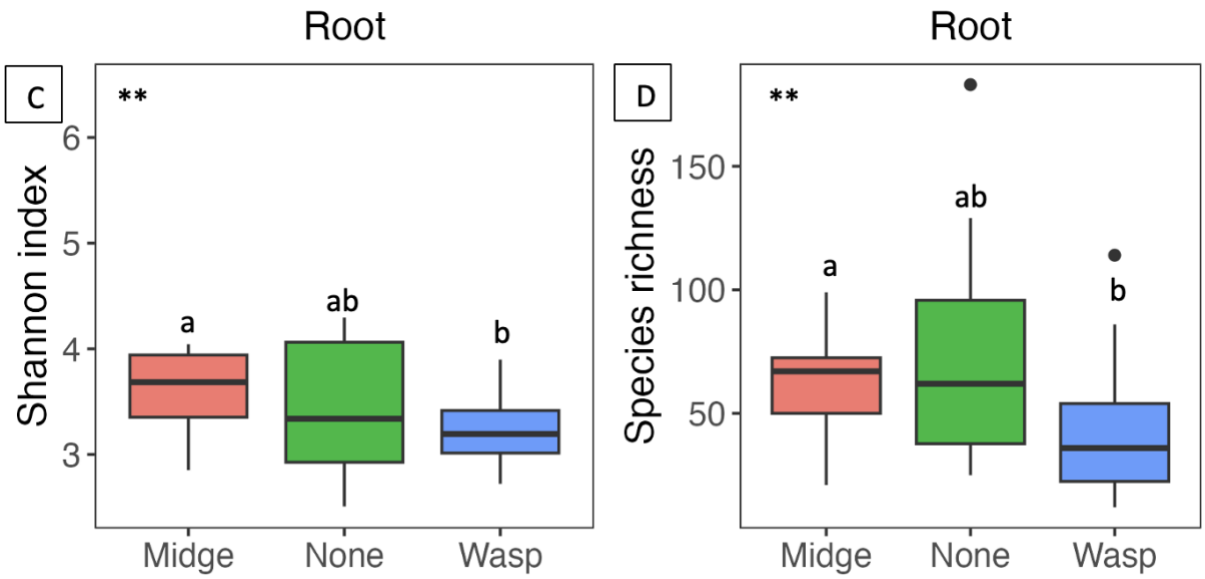



Figure 4: Alpha diversity (Shannon index, A and C) and richness (B and D) of bacteria in rhizosphere and root samples. No differences in diversity (A: Shannon diversity: Kruskal-Wallis $X^2 = 0.9074$, df = 2, p-value = 0.6353) or richness (B: species richness: Kruskal-Wallis $X^2 = 2.0015$, df = 2, p-value = 0.3676) were observed in rhizosphere samples. Differences in diversity (C: Shannon diversity: Kruskal-Wallis $X^2 = 7.489$, df = 2, p-value = 0.0236) and richness (D: species richness: Kruskal-Wallis $X^2 = 8.062$, df = 2, p-value = 0.0178) were observed in root samples.

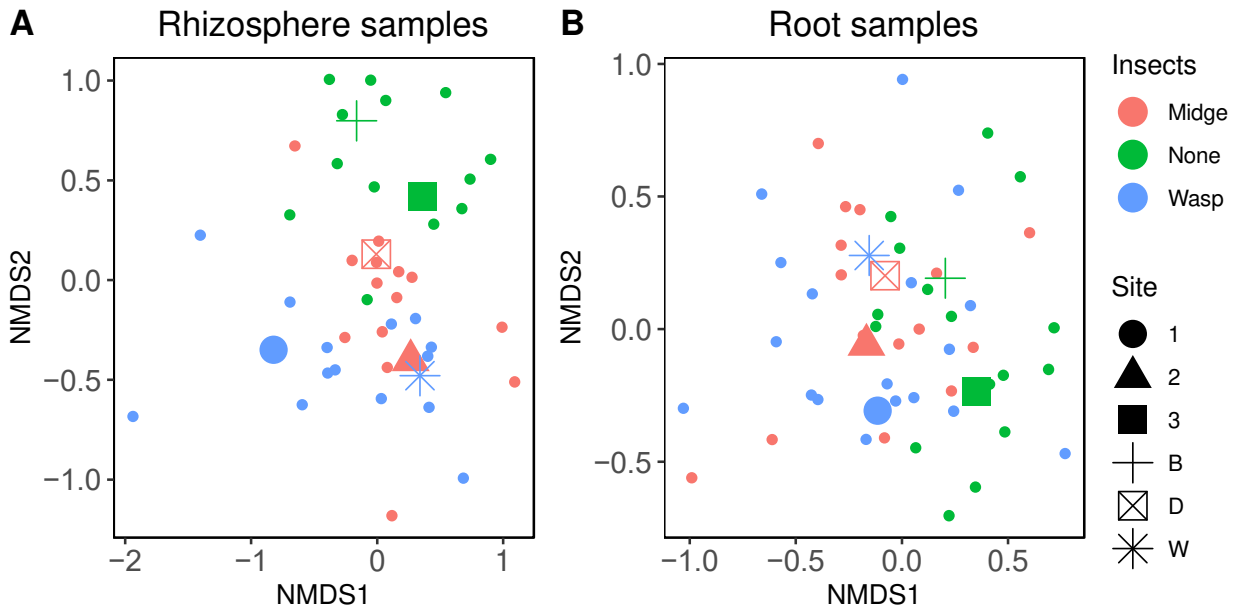
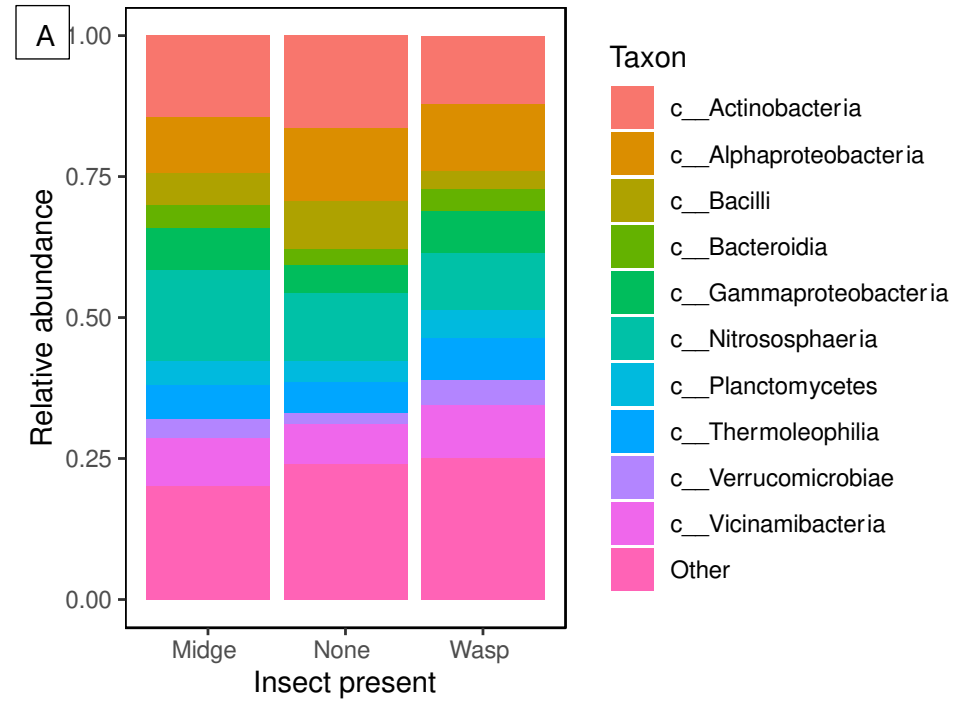



Figure 5: NMDS plots of beta diversity for rhizosphere and root samples. PerMANOVA indicated differences in beta diversity when looking at rhizosphere when looking at insect establishment levels (F = 4.0093; df = 2; R2 = 0.1490; *p* = 0.001) and sites (F = 3.5963; df = 3; R2 = 0.2005; *p* = 0.001), stress = 0.1955, A). For root samples, perMANOVA shown significance when comparing diversities between insect establishment levels (F = 2.3557; df = 2; R2 = 0.0835; *p* = 0.001), and sites (F = 2.5574; df = 3; R2 = 0.1361; *p* = 0.001), stress = 0.2403, B.



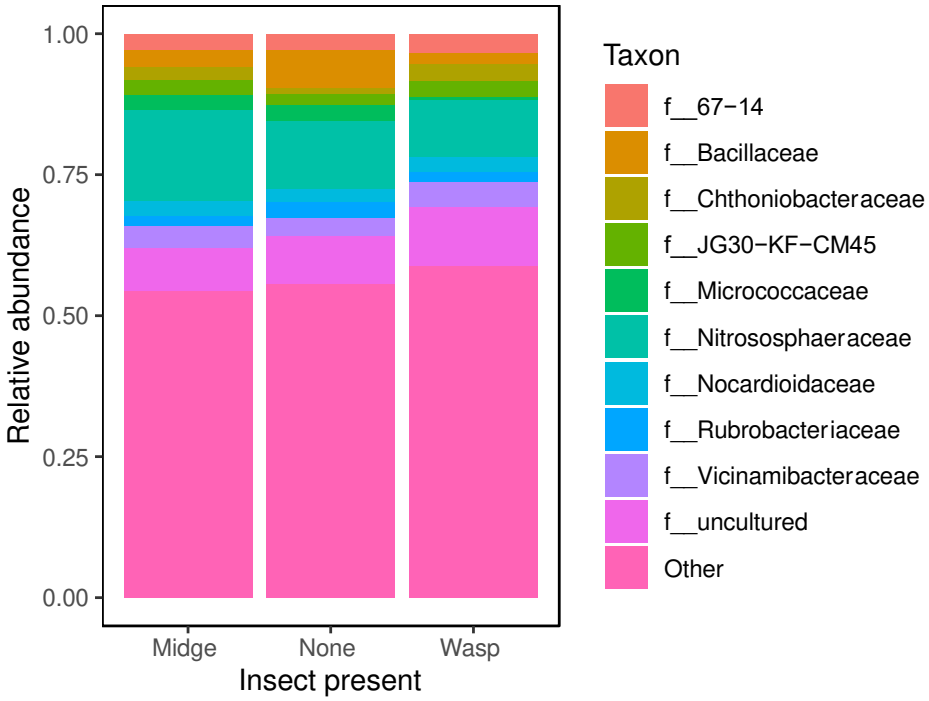
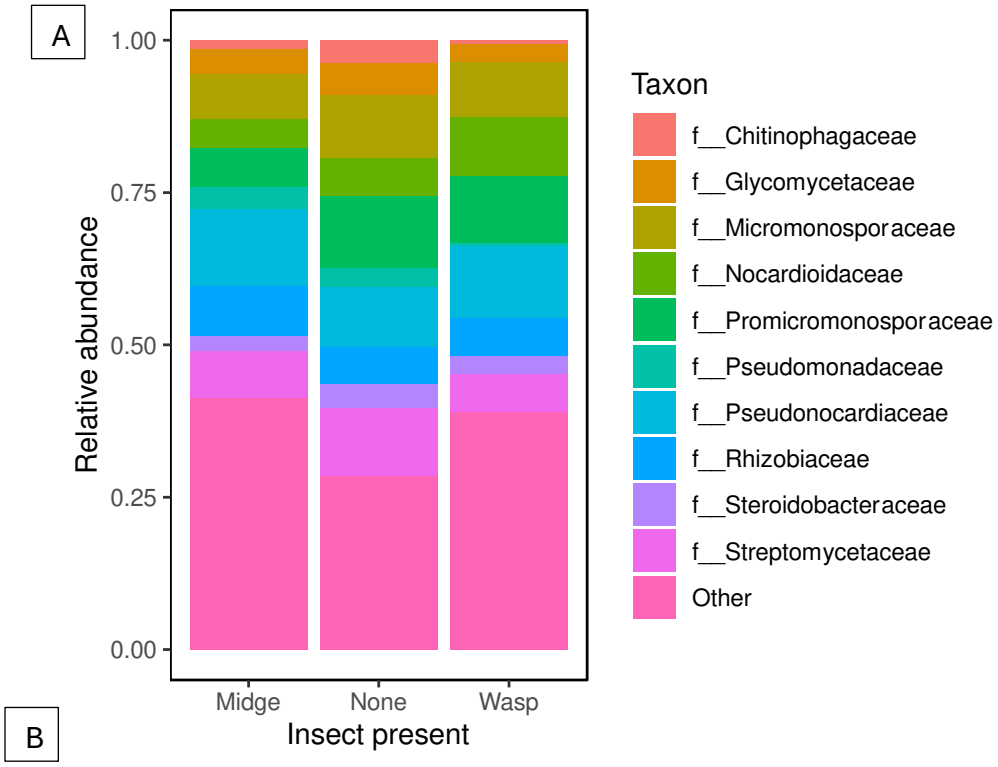



Figure 6: Relative abundance of most abundant bacterial taxa for rhizosphere samples at class (A) and family (B) level.



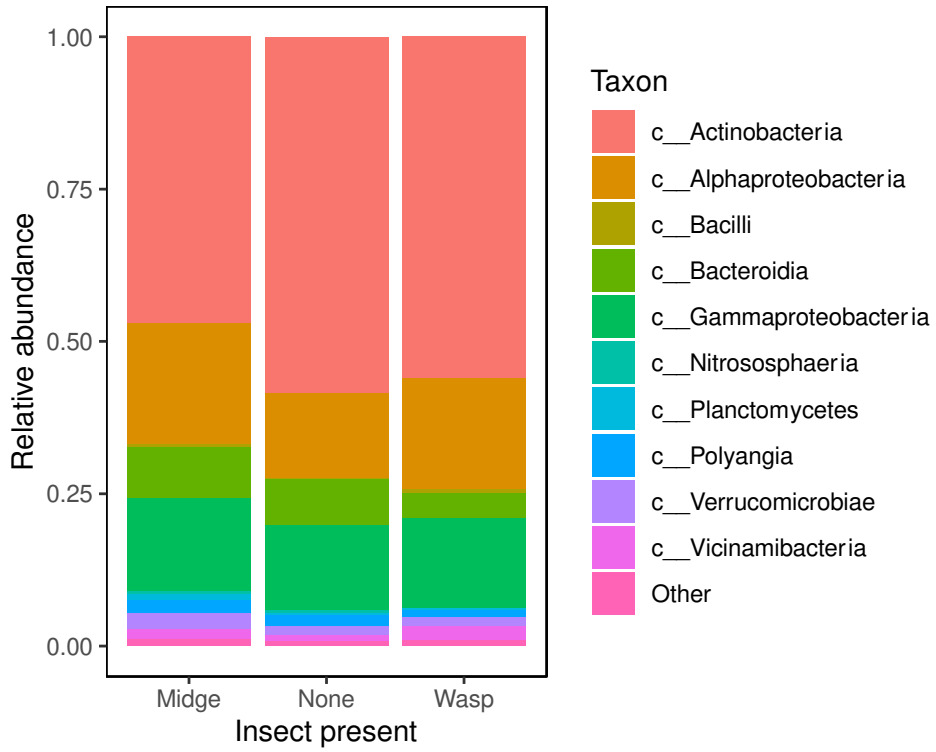



Figure 7: Relative abundance of most abundant bacterial taxa for root samples at class (A) and family (B) level.

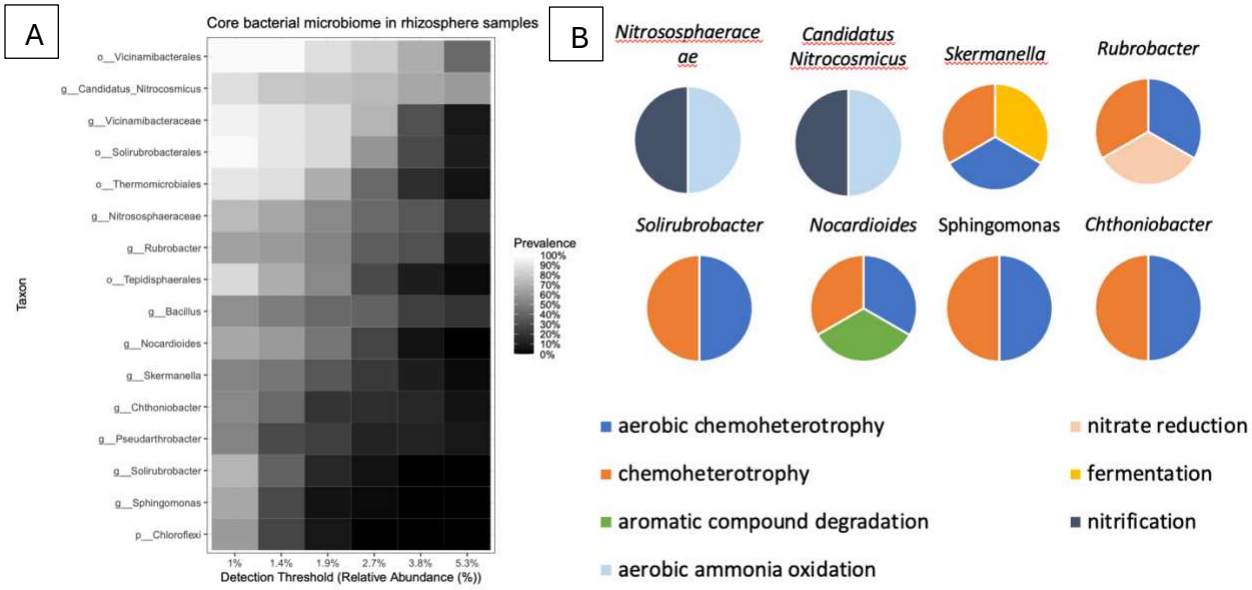



Figure 8: A: Core bacterial microbiome at genus-level for rhizosphere samples. Taxonomies are represented in y-axis and detection thresholds in x-axis. Each block represents the prevalence of each taxon at which detection threshold. B: Functions associated with taxa in core microbiome matched to FAPROTAX database.

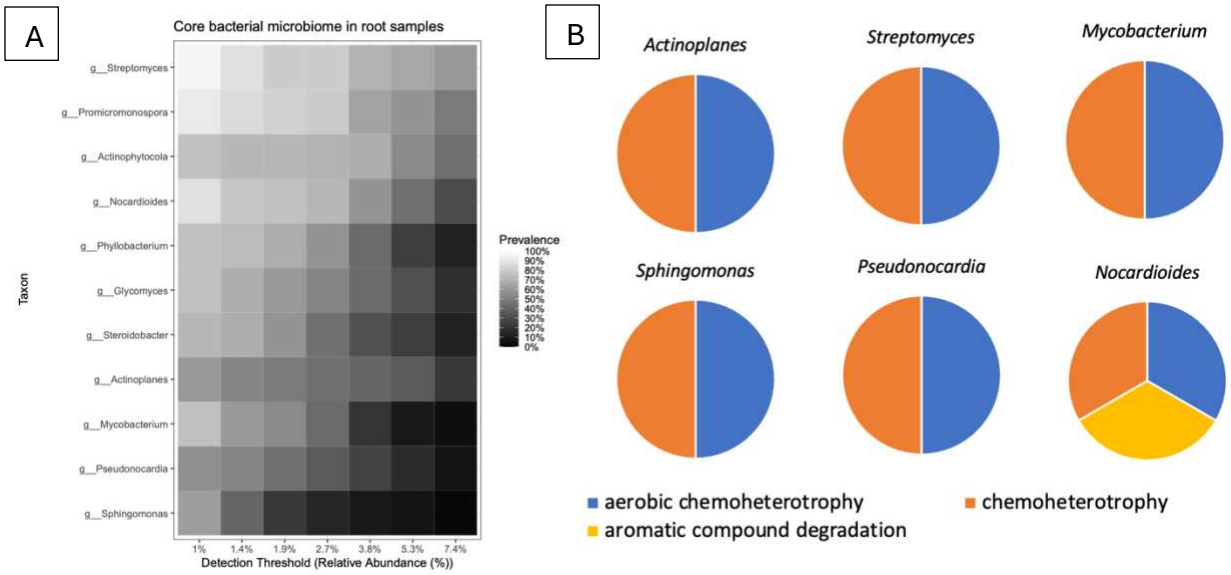
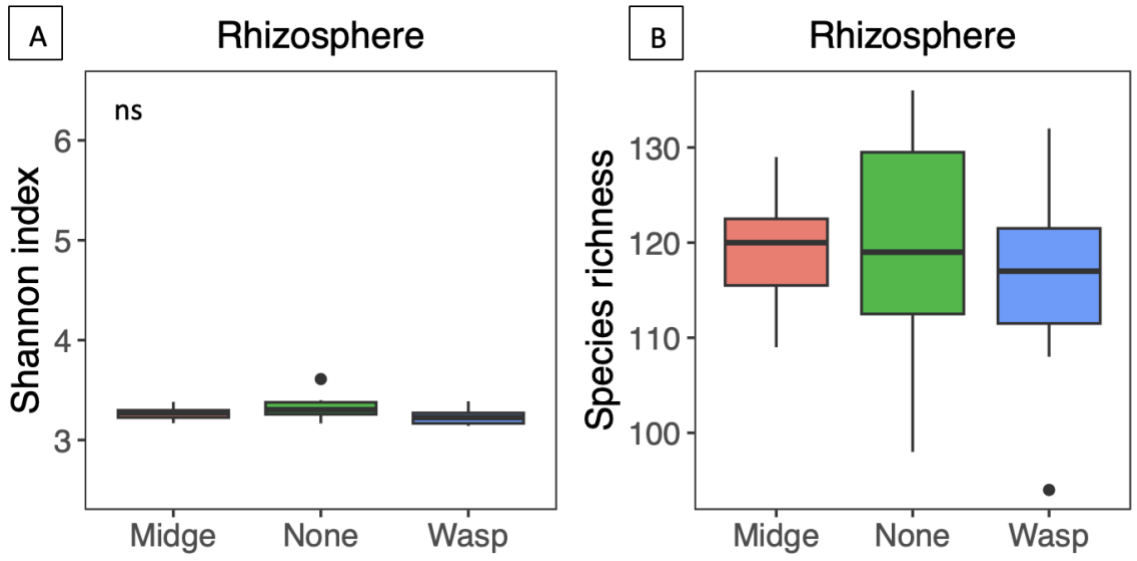



Figure 9: A: Core bacterial microbiome at genus-level for root samples. Taxonomies are represented in y-axis and detection thresholds in x-axis. Each block represents the prevalence of each taxon at which detection threshold. B: Functions associated with taxa in core microbiome matched to FAPROTAX database.

Fungal communities:



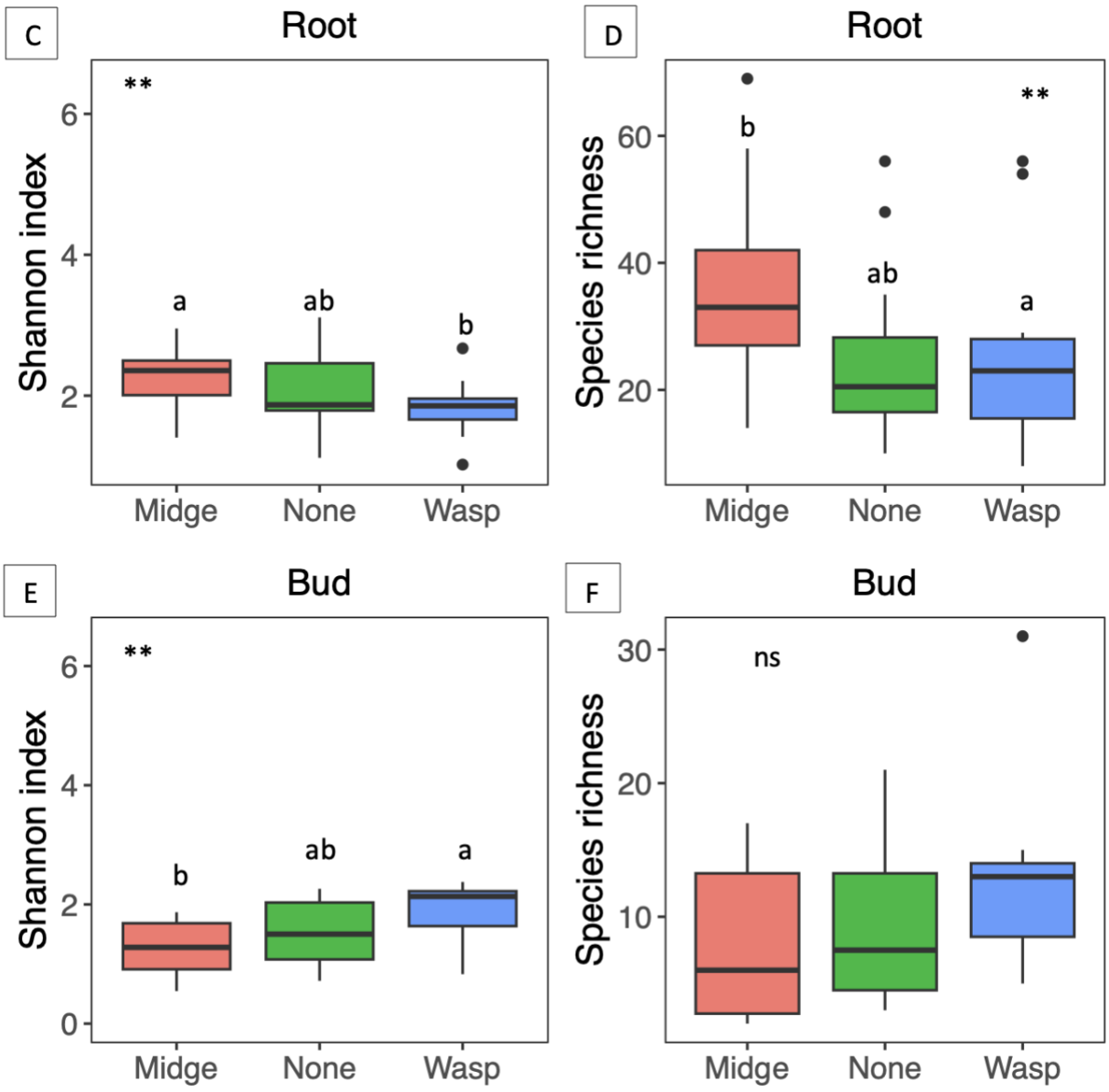



Figure 10: Alpha diversity (Shannon index, A, C, and E) and richness (B, D, and F) of fungi in rhizosphere, root, and bud samples as a function of insect treatment (midges, wasps, no insect). No differences were observed among rhizosphere samples taken from midge, wasp, or no insect sites in terms of diversity (A: Shannon diversity: Kruskal-Wallis $X^2$ = 2.991, df = 2, p-value = 0.2242) or richness (B: species richness: Kruskal-Wallis $X^2$ = 0.514, df = 2, p-value = 0.773) for rhizosphere samples. Root samples from wasp sites were significantly less diverse (C: Shannon diversity: Kruskal-Wallis $X^2$ = 7.2654, df = 2, p-value = 0.0264) and species rich (D: species richness: Kruskal-Wallis $X^2$ = 8.938, df = 2, p-value = 0.0115) than midge samples. Bud samples from midge sites were significantly less diverse than midge samples (E: Shannon diversity: Kruskal-Wallis $X^2$ = 8.5903, df = 2, p-value = 0.0136). No differences were observed among the three insect treatments in terms of species richness (F: species richness: Kruskal-Wallis $X^2$ = 3.376, df = 2, p-value = 0.1849,).

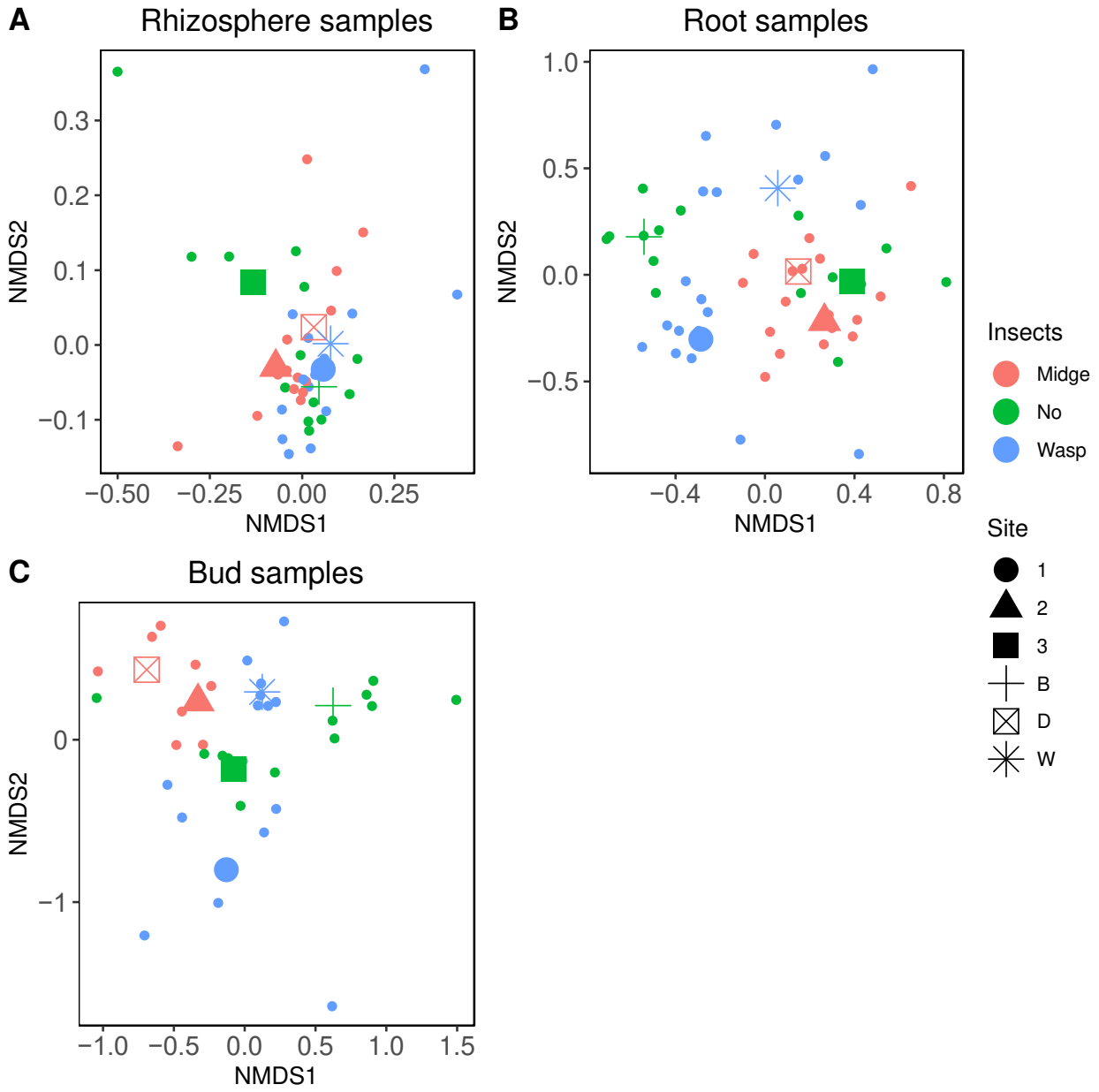



Figure 11: NMDS plots of beta diversity. PerMANOVA indicated no differences for rhizosphere samples when looking at insect treatment (A: F = 1.0109; df = 2; R2 = 0.0504; p-value = 0.162), but seen when looking between sites (F = 1.0245; df = 2; R2 = 0.0766; p-value = 0.035, stress = 0.1415). For root samples, differences were observed between insect treatments (B: F = 2.3132; df = 2; R2 = 0.0796; *p*-value = 0.001) and sites (F = 2.4824; df = 3; R2 = 0.1282; *p*-value = 0.001), stress = 0.2021]) and bud samples insect establishment levels (C: F = 1.8034; df = 2; R2 = 0.0935; p-value = 0.001) and sites (F = 1.9929; df = 2; R2 = 0.1549; p-value = 0.001), stress = 0.1423].

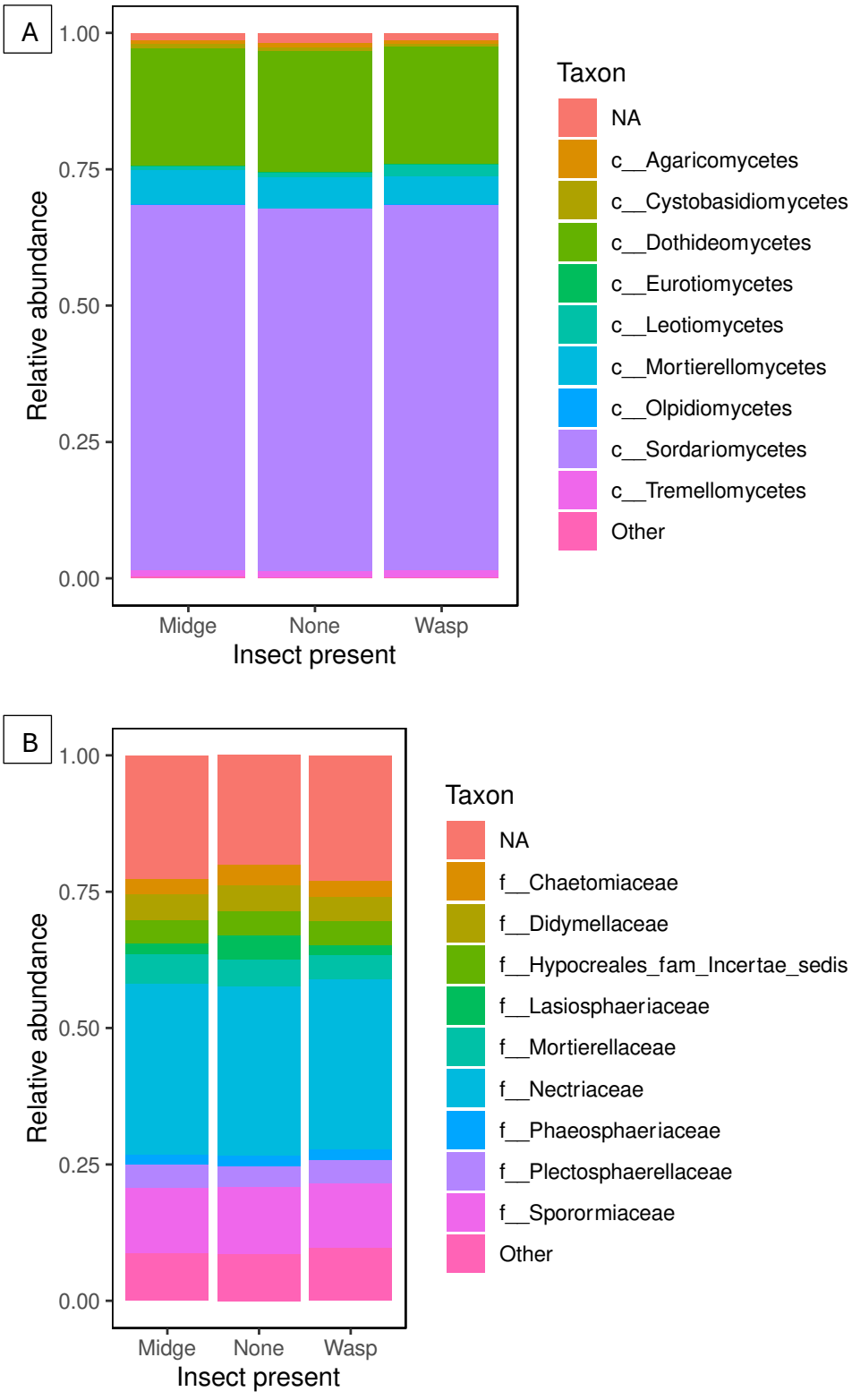



Figure 12: Relative abundance of most abundant fungal taxa for rhizosphere samples at class (A) and family (B) level.

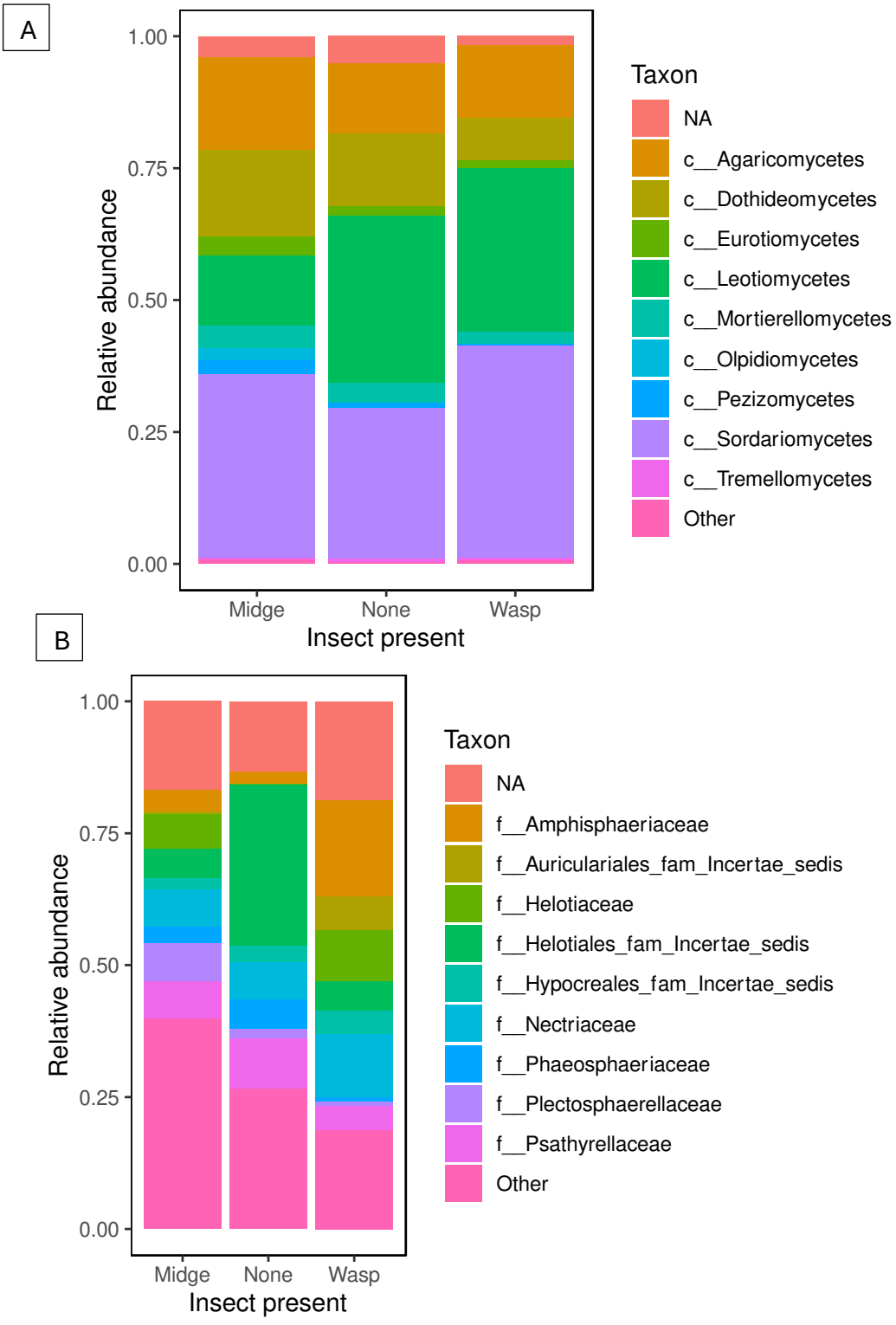



Figure 13: Relative abundance of most abundant fungal taxa for root samples at class (A) and family (B) level.

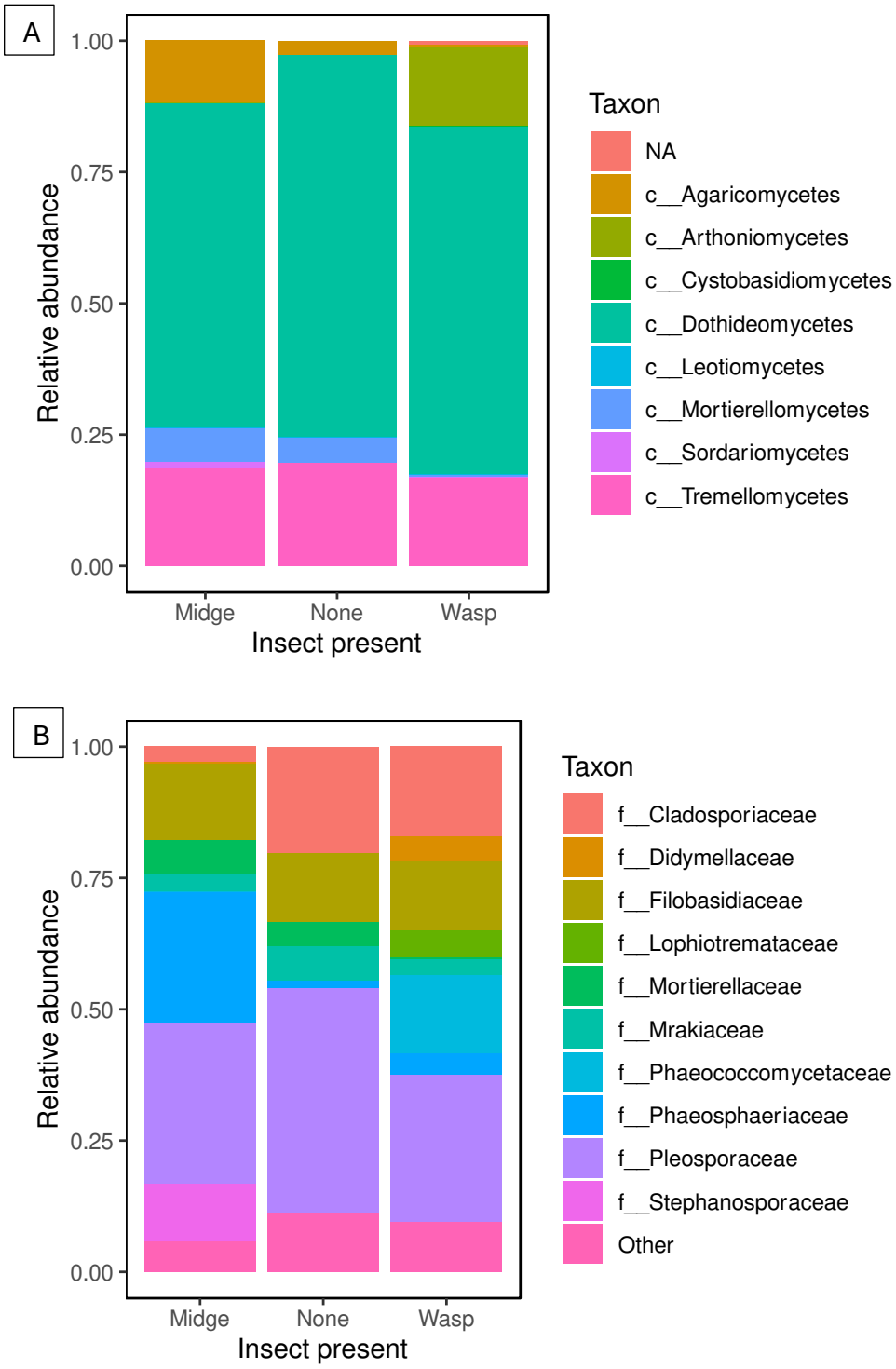



Figure 14: Relative abundance of most abundant fungal taxa for bud samples at class (A) and family (B) level.

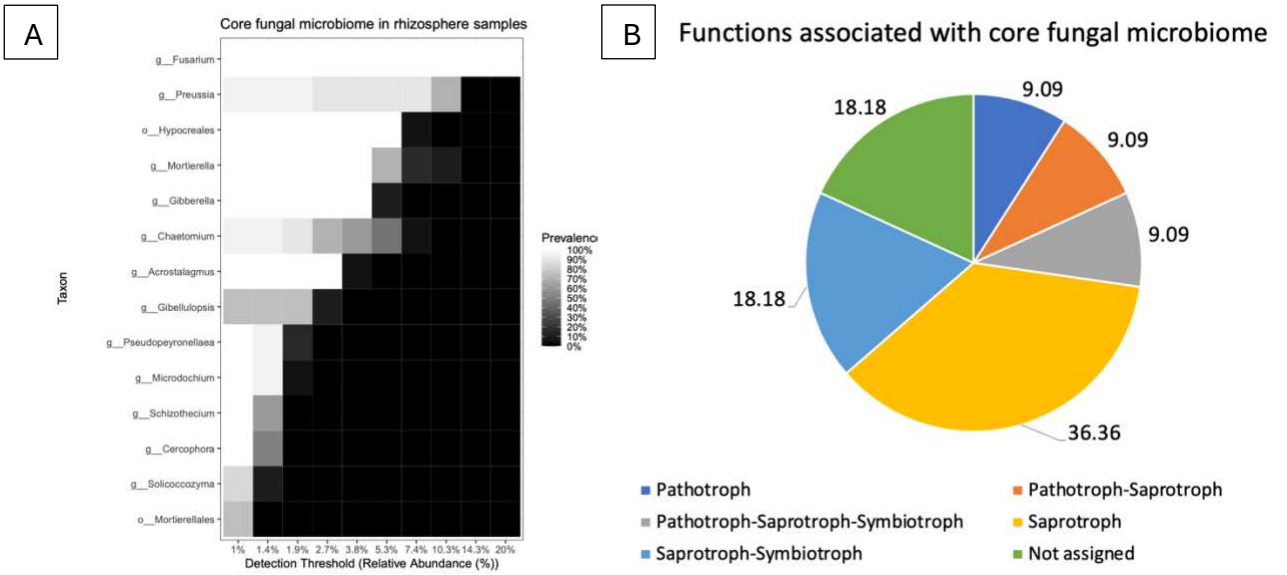



Figure 15: Core fungal microbiome at genus-level for rhizosphere samples. Taxonomies are represented in y-axis and detection thresholds in x-axis. Each block represents the prevalence of each taxon at which detection threshold.

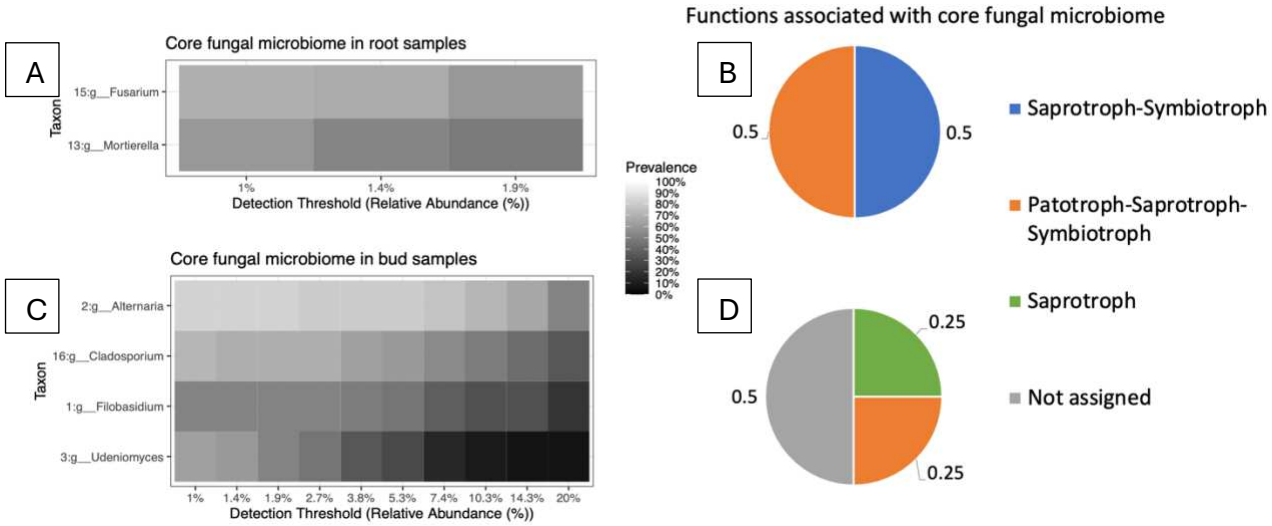



Figure 16: Core fungal microbiome at genus-level for root samples. Taxonomies are represented in y-axis and detection thresholds in x-axis. Each block represents the prevalence of each taxon at which detection threshold.

Table 1: Genus of interest (bacteria in light blue, fungi in light green), sample type, *p*-value, and relative abundance of each taxon for midge, none, and wasp-associated sites.

| Genus | Sample type | *p*-value | Midge | None | Wasp |
|---|---|---|---|---|---|
| ***Pseudomonas*** | Rhizosphere | 0.316 | 0.003 | 0.001 | 0.002 |
| ***Bacillus*** | Rhizosphere | 0.401 | 0.029 | 0.063 | 0.018 |
| ***Streptomyces*** | Rhizosphere | 0.293 | 0.01 | 0.013 | 0.009 |
| ***Pseudomonas*** | Root | 0.001 | 0.036 | 0.03 | 0.005 |
| ***Bacillus*** | Root | 0.532 | 0.005 | 0.001 | 0.007 |
| ***Streptomyces*** | Root | 0.117 | 0.077 | 0.112 | 0.062 |
| ***Trichoderma*** | Rhizosphere | 0.747 | 0.001 | 0.001 | 0.001 |

# CHAPTER 3: Impacts of biological control agents on Russian knapweed

## Introduction

Introduction of two or more species of herbivores to manage a single invasive plant species is a common practice in classical biological control (e.g. Denoth et al., 2002; Seastedt et al., 2007); however, whether adding more than one biological control agent will indeed improve the management of the invasive plant continues to be strongly debated (Denoth et al., 2002). The introduction of several agents is commonly associated with a cumulative stress model, where the addition of more agents will result in increased stress and, therefore, increased management success of the invasive plant (e.g., Seastedt et al., 2007). Another common perception is that by adding more agents, there will be a higher success rate of the biological control program. This latter concept has been likened to a lottery, where each agent is a "ticket" and the more "tickets" released, the higher the chances that at least one of those agents will be able to manage the invasive species (Denoth et al., 2002). This approach can be risky because introduced biological control agents can consume non-target organisms or interfere with native food webs, indirectly disrupting associations between native species through plant-mediated effects (Blossey et al., 2018; Pearson and Callaway, 2005). Therefore, it is important to understand how each agent can impact the target pest species and how it might interact with other community members including other biological control agents. Some biocontrol agents, e.g., the gall-forming Diptera, are more frequently associated with antagonistic effects towards other

agent introductions, possibly because they often target highly defended reproductive or meristematic tissues, leading to negative associations with herbivores (Stephens et al., 2013). How plants respond to herbivory can shape plant-mediated interactions between insect herbivores (Wurst and van der Putten, 2007). Plant responses can reduce the likelihood of subsequent attacks by priming or inducing defense mechanisms (Anzano et al., 2022; Erb et al., 2012). Alternatively, interactions between herbivores can be synergistic if the changes to plant mechanisms end up drawing resources to plant tissues that are used by subsequent herbivores (Soler et al., 2012). Understanding such relationships is fundamental for biological control programs, since in several systems multiple biological control agents are introduced and interactions between these insect herbivores are mediated by their invasive host.

The consequences of plant-mediated interactions can also be impacted by abiotic factors that change the quality of the host plant, including nutritional quality of consumed tissues or the ability the plant to induce defenses in response to herbivory (Anzano et al., 2022; Lin et al., 2023). One very important abiotic factor is water stress. Water stress can alter plant resource allocation patterns between sources and sinks (e.g., apical meristems, Rodrigues et al., 2019), thereby differentially affecting herbivore feeding guilds that preferentially feed on source or sink plant tissues. Additionally, responses to abiotic stressors can indirectly change plant suitability as a host for herbivorous insects (Saska et al., 2023; Valim et al., 2016). For instance, collard greens (*Brassica oleracea* var. *acephala*) produced thicker waxy layer when exposed to drought conditions, which reduced the performance of chewing and sap-sucking herbivores (Valim et al., 2016). Plant-mediated

interactions between herbivores may also shift from competitive to synergistic under abiotic stress (the stress gradient hypothesis; Bertness & Callaway, 1994; Chamberlain et al., 2014). For instance, when two herbivores, a root feeding beetle (*Agriotes lineatus*) and a leaf-mining fly (*Chromatomyia syngenesiae*), feed on *Sonchus arvensis* under lower water availability, the presence of the root feeder increased the weight of the leaf-miner whereas no effects were observed in control watering (Staley et al., 2008). Additionally, the degree of water stress can impact how plants will respond to herbivory. In low water stress, plants response mechanisms are primed (Lin et al., 2023). However, in intense water stress, plants' response is variable (Lin et al., 2023). Differences in host plant suitability determined by water stress can lead to differential insect establishment success and, therefore, biological control success.

Russian knapweed is an invasive perennial noxious weed that persists in same location for several growing seasons due to rhizomatous growth (Gaskin and Littlefield, 2017). To help manage this species, two insect biological control agents have been released: a meristem gall midge (*Jaapiella ivannikovi*) and a stem gall wasp (*Aulacidea acroptilonica*). Insects induce gall formation in different tissues of Russian knapweed and are thought to reduce overall plant fitness in their native range (Djamankulova et al., 2008). However, further investigation is needed to better understand the long-term effects of these insects on Russian knapweed populations in the introduced range.

Most galls become resource sinks for plants (Oliveira et al., 2016) and can reduce long-term fitness of perennial plants (Prade et al., 2016). We predict that galls weaken the rhizomes through reduction of available resources at the end of the growing season,

leading to weakened regrowth in the beginning of the following season. Because two insects have been approved to manage Russian knapweed, they are frequently simultaneously introduced. However, it is not known if these insects can indirectly interact through plant-mediated functions. Additionally, since Russian knapweed can invade sites that vary in terms of water availability (EDDMapS 2024), drought stress can affect the establishment success of the biological control agents. Therefore, the goal of this paper is to investigate (1) if plant-mediated interactions between biological control agents affects their establishment success; (2) how drought stress shifts plant-mediated interactions between biological control agents; and (3) if the combination of insect introductions and watering levels change the fitness of Russian knapweed. To address these goals, garden experiments were setup in two locations in Colorado (Palisade and Fort Collins). Each common garden was divided into two water treatments: low and high. In each location, 100 Russian knapweed plants were planted and caged to enable distinct insect and water stress treatments. Gardens were kept for two years, and insect establishment and plant fitness were assessed.

## Methods

### Garden experiment locations:

Russian knapweed from four populations were grown in two common gardens: at the Agricultural Research, Development and Education Center at Colorado State University (ARDEC - 40°39'25.0"N; 104°59'40.8"W) and at the Palisade Insectary (Palisade, CO - 39°06'48.6"N 108°21'01.0"W) for two years (2021 and 2022). We chose these locations

because they are representative of sites where knapweed can be found. Soil conditions in each site are classified as Kim loam and Turley clay loam for ARDEC and Palisade, respectively (USDA Web Soil Survey). Annual precipitation data for each location and each year are presented in table 2. In each garden, 25 Russian knapweed plants belonging to each of four populations were planted (100 plants total). Two populations were collected from the Palisade area [near Cameo (39°08'53.6"N 108°18'58.7"W) and Fruita (39°09'50.0"N 108°46'53.4"W), Colorado] and two from ARDEC area [near Fort Collins (40°40'24.8"N 104°58'55.6"W) and Denver (39°50'14.1"N 104°43'21.3"W), Colorado]. Both gardens were split into two watering treatments: low and high. We decided to irrigate even the low watering level to enhance Russian knapweed establishment and persistence in the area.

Insect source populations:

Wasps and midges used in the common garden experiments were collected from knapweed sites where both insects have successfully established in Mesa County, Colorado. For both insects, galls collected from field sites were brought to a lab, excess plant material was removed, and galls were grouped into 200mL plastic containers (~150 wasp galls or ~ 10 midge galls per container) and covered with mesh until insect emergence. One day after emergence, we expected that all females had mated. Midges and wasps were sorted into groups of five mated females small (10ml) plastic souffle cups (10mL).

Insect treatments:

Plots were established in July of 2020 and in June of 2021. Cages (0.5m x 0.5m x 1m) were placed on top of plants to prevent other herbivores from entering the cages and to contain midges and/or wasps added to each treatment. We established five insect treatments per garden (20 cages per treatment): midges only (10 adult females per cage), wasps only (10 wasp females per cage), midge + wasp (substitutive: 5 midges + 5 wasps), midge + wasp (additive: 10 midges + 10 wasps), control (no insects released). The substitutive treatment kept the number of released insects the same as the midge-only or wasp-only treatments, whereas the additive treatment kept the number of wasps and midges the same as the wasp-only and midge-only treatments. The combination of substitutive and additive treatments allowed us to compare the effects of insect number and insect identity on the performance of knapweed. Insects were introduced to treatment cages twice during the experiment (at the beginning of each growing season).

Data analyses:

For each cage, aboveground plant biomass, height of tallest stem, number of stems, number of flowers, and number of midge and wasp galls were collected at the end of each year. Rainfall conditions at the Palisade and ARDEC common garden sites differed significantly making it difficult to draw meaningful comparisons between drought stress and irrigation treatments between the two gardens. Therefore, data were analyzed separately for the two common gardens. To test if water and/or insect treatments affected

the growth rate of Russian knapweed, we fitted mixed models for each response variables with water and insect treatments as fixed predictors and plant population as a random effect. Count data were analyzed using a negative binomial correction for over dispersed data with the function “glmer.nb”, while continuous data were analyzed using the function “lmer”, both from the package “lme4” (v.1.1-34). Plant biomass for both locations and both years were transformed using log + 1. First, we tested the fit of complete models (including insect treatment and watering levels as fixed effects and plant population as a random effect) and compared them with reduced models that did not include one of the fixed effects. Next, we tested if treatments and interactions (insect * water treatment) were significant using the function “mixed” (method = Kenward-Roger) from package “afex” (v.1.3-0). Further evaluation of estimated marginal means (EMMs) were calculated and pairwise comparisons were performed (“emmeans” package – v.1.8.8).

To test whether insect and water treatments affected the number of galls (each insect separately and together), we fitted a model containing the number of galls of each insect as response variable and water treatment and filtered insect treatments (we only included treatments that could contain midges when analyzing midge galls, for example) as fixed effects and plant population as random effect. Additionally, we wanted to understand if plant fitness parameters were associated with number of galls, meaning that plants had more galls because insects had more tissue to gall. To do so, we tested the correlation between plant fitness parameters (biomass, number of stems, height of tallest stem, and number of flowers) and number of each insect gall as well as total number of

galls using Pearson's correlation values obtained with the function "ggpairs" from package "GGally"(v.2.2.0).

Russian knapweed plants can persist in the same location for several generations because of its rhizomatous growth. Because each winter the above ground tissue senesces, the belowground rhizome persists and produce new stems once conditions are favorable. Although we were not able to retrieve rhizomes after the completion of the experiment, the number of stems can be a good estimate of rhizome size and condition, since bigger rhizomes will have higher number of buds that will develop into stems compared to smaller rhizomes. Therefore, we estimated the relative growth rate (RGR) of rhizomes in different water and insect treatments by comparing the number of stems at the end of each year using an RGR equation (see equation below). Outputs from the RGR equation were used as response in mixed model with insect and water treatments as fixed effects and plant population as random effect.

$$Relative\ Growth\ Rate\ (RGR) = \frac{(Number\ of\ Stems\ 2022) - (Number\ of\ Stems\ 2021)}{(Number\ of\ Stems\ 2021)}$$

Results

Plant-mediated effects between water treatment and insect introductions:

To investigate the relationship between insect galls, water availability, and insect treatments, we separated total treatments into midge releases (either midges alone and

with wasps) and wasp releases (either alone and with midges) and tested the effect of water and insect treatments on numbers of galls of each insect. The goal of these analyses was to test whether introducing one species or both in addition to manipulating watering levels impacted the gall formation success of each insect. Water availability had a significant impact on the number of midge and wasp galls in both locations; however, patterns varied between years. High water availability was correlated with an increase in midge galls in year 1 (2021) at the Palisade common garden. For first year midge introductions in Palisade (2021), introduction of midges resulted in nearly eight times the number of galls in high water treatments [Midge / (Midge + Wasp – additive): ratio = 7.82, z-ratio = 2.640, p-value = 0.022; Midge / (Midge + Wasp – replacement): ratio = 15.04, z-ratio = 3.428, p-value = 0.002 – Figures 17A and 20A]. However, opposite trends were observed in low watering level, since higher number of midge galls were observed when midges and wasps were introduced to cages, regardless of in replacement or additive fashion [Midge / (Midge + Wasp – additive): ratio = 0.05, z-ratio = -3.648, p-value < 0.001; Midge / (Midge + Wasp – replacement): ratio = 0.13, z-ratio = -2.551, p-value = 0.029 – Figures 17A and 20A]. Similar patterns were observed in the second year in high watering level [Midge / (Midge + Wasp – replacement): ratio = 383.99, z-ratio = 3.634, p-value < 0.001 – figures 18A and 20A], but not in low [Midge / (Midge + Wasp – replacement): ratio = 0.2393, z-ratio = -1.049, p-value = 0.546; Midge / (Midge + Wasp – additive): ratio = 0.0541, z-ratio = -2.094, p-value = 0.091 – Figures 18A and 20A]. Additionally, in both years nearly 25 times the number of midge galls were observed in high watering level for midge only introductions (2021: High / Low: ratio = 24.44, z-ratio = 3.967, p-value < 0.001; 2022: High / Low: ratio = 24.69, z-ratio =

2.360, p-value = 0.018 – Figure 21), while low watering level led to higher number of galls when both insects were introduced in 2021 (Midge + Wasp – additive: High / Low: ratio = 0.16, z-ratio = -2.312, p-value = 0.021; Midge + Wasp – replacement: High / Low: ratio = 0.21, z-ratio = -1.987, p-value = 0.047 – Figure 21A) but only in replacement treatment in 2022 (Midge + Wasp – replacement: High / Low: ratio = 0.0154, z-ratio = -2.543, p-value = 0.011; Midge + Wasp – additive: High / Low: ratio = 0.2120, z-ratio = -1.159, p-value = 0.247 – Figure 21B). For wasp introductions, we did not see any treatment effects on wasp gall formation success in the first year (water treatment: df = 1, 54; F-value = 0.30; *p*-value = 0.589 – Figure 22A; insect treatment: df = 2, 53.73; F-value = 1.14; *p*-value = 0.326 – Figures 17B and 23A), likely because of low wasp establishment levels (Figure 18A). However, in the second year we see that low watering levels led to higher number of wasp galls [(High / Low): ratio = 0.227, z-ratio = -22.750, p-value < 0.001 – Figure 23B], in high watering level, we see that adding midges and wasps led to higher number of wasp galls in additive or replacement [(Midge + Wasp – additive) / Wasps only: ratio = 2.85; z-ratio = 6.456; *p*-value < 0.001; (Midge + Wasp – replacement) / Wasps only: ratio = 3.401; z-ratio = 7.689; *p*-value < 0.001 – figure 23B]. While running similar analyses for ARDEC data, no significant effects on midge gall analyses (water treatment: df = 1, 52.96; F-value = 0.62; *p*-value = 0.433; insect treatment: df = 2, 51.39; F-value = 0.97; *p*-value = 0.387 – Figures 19A, 24, and 25). We see that in high watering level, adding more wasps in addition to midges (additive treatment) led to higher number of wasp galls in comparison to replacement [(Midge + Wasp – additive) / (Midge + Wasp – replacement): ratio = 6.548, z-ratio = 2.815, p-value =

0.013 – Figure 19 and 26], but no differences were observed between replacement or additive treatments and wasps only.

To better understand how plant size was correlated with insect establishment success, we tested the correlation between plant parameters (biomass, percent cover, height of tallest stem, number of flowers, and number of stems) and number of insect galls (midge and wasp galls). In the Palisade common garden, high watering levels did not correlate with any plant-associated parameters (Tables 3 and 4). However, in low watering levels, we see that in 2021 – a year that midges did better than wasps – number of midge galls was directly correlated with number of stems (Pearson's correlation = 0.434 – Table 3). While in 2022 – a year that wasps did better than midges – number of wasp galls was directly associated with both height of tallest stem (Pearson's correlation = 0.490) and plant biomass (dry weight, Pearson's correlation = 0.463 – Table 4). At ARDEC in 2021, midge and wasp gall numbers are correlated with height of tallest stem regardless of the watering level (Tables 3 and 4). Additionally, in high watering levels, midge and wasp galls were positively associated with number of flowers (Pearson's correlation midge = 0.573, Pearson's correlation wasp = 0.578) and with the presence of the other insect (Pearson's correlation = 0.612). Midges were also correlated with number of stems (Pearson's correlation = 0.320) in high watering level. In low watering level, presence of midge galls was correlated with number of stems (Pearson's correlation = 0.577) and number of flowers (Pearson's correlation = 0.578). Additionally, height of tallest stem was correlated with both midge and wasp galls (Pearson's correlation midge = 0.470, Pearson's correlation wasp = 0.423 – tables 3 and 4).

Treatment effects on Russian knapweed fitness:

Compared to plants in the high water treatment, plants in the low water treatment did not grow as tall (High - Low: estimate = 5.65, t-ratio = 2.186, p-value = 0.030 – Figure 28) and had reduced number of stems (High/Low: ratio = 1.23, z-ratio = 2.415, p-value = 0.010 – Figure 29) in Palisade in the second year. Additionally, low watering levels led to reduction in plant biomass in Palisade (High – Low: estimate = 0.272, t-ratio = 2.084, p-value = 0.040 – Figure 31) and ARDEC (High – Low: estimate = 0.609, t-ratio = 3.013, p-value = 0.003 – Figure 32) in the first year. We did not analyze data from the ARDEC common garden in 2022. Severe winds dislodged many cages allowing midges and wasps to contaminate other treatment plots. Furthermore, Russian knapweed in many plots outgrew the cages and started to produce new shoots outside of the cages rendering our measures of biomass within the cage, height of the tallest stem, and the number of flowers difficult to interpret.

We did not find insect treatment effects plant growth in the first year in the Palisade garden, likely because of insect effects on plant fitness need to accumulate throughout years. When holding water and insect treatments constant, insect treatment was marginally significant in the second year (df = 1, 88.17; F = 4.78; p-value = 0.07). When averaging over each water treatment, we see that height of tallest stems in midge only treatments in high watering level was lower than treatments with midge and wasp-replacement (df = 89.7, t-ratio = -2.789, p-value = 0.049 – Figure 28), suggesting that the

presence of wasps may suppress midge effects on plant growth. Surprisingly, adding both insects, either in a replacement or cumulative fashion, did not impact plant growth compared to no insects regardless of location or watering level. Additionally, results of RGR analysis indicated that plants in low watering levels had lower growth rate (High – Low: estimate = 0.939, df = 1, 87.3, t-ratio = 2.271, p-value = 0.026 – Figure 30), but no insect treatment effects were observed.

## Discussion

Water levels shift plant-mediated interactions between insects, enhancing the establishment of biological control agents when introduced together. More specifically, low watering levels seem to promote synergistic interactions between insects, providing support for the Stress Gradient Hypothesis. High watering availability led to negative effects between the wasp and midge galls. Additionally, this study showed that interactions between water stress and presence of insects directly reduce the fitness of Russian knapweed during initial plant establishment (first year – 2021) and effects accumulate in subsequent year.

### Plant-mediated effects between water treatment and insect introductions:

Interactions between insects were asymmetric under conditions of high water availability, partially supporting our expectations. We hypothesized that in high watering level interactions between insects would be competitive (Stress Gradient Hypothesis,

Bertness & Callaway, 1994). For midge galls, our findings agreed with our expectations. The presence of wasps was associated with a lower number of midge galls in low stress scenarios (high watering level). However, where wasps had higher numbers of galls when midges were also present, regardless of stress scenario (high and low watering levels). Both insects were released in cages at the same time, and initial galls of midges and wasps were induced at the same time. However, different from wasps, midges are multivoltine, and able to complete at least three generations in one year. So in high water levels, plants may have been able to better respond to further gall formation. In another gall midge system, it has been reported that immatures of Hessian fly release a suite of proteins through salivary excretions and those proteins are responsible for reprogramming host tissue and formation of feeding sites (Chen et al., 2008; Shukle et al., 2009). The presence of these chemicals and formation of feeding sites for midges might also be beneficial for wasps, since there is a positive effect of presence of midges. However, wasps might compete for resources or trigger other types of responses that have a negative effect on the number of midge galls. In a microbiome investigation (Chapter 1), we found that Russian knapweed roots from wasp-associated sites had lower microbiome richness and diversity, as well as lowest abundance of taxa associated with plant response to herbivory, indicating that wasps trigger whole plant resistance mechanisms that might reduce root colonization (Franco et al. *unpublished data*). Such mechanisms could also have a negative effect on gall formation or non-preference by female midges. Observed results align with findings in the tall goldenrod system. Tall goldenrod (*Solidago altissima*) can perceive sex pheromones from males of the gall fly *Eurosta solidaginis* and respond by producing induced defenses

mechanisms that can be perceived by females of *E. solidaginis* and lead to non-preference, as well as enhanced plant response mechanisms to subsequent herbivory (Helms et al., 2013).

At low watering levels, our findings agree with the expectations from the Stress Gradient Hypothesis, where stressful environments lead to positive associations between interacting species (Bertness and Callaway, 1994). The nature of the relationship between gall forming insects shifted from competitive in high watering treatments to facilitative under water stress. More specifically, both midges and wasps benefitted from the presence of one another, leading to a higher number of galls in conditions of water stress. Plants commonly increase constitutive resistance traits, such as thickened cell walls, when under abiotic stress making it a poor host for herbivores (Saska et al., 2023; Valim et al., 2016). However, we saw that low watering level had higher numbers of midge and wasp galls compared to high watering level when both insects were introduced to the Palisade garden. These findings suggest that insects had synergistic effects when present together while Russian knapweed was under water stress. Additionally, water stress facilitated gall formation of insects, possibly through reduction of plant defense mechanisms or synthesis of anti-herbivory chemicals. The patterns observed in our study are similar to those observed in Brassicaceae systems (Kuczyk et al., 2021; Valim et al., 2016). The total amount of glucosinolates, an anti-herbivore chemical, in *Brassica oleracea* var *acephala*, occurred at much lower concentrations in plants cultivated under water stress (Valim et al., 2016). Similar patterns have been observed in studies testing the effects of water and temperature on *Sinapis alba* (Kuczyk et al., 2021).

Wasp galls were frequently positively correlated with height of tallest stem, while midge galls are frequently positively correlated with number of stems. These findings corroborate with observations of oviposition strategies of midges and wasps (Franco, G.M. and Folks, C.C., *personal observation*). Wasps seem to find a suitable stem and lay several eggs throughout the same stem, therefore, longer stems lead to more available oviposition sites. While midges seem to prefer laying eggs on apical meristems, and therefore, increased number of stems leads to more apical meristems, which means more oviposition sites. However, midges can also lay eggs on lateral meristems, and this could be a plausible explanation for number of midge galls also be correlated with height of tallest stem at ARDEC. Additionally, at low watering levels, the number of wasp galls was positively correlated with plant biomass, suggesting that bigger plants provided more opportunities of gall formation for wasps. Similar trends were not observed for high watering levels.

Treatment impacts on Russian knapweed fitness:

Water availability is a key factor driving growth and reproduction of several plant species; therefore, plants have developed distinct strategies to cope with water deficiency. Changes in root structure, reduction of aboveground biomass, and shifts in metabolic processes are common adaptations to water stress (Gupta et al., 2020). In the case of Russian knapweed, we saw that low watering levels reduced plant biomass in the first year after plant establishment (2021). Additionally, reduced water availability also reduced the

relative growth rate (RGR) of Russian knapweed, suggesting slower establishment while under water stress. Several parameters need to be further investigated to better understand the impacts of drought on invasive species. For instance, the invasive spotted knapweed (*Centaurea stoebe*) had lower biomass when cultivated in dryer conditions (Bunn et al., 2023); however, when competition with a native grass was assessed in interaction with watering level and soil microbiome, spotted knapweed benefitted from dry conditions due to lower abundance of potential pathogens targeting spotted knapweed and higher targeting the native competitor (Bunn et al., 2023). Therefore, to better understand how plant fitness reflects the competitive success of invasive plants, additional interactions should be taken into consideration.

Insect treatments also impacted the growth of Russian knapweed in the second year in high watering level at Palisade garden, possibly due to accumulation of stress. For instance, we observed a reduction of height of tallest stem and number of stems in the low water availability treatment in the Palisade garden in the second year (2022). Additionally, we believe that we would see differences in growth rate in low watering level due to an interaction between insect establishment and water availability if we were able to continue the study for another year.

In conclusion, we see that water availability is a key aspect while investigating the interaction between Russian knapweed and the biological control agents. Low watering levels led to reduction of plant fitness and increased number of insect galls when introduced together. However, no significant effects of insect establishment were observed after two years of experiment in low watering level, but our findings indicate that longer

term impacts of insects could be present. In high watering level, the addition of midges alone led to negative impacts on Russian knapweed fitness, but adding wasps in addition to midges negated the midge impacts on Russian knapweed growth. Therefore, we conclude that adding both insects in dryer areas would be beneficial for managing Russian knapweed, but in more humid areas, adding midges only might lead to higher suppression of plant growth.

Figures and Tables:

Table 2: Total precipitation in mm in each location and each year. Data from the Colorado Agricultural Meteorological Network (CoAgMet).

| | Palisade | ARDEC |
|---|---|---|
| Total Precipitation 2021 | 148.7mm | 197.4mm |
| Total Precipitation 2022 | 176.5mm | 202.8mm |

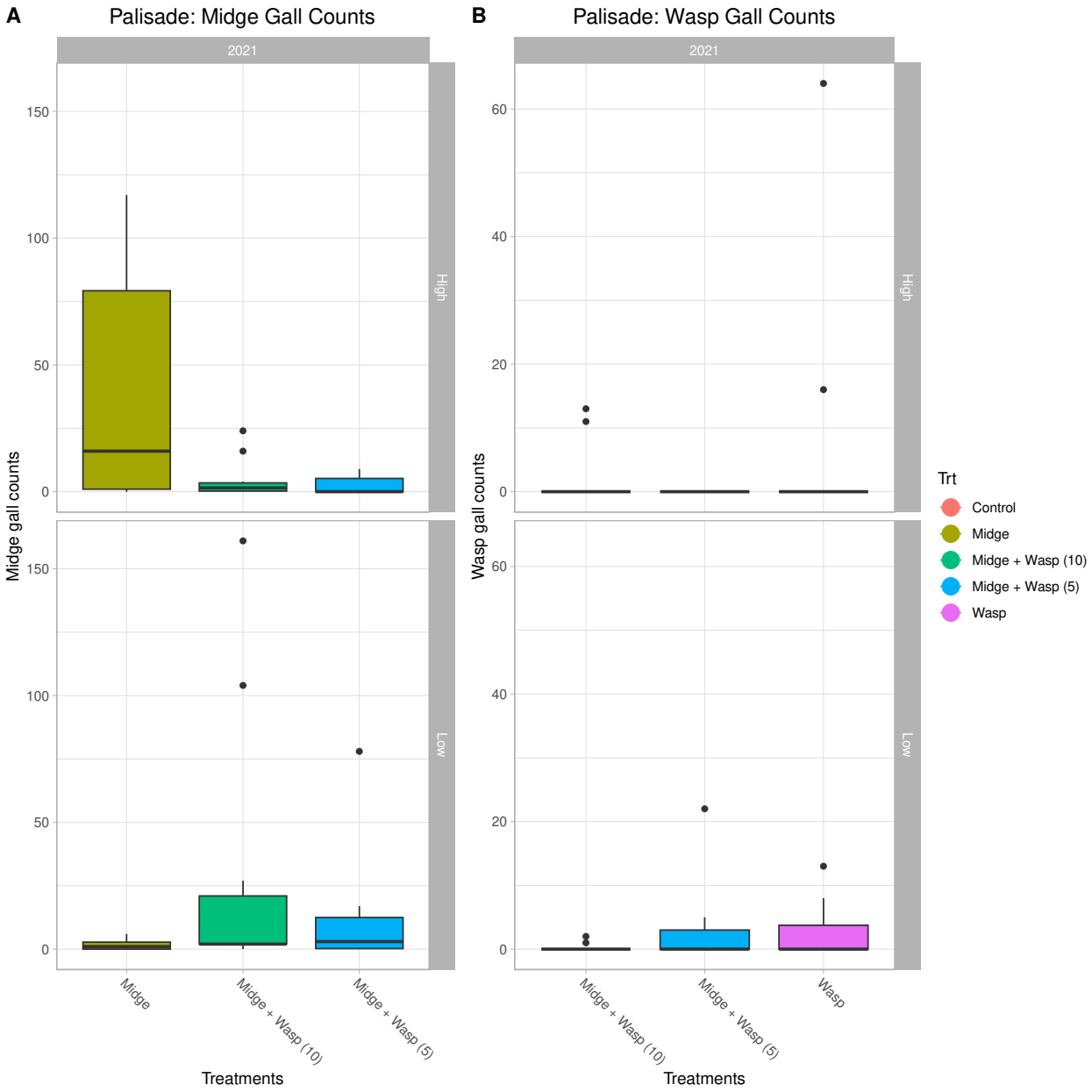



Figure 17: Number of midge (A) and wasp (B) galls in Palisade in the first year (2021). A: Overall number of midge galls were higher in high watering levels and in treatments where midges only were introduced, suggesting an antagonistic effect between midges and wasps in high watering levels [Midge / (Midge + Wasp – additive): ratio = 7.82, z-ratio = 2.640, p-value = 0.022; Midge / (Midge + Wasp – replacement): ratio = 15.04, z-ratio = 3.428, p-value = 0.002]. However, opposite trends were observed when looking at low watering levels, where addition of both insects led to higher counts of midge galls [Midge / (Midge + Wasp – additive): ratio = 0.05, z-ratio = -3.648, p-value < 0.001; Midge / (Midge + Wasp – replacement): ratio = 0.13, z-ratio = -2.551, p-value = 0.029]. B: Establishment of wasp galls

was low in 2021 due to known factors and no wasp establishment differences were observed (df = 2, 53.73; F-value = 1.14; *p*-value = 0.326).

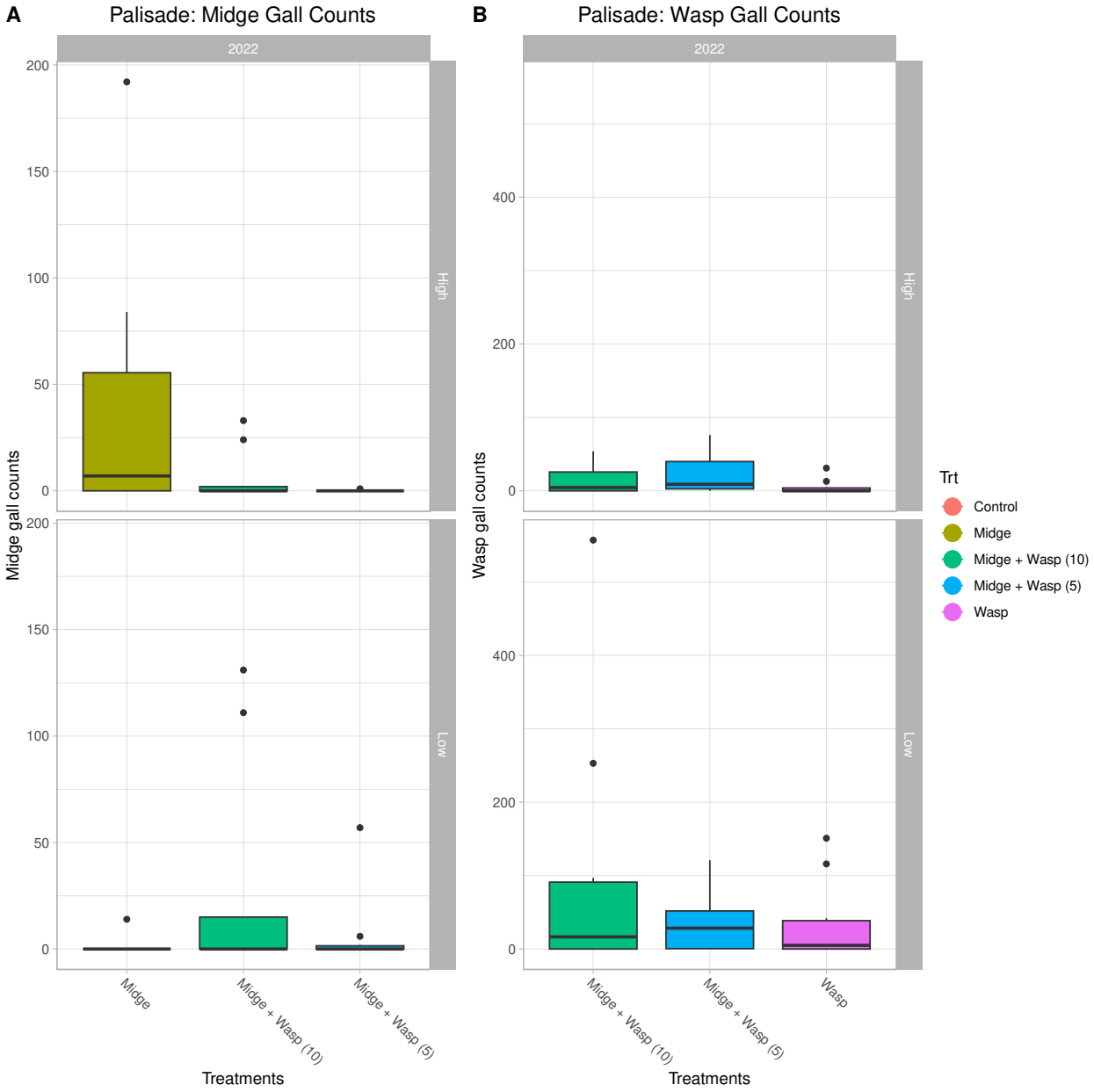



Figure 18: Number of midge (A) and wasp (B) galls in Palisade in the second year (2022). A: Year 2 findings (2022) were similar to year 1 (2021) for high watering levels but not low. Treatments where midges only were added in high watering level led to higher number of midge galls compared to midges and wasp – replacement [Midge / (Midge + Wasp – replacement): ratio = 383.99, z-ratio = 3.634, p-value < 0.001]. Number of midge galls did not change between insect treatments [Midge / (Midge + Wasp – replacement): ratio = 0.2393, z-ratio = -1.049, p-value = 0.546; Midge / (Midge + Wasp – additive): ratio = 0.0541,

z-ratio = -2.094, p-value = 0.091]. B: Addition to midges and wasps led to higher numbers of wasp galls in high watering levels [(Midge + Wasp – additive) / Wasps only: ratio = 2.85; z-ratio = 6.456; *p*-value < 0.001; (Midge + Wasp – replacement) / Wasps only: ratio = 3.401; z-ratio = 7.689; *p*-value < 0.001] and low [(Midge + Wasp – additive) / Wasps only: ratio = 3.734; z-ratio = 16.895; *p*-value < 0.001; (Midge + Wasp – replacement) / Wasps only: ratio = 1.528; z-ratio = 4.738; *p*-value < 0.001)], suggesting that the presence of midges is beneficial for wasps. Additionally, differences in number of wasps introduced were also observed, where the more wasps, more galls [(Midge + Wasp – additive) / (Midge + Wasp – replacement): ratio = 2.444; z-ratio = 14.158; *p*-value < 0.001].

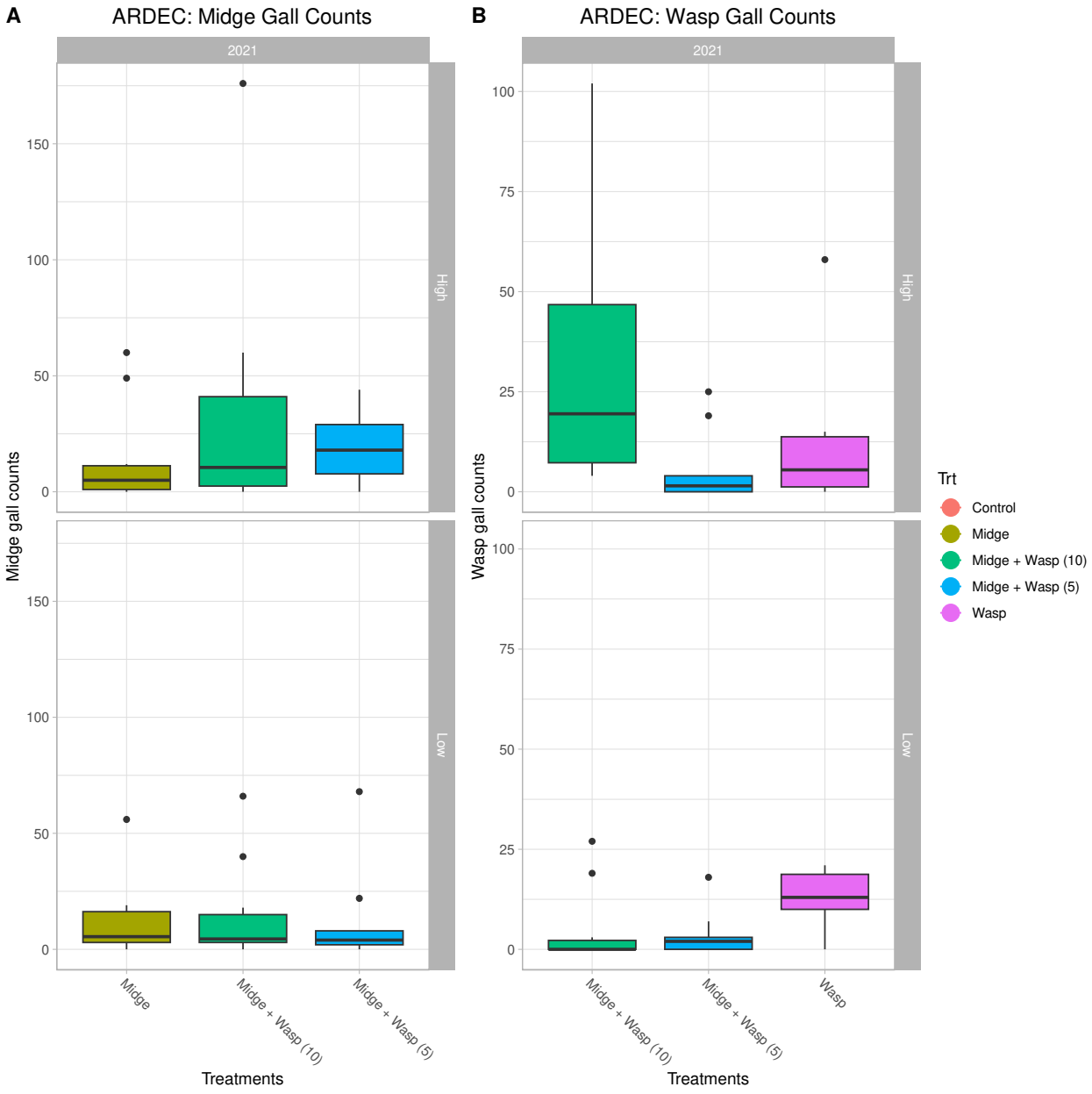



Figure 19: Number of midge (A) and wasp (B) galls in the first year (2021) at ARDEC. A: No differences were observed when comparing the number of midge galls between insect introductions in high or low watering levels (water treatment: df = 1, 52.96; F-value = 0.62; *p*-value = 0.433; insect treatment: df = 2, 51.39; F-value = 0.97; *p*-value = 0.387). B: For wasp galls in high water level, we see that adding both midges and wasps in additive manner led to higher number of wasp galls compared to replacement [(Midge + Wasp – additive) / (Midge + Wasp – replacement): ratio = 6.548, z-ratio = 2.815, p-value = 0.013], but no differences between wasps only or midges and wasps were observed. No

differences in number of wasp galls were observed in low watering level.

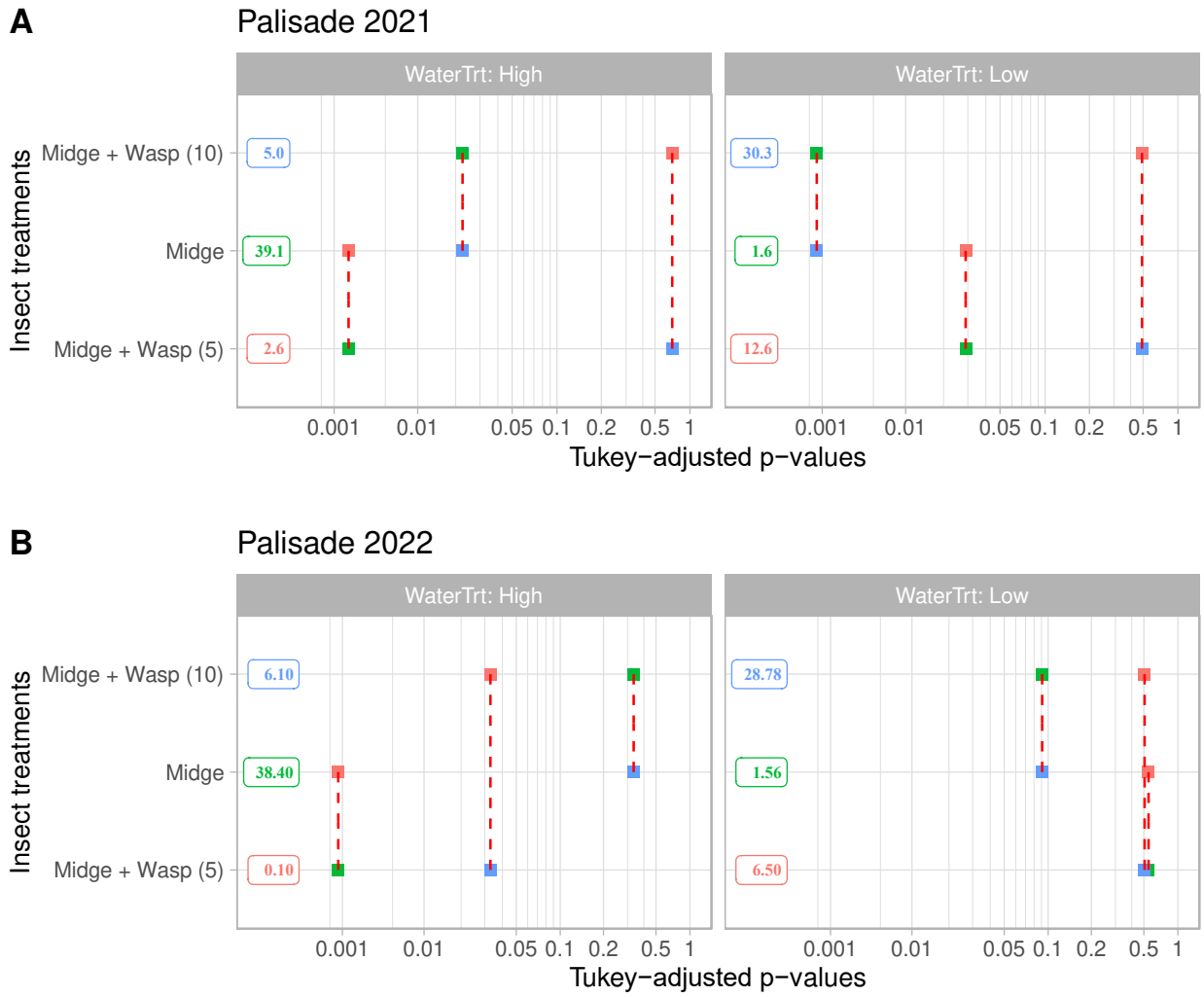



Figure 20: Comparison between number of midge galls in treatments that midges, midges and wasps – additive (midges + wasps 10), and midges and wasps replacement (midges + wasps 5) were established per watering level in the first (2021) and second year (2022).

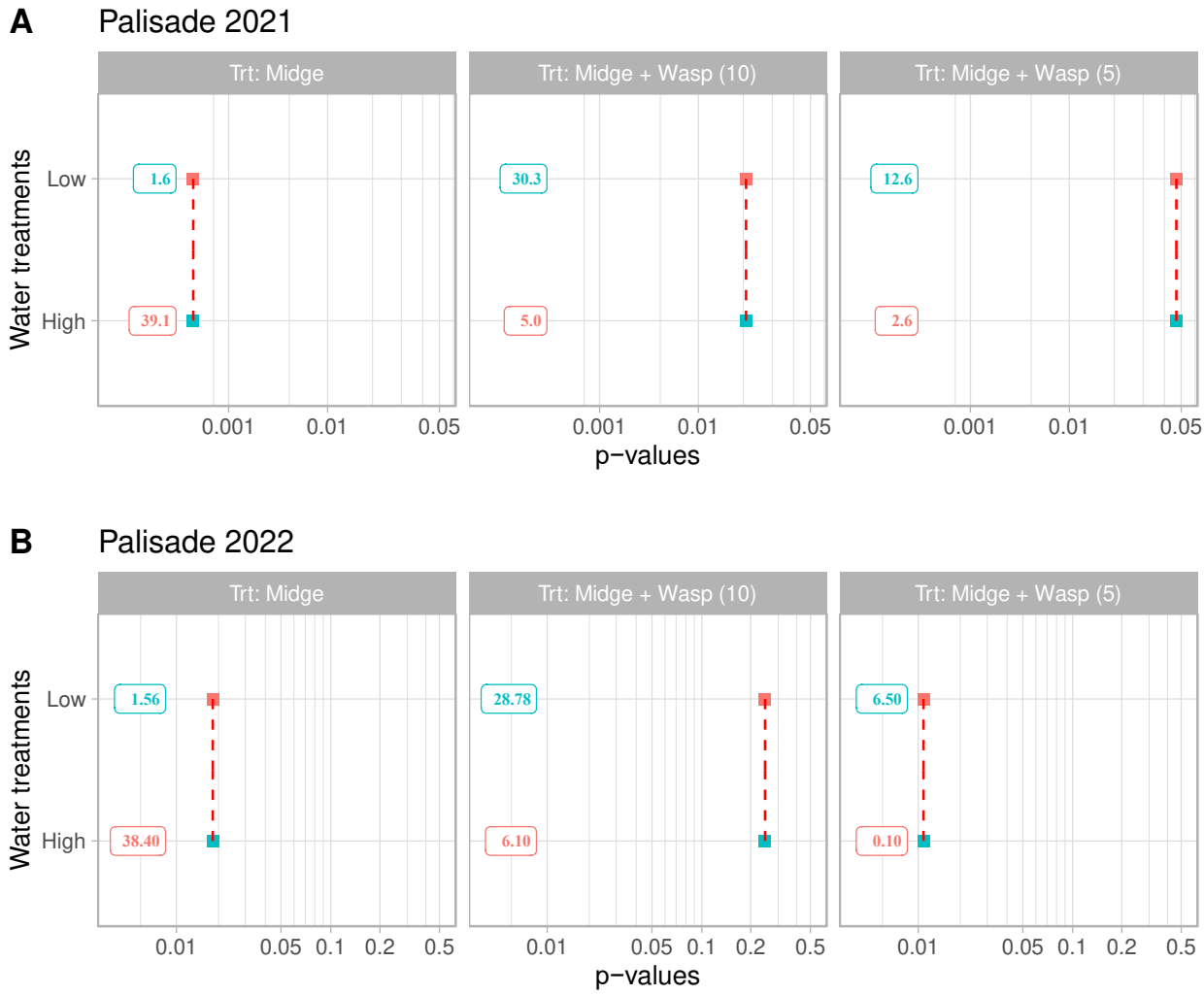



Figure 21: Comparisons between number of midge galls in distinct watering levels within each treatment that midge galls were present (midges only, midges and wasps – additive (midges + wasps 10), and midges and wasps replacement (midges + wasps 5)) in the first (2021) and second year (2022).

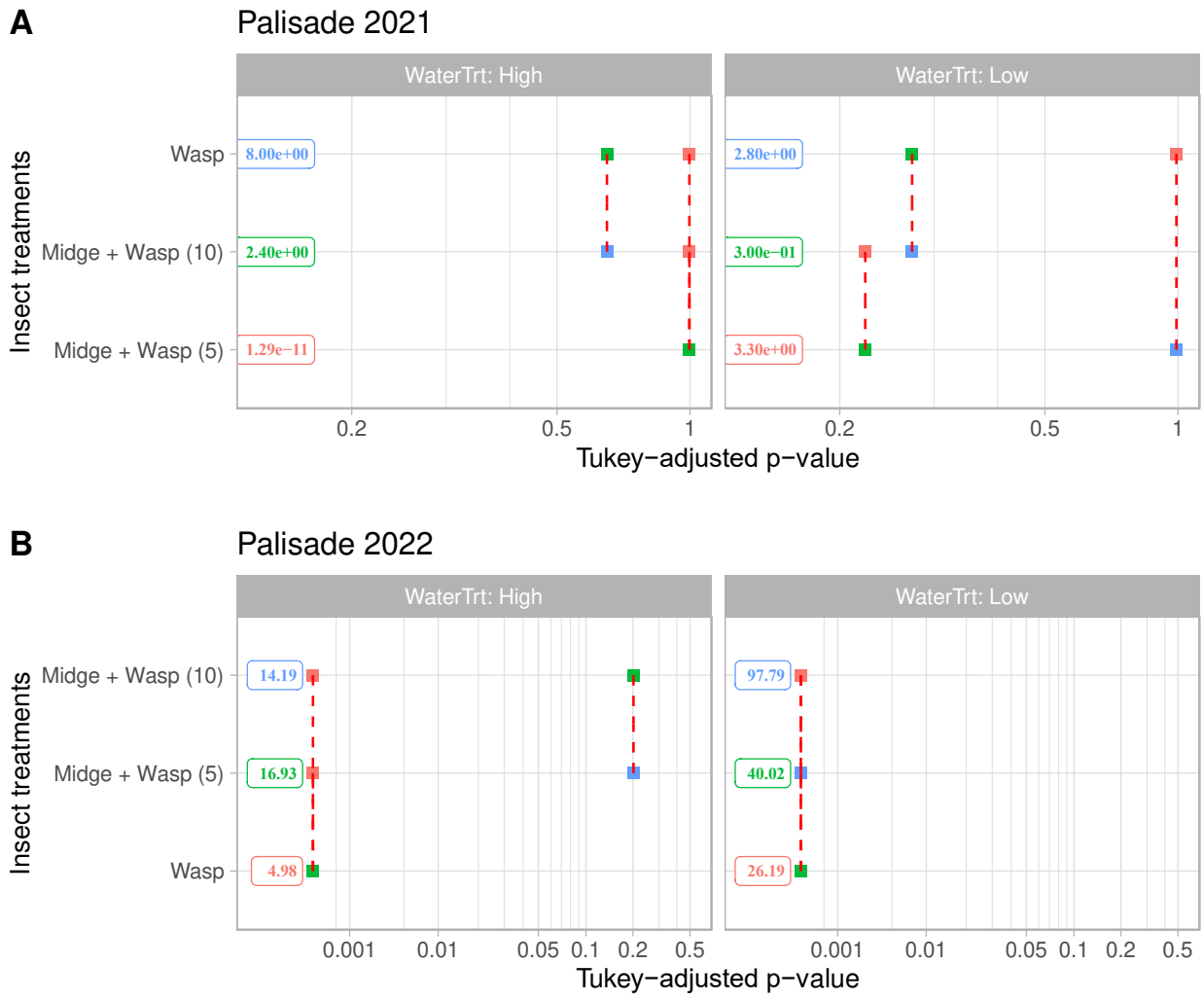



Figure 22: Comparison between number of wasp galls in treatments that wasps, midges and wasps – additive (midges + wasps 10), and midges and wasps replacement (midges + wasps 5) were established per watering level in the first (2021) and second year (2022).

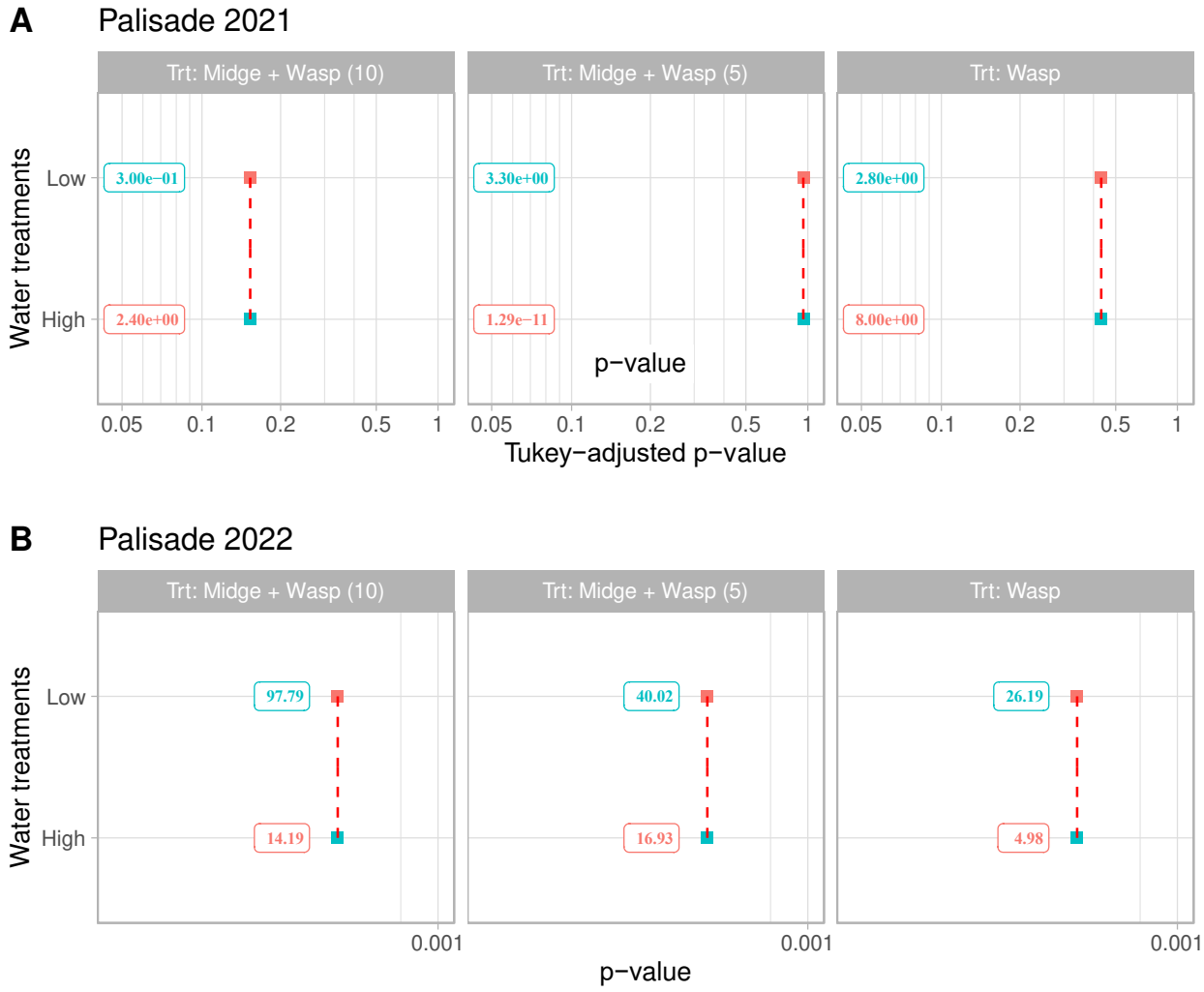



Figure 23: Comparisons between number of wasp galls in distinct watering levels within each treatment that wasp galls were present (wasps only, midges and wasps – additive (midges + wasps 10), and midges and wasps replacement (midges + wasps 5)) in the first (2021) and second year (2022).

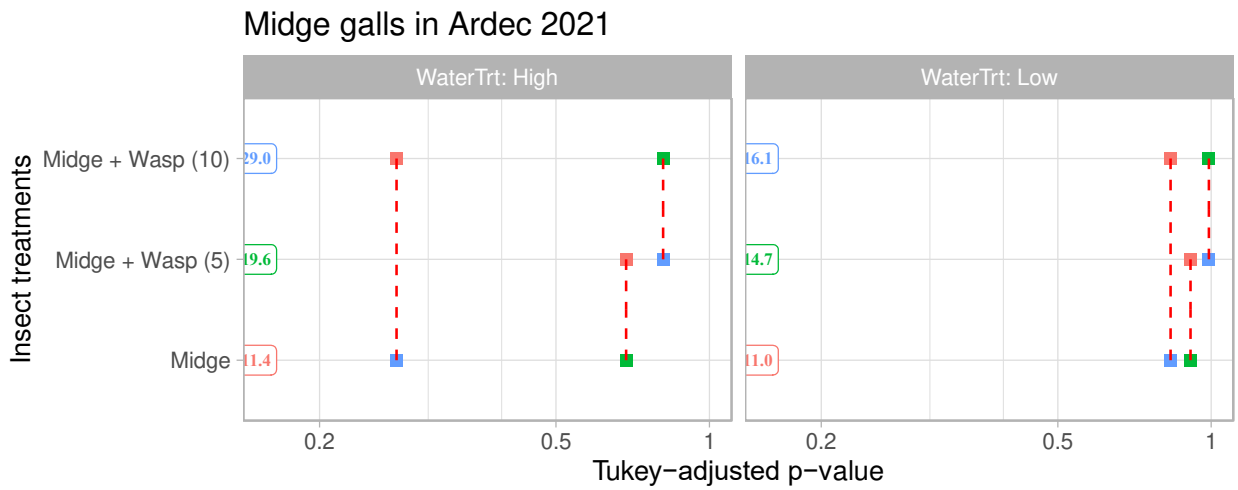



Figure 24: Comparison between number of midge galls in treatments that midges, midges and wasps – additive (midges + wasps 10), and midges and wasps replacement (midges + wasps 5) were established per watering level in the first year (2021).

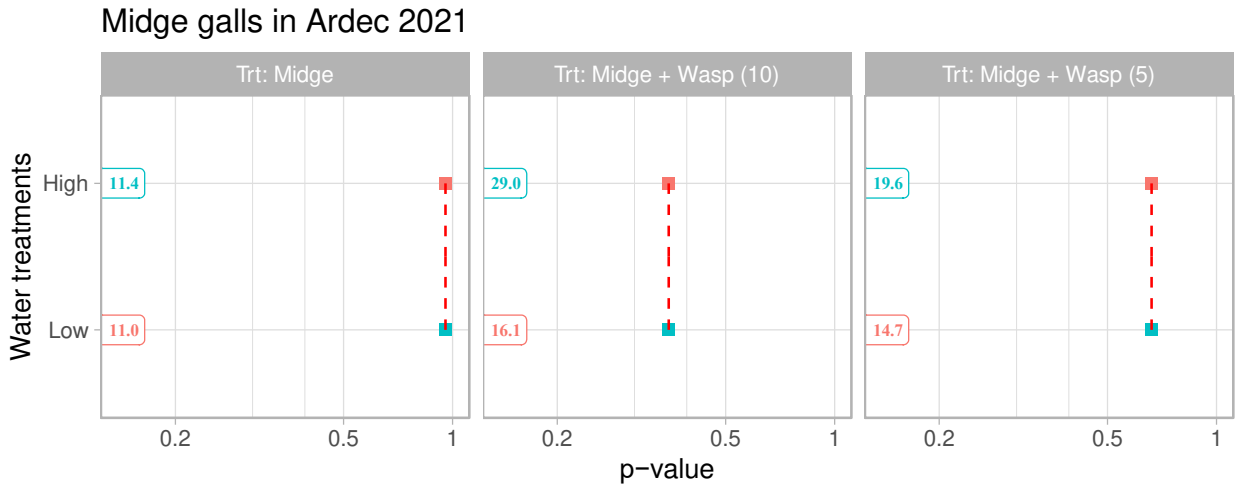



Figure 25: Comparisons between number of midge galls in distinct watering levels within each treatment that midge galls were present (midges only, midges and wasps – additive (midges + wasps 10), and midges and wasps replacement (midges + wasps 5)) in the first year (2021).

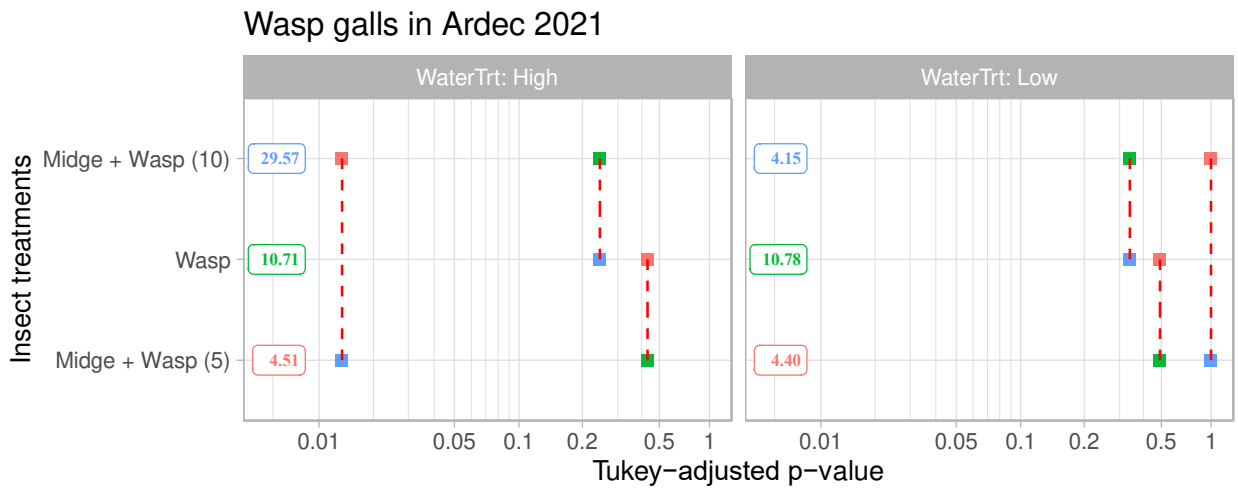



Figure 26: Comparison between number of wasp galls in treatments that wasps, midges and wasps – additive (midges + wasps 10), and midges and wasps replacement (midges + wasps 5) were established per watering level in the first year (2021).

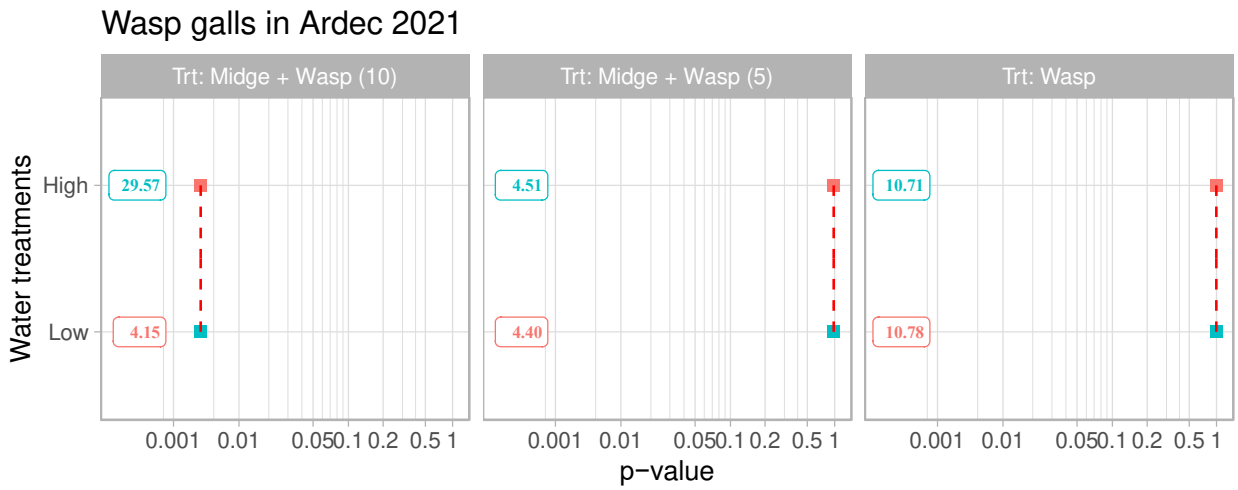



Figure 27: Comparisons between number of wasp galls in distinct watering levels within each treatment that wasp galls were present (wasps only, midges and wasps – additive (midges + wasps 10), and midges and wasps replacement (midges + wasps 5)) in the first year (2021).

Table 3: Correlation between number of midge galls and plant parameters in each location and year.

| Midge | Palisade | | | | ARDEC | |
|---|---|---|---|---|---|---|
| Year | 2021 | | 2022 | | 2021 | |
| Water level | High | Low | High | Low | High | Low |
| Tallest Stem | -0.120 | -0.077 | -0.145 | -0.266 | 0.404* | 0.470* |

| | | | | | | |
|---|---|---|---|---|---|---|
| Stem Number | -0.048 | 0.434* | 0.099 | 0.181 | 0.320* | 0.577* |
| Flower Number | -0.153 | 0.16 | 0.271 | -0.125 | 0.573* | 0.548* |
| Biomass | -0.146 | 0.161 | -0.107 | -0.192 | 0.160 | 0.535* |

Table 4: Correlation between number of wasp galls and plant parameters in each location and year.

| Wasp | Palisade | | | | ARDEC | |
|---|---|---|---|---|---|---|
| Year | 2021 | | 2022 | | 2021 | |
| Water level | High | Low | High | Low | High | Low |
| Tallest Stem | -0.032 | -0.075 | 0.171 | 0.490* | 0.323* | 0.423* |
| Stem Number | 0.171 | -0.224 | -0.085 | -0.061 | 0.277 | 0 |
| Flower Number | 0.115 | -0.105 | -0.37 | -0.048 | 0.578* | -0.067 |
| Biomass | 0.036 | -0.238 | 0.170 | 0.463* | 0.186 | 0.463* |

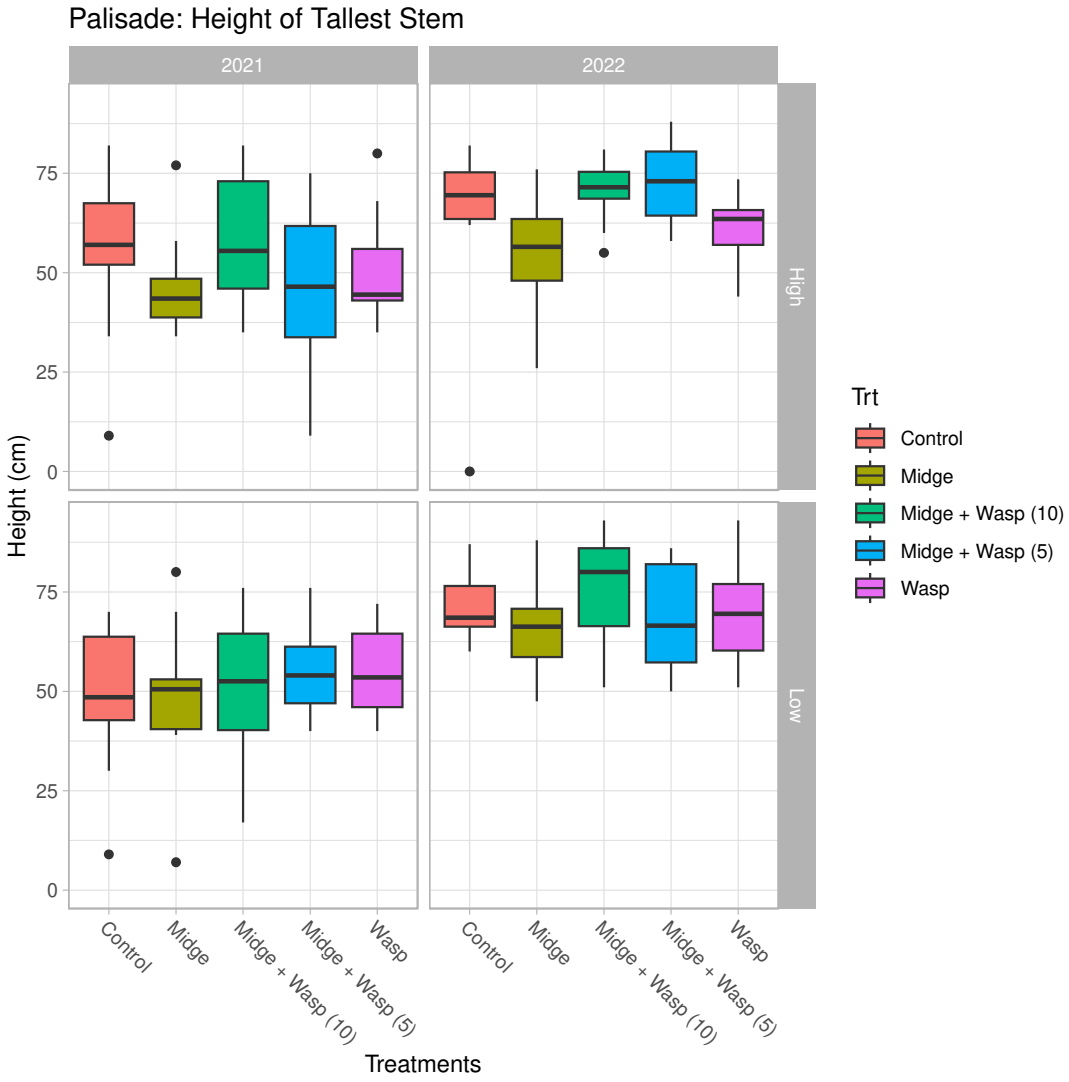



Figure 28: Height of tallest stems in each cage for the Palisade garden in both years and water treatments. Statistical differences were observed when comparing stem height between water treatments in the second year (2022). Where, High – Low: estimate = -5.65, t-ratio = -2.186, p-value = 0.03. Additionally, plants in midge only treatments presented shorted stems compared to midge and wasp – additive [Midge – (Midge and Wasp – additive): estimate = -11.37, t-ratio = -2.747, p-value = 0.05].

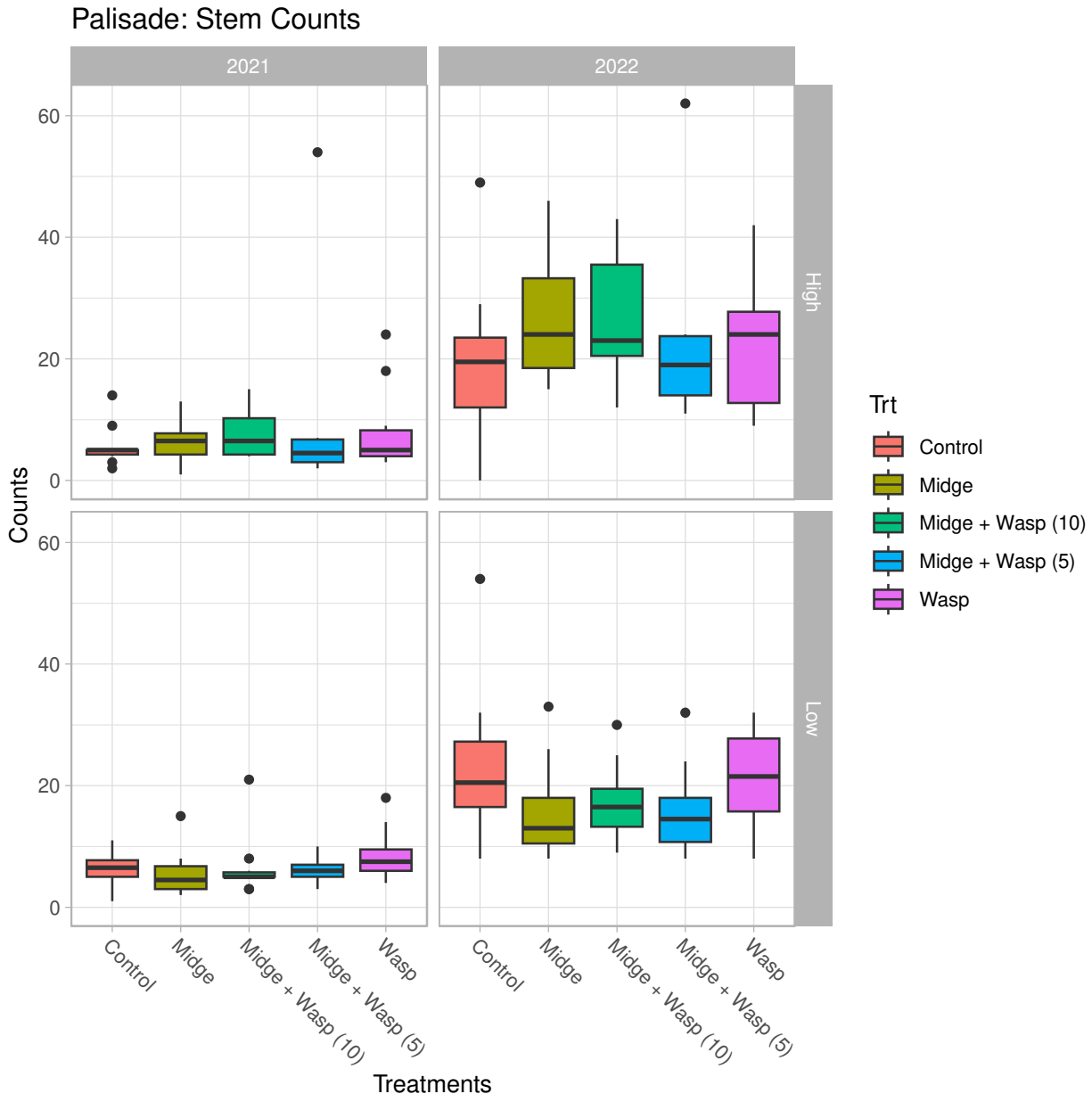



Figure 29: Number of stems in each year, water level, and insect treatments in Palisade garden. Statistical differences were observed when comparing the number of stems in the second year when comparing high and low watering levels (High/Low: ratio = 1.23, z-ratio = 2.415, p-value = 0.01).

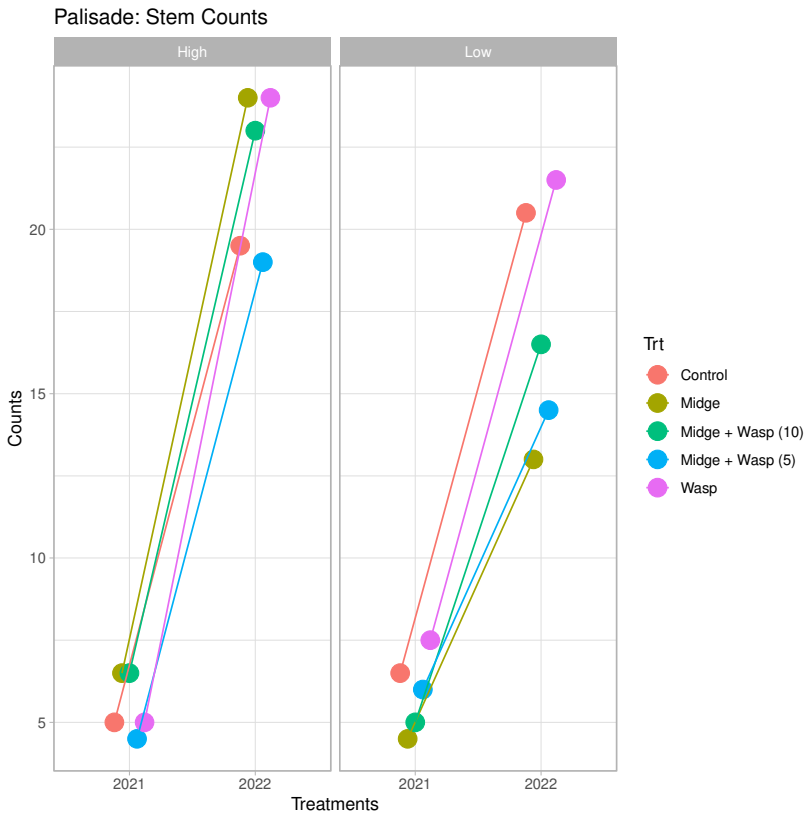



Figure 30: Number of stems in high and low watering levels in Palisade in both years. Low water level had lower stem growth rate compared to high water level (High – Low: estimate = 0.939, df = 87.3, t-ratio = 2.271, p-value = 0.0256).

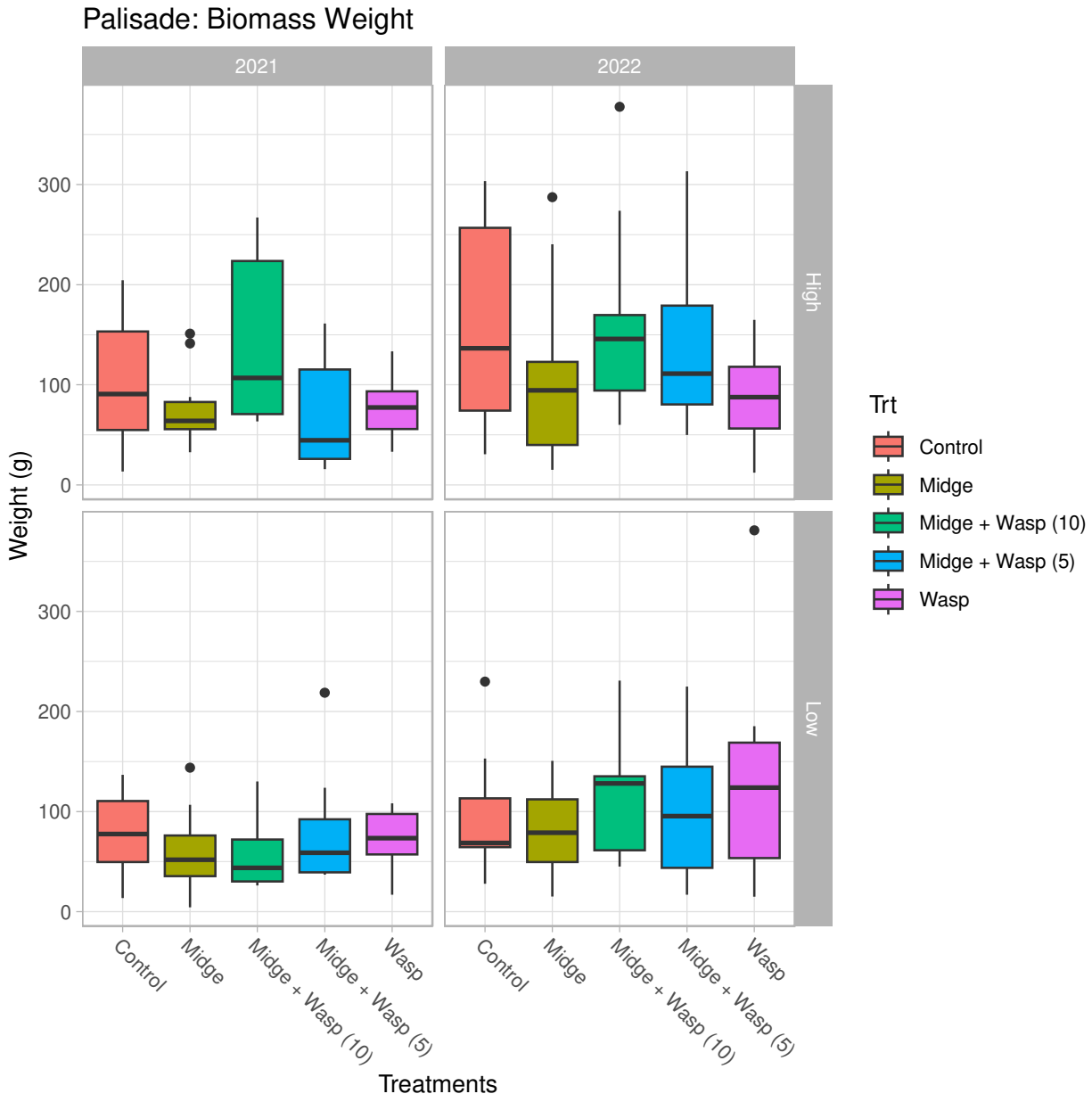



Figure 31: Biomass of Russian knapweed in each year, water level, and insect treatments in Palisade garden. Statistical differences were observed when comparing the biomass in the first year when comparing high and low watering levels (High – Low: estimate = 0.272, t-ratio = 2.084, p-value = 0.040), but no differences were observed in the second year (High – Low: estimate = 0.196, t-ratio = 1.521, p-value = 0.1319).

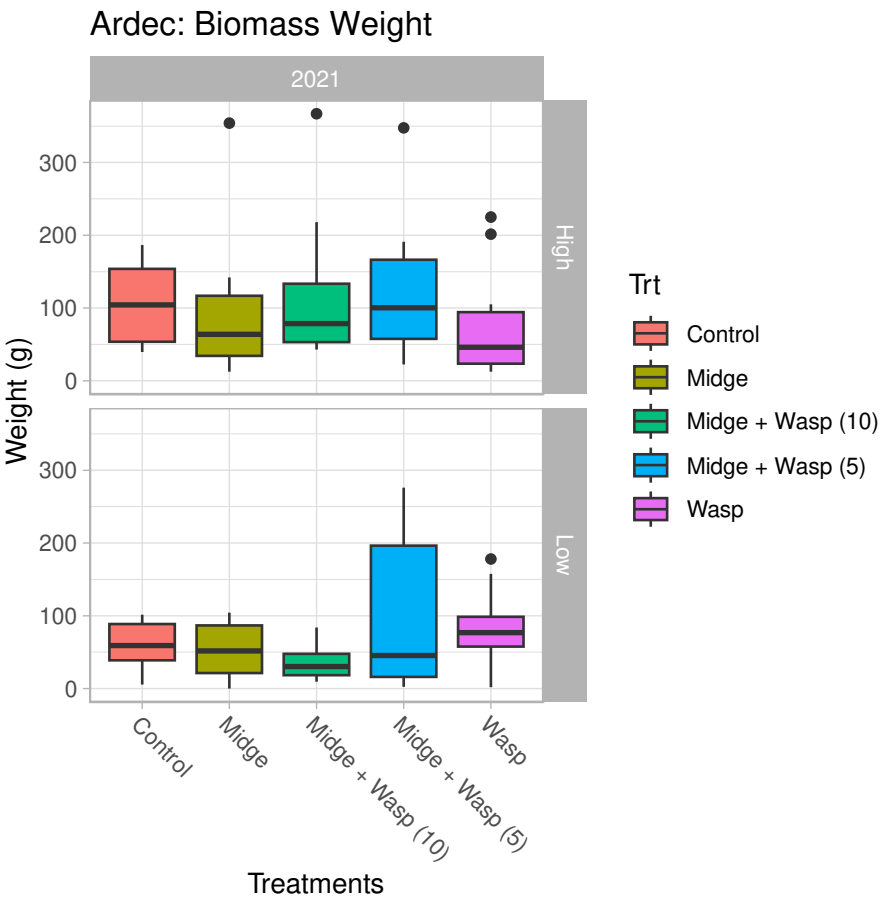



Figure 32: Biomass of Russian knapweed in each water level and insect treatments in ARDEC garden. Statistical differences were observed when comparing the biomass in the first year when comparing high and low watering levels (High – Low: estimate = 0.609, t-ratio = 3.013, p-value = 0.003).

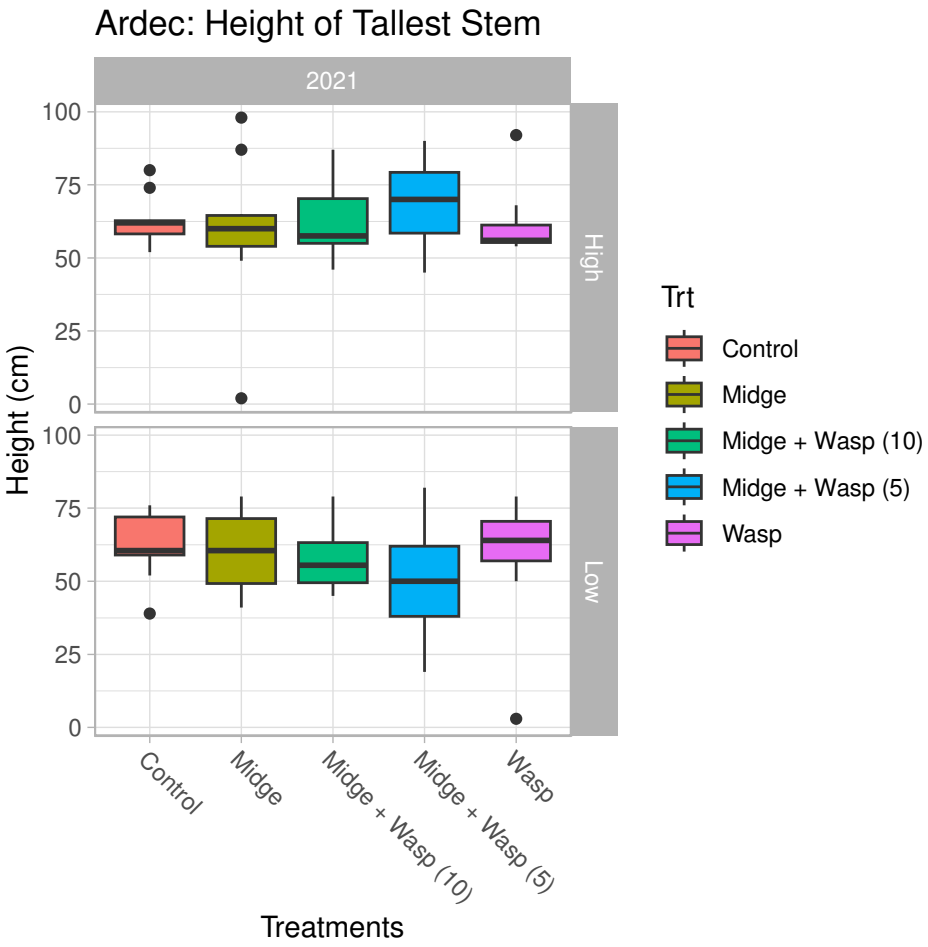



Figure 33: Height of tallest stems in each cage for the ARDEC garden in the first year for distinct water and insect treatments. No statistical differences were observed when comparing stem height between water treatments or insect treatments.

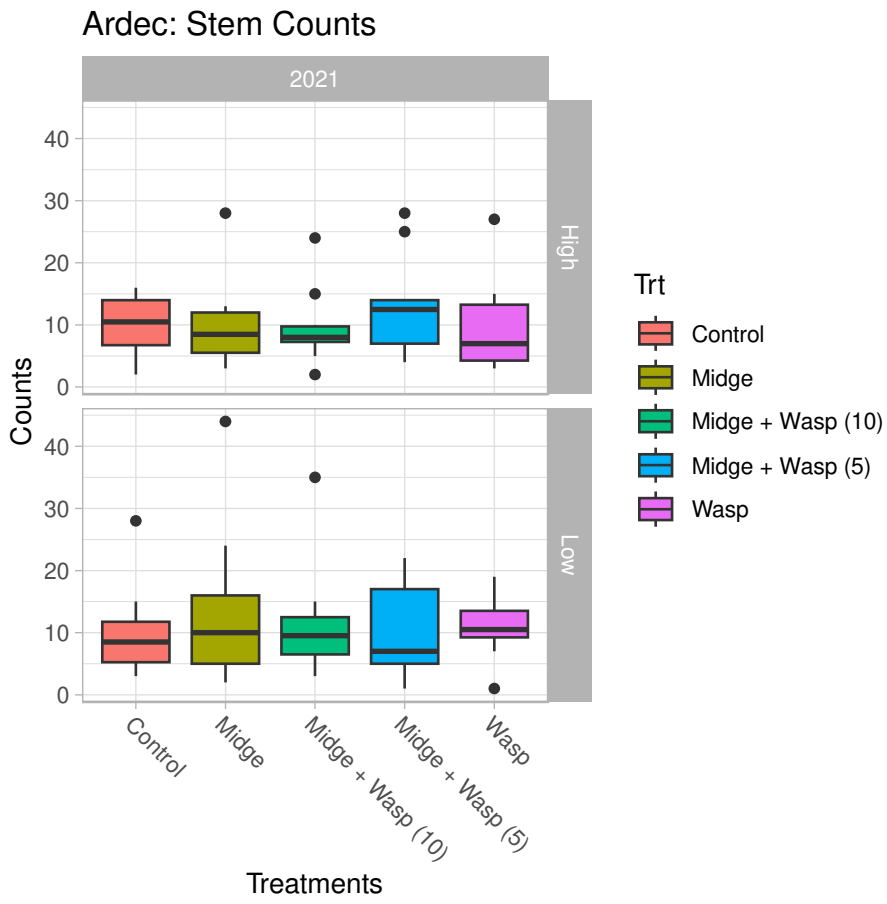



Figure 34: Number of stems in the first year for each water level and insect treatments in ARDEC garden. No statistical differences were observed when comparing the number of stems between treatment combinations.

# CHAPTER 4: Microbiomes associated with Russian knapweed in garden experiment

## Introduction

Once plants establish in new areas, interactions with local microorganisms can determine the plant's resilience to biotic and abiotic stressors. Beneficial associations between invasive plants and mycorrhizal fungi can facilitate invasive species' range expansion (Clavel et al., 2021), since mycorrhizal fungi can provide nutrients (mostly nitrogen and phosphorus), help with water retention in rhizosphere region, and reduce the abundance of pathogenic organisms (reviewed by Tedersoo et al., 2020). For instance, the invasive spotted knapweed *Centaurea stoebe* forms associations with arbuscular mycorrhizal fungi (AMF), and which can ameliorate drought stress (Bunn et al., 2023). Nitrogen cycling bacteria can also prove beneficial to invasive plants through increasing the availability of nitrogen, a frequent limiting resource impacting plant growth. The Sonoran Desert buffelgrass (*Pennisetum ciliare*) consistently recruits taxa that assist with nitrogen availability (Jara-Servin et al., 2023). Additionally, while interactions with potential pathogens could decrease plant fitness, it could also provide a competitive advantage against native plant species, since those pathogens likely co-evolved with native plants and might prefer them as hosts (Colautti et al., 2004; Dukes et al., 2019). Plant populations can also adjust to distinct levels of abiotic stress and form associations with symbiotic microorganisms (Pieterse et al., 2016). Such microbial associations should be considered while investigating range expansion of invasive plants.

Plant-microbiomes interactions can also improve resilience against subsequent disturbances, such as abiotic stresses and herbivory. Drought may shift plant-microbiome interactions (Trivedi et al., 2022) and change plant-mediated effects between microbiomes and insect herbivores. Associations with fungal guilds, such as mycorrhizal fungi, can shift plant responses to herbivory and ameliorate drought effects. Arbuscular mycorrhizal fungi can change plant response to herbivory by enhancing plant growth through increased nutrient acquisition, as well as increasing constitutive and induced resistance mechanisms (reviewed by Frew et al., 2022). In turn, herbivory can shift plant resource allocation and changing composition of root exudates, which enhances mycorrhizal fungi colonization (Xing et al., 2024). Additionally, associations between plants and mycorrhizal fungi can be enhanced under low water availability conditions (Bunn et al., 2023). Therefore, associations between plants and microbiomes change in response to drought and herbivory. Implications of such shifts need to be further investigated.

Classical biological control agents are frequently introduced to decrease the performance and competitive ability of invasive plants. However, relationships established between invasive plants and local microbiomes are frequently different from the relationships these plants have with microbiomes in their native range (Lu-Irving et al., 2019) and could impact plant response to biological control agents. While such changes in plant-microbiome interactions may have significant consequences for the success of biological control efforts, microbiome-mediated interactions between invasive plants and their classical biological control agents are understudied. Additionally, introductions of herbivores changes stress levels of plants and therefore reshape plant-associated

microbiomes through resource reallocation (Kong et al., 2016; Malacrinò et al., 2021). Studies investigating interactions between microbiomes, plants, and their herbivores are still in their infancy and further investigation is needed to understand how such interactions could be applied to management of invasive plants.

In this study, we explore the effects of herbivory and soil moisture availability on the interactions between soil microbiomes and the Russian knapweed (*Rhaponticum repens*). Russian knapweed is an invasive noxious weed commonly found throughout the western United States under a wide range of soil moisture conditions. Once it establishes in an area, Russian knapweed forms dense patches that suppress the growth of other plant species (Gaskin and Littlefield, 2017). Two biological control agents were introduced to manage Russian knapweed: a gall midge (Diptera: Cecidomyiidae: *Jaapiella ivannikovi*) and a gall wasp (Hymenoptera: Cynipidae: *Aulacidea acroptilonica*). Both biological control agents lead to gall formation on plant tissues and reduce plant growth in the native range (Djamankulova et al., 2008). However, establishment success of biological control agents is variable across water availability ranges. We hypothesize that adaptations to drought tolerance, such as associations with microbiomes, could play an important role in plant response to biotic and abiotic stress. To test our predictions, we established a garden experiment to explore the interaction between water availability and herbivory and their effects on knapweed growth and reproduction. This experiment was conducted in Palisade – Colorado, an area with low rainfall. We established two drought amelioration treatments by irrigating half of the plots in high and low frequency. We hypothesized that Russian knapweed would form associations with drought tolerant bacterial and fungal taxa to assist

with stress amelioration at low water availability, while high water availability would lead to shifts in microbiome community against taxa associated with drought. We also hypothesized that associations with stress amelioration taxa in low watering level would affect the establishment of biological control agents; however, the direction of such interaction could either enhance or decline the establishment of biological control agents. The results of this experiment will help our understanding of the role of microbiomes on mechanisms of plant response to drought and herbivory.

## Methods

### Sample collection:

A common garden was setup to evaluate the long-term effects of the two gall-forming biological control agents and two watering levels (high and low) on four populations of Russian knapweed. Fifty knapweed plants were planted in two plots (100 plants total) and in each plot was established a distinct watering regime (high or low). Cages (0.5*0.5*1.0m, W*L*H) were placed on each plant to prevent outside herbivory and contain desired insect treatments. Insect treatments consisted of midges or wasps only, midges and wasps additive or replacement, and no insects, making a total of five insect treatments with 10 replicates per watering level. The garden was located at the Insectary of the Colorado Department of Agriculture in Palisade, Colorado and was maintained for three years. Where at the end of each year, aboveground biomass was removed, and in the beginning of each growth season, insect treatments were re-established. In year 2, we

collected six bulk soil samples from each watering level and rhizosphere samples from plants that had midge-only (high = 6 cages, low = 4 cages), wasp-only (high = 6 cages, low = 7 cages), no insects (high = 6 cages, low = 5 cages), and both insects (high = 5, low = 5) in the second year from both watering levels. Number of samples were limited by insect establishment in second year. Rhizosphere soils were collected by digging a hole next to Russian knapweed plants following the roots until approximately 0.1m-deep and soil in contact with roots were collected with a collection tube (5mL). Samples were kept on dry ice and then placed in a -80˚C freezer until processing. DNA was extracted from samples using a using MoBio PowerSoil kit (MoBio, Carlsbad, CA, USA) and library and sample preparation were conducted targeting the V3-V4 regions of 16S (bacterial) and ITS1-ITS2 (fungal) amplicons following protocols of the "Earth Microbiome Project" (Thompson et al., 2017). After cleaned and pooled, samples were sent to be sequenced at the Cooperative Institute for Research in Environmental Sciences at the University of Colorado – Boulder. Samples were sequenced with an Illumina MiSeq series.

Bioinformatics:

The bioinformatics of the sequenced data for this study was conducted using Qiime2 (v. 2023.5, Boylen et al. 2019). Samples were denoised using the dada2 protocol for paired reads (Callahan et al., 2016) and matched with the SILVA (v138.1, Quast et al. 2013) and UNITE (v9, Abarenkov et al. 2023) databases for bacterial and fungal samples, respectively with a 99% confidence level using the function "feature-classifier classify-

sklearn" (qiime2, Bokulich et al. 2018). Samples classified with confidence lower than 97% of similarity were removed.

Data analysis:

Amplicon sequence variants (ASVs) and taxonomy tables generated with Qiime2 were exported and imported into RStudio (v.2023.06.2, Posit Team 2023). Initial removal of low abundance reads (rare ASVs, total abundance across samples lower than 30) and samples with low abundance sequences (less than 5) were filtered out. Filtered tables were imputed into a mctoolsr project ("mctoolsr" package, v.0.1.1.9, Leff 2016). Data were normalized using the cumulative sum scaling method with functions from "metagenomeSeq" package (v.1.42.0, Paulson and Talukder 2017) and normalized data were imported to a new mctoolsr project.

Community parameters, such as alpha and beta diversities, were calculated from the non-normalized and normalized data, respectively, using the "vegan" package (v.2.6-4, functions "diversity" for alpha diversity and "adonis2" for perMANOVA, Oksanen et al. 2022). Community diversity and richness estimates were correlated with growth parameters of Russian knapweed (biomass, number of stems and flowers, and height of tallest stem) using function "ggpairs" from package "GGally" (v. 2.2.0, Schloerke et al. 2024). Non-metric multidimensional scaling (NMDS) values were calculated and plotted with "mctoolsr" package, as well as taxa abundance plots and relative abundance of desired taxa. Data tables were merged to phylum and genus levels (package "phyloseq",

v.1.44.0) and differential abundance analyses of taxa were performed using the package "metagenomeSeq".

Functions associated with taxa were predicted with FAPROTAX (Louca et al. 2016) and FUNGuild (Nguyen et al., 2016) for bacterial and fungal communities, respectively. Outputs generated with FAPROTAX were analyzed for differential abundance of functions in distinct treatment levels. Pairwise comparisons were conducted using the function "DESeq" from "DESeq2" R package (v. 1.40.2). Outputs from FUNGuild were processed and guilds assigned that contained "plant pathogen" as a possibility were identified as plant pathogens (at any confidence level), while guilds assigned as "mycorrhizae" (at confidence of probable or highly probable) were assigned as mycorrhizae. Other guilds were agglomerated and assigned as "other". Updated values were used as response for mixed models with water and insect treatments as fixed effects and Russian knapweed population as random effect. Effects of Russian knapweed populations towards fungal guilds were tested, but no statistical significance ($p$=0.05) was observed.

## Results

### Bacterial communities:

A total of 2,006,150 reads were obtained from sequencing and inputted to the dada2 pipeline. After filtering based on quality 1,698,320 reads were retained, 1,570,670 passed the denoising filter, 1,393,225 merged forward and reverse reads, and a total of 1,373,137 reads were classified as non-chimeric. Taxonomic classification was conducted

and samples with 97% confidence level or higher were retained and inputted into R for analysis. After this first-step cleaning, all reads in blank samples were removed (majority were chimeric reads). We additionally filtered out rare amplicon sequence variants (ASVs, total abundance lower than 30) and ASVs that were classified as chloroplast or mitochondria. After all the filtering was conducted, a total of 814,247 reads were retained.

Rhizosphere samples had higher Shannon diversity indices compared to bulk soil (Rhizosphere – Bulk: estimate = 0.5, df = 51, t-ratio = 4.347, *p*-value < 0.001), as well as higher species richness (Rhizosphere – Bulk: estimate = 104, df = 51, t-ratio = 4.130, *p*-value < 0.001). Population and insect treatments did not impact Shannon diversity and species richness measures for rhizosphere samples (table 05, figures 35 and 36), and water treatment did not impact species richness and diversity in bulk soil samples (table 06, figure 37). Water availability negatively affected species diversity in rhizosphere samples (df = 3, F-value = 4.5396, *p*-value = 0.045 – table 05), but not species richness (df = 3, F-value = 4.5396, *p*-value = 0.177 – table 05). Additionally, no correlations were observed when contrasting plant fitness parameters and bacterial species richness and diversity. While looking at dissimilarities between communities in rhizosphere of Russian knapweed, water availability was the main factor impacting bacterial communities (df = 1, R2 = 0.086, F-value = 4.131, Pr(>F) = 0.001, stress = 0.139 – table 07, figure 38), while neither insect treatments (df = 3, R2 = 0.071, F-value = 1.135, Pr(>F) = 0.210 – table 07), population (df = 3, R2 = 0.064, F-value = 1.022, Pr(>F) = 0.421 – table 07), nor any of the tested interactions led to significant changes. Water treatment was a marginally significant factor when

comparing dissimilarities of bacterial communities in bulk soil samples (df = 1, R2 = 0.1368, F-value = 1.5846, stress = 0.071, *p*-value = 0.058 – table 07, figure 38).

Bulk soil samples were predominantly composed of the phyla Crenarchaeota, Actinobacteriota, Proteobacteriota, Firmicutes, Acidobacteriota, Bacteroidota, Chloroflexi, and Verrucomicrobiota, with no differences between high and low water treatments (figure 05). Additionally, we tested for differential abundance of taxa between rhizosphere and bulk soil of high and low watering levels. In high watering level, the phyla Chloroflexi, Myxococcota, Acidobacteriota, WPS-2, Bdellovibriota, WS2, RCP2-54, and Planctomycetota were more abundant in rhizosphere samples, while Actinobacteriota was higher in bulk soil samples (figure 38). In low watering level, the phyla Entotheonellaeota, Verrucomicrobiota, Acidobacteriota, Planctomycetota, and RCP2-54 were more abundant in rhizosphere samples, while abundance of Abditibacteriota was higher in bulk soil samples (figure 41). Rhizosphere samples were predominantly composed of Proteobacteriota, Crenarchaeota, Acidobacteriota, Actinobacteriota, Firmicutes, Chloroflexi, Bacteroidota, and Planctomycetota (figure 42). While testing for differential abundance of phyla between high and low watering treatments, we saw differences in taxa with low relative abundance (less than 0.01%). Results of this analysis showed higher abundance of the phyla Dependentiae and WPS-2 in high watering level, and Entotheonellaeota in low watering level (figure 43). No differences were observed when comparing relative abundance of taxa associated with distinct Russian knapweed populations in high and low watering levels.

We further investigated functions associated with taxa with FAPROTAX and ran differential abundance analyses on each of the desired comparisons. In high watering level, we saw higher differential abundance of 18 predicted functions towards rhizosphere samples, while only one (nitrate reduction) being more abundant in bulk soil samples (figure 44). Similar patterns were observed in low watering level, where six predicted functions were higher in rhizosphere samples, while only two (aromatic compound degradation and nitrate reduction) were higher in bulk soil samples (figure 45). Functions associated with the nitrogen cycle were more abundant in rhizosphere samples of Russian knapweed in high watering level than in bulk soil samples, no distinctions in functions associated with the nitrogen cycle. While further investigating rhizosphere samples only we see that samples from high watering levels had higher abundance of functions associated with manganese oxidations (figure 46). Additionally, we investigated comparisons between insect presence and watering levels. We only see differences when comparing no insects and midges only, where midge only plants had higher abundance of photosynthetic cyanobacteria and oxygenic photoautotrophy.

Fungal communities:

We chose to only use forward reads for ITS amplicons due to low overall quality of reverse reads. A total of 932,674 forward reads were obtained from sequencing, 720,163 passed the quality filter, 686,210 were denoised, and 676,425 were classified as non-chimeric. Taxonomy classification was conducted, samples with at least 97% confidence

level were retained, and inputted into R for analysis. After the first-step cleaning, all reads in blank samples were removed (majority were chimeric reads). We additionally filtered out rare ASVs (total abundance lower than 30). After filtering, a total of 240,263 reads were retained.

Sample type was also a key aspect when investigating species diversity (df = 1, F-value = 33.2364, Pr(>F) < 0.001) and richness (df = 1, F-value = 21.0109, Pr(>F) < 0.001) for fungal samples. Rhizosphere samples had higher Shannon diversity indices compared to bulk soil (Rhizosphere – Bulk: estimate = 0.96, df = 51, t-ratio = 5.755, *p*-value < 0.001), as well as higher species richness (Rhizosphere – Bulk: estimate = 17.7, df = 51, t-ratio = 4.600, *p*-value < 0.001). Within same sample types, water and insect treatments did not impact Shannon diversity and species richness measures for rhizosphere (table 01, figure 13) and water level did not impact bulk soil samples (table 06, figure 48). However, Russian knapweed population identity was a significant factor impacting species diversity (df = 3, F-value = 3.2322, *p*-value = 0.043 – table 05, figure 49), but not species richness (df = 3, F-value = 1.7488, *p*-value = 0.1878 – table 05, figure 49). While testing for correlations between plant fitness parameters and fungal species richness and diversity, we observed that positive correlations between biomass (0.315) as well as tallest stem (0.318) and species richness were observed when grouping high and low watering levels. Furthermore, while separating watering levels, high watering level showed correlation between flower number and species richness (0.461). No correlations in low watering levels were observed. While looking at dissimilarities between communities in rhizosphere of Russian knapweed, water treatments (df = 1, R2 = 0.0468, F-value = 2.0630, Pr(>F) = 0.001, stress =

0.2494 – table 03, figure 50) and interactions between water and insect treatments (df = 3, R2 = 0.0760, F-value = 1.1177, Pr(>F) = 0.052, stress = 0.2494 – table 07, figure 50) seemed to be the main factors impacting fungal communities, while insect treatments, plant populations, and other interactions did not lead to significant changes. Water treatment was not a significant factor when comparing dissimilarities of fungal communities in bulk soil samples (df = 1, R2 = 0.0942, F-value = 1.0401, *p*-value = 0.155, stress = 0.1595 – table 07, figure 5-).

Bulk soil and rhizosphere samples were mainly composed by Ascomycetes (figures 51 and 52) and no differences in relative abundances were observed in the phylum level. While looking at differential abundance plots of fungi in rhizosphere and bulk soil samples, we did not see any statistically significant changes at the phylum level and *p*=0.05. However, significant differences were observed at the genus level. In high watering level, we saw increased abundance of the genera *Acremonium, Iodophanus, Thermomyces, Conocybe,* an uncertain fungus*, Serendipita, Geosmithia, Paraphoma, Peziza, Naganishia,* and an uncertain *Agaricomycetes* in rhizosphere samples, while *Aspergillus* was much higher in bulk soil samples (figure 53). In low watering levels, we observed higher abundance of *Paraphoma, Acremonium, Serendipita, Geosmithia,* and an uncertain *Agaricomycetes* in rhizosphere samples (figure 54). Additional analyses were conducted to find differentially abundant taxa in rhizosphere samples from high and low watering levels (figure 55). Results suggested no differences in the phylum level; however, at the genus level, most significances observed (*p* = 0.05) were towards high watering levels (14 out of 18). The genera *Peziza,* an uncertain genus in the Pezizales family, *Conocybe,* an uncertain

Pleosporales, *Hormonema, Ascobolus, Iodophanus,* and uncertain Pyxidiophorales, *Hyalorbilia, Alternaria, Stagonospora,* an uncertain Ascomycete, *Idriella,* and *Knufia* were more abundant in high watering level, while *Acremonium, Serendipita, Scutellinia,* and an uncertain genus in the Ascodesmidaceae family were more abundant in rhizosphere samples of low watering level plants. While testing the effects of Russian knapweed population onto rhizosphere microbiomes, we did not see any differences in relative abundance in any taxonomic level.

We also investigated differences in relative abundance of three predicted guilds in the fungal community: mycorrhizae, potential plant pathogens, and other (figures 56, 57, and 58) Differences in relative abundance of mycorrhizae was higher in rhizosphere samples compared to bulk soil (Rhizosphere – Bulk: Estimate = 0.0125, df = 51, t-ratio = 2.859, p-value = 0.0061 – figure 56). No additional differences in predicted guilds were observed when comparing rhizosphere and bulk soil samples. While looking at rhizosphere samples only, we see that watering treatment was a significant factor in every guild. Water treatment negatively impacted the relative abundance of mycorrhizae (df = 1, 34.84, F-value = 6.39, p-value = 0.016 – figure 56). High watering level had fewer mycorrhizal associations than low watering level (Low – High: Estimate = 0.0123, df = 34.8, t-ratio = 2.528, p-value = 0.0162), as well as the presence of other guilds (df = 1, 34.84, F-value = 5.56, p-value = 0.024 – figure 57). Low watering level had higher relative abundance of other guilds than high watering level (Low - High: Estimate = 0.0819, df = 34.8, t-ratio = 2.358, p-value = 0.0241). Lastly, it also impacted the presence of potential plant pathogens (df = 1, 34.84, F-value = 7.98, p-value = 0.008 – figure 58). High watering level had higher relative

abundance of potential plant pathogens than low watering level (High - Low: Estimate = 0.0943, df = 34.8, t-ratio = 2.825, p-value = 0.0078). Neither insect treatments nor plant populations led to additional differences in rhizosphere samples.

## Discussion

Russian knapweed commonly invades areas with varying levels of water availability. We chose to conduct this experiment in Palisade – Colorado because it reflects one of the most arid locations that Russian knapweed invades, and we would be able to provide stress amelioration through irrigation, and therefore evaluate the effects of water stress amelioration on rhizosphere microbiome. In other analyses (chapter 2 of this dissertation), we found that high watering levels significantly enhanced growth parameters of Russian knapweed. However, in this chapter, we investigated if, after two years of establishment of watering treatments in conjunction of distinct levels of herbivory, it would impact the microbiome in the rhizosphere of distinct populations of Russian knapweed. Surprisingly, we found that rhizosphere microbiome of Russian knapweed is richer and more diverse than bulk soil samples; however, similar patterns were observed when testing the effects of sample type (rhizosphere x bulk soil) in Agave plants in desert locations (Coleman-Derr et al., 2016), making this a commonality in arid environments. In addition to sample type, we found that water was also an important factor impacting microbiome composition in rhizosphere samples but not in bulk soil samples. Such finding agrees with other research on the effect of external environmental factors on rhizosphere microbiome (Peiffer et al.,

2013). Fields with high and low watering treatments were side-by-side and no differences in microbiome community were observed in bulk soil samples. However, high watering level led to a reduction of bacterial diversity but not richness and no changes in fungal richness and diversity in rhizosphere samples. These findings go against our expectations, since reduction of water availability has been reported to decrease species richness and diversity (Maestre et al., 2016).

Most abundant bacterial phyla in rhizosphere and bulk soil samples were Proteobacteria, Crenarchaeota, Acidobacteria, Actinobacteria, Firmicutes, Chloroflexi, Bacteroidota, and Planctomycetota and most abundant fungal phylum was Ascomycota, which are common taxa associated with drylands around the world (Maestre et al., 2016). Even though water was added to plants and provided some stress amelioration in high watering level, it did not lead to significant changes in relative abundance of taxa associated with arid areas, leading to the conclusion that two years was not enough for making substantial changes in microbiome community, even though growth of Russian knapweed rapidly responded to water amendments.

While discrepancies in differential abundance of taxa between rhizosphere samples in distinct watering treatments were detected in low abundance taxa (less than 0.1% relative abundance), their relevance in the community still needed to be further investigated. Higher abundance of the phyla Dependentiae and WPS-2 were observed in high water soils, and the ecological significance of these phyla is still unclear. All described isolates from bacteria in the phylum Dependentiae are parasitic of protists (reviewed by Weisse et al., 2023); while WPS-2 (or Eremiobacterota) is a potentially a scavenger phylum

commonly associated with Chloroflexi and Actinobacteria, and commonly found in low relative abundance in bare soil or associated with vegetation (Sheremet et al., 2020). While higher abundance of the phylum Entotheonellaeota was found in low watering treatment, abundance of this phylum has been negatively correlated with nitrate nitrogen (An et al., 2022) and positively correlated with soil C:N (Ren et al., 2020). This suggests that rhizosphere samples in low water level had less available nitrogen than high water level.

We additionally checked for functions associated with taxa found in samples and conducted a differential abundance analysis in relation to sample type, water treatment, plant population or insect treatments. Plant population did not lead to changes in rhizosphere community, going against previous findings on other plant species (Bowen et al., 2017). For bacterial samples, we see that functions associated with nutrient cycling, parasitic or saprotrophs, and predatory were more abundant in rhizosphere samples compared to bulk soil. Functions associated to nitrogen cycling were particularly higher in rhizosphere samples of high water level in comparison to bulk soil samples. This finding agrees with other studies showing that taxa associated with nitrogen cycling are more abundant in habitats with higher water availability (Cregger et al., 2014). While looking at rhizosphere samples only, we did not see many distinctions between functions associated with high or low water levels. In fact, only one function seems to be differentially abundant between those two treatments and it is manganese oxidation. Manganese oxidation is crucial for the manganese cycle as well as changes other rates of nitrogen cycling through catalytic properties (Luther II et al., 1997), such changes can lead to reduction of available nitrogen for plants due to increase of denitrification.

Water availability in rhizosphere samples played an important role in abundance of fungal guilds. Abundance of mycorrhizal fungi was inversely proportional to water level, while presence of protentional pathogens was positively associated with high water level. Species classified as mycorrhizae belonged to the genera *Serendipita* and *Sebacina*, which have been reported as ectomycorrhizal species that assist with water retention near roots and drought tolerance (Ghimire and Craven, 2011; Hosseini et al., 2023), plant protection (Sarkar et al., 2019), and plant growth promotion (Ghimire et al., 2009). Higher abundance of ectomycorrhizal fungi has been correlated to lower abundance of potential pathogens (as reviewed by Tedersoo et al., 2020), as observed in the current study. Rhizosphere microbiomes of spotted knapweed, *Centaurea stoebe*, had higher abundance of arbuscular mycorrhizal fungi and lower abundance of potential pathogens when growth in dry pots and in competition with *Bromus marginatus* (Bunn et al., 2023). Such dynamics impacted increased the competitive ability of *C. stoebe* in competition with *B. marginatus* (Bunn et al., 2023). Although we did not test the competitive ability of Russian knapweed against other plant species in the presence of ectomycorrhizal fungi, we hypothesize that such associations may increase Russian knapweed's competitive ability against plant species. Future research on this topic might be relevant while attempting to advance knowledge on success of invasive plants.

While microbiomes associated with Russian knapweed did not show changes according to presence of biological control agents, plant-microbiome associations could have led to changes of Russian knapweed as a host for both gall-forming insects. In each watering level, Russian knapweed was able to form associations with distinct taxa to

obtain required nutrients. In high watering treatment, we see evidence of higher abundance of nitrogen and functions associated with the nitrogen cycle, although, it also revealed higher relative abundance of potential pathogens. Meanwhile, in low watering treatment we see higher abundance of mycorrhizal fungi, which can provide nitrogen and phosphorous to host plants (reviewed by Tedersoo & Bahram, 2019). Such functions could shift the quality of Russian knapweed as a host for herbivores. Herbivory damage on Russian knapweed was measured by number of galls of each insect, midges (*Jaapiella ivannikovi*) and wasps (*Aulacidea acroptilonica*), at the end of the growing season. Low watering level was associated with higher number of insect galls and a synergistic relationship between both insects (Folks and Franco et al. *unpublished data*). While in high watering levels relationships between insects were asymmetric. Wasps benefitted from presence of midges, and midges benefitted from absence of wasps (Folks and Franco et al. *unpublished data*). These results go against our original hypothesis that regardless of watering levels, relationships formed by Russian knapweed would sustain its quality as a host for biological control agents. The Stress Gradient Hypothesis suggests that in high stress environments, interactions between competing species switch from neutral/negative towards facilitative (Bertness and Callaway, 1994). Therefore, we conclude that associations with mycorrhizae in low watering level might have ameliorated some of the stress associated with low water availability, but still was not enough to match benefits of higher water availability.

In conclusion, we see that water availability is a key aspect shaping rhizosphere microbiome of Russian knapweed, leading to higher variation than presence of herbivores

or plant population. Although presence of taxa commonly associated with arid environments were predominant in both watering treatments, increased presence of mycorrhizal fungi in low watering level and potential plant pathogens in high watering level leads to the conclusion that drought amelioration did change plant-microbiome interactions in distinct watering treatments. Additionally, the flexibility between associations with distinct microbiomes under levels of abiotic factors sheds light on why Russian knapweed is such a successful invader in habitats with varying levels of water and nutrients.

Figures and Tables:

Table 05: Results from mixed models for rhizosphere samples.

| | | Treatment | Df | Richness | | Shannon diversity | |
|---|---|---|---|---|---|---|---|
| | | | | F-value | *p*-value | F-value | *p*-value |
| Fungi | Rhizosphere | Population | 3 | 1.7488 | 0.1878 | 3.2322 | 0.043 |
| | | Water | 1 | 0.2842 | 0.5996 | 0.2761 | 0.605 |
| | | Insect | 3 | 0.1262 | 0.9435 | 0.2159 | 0.884 |
| | | Water*Insect* Population | 14 | 1.0037 | 0.4839 | 1.0185 | 0.472 |
| Bacteria | Rhizosphere | Population | 3 | 0.8077 | 0. 504 | 0.4919 | 0.692 |
| | | Water | 1 | 1.9449 | 0.177 | 4.5396 | 0.045 |
| | | Insect | 3 | 0.5069 | 0.682 | 0.7541 | 0.754 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Water*Insect* Population | 14 | 0.5383 | 0.882 | 0.3261 | 0.326 |

Table 06: Results from models for bulk soil samples.

| | | | | Richness | | Shannon diversity | |
|---|---|---|---|---|---|---|---|
| | | Treatment | df | F-value | *p*-value | F-value | *p*-value |
| Fungi | Bulk | Water | 1 | 1.1948 | 0.3 | 0.4959 | 0.4974 |
| Bacteria | Bulk | Water | 1 | 0.5471 | 0.4765 | 0.0807 | 0.7821 |

Table 07: Adonis results for bacterial and fungal communities.

| | | Treatment | df | R2 | F-value | Pr(>F) |
|---|---|---|---|---|---|---|
| Bacteria | Rhizosphere | Water | 1 | 0.086 | 4.131 | 0.001 |
| | | Insect | 3 | 0.071 | 1.135 | 0.210 |
| | | Population | 3 | 0.064 | 1.022 | 0.421 |
| | | Water*Insect | 3 | 0.075 | 1.207 | 0.122 |
| | | Water*Population | 3 | 0.066 | 1.064 | 0.332 |
| | | Insect*Population | 8 | 0.199 | 1.194 | 0.113 |
| Fungi | Rhizosphere | Water | 1 | 0.0468 | 2.0630 | 0.001 |
| | | Insect | 3 | 0.0760 | 1.1171 | 0.065 |
| | | Population | 3 | 0.0732 | 1.0776 | 0.134 |
| | | Water*Insect | 3 | 0.0760 | 1.1177 | 0.052 |
| | | Water*Population | 3 | 0.0690 | 1.0142 | 0.418 |
| | | Insect*Population | 8 | 0.1829 | 1.0084 | 0.424 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Fungi | Bulk | Water | 1 | 0.0942 | 1.0401 | 0.155 |
| Bacteria | Bulk | Water | 1 | 0.1368 | 1.5846 | 0.058 |

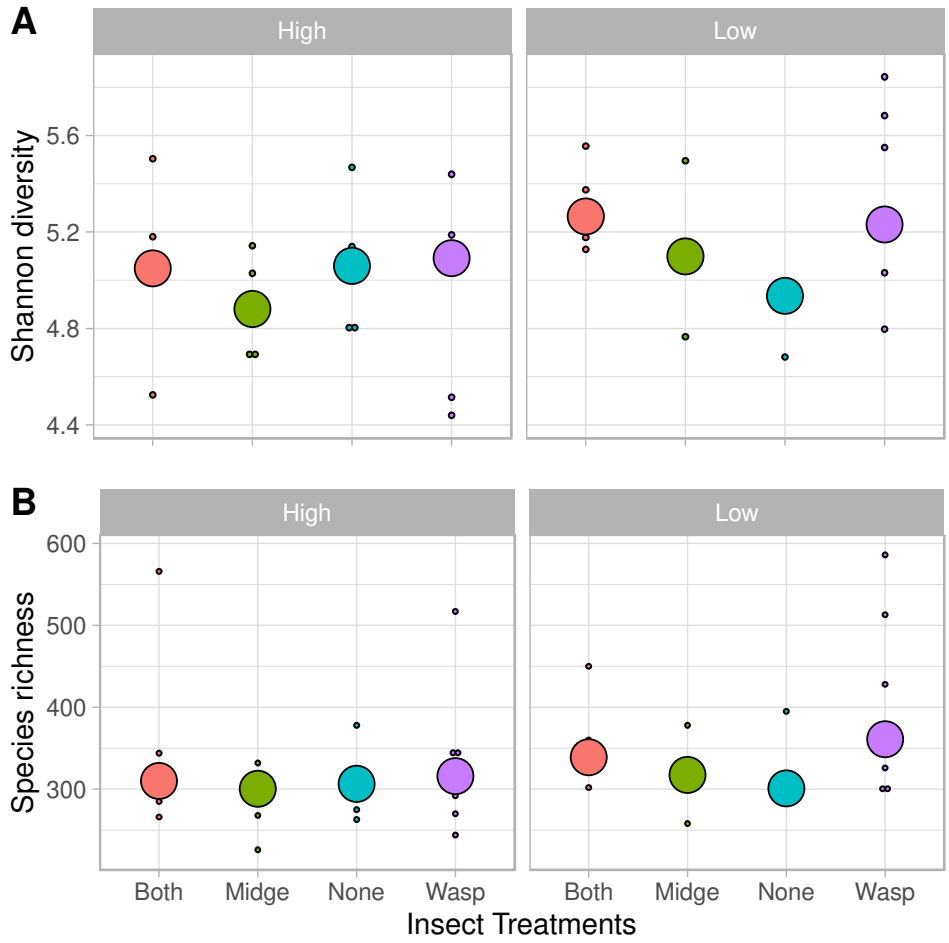



Figure 35: Mean diversity and richness of bacterial communities in rhizosphere samples in each insect and water treatments.

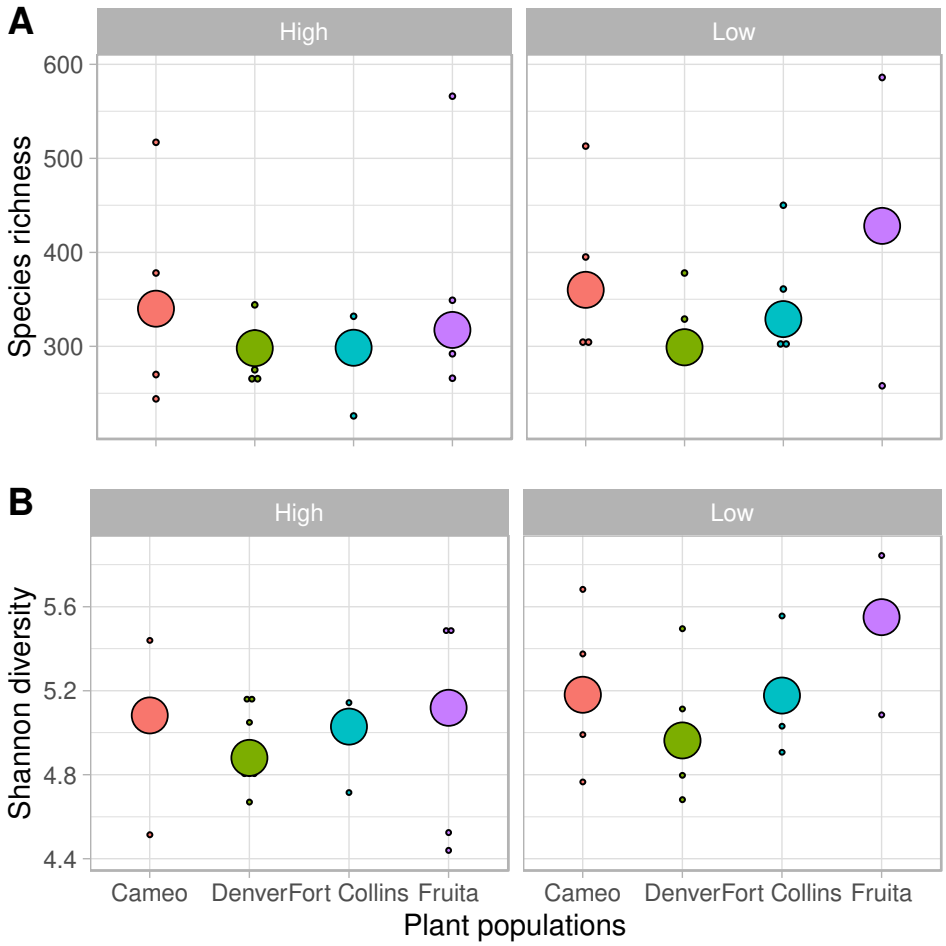



Figure 36: Mean diversity and richness of bacterial communities in rhizosphere samples in each Russian knapweed population and water treatments.

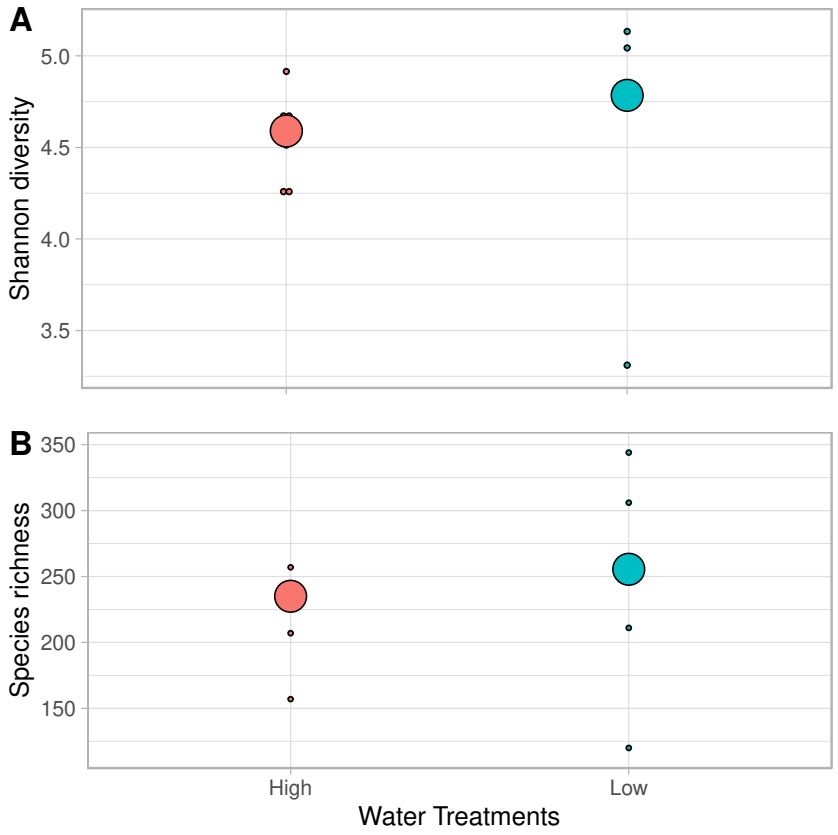



Figure 37: Mean diversity and richness of bacterial communities in bulk soil samples in each water treatment.

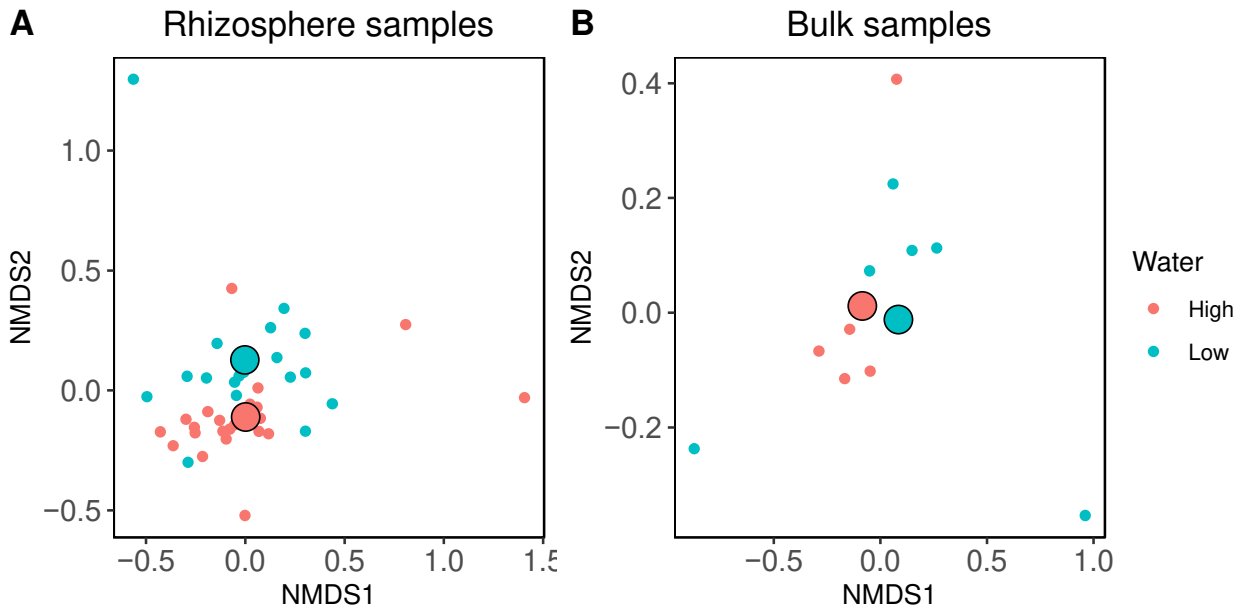



Figure 38: Nonmetric multidimensional scaling ordinations of bacterial community dissimilarities among water treatments (Bray-Curtis dissimilarities). A: For rhizosphere samples (stress = 0.139). B: For bulk soil samples (stress = 0.071).

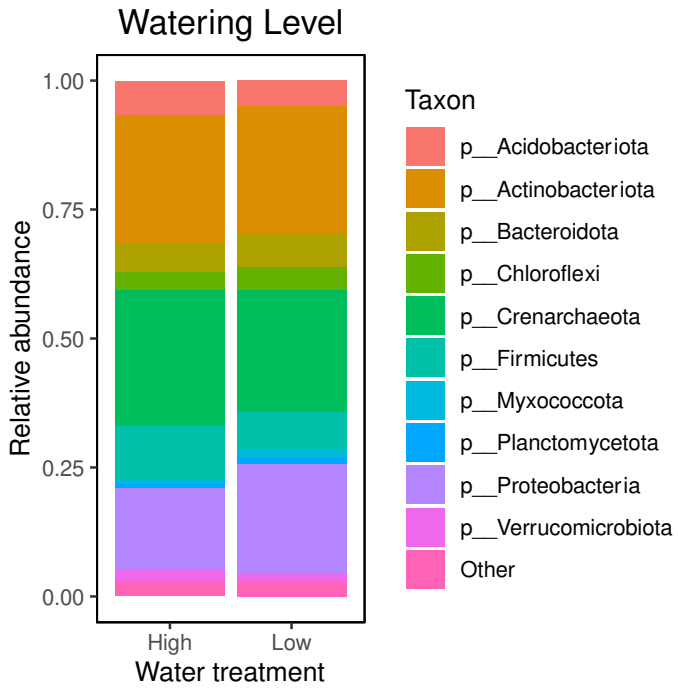



Figure 39: Relative abundance of most abundant phyla in bulk soil samples.

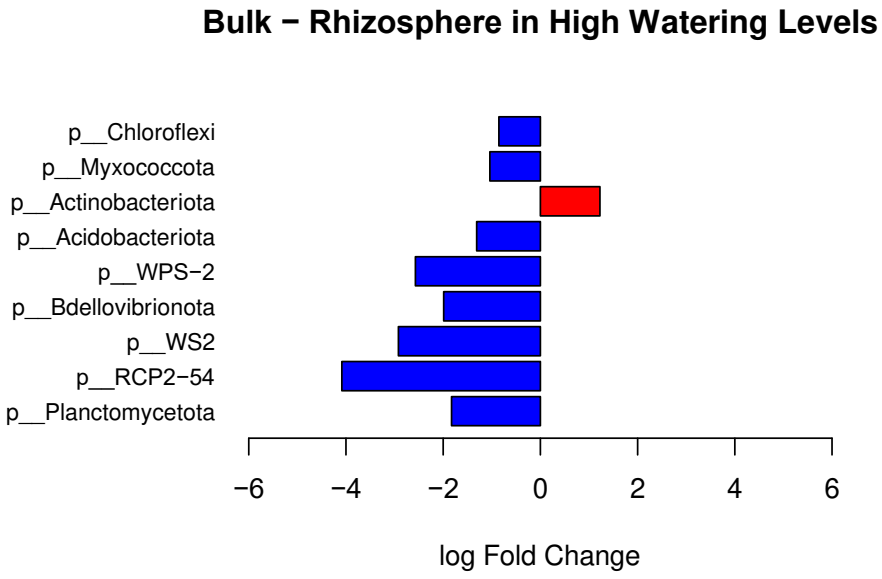



Figure 40: Differential abundance of phyla when comparing bulk soil and rhizosphere samples in high watering level. Bars in red show phyla with significantly higher abundance ($p < 0.05$) in bulk soil samples, while blue bars show phyla with significantly higher abundance ($p < 0.05$) in rhizosphere samples.

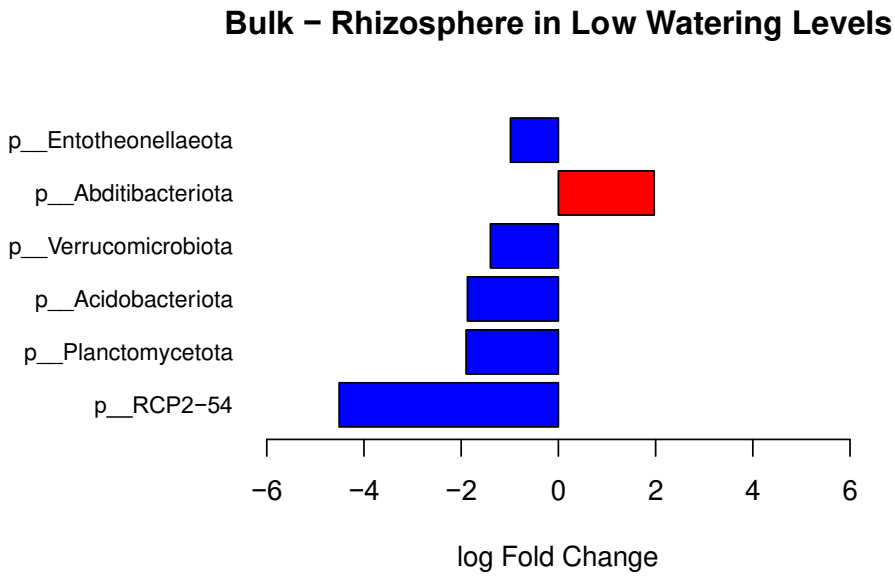



Figure 41: Differential abundance of phyla when comparing bulk soil and rhizosphere samples in low watering level. Bars in red show phyla with significantly higher abundance ($p < 0.05$) in bulk soil samples, while blue bars show phyla with significantly higher abundance ($p < 0.05$) in rhizosphere samples.

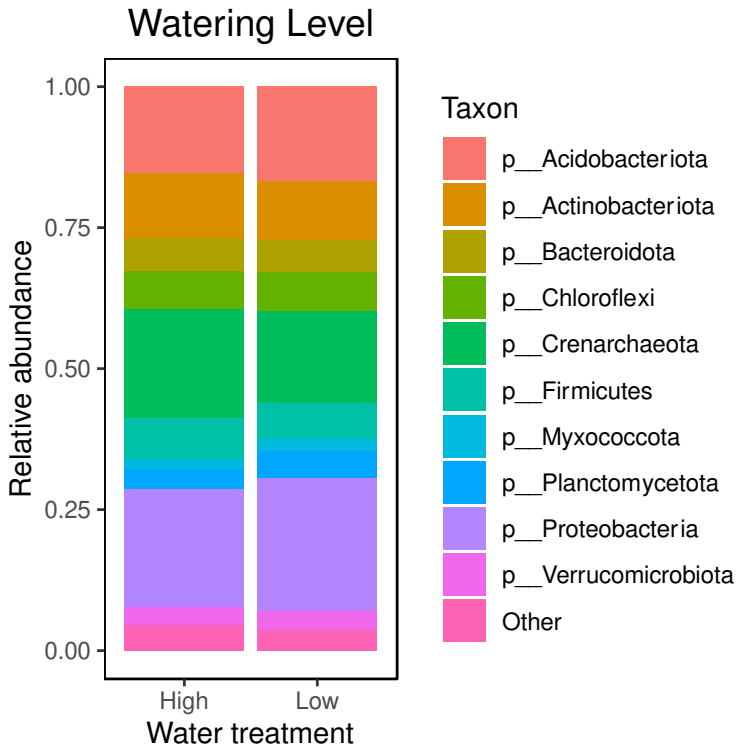



Figure 42: Relative abundance of most abundant phyla in rhizosphere samples.

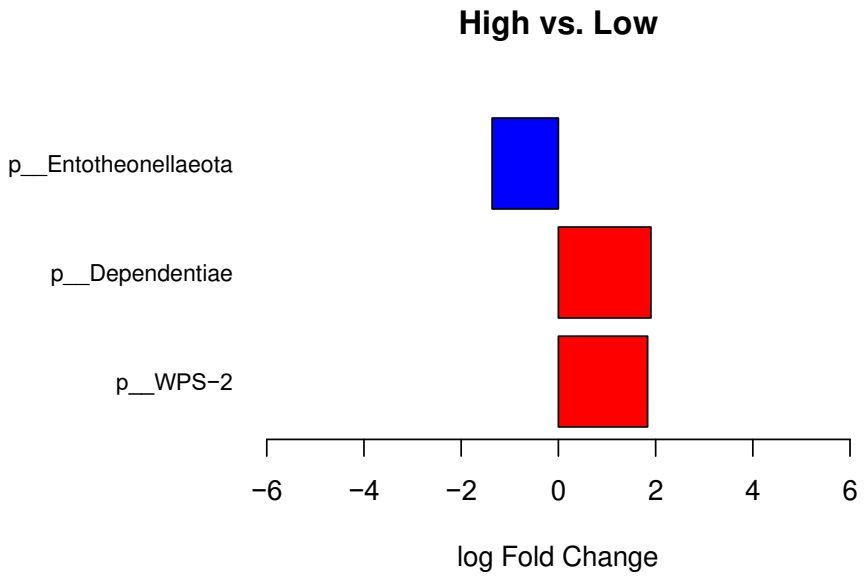



Figure 43: Differential abundance of phyla in rhizosphere samples of Russian knapweed under high vs. low watering levels. Red bars show phyla enriched in high watering levels, while blue bars show taxa enriched in low watering levels.

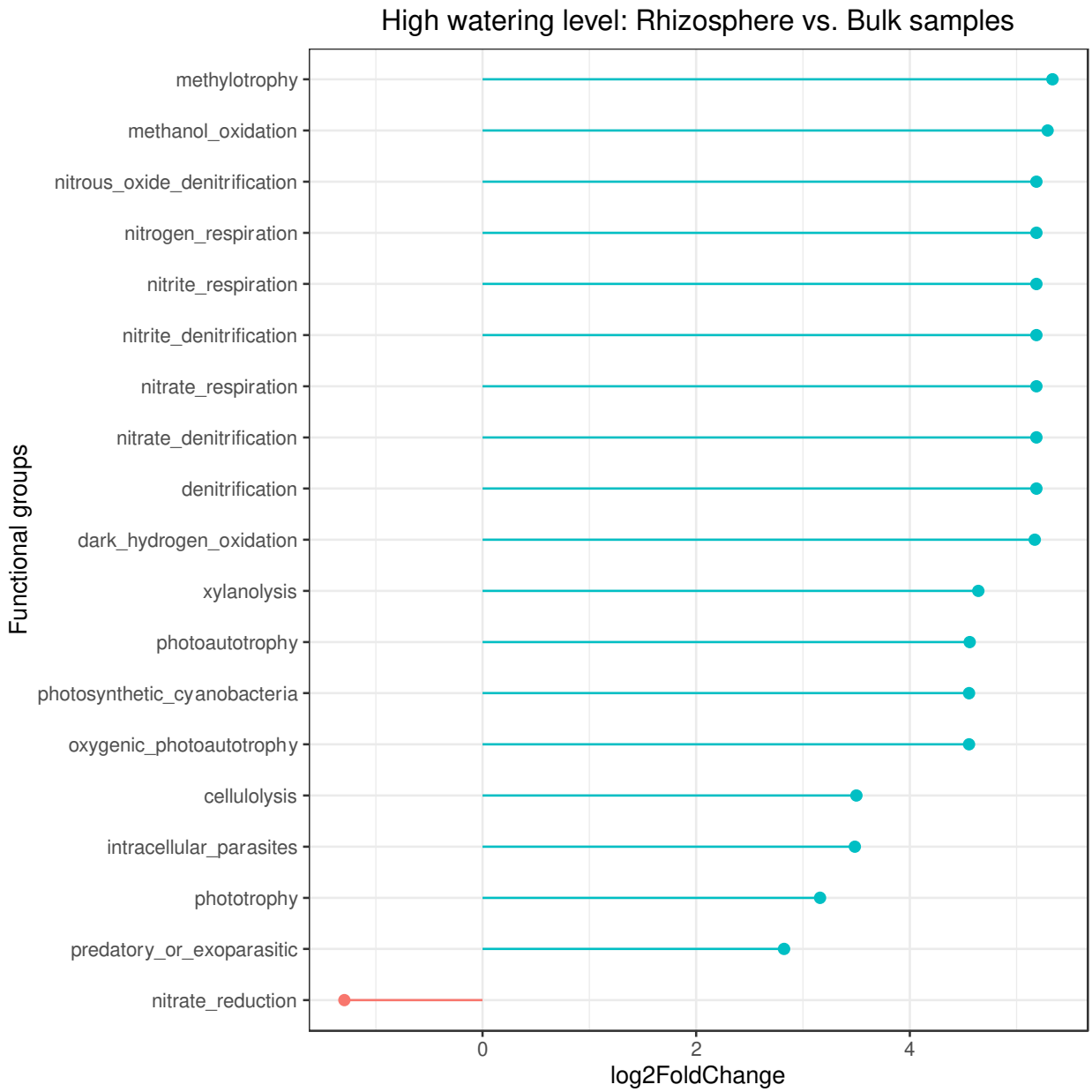



Figure 44: Differential abundance of functions associated with bulk soil (in red) and rhizosphere (in blue) samples in high watering level.

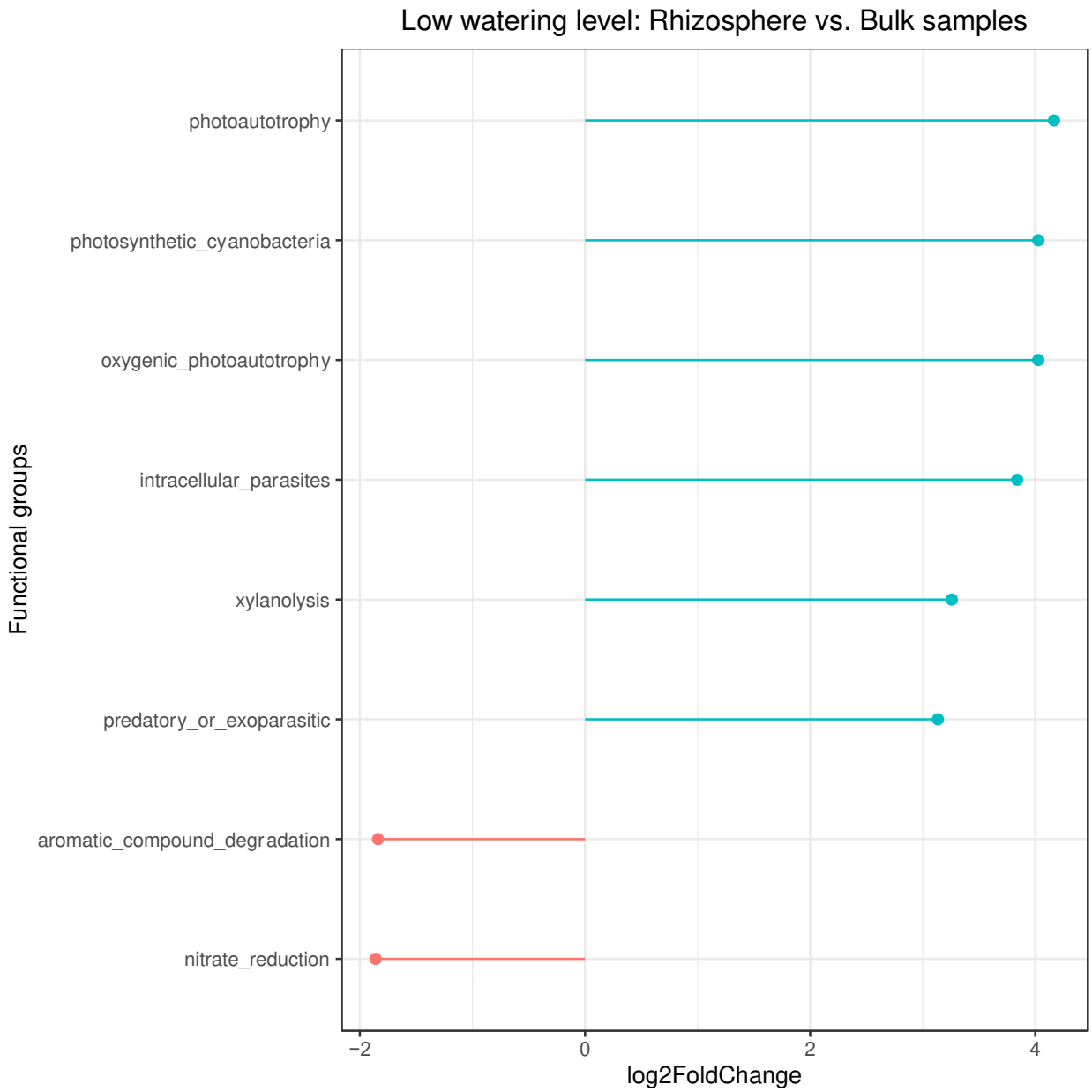



Figure 45: Differential abundance of functions associated with bulk soil (in red) and rhizosphere (in blue) samples in low watering level.

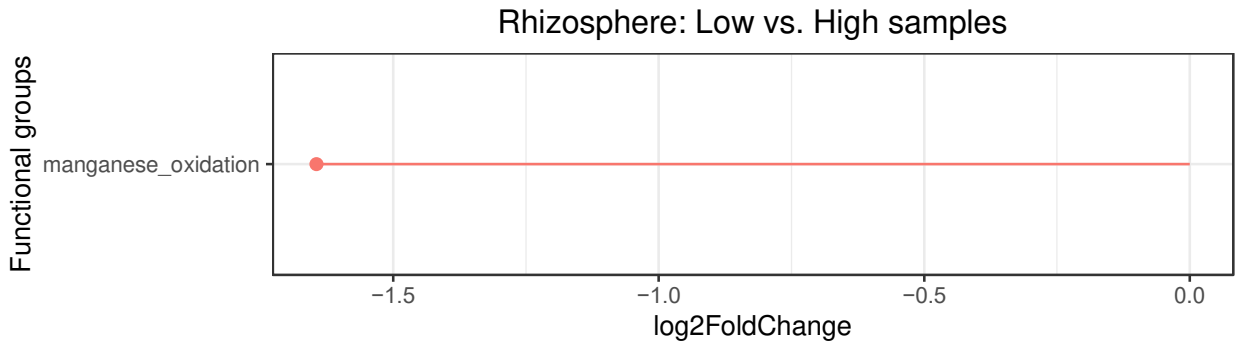



Figure 46: Differential abundance of functions associated with rhizosphere samples from high (in red) and low (in blue) watering levels.

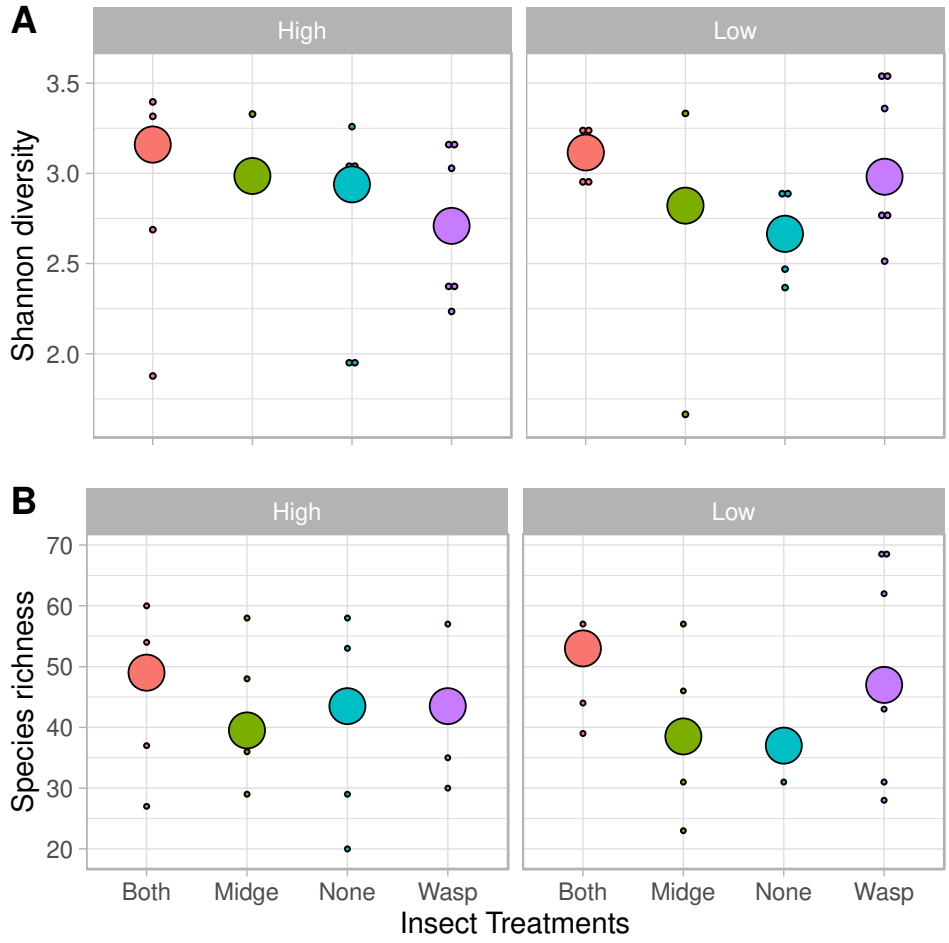



Figure 47: Mean diversity and richness of fungal communities in rhizosphere samples in each insect and water treatments.

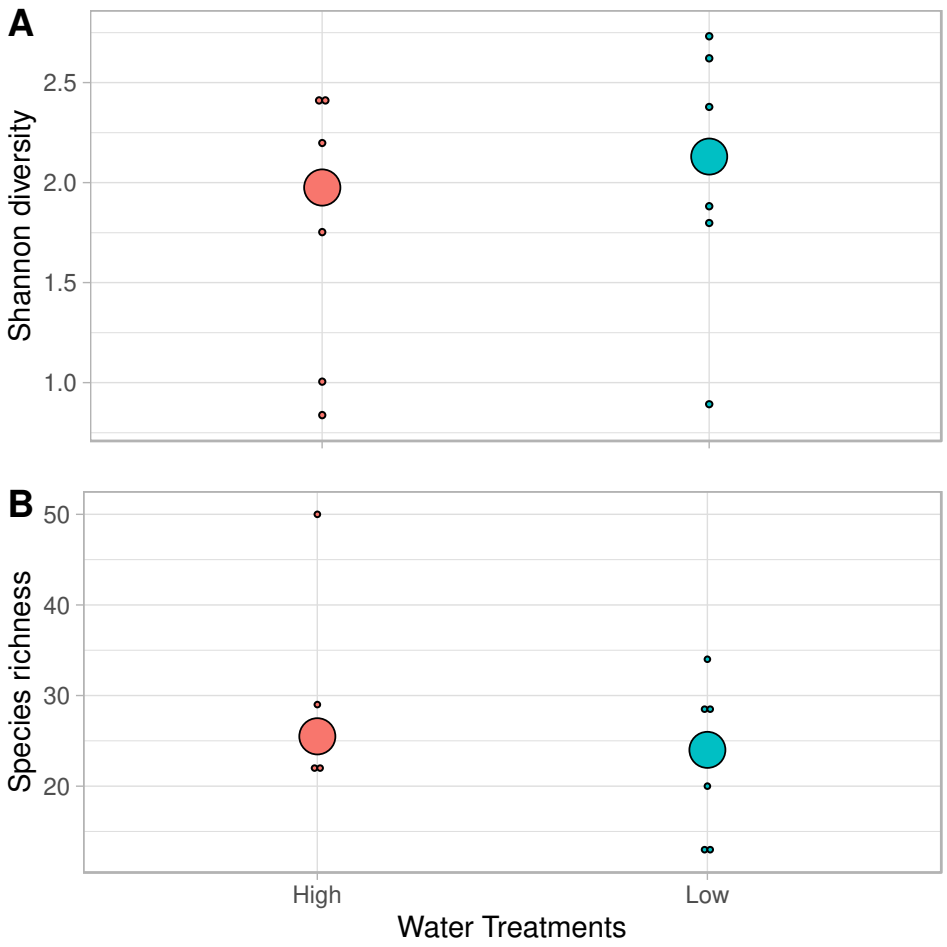



Figure 48: Mean diversity and richness of fungal communities in bulk soil samples in each water treatment.

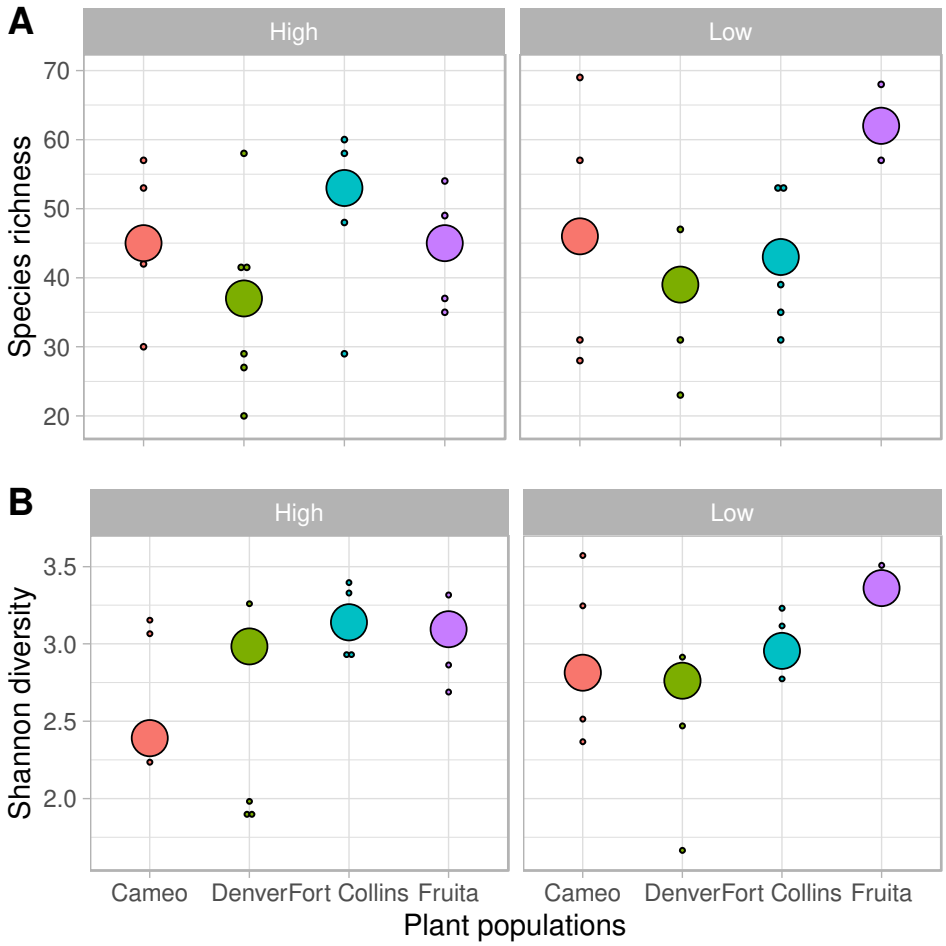



Figure 49: Mean diversity and richness of fungal communities in rhizosphere samples in each Russian knapweed population and water treatments.

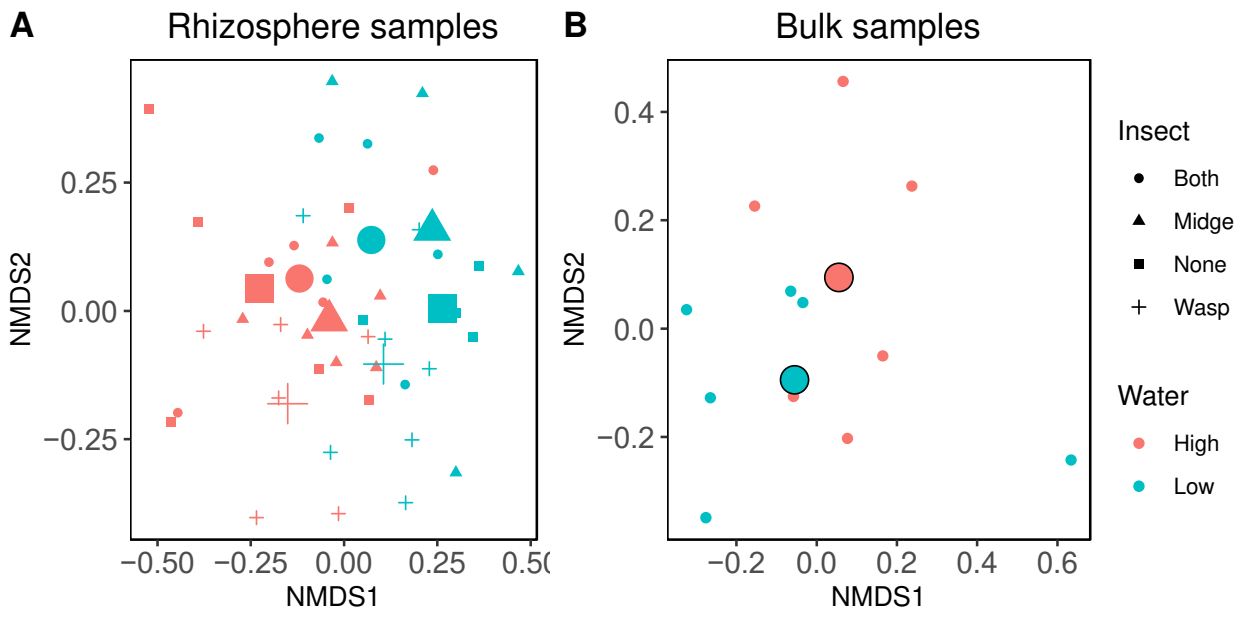



Figure 50: Nonmetric multidimensional scaling ordinations of community dissimilarities among water treatments (Bray-Curtis dissimilarities). A: For rhizosphere samples (stress = 0. 2494). B: For bulk soil samples (stress = 0. 1595).

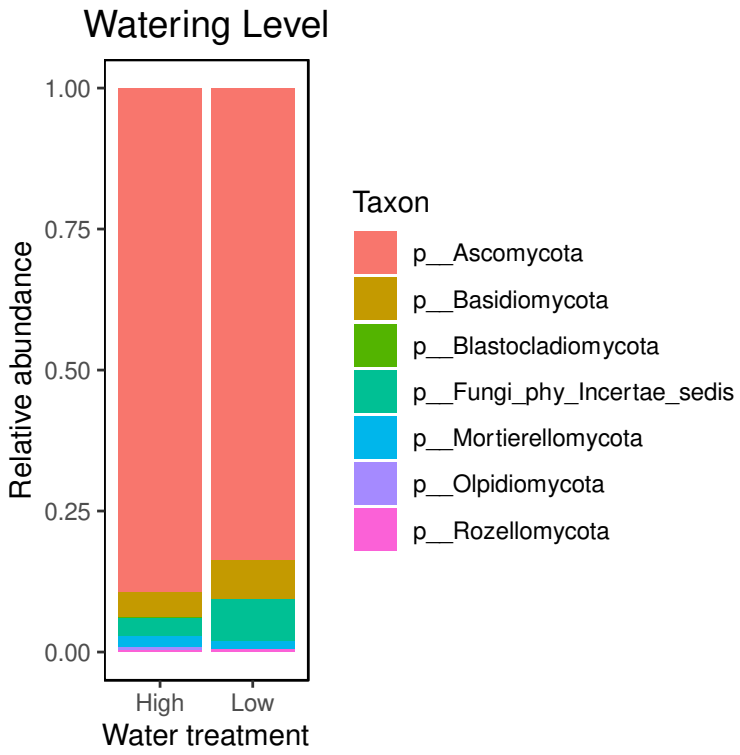



Figure 51: Relative abundance of most abundant fungal phyla in bulk soil samples.

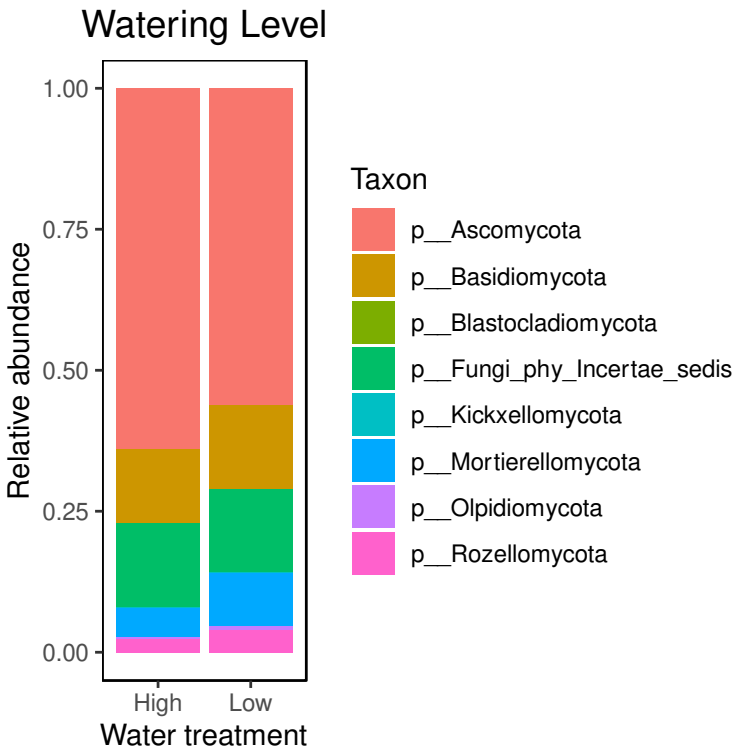



Figure 52: Relative abundance of most abundant fungal phyla in rhizosphere samples.

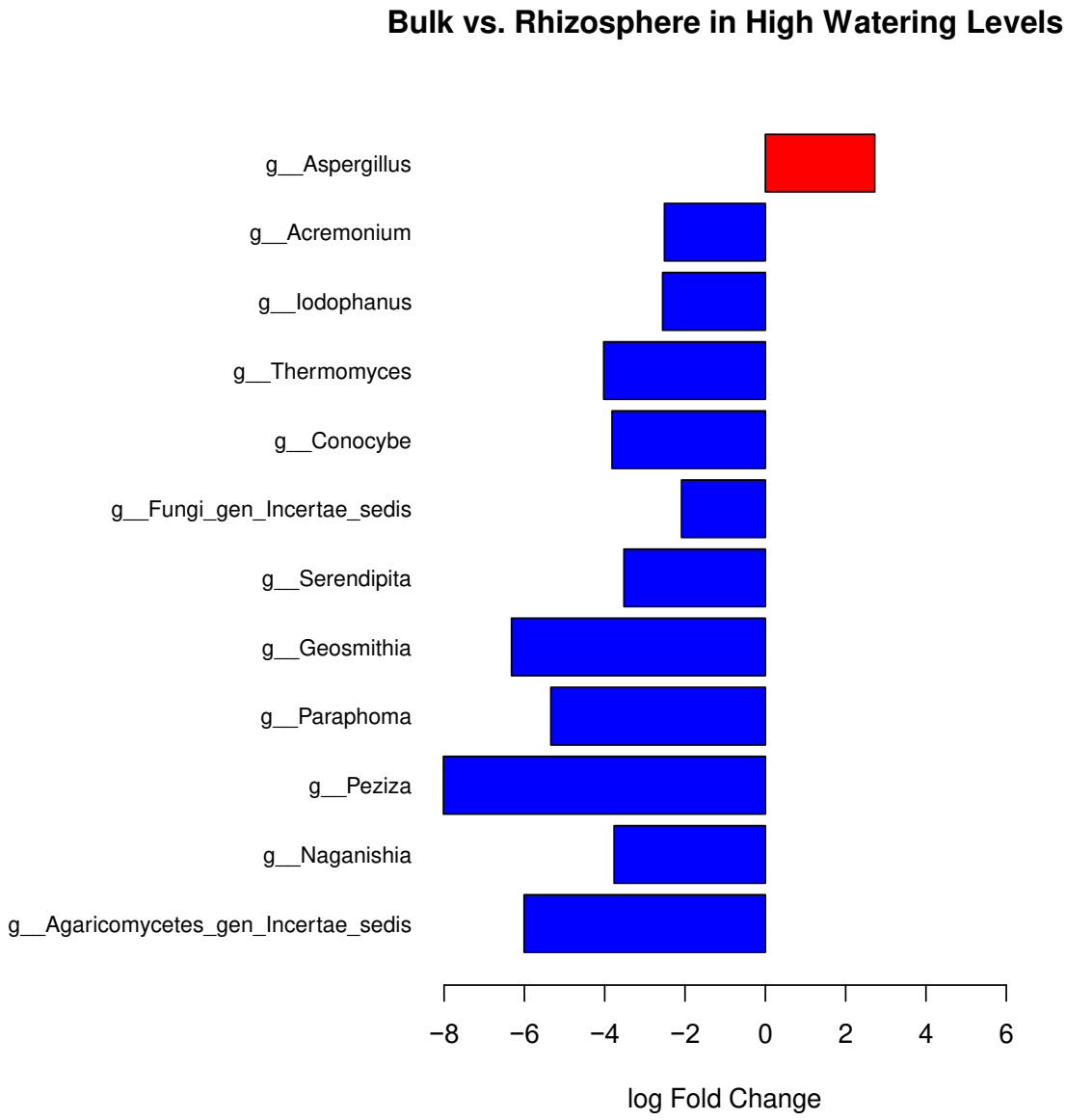



Figure 53: Differential abundance of genera when comparing bulk soil and rhizosphere samples in high watering level. Bars in red show phyla with significantly higher abundance ($p < 0.05$) in bulk soil samples, while blue bars show phyla with significantly higher abundance ($p < 0.05$) in rhizosphere samples.

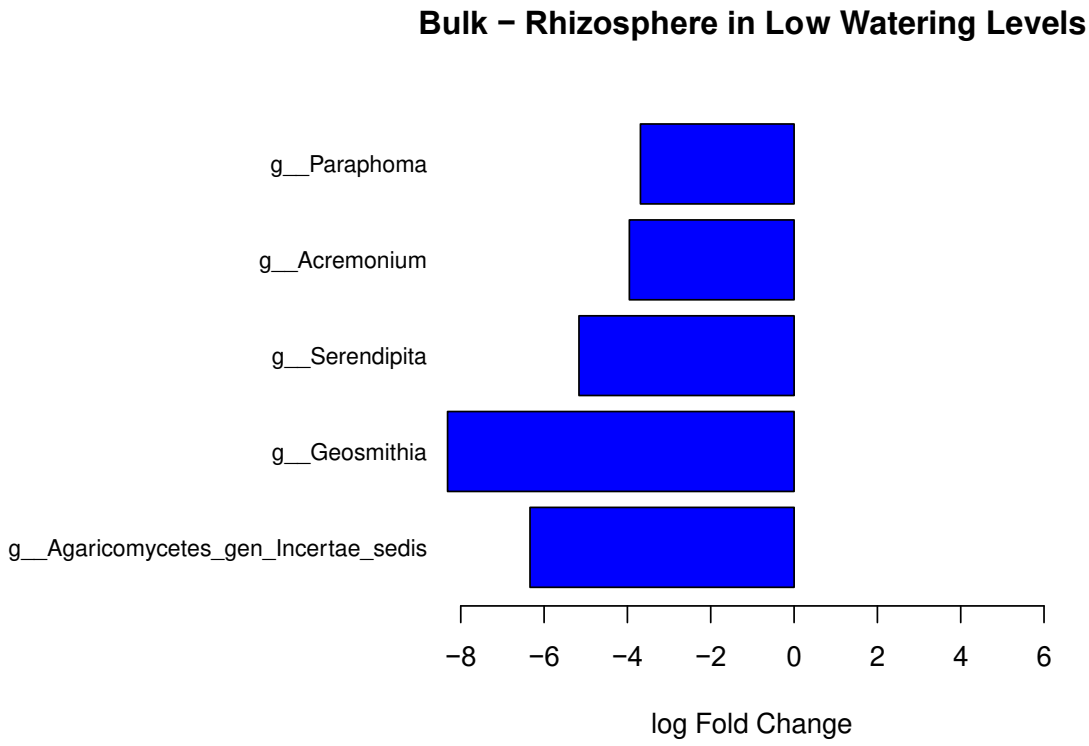



Figure 54: Differential abundance of genera when comparing bulk soil and rhizosphere samples in low watering level. Bars in red show genera with significantly higher abundance ($p < 0.05$) in bulk soil samples, while blue bars show phyla with significantly higher abundance ($p < 0.05$) in rhizosphere samples.

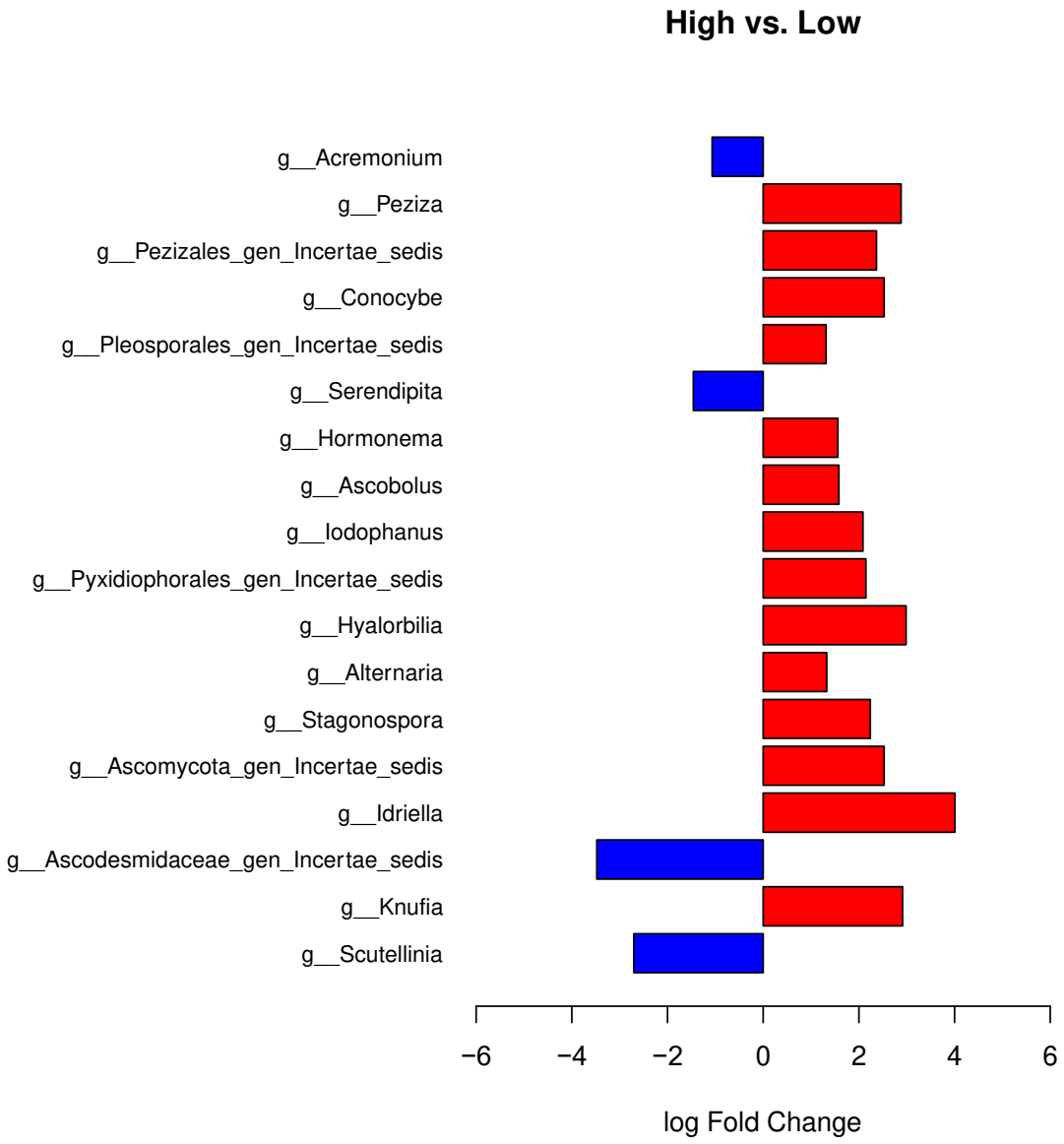



Figure 55: Differential abundance of genera when comparing rhizosphere samples from high and low watering levels. Bars in red show genera with significantly higher abundance ($p < 0.05$) in high watering level, while blue bars show genera with significantly higher abundance ($p < 0.05$) in rhizosphere samples of low watering level.

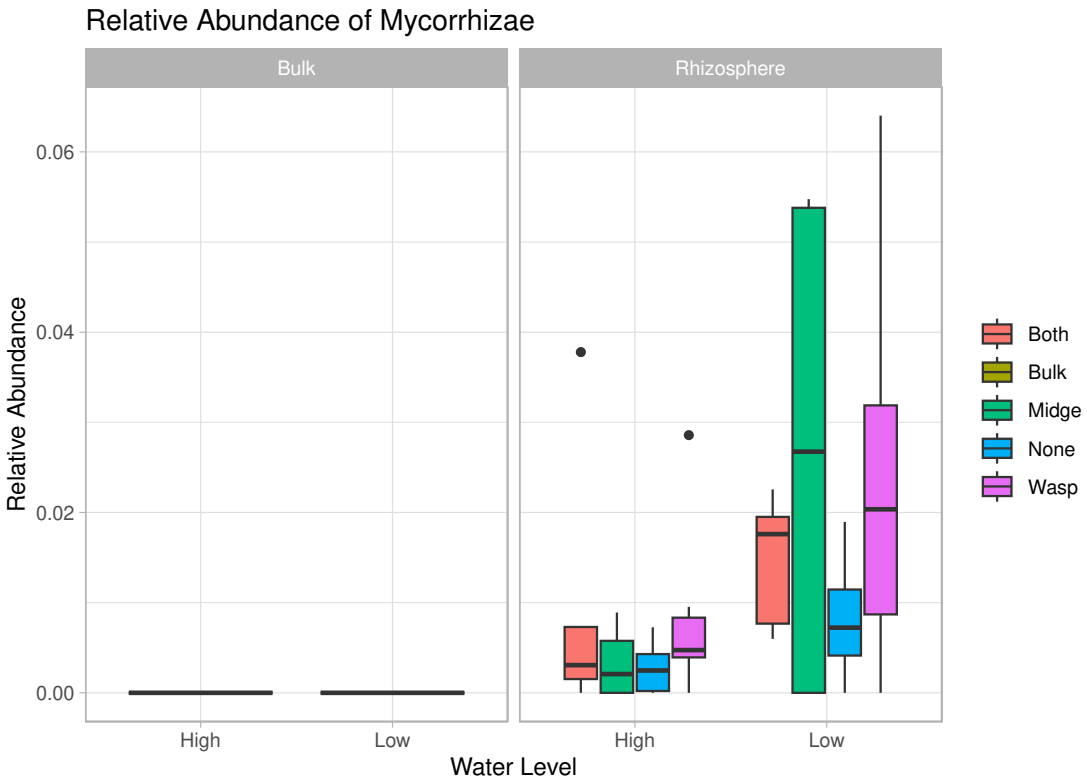



Figure 56: Relative abundance of potential mycorrhizal fungi in bulk soil and rhizosphere samples. Within rhizosphere samples, distinctions were observed when comparing different insect establishment levels. Abundance of mycorrhizal fungi was much higher in rhizosphere samples than bulk soil (Rhizosphere – Bulk: Estimate = 0.0125, df = 51, t-ratio = 2.859, p-value = 0.0061). Additionally, abundance of mycorrhizae was much higher in low watering level compared to high (Low – High: Estimate = 0.00985, df = 45, t-ratio = 2.710, p-value = 0.0095).

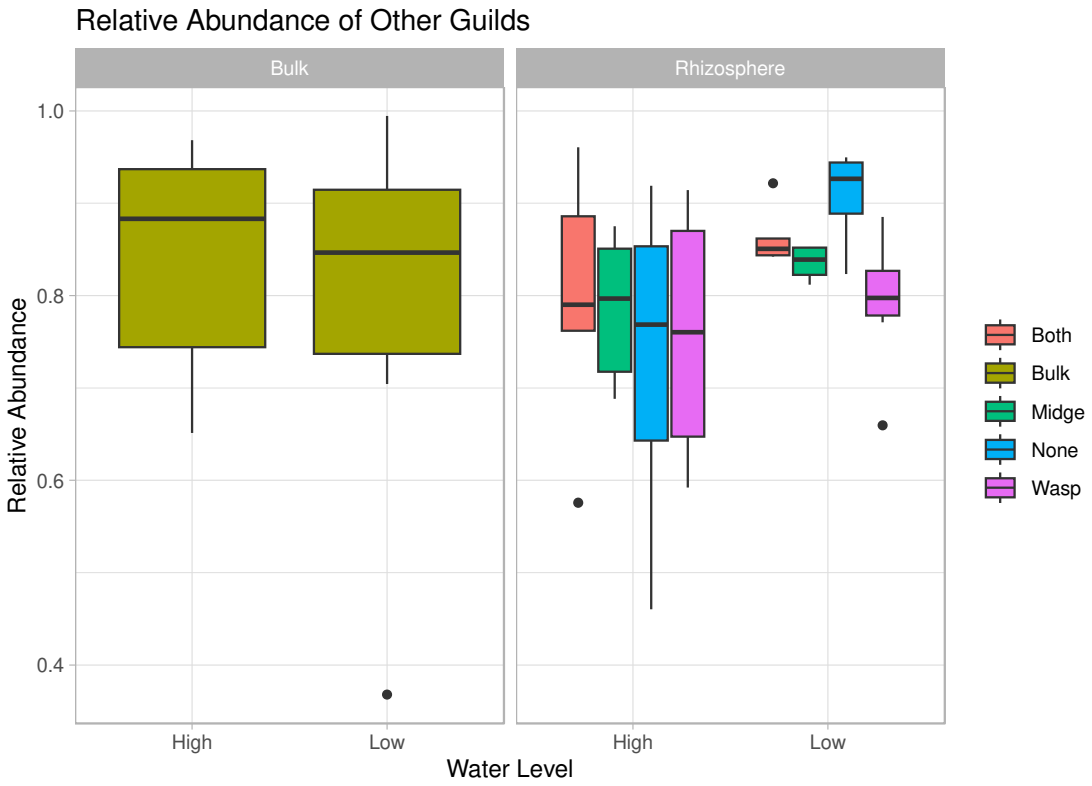



Figure 57: Relative abundance of guilds other than potential plant pathogens and mycorrhizal fungi. Statistical significances were observed when comparing relative abundances in rhizosphere samples (Low - High: Estimate = 0.0819, df = 34.8, t-ratio = 2.358, p-value = 0.0241).

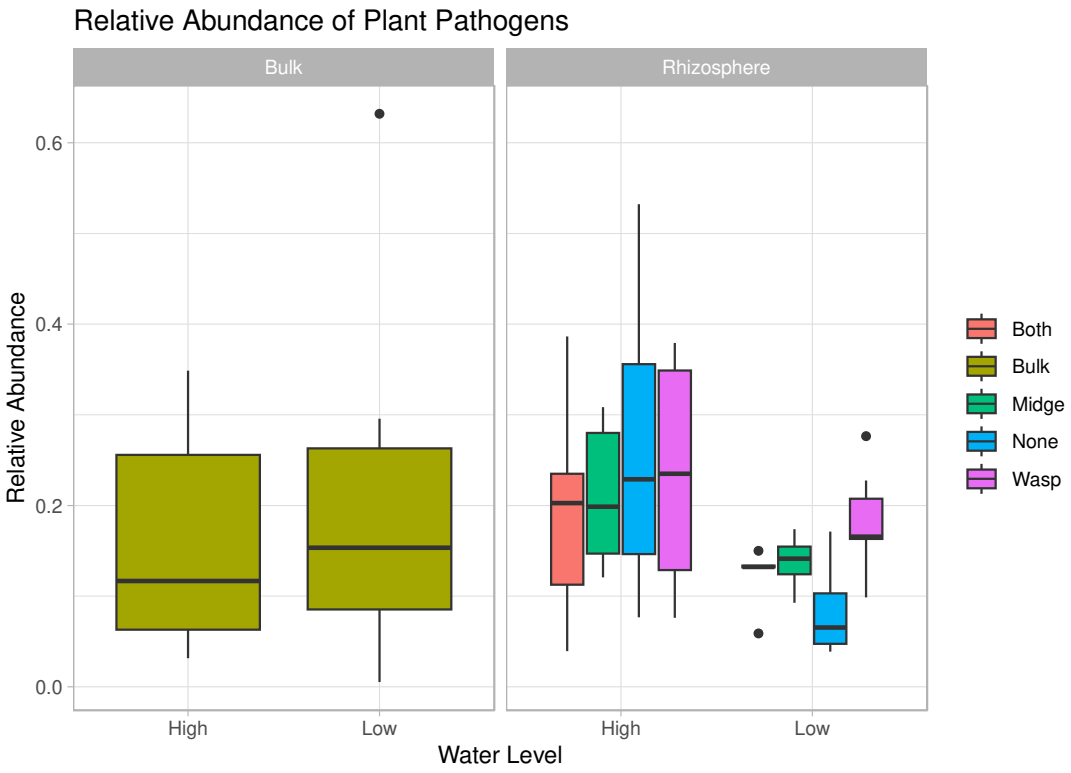



Figure 58: Relative abundance of potential plant pathogens in bulk soil and rhizosphere samples. Statistical differences were observed when comparing relative abundance of potential plant pathogens high and low watering levels (High - Low: Estimate = 0.0943, df = 34.8, t-ratio = 2.825, p-value = 0.0078).

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