Examining the Effects of Herbivory on Aboveground Growth and Induced Defenses in *Eucalyptus globulus*

Kesaaraa Wijeyewickrema

Abstract *Eucalyptus globulus* is an invasive species found in California. The reasons for invasive spread are complex and interrelated. However, as *E.globulus* escaped natural enemies for the first 150 years following its introduction to California, escape from natural enemies may have played a significant role in its expansion. To examine the extent of the role of enemy release in the spread of *E.globulus* this study looks at the effect of herbivory on growth and examines a possible resource trade-off between growth and defense due to the cost of producing defensive compounds. A field experiment was conducted in Pinole, California using enemy exclosures to determine above ground differences in growth of protected and unprotected seedlings, and to examine differences in concentration of terpenes between protected and unprotected seedlings as evidence for induced defenses. Leaf samples were collected at the end of the field experiment and were examined using Gas Chromatography- Mass Spectrometry (GC-MS). The study found that protected seedlings were able to grow greater than unprotected seedlings suggesting that *E.globulus* underwent regulatory release from enemies when first introduced to California. Induced defenses were also demonstrated, indicating that *E.globulus* may have benefited from low investment in defense when first introduced.

Introduction

The Continental United States of America harbors more than 3000 exotic species (Kartesz and Meacham 1999, Fine 2002). Although there is some accidental introduction, most exotic species have been introduced deliberately through trade and travel, and it is expected that the number of invasive species will continue to increase around the world (Moles et al.2008). Invasive plants are plants that originate in one environment and are introduced to another, where they are able to out compete native plants for resources enabling them to grow rapidly. The wide spread growth of introduced plants can cause severe ecological damage and can be economically costly to control. For example, invasive grasses produce highly inflammable litter that increases the occurrence of annual wildfires (D'Antonio and Vitousek, 1992). By enabling annual wildfires and other disturbances uncommon to native systems, introduced plants are able to alter ecosystems ensuring their continued dominance (Fine 2002). Understanding the factors that aid the invasive spread of introduced plants is vital to their control and eradication.

Eucalyptus globulus commonly known as blue gum eucalyptus is an invasive species found in California. *Eucalyptus globulus* is one of more than 700 eucalyptus species. It is a native of Australia, found mainly in Tasmania and the Southeast of state Victoria. *E.globulus* is a fast growing species that prefers Mediterranean climates and wet soils (Esser 1993). Individuals were ornamentally introduced to California in 1853, and later gained commercial value as timber due to their fast growth rates. Although initially cultivated in plantations, no longer controlled and maintained they have expanded and have become an invasive problem.

East Bay Regional Parks faced with the invasive spread of *E.globulus* use controlled burnings and chemical applications as an effort to control the continued expansion of *E.globulus* (JH 2008, pers. comm.). The ability to store soil nutrients in litter and surface soils under the tree, and the ability to inhibit other plant growth by leeching chemicals into the soil from leaves and bark (allelopathy) causes damage to nearby plants (Davidson, 1993). The economic cost of controlling *E.globulus* and the ecological damage caused by it make it important to understand how *E.globulus* may have expanded to become invasive in California.

The hypothesis on life history traits of a plant and the enemy release hypothesis are among the many hypotheses attempting to explain the invasive spread of introduced plants. The hypothesis of life history traits, predicts that plants with certain characteristics such as reproduction sexually and asexually, rapid growth and ability to adapt to environmental stress will become invasive in introduced areas (Sakai et al. 2001). However, few empirical data exists to refute or support this list of characteristics (Kolar and lodge 2001). Thebaud et al. (1996). examined two introduced Conyza species with similar life history traits, but found that their invasiveness differed. Other studies have found that introduced plant species that do become invasive have only a few of the traits listed (Williamson and Brown, 1986, Roy 1990).

The enemy release hypothesis predicts the invasive spread of introduced plants that escape their natural enemies (Keane and Crawley 2002). Plants that are introduced to new environments experience low-level regulation from their natural enemies who are not present in the new environment (insects, mammals and other herbivores). This decrease in regulation enables introduced plants to allocate more resources to their growth and less to their defense. The opportunity to allocate more resources to growth gives introduced plants a competitive advantage over native species enabling it to out compete natives and become invasive.

Agrawal et al.(2005) testing the Enemy Release Hypothesis on congeneric plant pairs (plants of the same genus) found that the invasive species experienced less damage from herbivores than the native species. The findings conflicted with their 2002 study that found the opposing result (Agrawal et.al. 2003). They believe that one reason for the conflicting result is the changing herbivore fauna, and feel that studies examining the Enemy Release Hypothesis must look at herbivore effects over multiple years to obtain an accurate effect.

Colautti et al. (2004) reviewed studies testing the Enemy Release Hypothesis and were skeptical of the findings. They believe most of the studies reviewed do not differentiate between two types of enemy release, regulatory release and compensatory release. Regulatory release results in the loss of enemies during the invasion process resulting in direct changes to plant fitness or biomass. Compensatory release is when the loss of enemies results in an indirect release, as the limited resources utilized for defense are reallocated elsewhere. Both types of release are not mutually exclusive and can be seen where an introduced plant is released from an enemy that caused significant damage and elicited a defense.

Apart from life history traits and Enemy Release, fluctuating resources in an environment has gained prominence as an important theory explaining invasive spread (Davis et al. 2000). Disturbances in an environment may lead to an increased availability in resources that make a community vulnerable to invasion. Introduced species are known to readily invade disturbed tropical forests (Fine 2002).

While theories on invasive spread provide insight and a framework for understanding the mechanisms that enable introduced species to become invasive, researchers are increasingly leaning towards interrelated causes for invasive spread. Blumenthal (2008) argues that high resource availability and Enemy Release may interact to provide introduced species the opportunity to become invasive. Thebaud et al. (1996) believe that a variety of ecological factors, namely competition with neighboring plants, resource availability and herbivore pressure interact to help introduced species become invasive. Interactive effects may create “invasion opportunity windows”, times when introduced species make advances in native communities (Johnstone 1986).

Considering the fact that *E.globulus* was free from its natural insect enemies for the first 150 years following its introduction, Enemy Release may have played a significant role in its expansion (Paine and Millar 2002). 15 of its insect enemies were accidentally introduced into California only within the past 20 years. Although, several studies have found that native enemies can rapidly colonize introduced plants (Strong et al. 1984, Auerbach and Simberloff 1988) *E.globulus* is thought to have been relatively free from native insect damage (Paine and Millar 2002). This may be due to that fact *Eucalyptus* species are known to produce defensive compounds in response to herbivory (Eyles et al. 2003a; 2003b, Rapley et al.2008).

The production of these chemicals can be costly due to complex biosynthetic pathways (Cheng et al. 2007). The need to construct specialized storage due to auto-toxicity adds to the cost associated with defensive compounds. Auto-toxicity is the idea that the chemicals plants produces for defense against herbivores can negatively affect the fitness of the plant itself (Karban and Meyers 1989).

Plants may reduce the cost of producing chemical defenses by only manufacturing them after herbivory, also known as induced defense. After observing larvae chewing off partially eaten leaves in *Eucalyptus,* Edwards and Wanjura (1989) hypothesized that this behavior was an attempt to stop an induced response from the plant. A study done by Rapley et al. (2008) in Southern Tasmania looking at the damage caused by the autumn gum moth on *E.globulus* found an increase in concentration of foliar tannins. Changes in plant secondary metabolites have been recorded as a response to wounding and disease in the bark of *E.globulus* (Eyles 2003a; 2003b). Some studies have observed constitutive defenses (Klemola et al. 2003, Haukioja 2005), however, induced defenses are considered to be more beneficial and cost efficient (Karban and Meyers 1989, Agrawal and Karban 1999).

Terpenes are a commonly found defensive compound in the foliage of *Eucalyptus*. Although there are other defensive compounds such as phenols, tannins and foliar waxes found in *Eucalyptus,* terpenes are the most structurally diverse group of secondary plant metabolites and are thought to be more involved in defense (Gershenzon, 1994). Secondary plant metabolites are defined as compounds whose production is restricted to certain plant groups, and are not necessary for respiration and photosynthesis. Monoterpenes and certain sesquiterpenes are volatile compounds released from a plant after herbivore attack. Monoterpenes are colorless, lipophilic substances that are associated with the characteristic odor of most plants. These volatile compounds attract anthropods that subsequently attack the herbivore. Diterpenes and other sesquiterpenes act as toxins on the herbivore, and are involved in direct defense (Cheng et al. 2007). Terpenes are compounds formed by the union of two or more five carbon units known as isoprenes. Terpenes are grouped according to the number of isoprene units present. However, only the lower terpenes, monoterpenes (two isoprene units), sesquiterpenes (three isoprene units), and to a much lesser extent diterpenes (four isoprene units) are volatile in nature (Vaughn, 2001).

In eucalyptus, terpenes accumulate in secretory cavities and secretory ducts that are internal structures with large spherical intercellular spaces lined with a layer of specialized secretory cells (Gershenzon, 1994). Terpenes are stored under pressure within these cavities. When an herbivore severs a cavity or a duct the terpenes rush to the surface creating a high concentration of terpenes at the point of attack.

The object of this study is to test the possible contribution of the Enemy Release Hypothesis to the expansion and invasive growth of *E.globulus* in California. In order to test how the Enemy Release Hypothesis may have played a role in the invasive spread of *E.globulus* I will examine the regulatory release with the use of insect exclosures and compare the growth of protected and unprotected saplings. I will also examine the compensatory release by testing for induced defenses in unprotected seedlings as a response to herbivory. I hypothesize that there is a resource trade-off between seedling growth and defense if unprotected seedlings are observed to grow less than protected seedlings and have high concentrations of terpenes as compared to the protected seedlings.

Methods

In order to test if plants protected from herbivory are able to allocate more resources to growth than those unprotected from herbivory I conducted a field experiment. I chose to conduct the experiment at Point Pinole Regional Park as it primarily consists of *E.globulus* stands, and I was able to easily access seedlings of appropriate height. The environment at Pinole Regional Park is also believed to be similar to native habitat of *E.globulus* (JH 2008, pers. comm). I received a permit from the East Bay Regional Parks to carry out the field experiment.

The experiment lasted from December 12th 2008 until April 2nd 2009. I chose to perform a paired experiment in which I had 15 pairs of protected and unprotected seedlings, a total of 30 seedlings. Each pair was of similar height and located within 10 feet of each other. A paired design was chosen to minimize effects of uncontrolled variables such as differences in water, sunlight and nutrient availability. Each pair was between 39cm and 72cm tall. Over this time period I visited the seedlings 5 times to measure height of each seedling and make estimates of the number of insects found on each seedling. The specific dates I visited the field site to collect data are December 12th 2008, February 7th 2009, February 21st 2009, March 14th 2009, and April 2nd 2009. Height of seedlings was measured using a measuring tap. Both non-native and native insects were counted. Insect numbers were estimated at each visit by counting the number of insects visible on the leaves. The insects were not removed from the seedlings to be identified as that may have affected the induced response that I was trying to measure. Instead insects were identified in the field by my mentor Prof. Paul Fine.

To construct the exclosures for the protected and unprotected seedlings I needed PVC pipe, netting, zip ties, thin metal wire and binder clips.

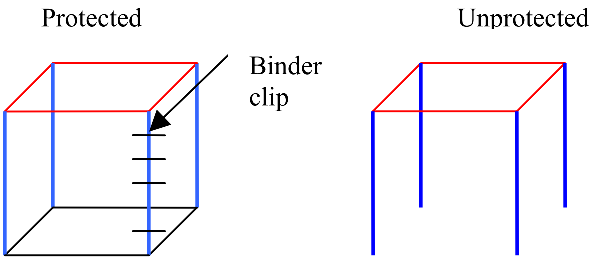


Figure 1: enemy exclusion. 4 PVC pipes are indicated in blue. Wire on top is indicated in red

PVC pipes cut to one meter were used for the frame of the cages (Fig.1). Each pipe was placed one meter apart, creating a cube. The four pipes were connected on top with thin metal wire to create a roof. Frost protection netting from American horticulture with width of seven feet and length of 250 feet was bought from American Horticulture (www.americanhort.com) to protect seedlings from insects.

The netting was cut into two ten feet strips for the protected cage. Each strip of netting was draped over the wire roof to cover the 4 sides. The netting was secured to the wire and the PVC pipe using zip ties. As the 2 strips of netting would over lap on the roof, the top most strip of netting was cut to make sure that there would only be one layer of netting on the roof. If not each seedling would be provided with twice the amount of shading on top than it would be getting from the 4 sides of the exclosure. In order to make measurements one side of the netting was secured to a PVC pipe using binder clips. This side would act as a flap that could be opened for taking measurements. The netting was secured to the ground using metal wire folded in two to create a hook, which was then driven into the soil. This made sure the edges of the netting were nailed down. After speaking with the superintendent of the park about the pests they had observed in the past it was determined that no pests could get in through the soil, and nailing down the edges would be sufficient.

For the protected exclosure the PVC pipes and the wire roof were assembled in the same manner, but the roof was the only part netted using a 1m x 1m piece of netting. The netting allows 80% of light through. The roof was netted to account for any difference in shading of the protected and unprotected seedlings by the netting.

To measure an induced defense in unprotected seedlings the largest youngest leaf on each seedling was broken off at the end of the study. I did not collect leaves at the beginning to establish a baseline level of terpenes as Rapley et al. (2008) believed by doing so they may have induced a response in their study seedlings. Leaves were not collected during the study to look at a trend as that may have affected the plants photosynthesis and therefore growth. It was assumed that looking at the end concentrations would indicate if there was an induced response (Eyles et al. 2003a; 2003b, Rapley et al.2008). Each leaf was stored in a zip lock bag and preserved in a -80F freezer (Rapley et al. 2008).

Gas Chromatography-Mass Spectrometry (GCMS) was used to analyze the difference in concentration of terpenes in the leaves of the protected and unprotected seedlings. Several steps were taken to prepare samples for GCMS. A one-hole puncher was used to take the same amount from each leaf to create samples. Each circular leaf sample was quickly put in a labeled glass 3ml vial and the top was screwed on. When all samples were in their respective vials, 2ml of a mixture of Dichloromethane (DCM) and the internal standard Dodecaine were added to each vial. The addition of the DCM and Dodecaine was done by unscrewing the cap and quickly pipeting the mixture into the vial. The vials were kept open for the shortest time possible as terpenes are extremely volatile.

An internal standard is used to quantify concentrations of compounds in samples run through a GCMS. It is a known concentration of a compound known not to be in the samples being tested. The concentration of the internal standard used for this study was 10ng/microlitre.

The results for the height measurements were analyzed using a Repeated Measures ANOVA since I was looking at the same seedlings over a period of time; therefore, the height measurements obtained at different times would not be independent of each other. The terpene concentrations were analyzed using a paired t-test.

Results

At the conclusion of the field experiment the protected seedlings had grown an average of 30% greater than the unprotected seedlings. This result indicates that seedlings protected from herbivores are able to allocate more resources to their growth than seedlings exposed to herbivores.

The protected and unprotected seedlings begin at the same height as seen in (Fig. 2). At the second time when height was measured, as indicated by t2 on the graph, the protected seedlings had already grown larger than the unprotected seedlings. This difference in growth is consistent until the end of the experiment. The difference in height between both groups was found to be significant ($P<0.001$, F-stat = 39.461)

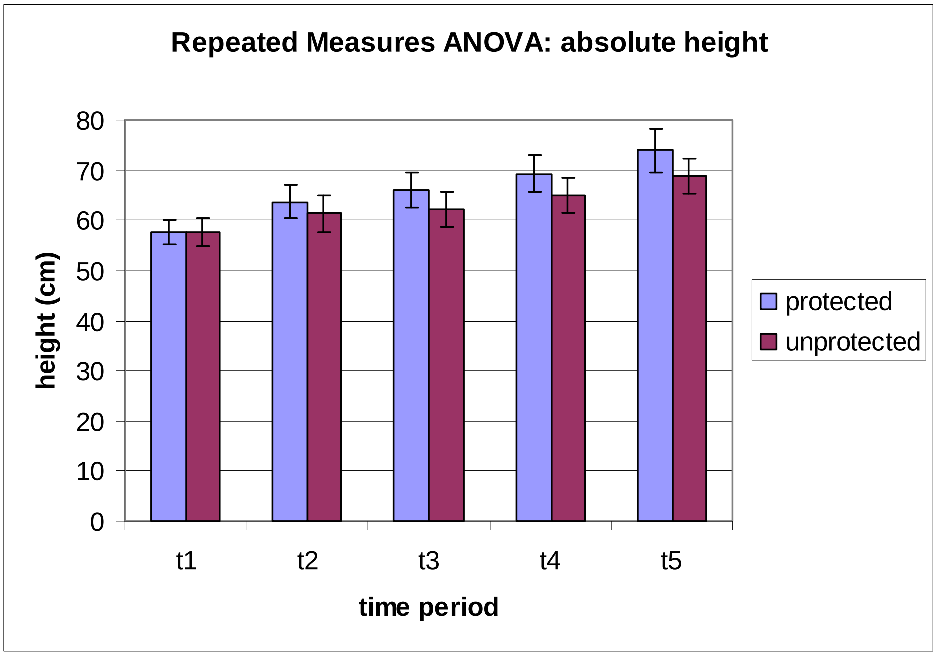


Figure 2: The graph depicts the absolute mean height of protected and unprotected groups at each collection time. The error bars indicate the standard error. Protected and unprotected seedlings start at the same height; however, protected seedlings continue to grow greater than unprotected seedlings

(Fig. 3) further illustrates the difference in height between protected and unprotected groups. It can be seen that the difference in height between the 2 groups was growing with time.

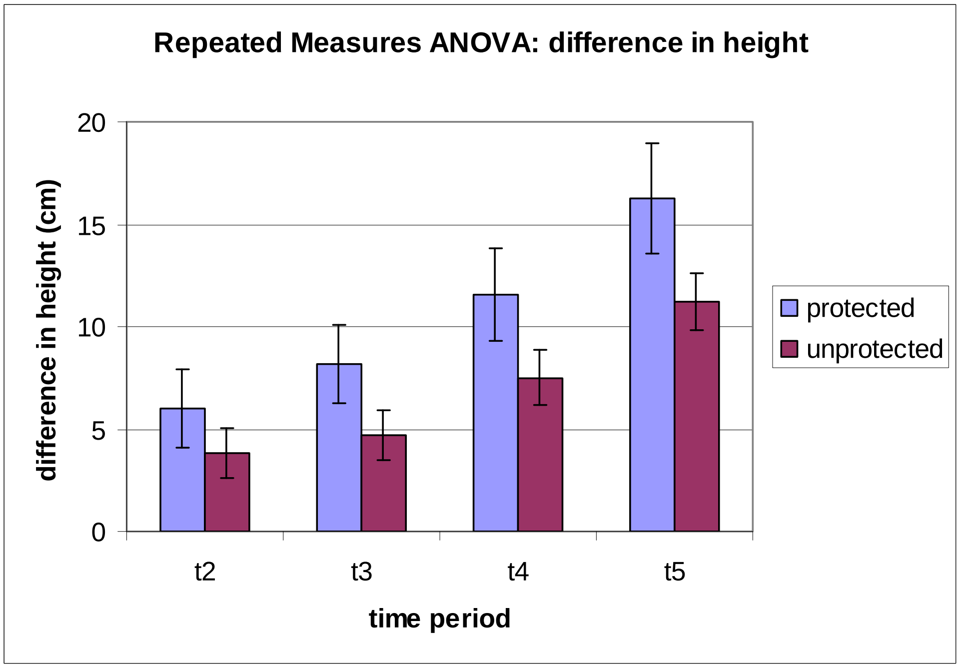


Figure 3: mean difference in height ±SE. Histograms further illustrate the difference in growth between protected and unprotected seedlings.

Evidence was found for induced defenses in seedlings when terpene concentrations between protected and unprotected seedlings were compared. Monoterpenes and sesquiterpenes were found in the leaf samples, but no diterpenes. (Fig. 4) shows the difference in concentration of monoterpenes between protected and unprotected seedlings. Monoterpenes were found to be significantly higher in unprotected seedlings than protected seedlings (P<0.042, t-stat = 2.725).

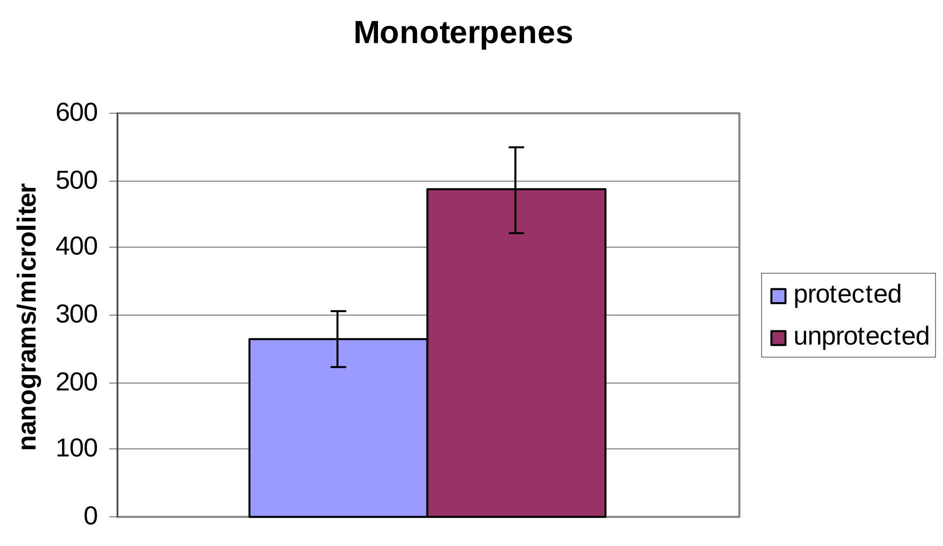
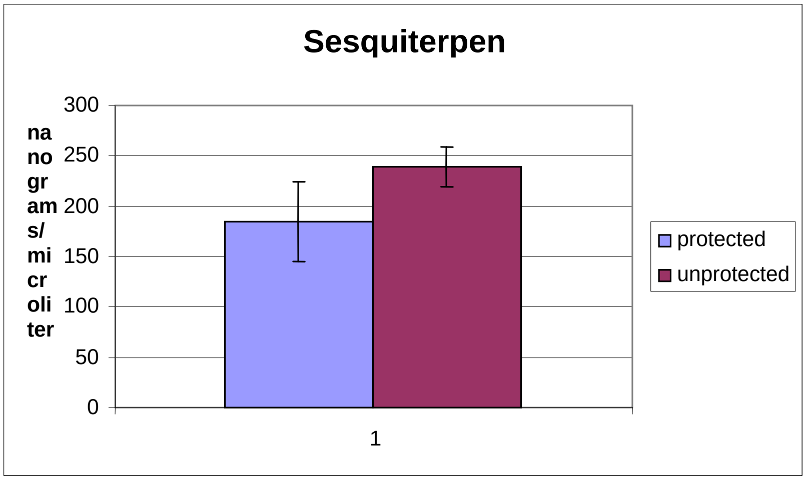
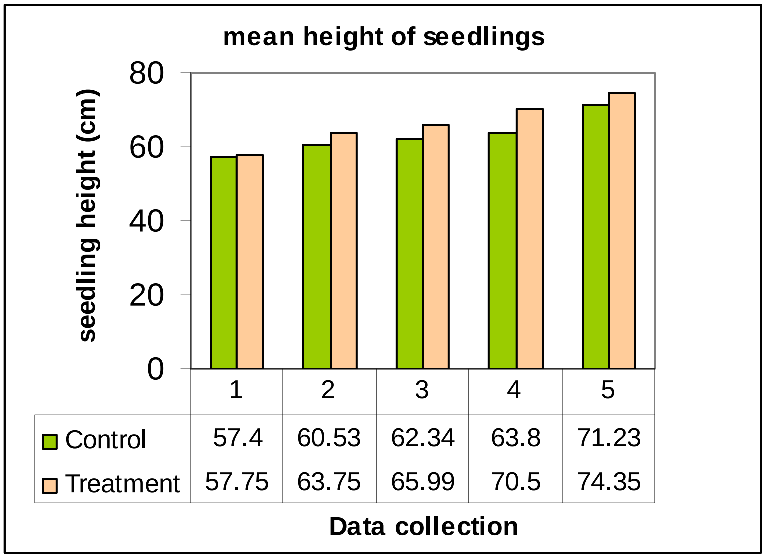


Figure 4: monoterpenes concentrations in nanograms/microliter are shown by the histogram. Error bars indicate means (± SE). Unprotected seedlings have a greater concentration of monoterpenes than protected seedlings

Sesquiterpene concentrations were higher in unprotected seedlings similar to monoterpenes (Fig. 5). However the findings were not significant (P< 0.194, t-stat = 1.499).

	~~13~~	~~57.4~~	~~60.5307692~~	~~62.33846~~

 ~~C~~				
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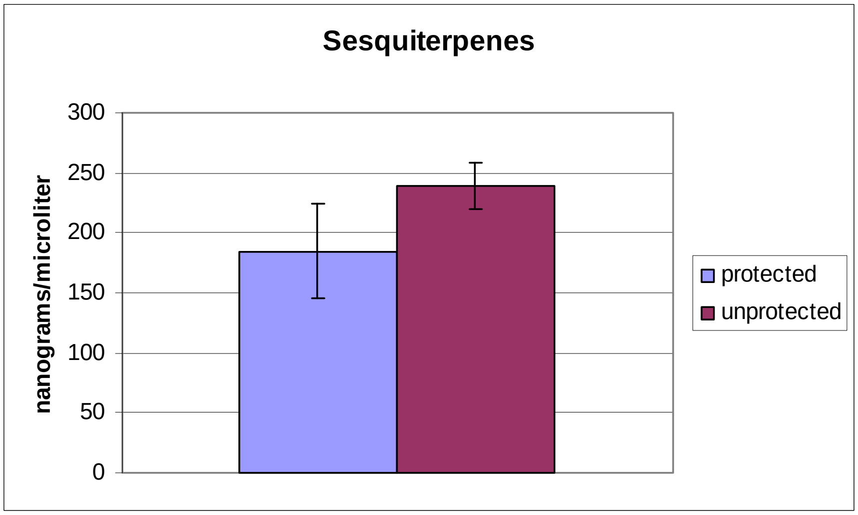

Figure 5: mean concentration of sesquiterpenes in protected and unprotected seedlings ± SE. Although sesquiterpenes were higher in unprotected the result was not significant.

Only one type of insect was found to be attacking the study seedlings. The insect was *Eucalyptus psyllid* a scale insect native to Australia (PF 2009, pers comm). No correlation was found between insect numbers and terpene concentrations.

Discussion

Protected seedlings were shown to grow greater than unprotected seedlings (Fig. 2, Fig. 3). The result indicates that protected seedlings were able to allocate more available resources to growth than seedlings exposed to herbivores. This suggests that when *E.globulus* was first introduced to California it underwent a regulatory release which enabled a direct investment in its growth and expansion.

The result also suggests herbivory as a means of controlling the future expansion and invasive spread of *E.globulus.* Examples of successful biological control of other invasives using insects include Cacti in Australia and South Africa controlled by mealybugs, and the control of the floating fern Salvinia in Australia by the weevil *Cyrtobagous salviniae* (Hosking et al. 1988, Moran et al. 1991, McFayden 1998). However, damage to non-target plants due to the introduction of insects is an important concern. The wasp *Cactoblastis cactorum* introduced to the Caribbean to control native cactus *Opuntia triacantha* has spread to the Florida Keys and is threatening native *Opuntia spinosissima* which has already been reduced due to development (McFayden 1998). In the cases reviewed by McFayden (1998) where non-target plants have been damaged the economic loss has been minor and temporary,and the benefits have been much greater.

Evidence for inducible defenses in *E.globulus* was also observed (Fig. 4). The concentration of monoterpenes in the unprotected seedlings was almost double the amount found in the protected seedlings. While Rapley et al. (2008) and Eyles et al. (2003a; 2003b) have found inducible defenses in *E.globulus* other studies have found inducible defenses in other plant species. For example, phenolic compounds induced in birches by the larval feeding of *Epirrita autumnata* (Kaitaniemi *et al*. 1998), phenolic compounds induced in quaking aspen after the larval feeding of *Choristoneura conflictana* (Clauson *et al*. 1991), tannins in oak after larval damage by *Lymantria dispar* (Schultz and Baldwin 1982; Rossiter *et al*. 1988) and terpenoids and phenolics in conifers after attack by bark beetles (Raffa 1991).

It is important to note that Rapley et al. (2008) saw an increase in foliar tannins following damage by the autumn gum moth which feeds by chewing on the foliage. Eyles et al. (2003a, 2003b) found increases in a range of compounds that included tannins and terpenes following damage to the lesion margin of bark by a fungal infection. Differences in the types of induced defenses seen in *E.globulus* are in keeping with the idea that *Eucalyptus* species experience variable herbivory (Herms and Mattson 1992). Differences in type of compounds induced may be evidence that plants have different defense mechanisms for different enemies. It would be interesting to look at different natural enemies of *E.globulus* in the future to test if they induce different defenses. Different defenses may have different levels of cost, and therefore, may affect the fitness of the plant differently (Colautti, 2004).

Several studies have been able to provide evidence for a resource trade-off between defense and other fitness parameters (Fritz and Simms 1992, Bergelson and Purrington 1996, Strauss and Agrawal 1999, Fine et al. 2006). Although this study provides evidence for regulatory release where protected seedlings were able to grow greater than unprotected seedlings, a correlation between number of insects and terpene concentrations have not been found as evidence for a compensatory release. Therefore, it cannot be conclusively said that the protected seedlings were growing greater due to increased investment in growth and fewer investments in defense.

When examining invasive success it is important to take into account competition with native plant species in the community being invaded (Colautti 2004). Atsatt and O'Dowd (1976) have pointed out the importance of the different types of plants in the invaded community that may attract or repel herbivores. Natural enemies of *E.globulus* that have been introduced to California may prefer natives over *E.globulus* as natives may not have developed defenses against them (Colautti 2004). *E.globulus* is known to use allelopathy and to store resources in the soil near it as a means of competing with plants in the invaded community (Davidson 1993). Like other studies looking at invasive spread have found, it is more than likely competition with other plants have played a role in the expansion of *E.globulus* in California. Future research looking at the invasiveness of *E.globulus* in California or elsewhere should take competition for resources with other plants and enemy impact on neighboring plants into account.

Finally, the netting used in this study may have caused a large enough shading effect to impact the amount of terpenes released in response to herbivores. A few studies have been able to prove that terpene emission is regulated by light, with the highest amount emitted during the day (Lu et al. 2002, Dudareva et al. 2004). If the netting did provide a shading effect great enough, the amount of terpenes being emitted in the unprotected seedlings may have been lower than seedlings not used in the study. This would have made the unprotected seedlings more susceptible to insect attack than surrounding seedlings and would have had a larger impact on the growth of the seedling.

The netting may have also shaded the protected seedlings that may have influenced their growth. Greater growth may have been observed in the protected seedlings because they were growing towards the available sunlight. If this was happening the protected seedlings would have experienced a decrease in biomass (CD 2008, pers. Comm.). Therefore, it is important to look at different parameters of growth such as stem diameter and number of new leaves in addition to height.

Conclusion This study was able to demonstrate that *E.globulus* seedlings are able to invest more resources in growth in the absence of herbivores, undergoing a regulatory release. Induced defenses were found in unprotected seedlings suggesting that *E.globulus* may not invest in defense when herbivores are absent. The findings of this study suggest that *E.globulus* may have benefited from escape from natural enemies following the first 150 years after its introduction in California. However, it is important to keep in mind that several ecological factors interact to enable introduced species to become invasive. Therefore, it cannot be said that the Enemy Release Hypothesis is the only explanation for the continued expansion of *E.globulus* in California.

Acknowledgements

I thank Prof. Paul Fine who has mentored me for the past year; the instructors and graduate students of ES 196B for all their guidance; Dr. Alissa Myrick of the Biology Scholars Program for the grant and support through out the project; Christopher DiVittorio for help with the exclosures, statistics, and general advice; John Hitchen and all the staff at Pinole Regional Park, and All my friends who helped me in the field.

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