Effects of Drought Stress on Floral Hemp (Cannabis sativa L.) Agricultural Systems

by

Gary Wayne Morgan Jr

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Approved by

Alvaro Sanz-Saez, Chair, Assistant Professor of Crop, Soil and Environmental Sciences
Katelyn Kesheimer, Department of Entomology and Plant Pathology
Jeanine Davis, Department of Horticultural Science, North Carolina State University

Abstract

Hemp (*Cannabis sativa* L.) is a crop of renewed interest in modern agriculture. However, for it to be considered hemp and not marijuana, it must have a total THC concentration of no greater than 0.3%. Drought is a common abiotic factor that can affect many locations globally, therefore it is important to conserve water resources when possible. Drought stress is believed to affect hemp at the production and physiological levels as well as can potentially increase THC concentrations. The objective of this Master Thesis was to determine the effects of drought stress intensities and timings on yield and cannabinoid concentrations, carbon assimilation and light capture mechanisms, as well as arthropod communities. Two cultivars of hemp, BaOx and Cherry Mom were planted in a greenhouse in early July and harvested in early October of 2021 and 2022. Moderate water stresses (30-50% soil water content) were found to not affect final yields, cannabinoid concentrations, or many carbon and light capture mechanisms within the crop. However, more intense drought stresses can negatively impact these parameters. In terms of arthropods this study found drought stresses do not significantly affect insect populations, but 'Cherry Mom' may be more susceptible to pests. These findings suggest that it is possible to cultivate a healthy and productive hemp crop while significantly reducing water use. This can lead to increased sustainability in terms of hemp production systems.

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CHAPTER 1: Literature Review

HISTORY AND USES OF HEMP

Hemp (*Cannabis sativa* L.) is a multipurpose crop native to central Asia that has been cultivated for millennia (McPartland et al., 2019). Evidence suggests that hemp was grown in a variety of locations in ancient times, including hemp seed consumption dated to 10,000 BP (Before Present) in Japan, fiber utilization in China circa 5,600 BP (McPartland et al., 2019) and as reported by Bradshaw et al. (1981), and fossil evidence of *Cannabis sativa* utilization in England dating back to 2,000 BP. There are at least 25,000 known uses of hemp involving fiber, seed, and cannabinoid products (Schluttenhofer and Yuan, 2017).

Traditionally, hemp has been grown for fiber and seed products. The term *canvas*, a common textile, originates from the Arabic word *kannabis*, which translates to hemp, and has been mentioned in Greek literature dating back to the fifth century (Addlesperger, 2015). The stalks of hemp are highly fibrous, primarily composed of bast fiber and hurd (Schluttenhofer and Yuan 2017). The bast fibers are used for papers and fabrics (Addlesperger, 2015; Schluttenhofer and Yuan 2017) and the hurd is typically used for animal bedding (Schluttenhofer and Yuan, 2017). More recently, an increased interest in the production of bioethanol from hurd (Gonzalez-Garcia et al., 2012).

Hemp seeds are nutrient dense, containing 30% protein, 25% starch, and 30% oil, with 90% of the oil being polyunsaturated fatty acids (Schluttenhofer and Yuan, 2017). In addition to its uses as fiber and food, there is evidence of hemp's potential in phytoremediation of heavy metals. It is widely known that mining can be detrimental to the environment, oftentimes releasing hazardous chemicals into the areas surrounding the mining activities. Husain et al. (2019) suggest that hemp plays a role in the phytoremediation of mined land, reporting that hemp grown in soils contaminated with nickel produced leaves containing 2.54 times more nickel compared to a control group. The study suggests that hemp may be a suitable plant for phytoremediation purposes in soils also contaminated with lead or cadmium (Husain et al., 2019).

The history of hemp as a cornerstone crop with a variety of uses throughout many civilizations is well-established. Renewed interest in modern hemp cultivation has not only resurrected these once common uses but has facilitated the rise of new and innovative uses. Medicinal usage of hemp has a similarly long and rich history. Ancient cultures, such as the Scythians, used hemp for medicinal purposes (Addlesperger, 2015). Medicinal use of hemp has been receiving renewed interest as of recent. Medicinally important cannabinoids have been attributed to mitigating pain and nausea stemming from chemotherapy (Agar, 2018). Cannabidiol (CBD), the most prominent cannabinoid produced by hemp, has been studied for potential medicinal uses and been approved for orphan drug status in eleven disorders, including glioma multiforme, brain cancer, and pediatric schizophrenia (Schluttenhofer and Yuan, 2017). Cannabidiol, specifically, has shown great promise in the treatment of numerous conditions and diseases such as healing heart, liver, and kidney diseases by interacting with various cellular systems and protecting the organs against oxidative stresses related to the activity of specific proteins and reactive oxygen species (Pacher et al., 2019). The same mechanism that CBD uses to combat oxidative stresses, has been implicated in the potential treatment of neurological disorders, most notably Parkinson's disease and Alzheimer's disease (Pacher et al., 2019).

The medicinal properties of cannabinoids are not limited to CBD; the chemically similar but, psychoactive cannabinoid tetrahydrocannabinol (THC) has shown success in the treatment of ailments such as multiple sclerosis. However, THC must be administered in high enough quantities that psychoactive side effects are induced (Pacher et al., 2019). The psychoactive properties of

THC are a primary reason the compound and plants that produce it are illegal compared to CBD, a non-psychoactive cannabinoid (Pacher et al., 2019; Schlosser, 1994). In fact, the psychoactive effects of THC are only a small part of why the compound and THC producing plants are illegal (Schlosser, 1994). Schlosser explains that in the early 1900's, marijuana was a common recreational substance for migrant workers from Mexico. These migrants, who were not favored by many southwestern United States locals, were looked down upon for their use of marijuana, ultimately allowing for preconceived stereotypes and prejudices to be developed (Schlosser, 1994). These preconceived and incorrect notions allowed for many local and state laws to be written prohibiting marijuana use, ultimately paving the way for marijuana legislation that still prevails to this day at the federal level (Schlosser, 1994). This xenophobic ideology of the early 1900s produced massive changes to the agricultural landscape as we know it today, by prohibiting hemp for nearly a century; a crop which had been at the forefront of American agriculture (Schlosser, 1994).

HEMP LEGAL ISSUES

In 1937, Congress passed the Marihuana Tax Act, which made it illegal to produce the crop unless a farmer was registered and agreed to pay a levy of one dollar per ounce of *Cannabis* produced (Addlesperger, 2015). This made hemp production no longer financially viable for farmers (Addlesperger, 2015). In addition, some states did explicitly outlaw the production of all *Cannabis* (Addlesperger, 2015). In 1970, hemp was officially designated as a Schedule I controlled substance via *The Federal Comprehensive Drug Abuse Prevention & Control Act* (Ellison, 2021). Hemp production was not permitted in the U.S. by federal law until the 2018 Farm Bill was signed into law (Mattingly, 2020). Following the 2018 federal legalization of hemp, more than 90,000 acres of hemp were planted throughout the United States, which is the most hemp grown since 1943 when 146,200 acres were planted (Mark et al., 2020).

In 2021, North Carolina had 13,987.32 acres of farmland and 6,895,270.59 square feet of licensed greenhouse area licensed for hemp (Paul Adams, NC Department of Agriculture & Consumer Services Industrial Hemp Program, personal communication, July 8, 2021). In 2021, Alabama had 570 acres of licensed farmland and 294,096 square feet of licensed greenhouse area dedicated to hemp production (National Agricultural Statistics Service 2022). Hemp and marijuana are the same plant, only differing in the level of THC produced. Hemp can only contain a maximum of 0.3% total THC, the psychoactive substance, on a dry weight basis (Skorbiansky et al., 2021). The final federal hemp rule dictates crop harvest must take place no later than thirty days post-compliance testing and set a negligence threshold at 1% total THC (Agricultural Marketing Service USDA, 2021). In the event of exceeding the 0.3% threshold, farmers are required to destroy the non-compliant crop (Skorbiansky et al., 2021) or remediate it (Agricultural Marketing Service USDA, 2021). According to the Agricultural Marketing Service, under the interim rule in 2020, 730 out of 6,166 USDA licensed acres (11.8% of all crops) had to be destroyed after exceeding this threshold (Agricultural Marketing Service USDA, 2021). Crop destruction results in significant loss for the farmer, many times making the loss irrecoverable to the farmer, however, to minimize these losses the USDA now allows for an extended (30 days) pre-harvest testing window, remediation of material via a floral dilution and partial crop disposal (Agricultural Marketing Service USDA, 2021). However, a grower who exceeds the 1% THC negligence threshold three times in five years will have their license revoked for five years (Agricultural Marketing Service USDA, 2021).

The 2018 Farm Bill, which commercially legalized hemp production was not without complications. One issue was that states were allowed the option to continue to operate under the 2014 Farm Bill (Mattingly, 2020). This appeared to not be an issue because the federal testing requirement also tested for THC compliance (Skorbiansky et al., 2021). However, states that chose to operate under the 2014 Farm Bill, may had different testing methods or sampling protocols. Farmers marketing their products in different states could encounter difficulties due to unique state sampling requirements. For example, North Carolina tested between weeks three and five of the flowering phase and had no deadline to harvest (North Carolina Department of Agriculture & Consumer Services Compliance Officer, personal communication). According to North Carolina's prior pilot program hemp law regarding marketability read as, “Samples with a THC level equal to or below 0.3% THC shall require no further action and the area or harvested plant material from which the sample was obtained shall be released for marketing or further processing.” (NCDA&CS Industrial Hemp Commission, 2017). This meant under the outdated law North Carolina hemp flower products were legal due to their state-issued THC test reports, however, buyers did not feel comfortable accepting this material because third party testing may have shown non-compliant THC content. Now that states are transitioning to permanent programs approved by the USDA or under USDA supervised cultivation these issues will hopefully be resolved (Agricultural Marketing Service USDA, 2021).

Possible causes of non-compliance hemp crop

Since hemp’s recent legalization, regulatory testing has shown crops “go hot”, a term which describes a crop that exceeds the 0.3% THC threshold (Jackson et al., 2021). However, little research has been conducted as to what causes this to happen. This is often attributed to environmental factors. Research on marijuana has shown that application rates of nitrogen fertilizer can affect cannabinoid content in *Cannabis sativa* (Saloner and Bernstein, 2021). Their findings suggest that insufficient nutrient concentrations, specifically nitrogen, result in elevated cannabinoid concentrations tetrahydrocannabinolic acid (THC-A) and cannabidiolic acid (CBD-A) and as nitrogen levels increased, cannabinoid content decreased (Saloner and Bernstein, 2021). Defoliation by corn earworm, *Helicoverpa zea*, larvae has been shown to significantly increase cannabinoid content (Jackson et al., 2021). In laboratory conditions, both CBD and THC concentrations significantly increased under elevated insect pressure. In field trials, one cultivar (Cherry Blossom) had significantly different CBD and THC levels due to insect feeding; however, the cultivar "the Wife" did not show significant differences (Jackson et al., 2021).

Lighting may also impact the production of cannabinoids in hemp. Research has shown that artificial lighting can significantly impact cannabinoid content, depending on specific light spectra configurations (Islam et al., 2020). Many artificial light-emitting diode (LED) configurations resulted in a variety of changes in CBD, CBD-A, THC, and THC-A production, with elevated THC production in all artificial lighting scenarios (Islam et al., 2020). These findings ultimately suggest that farmers are potentially risking hemp crops above the legal THC levels, when supplementing crops with LED lighting in the flowering phase. High levels of heavy metals in the environment can also influence cannabinoid production (Husain et al., 2019). Cannabidiol was found to significantly increase in the presence of heavy metals in soils, and qRT-PCR revealed that CBD-A synthase expression was eighteen times higher relative to non-contaminated controls (Husain et al., 2019). It has been shown that hemp genetics can significantly impact THC content; showing some cultivars are predisposed to higher THC concentrations than others (Mechtler et al., 2004). A number of adverse environmental conditions, such as drought and insect pressure, could be contributing factors to non-compliant hemp. Caplan et al., (2019) demonstrated increased cannabinoid content in medical cannabis grown in drought stressed environments. This response of THC and CBD to drought stress may extend to hemp, where there may also be variations in drought responses between cultivars. It would also be noteworthy to determine if the combination of drought stress and insect pressure are related to increased CBD and THC in hemp, as each individual factor has previously shown significant increases in both cannabinoids.

DROUGHT STRESS IN CROPS

Stress can affect crops in many ways, and often these stresses negatively impact crop health and production. Crop stress can stem from both biotic and abiotic sources. Drought is a prominent abiotic factor that commonly affects both domesticated and non-domesticated plants in a number of ways.

Effect of drought on physiology and yield

Reduction in growth is a common result following drought stress due to lack of cell expansion in plants (Keipp et al., 2020). Turgor pressure aids cell expansion and growth by allowing cells to form specific regions of differentiation (Coussement et al., 2021). If turgor pressure is reduced beyond a critical point due to drought, this differentiation ceases, and the cells cease to expand, resulting in reduced plant growth (Coussement et al., 2021).

Plant growth is also affected by drought due to the reduction in the amount of carbon dioxide fixed by photosynthesis. Stomata regulate gas and water exchange with the outside environment. Under water stress, leaf water potential is reduced, and stomata are closed to reduce water loss (Dias and Bruggemann, 2010). When stomata close to conserve water, there is minimal gas exchange occurring with the outside environment which limits the amount of carbon dioxide available for photosynthesis (Dias and Bruggemann, 2010). This reduction in carbon dioxide via stomatal closure ultimately leads to a reduction in the rate of photosynthesis and biomass accumulation, and therefore growth (Hu et al., 2010).

Besides stomatal limitations, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo), the enzyme responsible for CO2 fixation, is greatly affected by drought, resulting in inhibition of the carboxylation function at about 47.5%-50% (variety dependent). However, functionality can be restored once drought stress subsides (Xu et al., 2020). RuBisCo concentration, along with functionality, has shown significant decreases under drought, which affects photosynthesis (Nagy et al., 2013). Significant decreases in RuBisCo concentrations of drought stressed wheat (*Triticum aesitivum*) suggest a physiologic feedback response for slowing metabolism during drought, however, some genotypes do not reduce RuBisCo concentrations making it possible to select for cultivars that could be more drought tolerant (Nagy et al., 2013).

Besides the carbon fixation processes of photosynthesis such as stomatal aperture and RuBisCo activity, drought can also affect the light energy harvesting reactions. Photosystem I and Photosystem II are each affected differently under drought, but these impacts on the individual photosystem components reduce overall photosynthesis (Leverne and Krieger-Liszkay, 2021; Xu et al., 2020). This reduction in the functionality of the photosystems is hypothesized to be correlated with an overall reduction in electron absorption and therefore energy that could be used for carbon dioxide fixation (Leverne and Krieger-Liszkay, 2021). As the photosystems reduce the amount of photons that they can absorb, the extra photons that are received by the chlorophyll are capable of producing reactive oxygen species (ROS) when the transport of electrons across thylakoid membranes cannot result in the production of NADPH (Pospisil, 2009). These ROS have been shown to be destructive to many components of cells, leading to decreased biologic functionality (Pospisil, 2009). This includes damage to the structural integrity of cells (membranes and cell walls), as well as damaging internal components of cells such as DNA and lipids through oxidation (Berni et al., 2019). Drought stressed alfalfa (*Medicago sativa* L.) demonstrates diminished Photosystem II functionality, however, Photosystem I appears to not be affected (Xu et al., 2020). On the other hand, Leverne and Krieger-Liszkay (2021) have shown that Photosystem II of spinach (*Spinacia oleracea*) is not affected as severely because a physiologic response to drought stress slows the flow of electrons to Photosystem II.

Changes in plant physiology that allow the plant to survive drought can have negative impacts on yield. For example, lentil (*Lens culinaris*) experiences approximately a 24% decrease in yield compared to well-watered controls (Farooq et al., 2017). Common bean (*Phaseolus vulgaris*), a very drought sensitive legume, has shown a 40%-87% decrease in yield depending on the timing of the drought (Farooq et al., 2017). Sunflower (*Helianthus annuus*) has also shown significantly reduced yields when exposed to a 40% field capacity drought (Keipp et al., 2020). Decreases in seed weight and oil weight, but not oil concentration is attributed to a lack of cellular growth during the seed growth and maturation phases, resulting in lower yields (Coussement et al., 2021; Keipp et al., 2020). This study found that the cell size of drought-stressed sunflowers was significantly smaller than the controls (115.0 ± 5.1 micrometers, 104.9 ± 3.1 micrometers for each drought variety) (Keipp et al., 2020). Grain filling in wheat, specifically the water-soluble carbohydrates, has been shown to be affected during drought through the rate of remobilization from the plant's stems to grain (Liu et al., 2020). Wheat genotypes vary in their ability to remobilize water soluble carbohydrates with an improved ability to remobilize water soluble carbohydrates first in the lower portions of the plants, with steadily decreasing water-soluble carbohydrate concentrations in upper main stem internodes (Liu et al., 2020).

DROUGHT STRESS IN CANNABIS

Caplan et al., (2019) found that drought stress applied to medicinal marijuana in the last two weeks of the growing cycle did not decrease the flower yield. However, studies in hemp have previously shown variable effects on yield, as some strains are unaffected by drought stress and other strains have exhibited significant losses in yields under stress (Babaei and Ajdanian, 2020). Well irrigated hemp trials (watered at 100% evapotranspiration) have shown significantly greater yields than trials with reduced irrigation (75% of calculated evapotranspiration) (Garcia-Tejero et al., 2019). In fact, the final yields of well-watered controls were 43%-64% times higher than the drought treatments (Garcia-Tejero et al., 2019). These decreases in *Cannabis sativa* yields have been attributed to decreases in the photosynthetic rate caused by stomatal closure (Caplan et al., 2019; Herppich et al., 2020). Several studies have shown that drought will significantly affect final yields of hemp (Amaducci et al., 2000; Babaei and Ajdanian, 2020; Garcia-Tejero et al., 2019; Herppich et al., 2020). However, these studies often speculate the effect of drought at varying growth stages. These studies did not provide a wide range of drought intensities during hemp development. More research is needed to understand the effects responses of variable degrees of drought at different phases of hemp reproduction.

DROUGHT EFFECTS ON SECONDARY METABOLITES

Effect of drought on production of secondary metabolites

Drought stress has been shown effect secondary metabolites and economically valuable compounds of medicinal plants (Kleinwachter and Selmar, 2015). Kleinwachter and Selmar (2015) have shown that nearly all major categories of economically valuable secondary compounds increase significantly when exposed to at least a modest drought stress, however, this drought stress is also shown to decrease final yields and therefore metabolite yields.

Many have hypothesized why there are significant increases of specific compounds, such as essential oils, in response to drought stress. Kleinwachter and Selmar (2015) propose that in drought stress the stomata close which restricts the uptake of carbon dioxide. The limited uptake of carbon dioxide means that the Calvin cycle functionality is diminished and there is an overabundance of NADPH and H^+ derived from the light reactions. This overabundance of NADPH and H^+ allows for the production of reduced compounds such as secondary metabolites (Kleinwachter and Selmar, 2015). Research from Mohammadi et al. (2019) shows that one economically important chemical derived from *Ferula assa-foetida*, known as thiourea, is produced as a means of destroying reactive oxygen species, such as hydrogen peroxide in times of drought. It is assumed that a similar biological mechanism is responsible for the increased essential oils found in drought-stressed *Ferula assa-foetida* (Kleinwachter and Selmar, 2015; Mohammadi et al., 2019).

Cannabis sativa, marijuana and hemp, has displayed unique abilities to alter physiology and secondary metabolites under varying degrees of drought. Evolutionarily, cannabinoids were a defense substance meant to protect the plant from herbivory, by deterring and, in some circumstances, incapacitating pests that fed on the plant (Kariñho-Betancourt, 2018). Caplan et al. (2019) found that the major cannabinoids such as CBD, CBDA, THC, and THCA were all significantly increased when medical cannabis (marijuana) was exposed to mild drought stress (Caplan et al., 2019). Excess NADPH derived from the light reactions could explain the increased CBD, CBDA, THC, and THCA concentrations observed by Caplan et al. (2019) as unused NADPH can be used to produce reduced secondary metabolites as referenced before (Kleinwachter and Selmar, 2015).

There has been limited research on hemp on the effects of drought on THC and CBD content and the relationship with photosynthetic parameters related to carbon capture and photosynthetic light reactions. A project performed under field conditions in Spain in fiber hemp showed that moderate drought stress resulted in a significant increase in the cannabinoids CBG and CBC with a slight increase in THC content (Garcia-Tejero et al., 2019). Drought stress studies on other crops have shown significant increases in secondary metabolite concentrations that may occur in *Cannabis sativa*, as well. An example of this phenomenon was demonstrated in black cumin (*Nigella sativa*). Black cumin has shown in trials that the concentration of economically valuable compounds, such as essential oils have the potential to be increased under drought (Bayati et al., 2020). If there are significant increases in cannabinoids, such as THC, and decreases in CBD, like in black cumin metabolites (Bayati et al., 2020), this could negatively impact hemp compliance. However, if decreases in THC and increases in CBD were to occur this could be a beneficial response for producers. Therefore, it is crucial to know if there is a significant relationship between the level of drought stress and the production of cannabinoids, in particular CBD and THC. It is also important to compare the effects of drought stress on photosynthesis, yield, and pest pressure response to find similarities or differences to the findings of Caplan et al. (2019), Babaei and Ajdanian (2020), and Jackson et al. (2021). Replicated, research-based information will allow us to recommend specific watering regimens, and potentially genetics, to hemp farmers that allow for to allow for high yields, compliance, and efficient water use.

EFFECT OF INSECT PESTS ON CROPS

Arthropods are a major biotic factor that can infest and harm many plants. Insects consume plants by a variety of methods and this is based on their specific mouthparts, such as piercing-sucking mouthparts (Stafford et al., 2012). Arthropods with piercing-sucking mouthparts consume nutrients from within the plant cells or vascular tissues (Stafford et al., 2012). This method of feeding supply pest arthropods with nutrition while negatively affecting the plants' overall health (Farag Mahmoud, 2013; Stafford et al., 2012). Research has shown the ability for arthropods to infest a wide array of economically important crops such as maize (*Zea mays* L.) (Brewbaker and Kim, 1979). Two common pests of maize are the earworm (*Helicoverpa zea* Boddie) larvae and the fall armyworm (*Spodoptera frugiperda*) larvae, which are known to destroy the ear (Brewbaker and Kim, 1979). Arthropod damage can also facilitate additional pest infestations, such as pathogens. When maize crops sustain insect damage, this can allow for the introduction of fungal pathogens like yellow mold (*Aspergillus flavus)*, which produce toxins that are extremely harmful to humans (McMillian et al., 1978). Cotton (*Gossypium hirsutum* L.), another economically important crop, can also be infested with *H. zea* as well as thrips (Thysanoptera) (Brook et al., 1992). Typical damage to cotton is characterized by destruction of reproductive structures in cotton such as buds and bolls produced by each plant (Brook et al., 1992). The destruction of these structures reduces overall yields if infested with these pests (Brook et al., 1992).

While arthropods naturally infest a number of crops, environmental factors can influence arthropod populations on the plant. Drought, a common abiotic stressor, has been known to influence significant increases in plant pests (Mattson and Haack, 1987). It is hypothesized that drought stressed plants are more palatable to phytophagous arthropods due to the concentration of nutrients supplied by the plant (Mattson and Haack, 1987). Therefore, the more concentrated the nutrients are in the plant the more nutritious the plant is to insects resulting in a greater pest population (Mattson and Haack 1987; Ajayi and Samuel-Foo 2021).

There are several pests that have been identified on hemp, existing across a range of arthropod orders; however, there is minimal research conducted on the relationship between these arthropods and drought (Ajayi and Samuel-Foo, 2021). Corn earworm is a major pest of hemp, and infestations may be exacerbated in drought conditions due to weakening of the plants' insect defense mechanisms (Ajayi and Samuel-Foo, 2021).

EFFECTS OF ARTHROPODS ON CANNABINOID CONCENTRATIONS

There is little research on how insect communities in hemp are affected by drought. However, in the event of infestation, recent research has shown that cannabinoid production in hemp can be significantly affected. Park et al. (2022) determined that there are significant reductions of the cannabinoids cannabigerol (CBG), cannabigerolic acid (CBGA), and CBD when infected by tobacco hornworm (*Manduca sexta*). On the contrary, other researchers have found a strong relationship between corn earworm populations and increased THC and CBD in some hemp cultivars (Jackson et al., 2021).

EFFECT OF DROUGHT ON ARTHROPOD POPULATIONS

It has been thoroughly shown that drought stress affects plants on a number of different levels, including causing increased insect pressure. It has been proposed that drought stressed plants provide concentrated nutrition to herbivorous insects, as well as provide a more hospitable environment compared to well-watered plants (Mattson and Haack, 1987). Natural defense mechanisms of plants have also shown to be negatively impacted during drought, facilitating greater pest pressures (English-Loeb, 1990). However, it is proposed that if drought stress becomes strong enough, insect populations will be negatively impacted (English-Loeb, 1990; Mattson and Haack, 1987). Physiologic changes to drought stressed plants, such as yellowing of foliage and increased nutrient content, are highly attractive to many pests due to the color and concentrated nutrients in the foliage (Mattson and Haack, 1987).

OBJECTIVES

Due to the prior legal status, *Cannabis sativa*, hemp and marijuana, has had very little research conducted using modern practices and techniques. Studies on other crops, however, provide insight into the responses of drought timing and intensities on yield and physiological response, as well as the crops' attractiveness to pests. Because of the potential benefits hemp presents to producers, a thorough examination of yield, cannabinoid accumulation response, physiological response, and arthropod plant interactions are warranted. Therefore, the objectives of this study are:

1. Investigate the effects of various drought levels and timing on hemp growth, photosynthesis, and yield.

2. Study the effect of drought levels and timing on hemp THC and CBD content at different stages of flower development to determine the optimum plant age to perform harvest to maximize CBD yield and reduce the risk of THC non-compliance.

3. Study the effect of drought levels and timing on hemp insect pest populations with the end goal of designing adequate pest management practices.

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CHAPTER 2

Moderate Drought Stress Minimally Affects Floral Hemp (*Cannabis sativa* L.) Yield and Cannabinoid Content While Severe Stress Decreases It

Wayne Morgan[1], Jagdeep Singh[1], Katelyn Kesheimer[2], Jeanine Davis[3], Alvaro Sanz-Saez[1]*

[1] Department of Crop, Soil and Environmental Sciences, 253 Funchess Hall Auburn University, Auburn, AL 36849, USA

[2] Department of Entomology and Plant Pathology, 301 Funchess Hall Auburn University, Auburn, AL 36849 USA

[3] Department of Horticultural Science, North Carolina State University, 455 Research Drive, Mills River, NC 28759

Abstract

Hemp (*Cannabis sativa* L.) is a new crop of interest for many farmers due to its myriad of potential uses in everyday life and its potential to grow in a range of different environments. However, in order for it to be legally designated as hemp and not marijuana, it must have a THC concentration of no more than 0.3%. If this is exceeded the crop must be remediated or destroyed, resulting in a financial loss to the producer. Therefore, the objective of the study was to examine the effects of various timings and levels of drought on hemp floral yield, water use efficiency, and cannabinoids concentrations. The hemp cultivars BaOx and Cherry Mom were planted in a commercial greenhouse setting in 2021 and 2022 at the beginning of July and harvested in the first week of October. It was determined that the water use efficiencies and total transpiration varied between cultivars and that moderate water stresses (30%-50% soil water content) could produce similar yields to the thoroughly irrigated treatments while using less water. Moderate drought intensities did not modify tetrahydrocannabinol (THC) or cannabidiol (CBD) levels, however, intense drought treatments led to decreased THC or CBD concentrations. Overall, the percent of THC responded to drought stress with minor decreases whereas the percent of CBD was significantly decreased in intense drought stresses. These findings suggest future water regimes may be revised to reduce water usage and maintain yields, leading to increased sustainability of the floral hemp agricultural system.

Keywords: drought intensity, floral hemp, CBD%, THC%, greenhouse

INTRODUCTION

Hemp (*Cannabis sativa* L.) is a re-emerging crop originated from Central Asia (McPartland et al., 2019). For millennia, hemp was utilized by ancient civilizations due to its nutritional, medical, and practical household uses (McPartland et al., 2019). The seed produced on female plants can be utilized for food and cosmetic purposes because it contains a wide variety of oils and nutritional components (McPartland et al., 2019; Strzelczyk et al., 2022). The flower has long been used medicinally by many civilizations because of the cannabinoids produced by the plant (McPartland et al., 2019; Strzelczyk et al., 2022). Modern uses of the cannabinoids derived from hemp are purported to treat a number of ailments ranging from neurological maladies to cancer (Pacher et al., 2019). During the 20^{th} century hemp production declined as many countries, including the United States, illegalized its production due to its similarities with marijuana (*Cannabis sativa*, they are the same species) (Schlosser, 1994).

Over time, hemp legalization gained popularity and in 2018 hemp production was federally legalized in the United States (Mattingly, 2020). Floral hemp, utilized for cannabidiol (CBD) production is a particularly popular option for cultivation purposes. The legalization of hemp allowed for extensive production of the crop in the United States exceeding 90,000 acres in the first year following re-legalization (Mark et al., 2020). However, for *Cannabis sativa* to be considered hemp and not marijuana, it must not exceed tetrahydrocannabinol (THC) levels of 0.3% total-THC on a dry weight basis (Skorbiansky et al., 2021). In 2020, following the national legalization of hemp production, 11.4% of the hemp crops planted were destroyed for non-compliant THC levels (Agricultural Marketing Service USDA, 2021). Due to high prices of hemp seed or cuttings and other agronomic inputs (fertilization, pesticides, lights, etc.), crop destruction due to non-compliant THC concentrations, could result in large economic impacts for the producer and economy (National Agricultural Statistics Service USDA 2022). Therefore, understanding the biological and environmental factors affecting THC accumulation in hemp is necessary to prevent these losses due to non-compliance (Skorbiansky et al., 2021; Suzuki et al., 2014).

Hemp, though phenologically and genetically diverse, has been shown to maintain cannabinoid accrual at similar ratios of CBD and THC in floral tissues regardless of stress imparted on the plants (Toth et al., 2021). Toth et al. (2021) suggest that cultivar and phenology influence cannabinoid levels more than environmental conditions. Therefore, it is hypothesized that cultivars that mature earlier or environmental conditions such as drought that accelerate the phenology of the plant may result in higher THC and CBD concentrations (Carlson et al., 2021; Toth et al., 2021). However, many producers believe drought stress increases THC and CBD contents, but the research results are scarce and contradictory on the subject as there has been limited academic research conducted as the crop was illegal in many countries until quite recently.

Water stress is the most important abiotic stress for crop production as it reduces yield on irrigated and rain-fed conditions (Araus et al., 2002; Boyer, 1982). For example, the 2012 drought, which affected much of the U.S. decreased yield significantly in many row crops and cost the economy approximately 30 billion dollars (Rippey, 2015). Yield reduction under drought is the consequence of plants closing their stomata to reduce transpiration and save water. This reduces the amount of C that is fixed through photosynthesis and, therefore, biomass accumulation and yield (Frederick et al., 2001; Gao et al., 2021; Sakoda et al., 2022; Zhang et al., 2022). The effect of drought on agricultural crops depends on the intensity of the drought (Gao et al., 2021) and the timing (Frederick et al., 2001). For example, in soybean and peanut, drought affects yields the most when is applied during early flowering or pod filling (Frederick et al., 2001; Zhang et al., 2022). Hemp yields are also influenced by drought stress (Garcia-Tejero et al., 2019, Gill et al., 2022), however, due to the limited research on the crop much is unknown about how drought timing affects yields. Research has shown marijuana to maintain yields if drought stresses are initiated in the final weeks of flowering. Though no studies on series of drought timings have been conducted on *C. sativa*, it appears to often times be cultivar specific in the response to drought stress in which greater irrigation results in higher biomass yields (Babaei and Ajdanian, 2020; Caplan et al., 2019; Garcia-Tejero et al., 2019).

Evolutionarily, it is believed that secondary metabolites are produced as a defense mechanism against stresses such as herbivory attacks (Jackson et al., 2021). Therefore, many believe that abiotic stress may trigger defense mechanisms in hemp to produce cannabinoids such as THC and CBD, as if the plant was being attacked by another organisms (Jackson et al., 2021). Physiologically, alteration of the concentrations of cannabinoids, such as CBD and THC, in plants under drought stress may be related to a reduction of C-fixation (Sakoda et al., 2022) that results in excess NADPH produced in the light reactions that could be used for the production of secondary metabolites such as cannabinoids (Kleinwachter and Selmar 2015).

While the definitive cause of THC increases in *Cannabis sativa* is unclear, many producers attribute it to environmental conditions like drought. Marijuana has been shown to significantly increase THC and CBD concentration within the floral portions of the plant when exposed to drought in the last two weeks of flowering (Caplan et al., 2019), whereas hemp did not have significant increases in CBD for different irrigation regimes (Garcia-Tejero et al., 2019). These discrepancies in experimental results make it challenging for hemp producers to grow their crops with the same confidence that they would with other commercial crops. This challenge for producers stems from the fact that the greatest returns can be obtained by maximizing cannabinoid production and yield through optimal agronomic procedures while maintaining compliant THC concentrations (Carlson et al., 2021; Garcia-Tejero et al., 2019; Toth et al., 2021). Since hemp must not exceed the THC threshold of 0.3% total THC, producers may turn to over-irrigation of their crops or harvest early in an attempt to maximize production as well as maintain the legal status of the crop (Skorbiansky et al., 2021). Due to a changing climate, it is essential agricultural water is conserved as increasing temperatures and droughts threaten water resources globally (Wreford and Adger, 2010). In terms of flower yield, over-irrigation may not be required as hemp can successfully reproduce at low soil water moisture levels (Gill et al., 2022). This illustrates the hemp plant's ability to adapt to low water environments and suggests an opportunity to decrease the irrigation and increase water use efficiency of the crop. For these reasons, the objectives of this study were: 1) Determine the effects of various timings and levels of drought on hemp floral yield, water use efficiency, and cannabinoids concentrations, 2) Test if there is cultivar variation to drought in yield and cannabinoid concentration, 3) Study if cannabinoid content changes due to the phenology of different cultivars and if the cannabinoid concentration and ratio changes with time and drought stress.

MATERIALS AND METHODS

Study design, setup, and management

Hemp cultivation trials were conducted in 2021 and 2022 in Zirconia, North Carolina (35.213349, -82.416249) under greenhouse conditions. The first-year seedlings were planted on July 3, 2021, and the second year were planted on July 1, 2022. Two cultivars of high CBD hemp, ‘BaOx’ and ‘Cherry Mom’, were hand planted into 19-liter nursery pots and plant starters. The plants were clones sourced from one parent plant per cultivar to ensure identical genetics. The soil mixture was a premix “living soil” sourced from Dirtcraft Organics (Dirtcraft Organics LLC, Marshall, NC, USA).

During the vegetative stage each plant was grown using standard cultivation procedures. The pots were watered individually, as needed, to maintain an adequate and consistent soil water content (SWC) of approximately 100%-70%. A Fluence SPYDR series LED lighting system (Fluence USA, Austin, TX, USA) was installed in the greenhouse to provide supplemental light to ensure the plants were maintained in the vegetative state until the intended flowering date. The lighting system was programmed to provide an 18-hour light cycle. An industrial fan with a diameter of 63.5 centimeters was used to maintain temperature and promote airflow. Onset HOBO UA-002-64 pendants (Onset Brands, Bourne, MA, USA) were placed inside and outside of the greenhouse to record environmental conditions throughout the trials (Supplemental Table 1). The plants were supplemented with a 10-20-20 fertilizer in equal increments to ensure each plant received the equivalent of 68 kilograms of Nitrogen per acre throughout the flowering cycle.

Each 19-liter nursery pot’s weight was recorded and tared using an Ohaus Range 3000 series scale (Ohaus Corporation, Parsippany, NJ, USA). The pots were each filled with approximately 5,400 grams of soil and each weight was recorded. The pots were watered each day for three days until the soil was fully saturated to ensure the soil in each container was at 100% SWC. Additionally, 10 tared paper bags were filled with approximately 1,000 grams of soil. The 10 bags were placed in a drying oven at 105-130℃ for 5 days to ensure complete moisture removal. The final soil weights were then recorded. The thoroughly dry soil and saturated soil weights were used to generate a SWC curve to calculate upper and lower SWC ranges for each pot and drought treatment.

In both years flowering was initiated by reducing lighting hours to natural sunlight hours (maximum 14 hours). In the first year, flowering was initiated on August 10, 2021. The plants were watered at the same schedule as the vegetative stage until August 17, 2021 when drought was initiated. The second year of trials were transitioned into reproduction on August 8, 2022, and drought was initiated on August 15, 2022. Starting at drought initiation, each pot was weighed to get an estimate of the weight of each plant. This measurement was used to calibrate the total pot weight for accurate SWC measurements. Additionally, all drainage holes of the pots were covered to ensure no water loss occurred during the drought. The pots were weighed every morning from the beginning of drought until harvest. If the pot was at or below the lower threshold for the drought treatment (see below), the pot was watered until the upper threshold was attained. Total plant transpiration was calculated by adding the amount of water used by the plant and recorded by daily weighting. Plants were harvested on October 5, 2021 and October 3, 2022 for year one and year two experiments, respectively.

Drought treatment specifics

Forty-two clones per variety were randomly planted into one of the seven drought treatments. Early drought stresses were initiated seven days post-flower initiation and late drought stresses were initiated 28 days post-flower initiation. Each year trials were planted in a randomized complete block design (RCBD) that contained six replications per treatment. The seven drought treatments were as follows: Control (100%-70% SWC), Early Extreme Drought Stress (EE) (10%-0% SWC), Early Intermittent Drought Stress (EI) (100%-0% SWC), Early Moderate Drought Stress (EM) (50%-30% SWC), Late Extreme Drought Stress (LE) 10%-0% SWC), Late Intermittent Drought Stress (LI) (100%-0% SWC), and Late Moderate Drought Stress (LM) (50%-30% SWC). The Extreme Drought Stress consisted of maintaining the pot SWC between 0%-10% during the whole length of the drought period. The Intermittent Drought stress plants were watered until capacity (100% SWC), then let dry until reaching 0% SWC, at which time they were irrigated to reach 100% SWC, repeating this cycle as many times as necessary during the drought period. Moderate Drought Stress treatments were maintained at a 50%-30% SWC during the whole drought period.

In the first year, the crop was treated with GRANDEVO CG (Marrone Bio Innovations, Inc, Davis, CA, USA) during vegetative development following the instructions on the label every seven days from planting until August to prevent severe pest outbreaks of mites and aphids. There was no need to apply GRANDEVO CG during the second season as there were significantly less pests than year one during the vegetative phase.

Cannabinoid testing

Each year, cannabinoid sampling began at day 35 post-flower initiation and occurred at 10-day intervals. Samples for cannabinoid analysis were collected by cutting approximately four centimeters of the second tallest cola from each plant. This sample was then cut and lightly trimmed to a weight of approximately 2-3 grams. The samples were placed in individual paper envelopes and were vacuum-sealed for shipping. ACS laboratories (Sun City Center, FL, USA) analyzed each sample for a 10-cannabinoid potency profile that included CBD and THC. Upon arrival at the lab, the samples were oven-dried to the desired moisture content. The samples were then analyzed via Ultra High-Performance Liquid Chromatography-Tandem Mass Spectrometry using a UV detector to quantify cannabinoid concentrations of the hemp flower (ACS Laboratory, Sun City Center, FL, USA).

Phenology Comparison

Each year, phenological comparisons were conducted on a weekly basis starting at day 42 post-flower initiation. Phenology sampling was conducted by viewing the pistils of the upper third of each plant. The plants maturity was then estimated on a scale of 1-5 with 1 being immature to 5 being the most mature. The ratings were as follows: 1 (0%-20% browned pistils), 2 (20%-40% browned pistils), 3 (40%-60% browned pistils), 4 (60%-80% browned pistils), and 5 (80+ % browned pistils).

Harvest

Multiple measurements were conducted at harvest. Each year at harvest all vegetative leaves were removed from each plant and were placed in a paper bag to dry. The plants were then hung in the greenhouse for seven days to dry to an adequate floral moisture content of approximately 5%-10%. The stem, stalk, and flower component of each subject was separated and weighed individually. Moisture content of each component was also recorded using a General MMD4E moisture meter (General Tools and Instruments, Secaucus, NJ, USA) so each sample could be corrected to 0% moisture content. Water Use Efficiency (WUE was calculated as: (Dry Flower Yield (g) / Total Water Transpired (g)) x 1000.

Statistical Analysis

All data during the trials were analyzed using RStudio (R Foundation for Statistical Computing, Vienna, Austria). Both years were analyzed separately as there was a significant year effect for all measured parameters. To determine the effects of drought and cultivar over all measurements, two-way Analysis of Variance (ANOVA) and Tukey's HSD test were utilized to determine if significant differences existed at individual sampling dates such as in the harvest data. Furthermore, a Repeated Measures ANOVA was conducted to determine if measurements differed significantly over the course of cannabinoid samplings each season. The AGRICOLAE package in R was utilized to determine significant Tukey differences among all the treatments. Graphics to visualize results were generated using the ggplot2 package and corrplot package in RStudio.

RESULTS

Water use efficiency related traits

Water Use Efficiency (WUE) was similar among cultivars in 2021, however, in 2022 'BaOx' showed a 16% higher WUE than 'Cherry Mom' (Table 1). In both years, the EE water stress treatment was the only one than showed a higher WUE in comparison with the control. In 2021, 'BaOx' grown under EE, EI, and LE water treatments showed a 72%, 54%, and 29% lower transpiration, respectively, than the control with EM, LI, and LM treatments showing similar transpiration as the control. For 'Cherry Mom', as the control treatment already showed a 43% lower transpiration than 'BaOx', the differences between water treatments was less significant, with only the EE treatment showing a significant 61% lower transpiration (Table 1). In 2022, the cultivar Cherry Mom showed a 17% greater total transpiration than 'BaOx' (Table 1). In both cultivars, total transpiration was found to be 75%, 56%, 43%, and 29%, respectively for 'BaOx' and 76%, 54%, 43%, and 31%, respectively for 'Cherry Mom' lower in EE, EI, LE, and LI treatments compared to the controls, with EM and LM treatments showing similar transpiration as the controls (Table 1).

Yield and Harvest Index

The cultivar BaOx showed higher flower yield than 'Cherry Mom' in 2021, but 'Baox' was more sensitive to drought with the EE, EI, LE, and LI treatments showing 51%, 51%, 41%, and 27% lower yields than the control, respectively (Figure. 1). Interestingly, there were no significant decreases for any water stress treatments as compared to the control for 'Cherry Mom', although the EE, EI, and LI treatment showed a 29%, 14%, and 11% reduction in flower yield respectively (Figure 1). In 2022, both cultivars resulted in significant decreases of 62%, 47%, and 46% in flower yield for EE, EI, and LE treatments, respectively in 'BaOx' and 54%, 39%, and 32%, respectively for ‘Cherry Mom’ (Figure 1). Interestingly, both the EM and LM treatments did not show a decrease in yield (Figure 1). This shows the potential to reduce water use substantially while still maintaining similar yields as control treatments, however, if water availability is reduced too much, yields can suffer significantly.

In 2021, ‘BaOx’ showed a 20% lower harvest index (HI) than ‘Cherry Mom’ (Figure 2). In ‘Cherry Mom’ drought decreased the HI 14% in the EE treatment, while in ‘BaOx’ drought only decreased the HI 17% in the LE treatment (Figure 2). In 2022, ‘Cherry Mom’ showed a 5% higher HI than ‘BaOx’ and the water stress (WS) only showed an insignificant decrease of 17% in the EE treatment in comparison with the control for ‘BaOx’ (Figure 2).

Phenology

In 2021, ‘Cherry Mom’ showed more mature pistils than ‘BaOx’ on all days analyzed (Table 2). In addition, ‘Cherry Mom’ showed full matured pistils in all water treatments at 49 days after flowering (DAF) while ‘BaOx’ did not show full brown pistils even at the time of harvest (55 DAF). This means that ‘Cherry Mom’, from a phenological perspective, was a faster maturing cultivar than ‘BaOx’. At 42 DAF, all water treatments with the exception of EM and LM showed a higher maturity score than the control in ‘Cherry Mom’. For ‘BaOx’ at 42 DAF only the LE treatment seemed to have more advanced maturity value than the control. As flowering and drought progressed in the 49 DAF measurement, the EE, EI, LE, and LI treatments showed a more advanced maturity than the control (Table 2). At harvest (55 DAF) all water treatments for ‘BaOx’ showed the same maturity than the control treatment although all of these values were slightly lower than the values for ‘Cherry Mom’.

The year 2022 showed similar overall trends with ‘Cherry Mom’ having a more advanced maturity than ‘BaOx’, except at 55 DAF in which both cultivars had near similar average phenology ratings (Table 2). At 42 and 49 DAF, ‘BaOx’ showed more mature flowers in the EE treatment in comparison with the control showing that although the flowers had matured from one date to the other, the differences between water treatments were maintained. However, at 55 DAF, the EE, EI, LE, and LI treatments showed more mature flowers than the control, EM, and LM treatments. For ‘Cherry Mom’ a similar trend was observed at 42 DAF with only the EE treatment showing faster maturity than the control. At 49 DAF, flower maturity accelerated, with EE, EI, and LE treatments showing faster maturity. At harvest (55 DAF), ‘Cherry Mom’ behaved similarly than ‘BaOx’ as the EE, EI, LE, and LI treatments showed more matured flowers than the control and the moderate drought treatments showed similar flower maturity than the control. This shows that moderate drought did not have an influence on flower maturity in both cultivars, meanwhile severe drought stresses accelerated the hemp phenology.

Cannabinoids concentrations and contents

Cultivar, water treatment, and DAF showed significant effects on THC and CBD percentage on a dry weight basis (THC% and CBD%) in 2021 and 2022. In addition, the interaction between DAF x WS and DAF x cultivar was also significant for THC% and CBD% in both years.

In 2021, CBD% in general was significantly higher in ‘Cherry Mom’ than in ‘BaOx’ early in the season but was only slightly higher at harvest (Figure 3; Table 3). This is believed to be related to the earlier maturity of ‘Cherry Mom’ compared with ‘BaOx’ (Table 2). In 2022, ‘Cherry Mom’ maintained higher CBD% throughout the season, and CBD% did not display a steep increase in concentration as the season advanced, unlike in 2021 (Figure 3; Table 3). In 2021, drought negatively affected CBD% of ‘Cherry Mom’ at all DAF (35, 45, and 55), whereas ‘BaOx’ was only affected by drought at 55 DAF (Table 3). At 55 DAF ‘Cherry Mom’ exhibited significant decreases of approximately 24% for each EE and LE treatments in comparison with the control but ‘BaOx’ showed decreases of 23%, 21%, 28%, and 21% in EE, EI, LE, and LI treatments, respectively (Table 3). These results indicate that ‘Cherry Mom’ may be a preferable strain as it produces higher CBD% but it is more sensitive to drought. Producers must maximize CBD% in flowers as this parameter in combination with flower yield will result in greater marketable oil yield.

In terms of CBD yield (g CBD plant^{-1}), the highest CBD yields occurred in plants experiencing less water stress (Figure 4). In 2021, ‘BaOx’ produced a 45% greater average CBD yield than ‘Cherry Mom’ which is similar to the flower yield differences observed between these cultivars (Figure 4). The ‘BaOx’ cultivar in EE, EI, LE, and LI treatments showed a 62%, 60%, 58%, and 44% lower CBD , respectively, than the control treatment (Figure 4). ‘Cherry Mom’ did not have any water treatment that were significantly different from the control, however, the EM treatment produced 13% greater CBD yield per plant of any ‘Cherry Mom’ water stress treatment (Figure 4). In 2022, ‘BaOx’ produced a 28% lower CBD yield than ‘Cherry Mom’ (Figure 4). For ‘BaOx’, the EE treatment showed 77% lower CBD yield than the control (Figure 4). ‘Cherry Mom’ produced similar results with the EE treatment, showing a 71% decrease in CBD yield (Figure 4). Additionally, the LI treatment of ‘Cherry Mom’, was 35% lower than the control (Figure 4). These results show that extremely low SWC can significantly limit CBD yields per plant through a decrease in flower yield and CBD%.

In general, in 2021, ‘Cherry Mom’ plants showed a significantly higher THC content than ‘BaOx’, showing much higher THC% early in the season and with only slightly higher values at the end of the season (Figure 4). This is probably related to the quicker flower maturity in ‘Cherry Mom’ in comparison with ‘BaOx’ (Table 2). In 2022, ‘Cherry Mom’ showed significantly higher THC values than ‘BaOx’ during the whole season without ‘BaOx’ increasing its THC% as quickly as in 2021 at the end of the season (Figure 4; Table 4). In 2021, drought did not affect THC% for ‘BaOx’ until 55 DAF (Figure 4; Table 4). At that time, only the LE treatment showed decreased THC% of 22% in comparison with the control and with the other drought treatments being similar to the control. For ‘Cherry Mom’, drought decreased 17% the THC% at 35 DAF in the EE treatment as compared to the control and at 45 DAF, there were not differences in THC% from the control (Table 4). At 55 DAF ‘Cherry Mom’ still showed higher THC% than ‘BaOx’ but the difference between them was reduced significantly (Figure 4; Table 4). For ‘BaOx’, the lowest THC% was found in the LE treatment with a 22% decrease; whereas ‘Cherry Mom’ showed THC% decreases of 11%, 11%, and 15% in the EE, EI, and LE treatments, respectively, even though they were not statistically different than the control (Figure 4; Table 4). In 2022 at 35 DAF, both cultivars showed significant decreased THC% of 22% (‘BaOx’) and 19% (‘Cherry Mom’) only in the EE treatment (Figure 4; Table 4). At 55 DAF, ‘BaOx’ only showed a significant decrease of 27% in the EE treatments, while ‘Cherry Mom’ showed significant decreases of 30%, 19%, 21%, and 22% in THC% in EE, EI, LE, and LI treatments, respectively, in comparison with the control treatment (Table 4). These results show that greater water stresses can result in lower THC% and phenology appears to be related to when these effects are found (Table 4; Table 2). Additionally, these results find that drought does not increase THC levels at any timing or intensity.

CBD to THC ratios (CBD:THC) were found to shift over time and with water stress each year of the study for both cultivars (Table 5). In both years of the study at 35 DAF, ‘BaOx’, showed a significantly lower CBD:THC than ‘Cherry Mom’ (Table 5). Each year at 35 DAF the significantly lower CBD:THC was found in the EE treatment as compared to the control (Table 5). In both cultivars, the control, EM, and LM treatments had the highest CBD:THC of any water treatment at 35 DAF (Table 5). In 2021, at 45 DAF the cultivars displayed similar average ratios, but greater water stresses once again reduced CBD:THC (Table 5). For ‘BaOx’, while no treatments were significantly different from one another, in general there were reduced CBD:THC as water stress increased (Table 5). ‘Cherry Mom’, however, followed a general pattern similar to ‘BaOx’ but had significantly lower CBD:THC in the EE and EI treatments in comparison with the control (Table 5). At 55 DAF in 2021, both cultivars maintained similar CBD:THC (Table 5). In both cultivars, the EE treatment showed a significantly lower CBD:THC and ‘BaOx’ also showed a significantly lower CBD:THC for the EI treatment at that time point (Table 5). In 2022, ‘Cherry Mom’ always showed a significantly higher CBD:THC than ‘BaOx’ during the whole season (Table 5). At 55 DAF, the control, EM, and LM treatments showed the highest CBD:THC and the EE treatment showed the lowest ratio in both cultivar (Table 5). The effect of water stresses appears to alter CBD:THC with the greatest, and often most significant changes in ratios, occurring with the greater and longer stresses.

DISCUSSION

Effect of drought on transpiration yield and water use efficiency

Plant evapotranspiration is linked to biomass accumulation and therefore yield. To fix carbon through photosynthesis, the plant needs to open the stomata and lose water (Staple and Lehane, 1954; Zhang et al., 2022). In this experiment, we observed that total transpiration, biomass accumulation, and flower yield showed a similar response to the different drought treatments showing the relationship between them. In addition, total transpiration varied between cultivars each year of study, as in 2021 'BaOx' showed much higher transpiration than 'Cherry Mom' likely due to the fact that 'BaOx' was a much larger plant than 'Cherry Mom'. The small size and transpiration of 'Cherry Mom' in 2021 was probably due to a weaker clone cut in 2021 or a bad adaptation of those clones to the greenhouse conditions, as in 2022 both cultivars showed similar biomass accumulations and drought responses. The larger size and transpiration of 'BaOx' in 2021 resulted in this cultivar being more sensitive to water stresses than 'Cherry Mom'. Similar to our study, experiments involving quinoa (*Chenopodium quinoa*) find that large, fertilized plants can suffer from drought stress because they quickly transpire the water available in the soil (Alandia et al., 2016). Oy the contrary, small canopy size results in lower transpiration and drought tolerance, although average yields are low (Barraclough et al., 1989).

In 'BaOx' in 2021 and in both cultivars in 2022, intense early (EE and EI) and late (LE) drought treatments resulted in yield decreases between 30%-60% due to the significant reductions in plant transpiration, which is linked with a reduction in photosynthesis and therefore biomass accumulation and yield (Alandia et al., 2016; Staple and Lehane, 1954; Zhang et al., 2022). Gill et al., (2022) observe biomass reduction of 60%-90% when watering is reduced between 50%-90% during the whole growing season starting at the vegetative stage. In the current experiment we observed lower yield reductions in our extreme treatments, likely because the drought stress was applied only during the flowering period and not from the vegetative period onward as in Gill et al., (2022). In both years of the experiment and for both cultivars, moderate stresses, early moderate (EM) and late moderate (LM), were similar in flower yield but showed 20%-33% lower water use than the well-watered control. Similar results were obtained in field experiments in a Mediterranean environment where a reduction of 25% of evapotranspiration does not result in yield reductions (Garcia-Tejero et al., 2019). Our results and those from Garcia-Tejero et al., (2019), suggest that floral hemp is tolerant to moderate drought stresses and that this fact can be used to increase the water use efficiency of the system and reduce overall water use while maintaining yields. In the current study we also showed that there is cultivar variation for WUE. In this case although 'BaOx' showed more yield sensitivity to drought due to its large size, it showed the ability to produce more flower weight per kg of water, thus WUE. Similar results have been described by Brouder and Volenec (2008), in which crops grown to larger sizes when grown under elevated CO_2 show higher WUE due to increased photosynthesis and thus biomass accumulation while transpiration is maintained.

Harvest Index (HI), which is calculated as flower (seed in seed crops) weight divided by total biomass, is used as a selection criterion for drought tolerant cultivars (Aranjuelo et al., 2013; Zhang et al., 2022). The cultivars that are able to partition more biomass to the reproductive organs are able to yield more under those stressful conditions (Assefa et al., 2013; Songsri et al., 2008). Harvest Index appeared to be greater in 'Cherry Mom' than 'BaOx' because 'Cherry Mom' tended to have a greater amount of flowering sites than 'BaOx', ultimately allowing for more flowers per stem than 'BaOx'. As 'Cherry Mom' was the cultivar which showed lower yield reduction under drought it appears that HI may still be a good selection criterion for drought tolerance although our results are premature as they were tested with only two cultivars.

Effects of drought on cannabinoid concentration and CBD yield

THC% and CBD% in this study tested higher than compliant samples (> 0.3%) likely because we collected a much smaller sample from each plant than in a regular compliance tests. Compliance sampling generally collects 5-8 inches of the main stem flower (AMS USDA, 2021), however, as we sampled three times per season from the same plants, we had to collect smaller samples (approximately 1.5 grams of fresh weight from the main stem flower) at each sampling date. Collecting 5-8 inches each date would have had a deleterious effect on our harvest parameters. This resulted in samples more concentrated in cannabinoids as we collected more flower tissue and less stem and leaves that would dilute the overall cannabinoid content. This method should be reasonable to the overall study and should not affect data interpretation because we are determining the effects of drought timings and intensities rather than comparing cannabinoid concentrations to other studies.

CBD and THC as cannabinoids tend to respond together and it has been previously illustrated that they respond in a similar but not necessarily identical matter (Stack et al., 2021; Toth et al., 2021). Cannabinoid concentration (THC and CBD) was found to increase over the course of the flowering period in both cultivars, however, the cultivars varied at the rate and timing of accumulation. This is believed to be related to the different phenology of the two tested cultivars as ‘Cherry Mom’ often showed a more advanced flower maturity than ‘BaOx’. In fiber hemp maturity changes between different cultivars (Amaducci et al., 2008) and that floral hemp also varies in concentrations and accumulation rates among different cultivars (Stack et al., 2021; Toth et al., 2021). This shows that the cultivars with faster maturity usually show a faster peak in THC and CBD levels, and that these levels are dependent of specific cultivars (Stack et al., 2021; Toth et al., 2021). Knowing how the different cultivars mature during the season can be very useful to producers as choosing a cultivar that produces the majority of its cannabinoids in the last weeks before harvesting may demonstrate greater chances of legal THC% for federal compliance when tested 30 days before harvest (Agricultural Marketing Service USDA, 2021).

Drought has been found to increase secondary metabolites in plants and therefore could increase THC and CBD as they are secondary metabolites (Kleinwachter and Selmar 2015). This is due to the reduction of C-fixation via reduced stomatal conductance and photosynthetic assimilation rates (Gill et al., 2022; Sakoda et al, 2022) resulting in excess NADPH from light reactions being used in the production of secondary metabolites (Kleinwachter and Selmar, 2015). This theory was hypothesized in Caplan et al. (2019) in which there were CBD and THC increases in marijuana plants grown under drought stress. However, it is possible that if a drought is very intense, there could be irreparable damage to the photosynthetic system resulting in reduced light reactions that decrease the amount of available NADPH (Gill et al., 2022) and therefore reduce the levels of CBD and THC (Kleinwachter and Selmar, 2015). In the current study, 'BaOx' did not show decreases in THC% or CBD% in any drought stress treatment until harvest. However, 'Cherry Mom' was more sensitive and showed significant decreases in both cannabinoids earlier on in flowering. This earlier effect of drought stress in 'Cherry Mom' is likely phenologically driven similarly to different accumulation rates and cannabinoid percentages found in some cultivars as illustrated by Stack et al., (2021) and Carlson et al., (2021). 'Cherry Mom' showed earlier maturity than 'BaOx', therefore the effect of drought was shown earlier in that cultivar. However, at the end of the season both cultivars were equally affected, showing reduction in cannabinoids (most notably CBD) in the most extreme water stress treatments.

In 2021, the greater increase of THC and CBD accumulation in 'Cherry Mom' from 35 to 45 DAF coincided with the earlier decreases seen in various drought stresses, whereas the greater increase in 'BaOx' from 45 to 55 DAF coincided with decreases in concentration of 'BaOx' at harvest that were not shown earlier for 'BaOx'. This variation in net cannabinoid percent and the time of peak concentration has been observed to be cultivar dependent as cultivars can accumulate cannabinoids differently and at a range of times during reproduction (Amaducci et al., 2008; Carlson et al., 2021; Stack et al., 2021; Toth et al., 2021). In this study we showed that drought tends to decrease CBD content in a similar way to THC and that this trend is cultivar dependent and likely related to the different phenologies of the cultivars. Decreases in THC% and CBD%, particularly in 'Cherry Mom', under intense drought stress and not under moderate water stress appears to be opposite of the significant increases exhibited in drought stressed marijuana (Caplan et al., 2019). This discrepancy could be because Caplan et al., (2019) uses a very moderate drought stress at the end of flowering, or that marijuana cultivars accumulate more THC than floral hemp.

Combining flower yield and CBD content to determine actual CBD yield is an important parameter for producers as this could represent the economic output of their operation. CBD content was often reduced in the more intense drought stresses at harvest for both cultivars and years of study. Yield was also reduced (many times significantly) in the more intense water stresses, similar to Gill et al (2022). When yield and CBD content is converted to grams of CBD produced per plant, it can be seen that the intense water stresses reduced CBD yield per plant, but the moderate stresses were always similar to the control treatments. This is once again the opposite of the findings of Caplan et al., (2019) in which the drought imposed in that study significantly increases cannabinoid yield (g m^{-2}). Ultimately our results showed that intense drought stress significantly lowered CBD yield and therefore could reduce producer income. However, moderate water stress resulted in final floral and CBD yields that were similar to the controls for each cultivar, which can allow producers to conserve water and increase the sustainability of the floral hemp growing systems.

The CBD to THC ratios can vary between cultivars but different environmental conditions can make it change (Toth et al., 2021). Extraction methods like super critical carbon dioxide, followed by cannabinoid separation via chromatographic means like centrifugal partitioned chromatography is an efficient method to separate cannabinoids into individual components (CBD, THC) (Hazekamp et al., 2004; Qamar et al., 2021). However, due to the extractor expenses and cost limitations it is essential to maximize CBD to THC ratios, limiting waste products like THC and maximizing CBD yield when using these techniques. A small shift in CBD:THC ratios could result in an appreciable CBD yield variance in the final product, therefore determining potential CBD oil available via ratios for secondary processing of crops after initial extractions is important. This is important because it can affect the bottom line of producers and extraction companies. Our study found that CBD:THC ratios shifted over time and decreased under water stress. Based on the responses of CBD and THC individually, we observed that THC didn't decrease under drought as significantly as CBD which resulted in the decrease of the CBD:THC ratio. This shows the need for producers to monitor for water stress as severe stress can alter the CBD:THC. Previous studies implementing different abiotic and biotic stresses (ethephon, flooding, powdery mildew, and wounding) but not drought, observe that CBD:THC ratio does not change with stress (Toth et al., 2021). The difference in the response of CBD:THC ratio to stress in our study could be due to the fact that different stresses could affect the ratios in a singular way and therefore drought may be the only stress that decreases the CBD:THC ratio. Another reason could be that the intensity of the stresses performed in Toth et al., (2021) may not be as strong as in our extreme and intermittent drought stresses which showed high impacts on water use and yield. This is supported by the fact that the plants grown under moderate drought stress did not show a decrease in the CBD:THC ratios.

CONCLUSIONS

In this study we demonstrated how varying intensities of water stress and timings of water stress affected floral hemp yields and cannabinoid content. Intense drought stresses reduced flower yield and CBD yield significantly while moderate drought (30%-50% of SWC) maintained yields and CBD and THC concentrations while only using two thirds of the water in comparison with the control. This study also showed that THC% did not increase with water stress but decreases significantly in severe drought treatments. The variation in THC% and CBD% not related to drought stress may be more related to the specific cultivar concentrations and the phenology effect on cannabinoid content. This study could have great implications for floral hemp systems as it appears to indicate that if the water status of the soil is monitored properly, we can save significant amounts of water while maintaining flower yields and producing a compliant crop.

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Figure 1. Flower yield (g) measured at post-harvest processing (55 DAF). Bars are color coded based on water stress intensity. Purple signifies control, red signifies extreme stress, green signifies intermittent stress, and blue signifies moderate stress. Lone points are outliers outside of the interquartile range. Different letters signify significantly different means. Different lowercase letters refer to differences between water treatments, while upper case letters show differences between cultivars. P-values are in the upper portion of the graphic denote significance of water stress, cultivar, and interaction. Abbreviations are as follows: C (control), EE (early extreme water stress), EI (early intermittent water stress), EM (early moderate water stress), LE (late extreme water stress), LI (late intermittent water stress), LM (late moderate water stress).

Figure 2. Harvest Index (HI) measured at post-harvest processing (55 DAF). Bars are color coded based on water stress intensity. Purple signifies control, red signifies extreme stress, green signifies intermittent stress, and blue signifies moderate stress. Lone points are outliers outside of the interquartile range. Different letters signify significantly different means. Different lowercase letters refer to differences between water treatments, while upper case letters show differences between cultivars. P-values are in the upper portion of the graphic denote significance of water stress, cultivar, and interaction. Abbreviations are as follows: C (control), EE (early extreme water stress), EI (early intermittent water stress), EM (early moderate water stress), LE (late extreme water stress), LI (late intermittent water stress), LM (late moderate water stress).

Figure 3. Linear representation of the accumulation of CBD over the course of the flowering cycle subjected to each water stress for each year of study. Line coloration denotes water stress subjected to each cultivar. Abbreviations are as follows: C (control), EE (early extreme water stress), EI (early intermittent water stress), EM (early moderate water stress), LE (late extreme water stress), LI (late intermittent water stress), LM (late moderate water stress).

Figure 4. CBD yield (grams per plant) measured at post-harvest processing (55 DAF). Bars are color coded based on water stress intensity. Purple signifies control, red signifies extreme stress, green signifies intermittent stress, and blue signifies moderate stress. Lone points are outliers outside of the interquartile range. Different letters signify significantly different means. Different lowercase letters refer to differences between water treatments, while upper case letters show differences between cultivars. P-values are in the upper portion of the graphic denote significance of water stress, cultivar, and interaction. Abbreviations are as follows: C (control), EE (early extreme water stress), EI (early intermittent water stress), EM (early moderate water stress), LE (late extreme water stress), LI (late intermittent water stress), LM (late moderate water stress).

Figure 5. Linear representation of the accumulation of THC over the course of the flowering cycle subjected to each water stress for each year of study. Line coloration denotes water stress subjected to each cultivar. Abbreviations are as follows: C (control), EE (early extreme water stress), EI (early intermittent water stress), EM (early moderate water stress), LE (late extreme water stress), LI (late intermittent water stress), LM (late moderate water stress).

Figure 6. Linear representation of the shift of CBD to THC ratios over the course of the flowering cycle subjected to each water stress for each year of study. Line coloration denotes water stress

subjected to each cultivar. Abbreviations are as follows: C (control), EE (early extreme water stress), EI (early intermittent water stress), EM (early moderate water stress), LE (late extreme water stress), LI (late intermittent water stress), LM (late moderate water stress).

Table 1. Water Use Efficiency and Total Transpiration

*WUE (g flower kg water $^{-1}$) *Total Transpired(kg)

		Year 2021		Year 2022	
Cultivar	**Treatment**	**WUE**	**Total Transpiration**	**WUE**	**Total Transpiration**
Baox	Control	0.95 cde	64.259 a	1.092 cd	50.179 ab
	EE	1.683 a	17.694 g	1.666 a	12.465 i
	EI	1.018 cde	29.493 efg	1.307 bc	21.889 ghi
	EM	1.214 bc	47.866 abcd	1.104 cd	36.933 bcdef
	LE	0.794 e	45.565 bcde	1.054 cd	28.451 efg
	LI	0.854 de	52.455 abc	1.042 cd	35.843 cdefg
	LM	0.981 cde	60.740 ab	1.08 cd	42.377 bcde
	mean	1.07	45.439 A	1.192 A	32.591 B
Cherry Mom	Control	0.84 de	36.649 cdef	0.79 d	57.984 a
	EE	1.466 ab	14.145 g	1.534 ab	13.647 hi
	EI	1.1 cde	23.712 fg	1.057 cd	26.705 fgh
	EM	1.162 bcd	29.663 efg	0.958 d	46.33 abcd
	LE	0.945 cde	34.868 def	0.934 d	33.223 defg
	LI	0.883 cde	30.336 efg	1.046 cd	40.238 bcdef
	LM	0.881 cde	39.606 cdef	0.864 d	48.205 abc
	mean	1.04	29.854 B	1.026 B	38.047 A
Water Stress (WS)		<.0001	<.0001	<.0001	<.0001
Cultivar(C)	p-value	0.422	<.0001	<.0001	0.0006
WS x C		0.17	0.005	0.426	0.859

Table 2. Phenological Maturity Comparison

	* Brown Pistil %	1) 0-20%	2) 20-40%	3) 40-60%	4) 60-80%	5) 80-100%	
			2021			2022	
Cultivar	**Treatment**	**DAF 42**	**DAF 49**	**DAF 55**	**DAF 42**	**DAF 49**	**DAF 55**
	Control	1.167 f	1.167 d	3.833 bc	1 e	2.5 de	3 c
	EE	1.5 ef	2.5 bc	4.667 ab	2.167 abc	3.833 abc	5 a
	EI	1.167 f	2.333 bc	4.167 abc	1.667 bcde	3.167 abcde	5 a
Baox	EM	1.167 f	1.833 cd	3.667 c	1.167 de	2.167 e	3.667 bc
	LE	2.333 de	2.833 b	4.333 abc	1 e	3.5 abcd	4.667 a
	LI	1.667 ef	2.167 bc	4.667 ab	1.167 de	3.5 abcd	4.667 a
	LM	1.167 f	1.167 d	3.833 bc	1.333 cde	2.667 cde	3 c
	mean	1.452 B	2 B	4.167 B	1.357 B	3.048 B	4.143
	Control	3 cd	5 a	5 a	1.833 bcde	2.667 cde	3.333 c
	EE	4.833 a	5 a	5 a	2.833 a	4.333 a	5 a
	EI	4.833 a	5 a	5 a	2.333 ab	4.333 a	5 a
Cherry Mom	EM	3.667 bc	5 a	5 a	1.833 bcde	2.5 de	3.5 bc
	LE	4.5 ab	5 a	5 a	2.167 abc	4 ab	4.667 a
	LI	4.5 ab	5 a	5 a	2 abcd	3 bcde	4.333 ab
	LM	3.5 bc	5 a	5 a	1.833 bcde	2.833 bcde	3.333 c
	mean	4.119 A	5 A	5 A	2.119 A	3.381 A	4.167
Water Stress (WS)		<.0001	0.0002	0.024	<.0001	<.0001	<.0001
Cultivar (C)	p-value	<.0001	<.0001	<.0001	<.0001	0.016	0.795
WS x C		0.0001	0.0002	0.024	0.718	0.085	0.418
Repeated Measures ANOVA							
DAF			<.0001			<.0001	
WS			<.0001			<.0001	
C			<.0001			<.0001	
DAF x WS	p-value		0.039			<.0001	
DAF x C			<.0001			<.0001	
WS x C			0.01			0.252	
DAF x WS x C			<.0001			0.256	

Table 3. Total CBD Content

		CBD (% dry weight)				
			2021		2022	
Cultivar	**Treatment**	**DAF 35**	**DAF 45**	**DAF 55**	**DAF 35**	**DAF 55**
	Control	6.897 d	10.922 e	19.667 ab	10.273 c	12.124 bcd
	EE	6.801 d	10.075 e	15.166 de	7.358 e	7.221 e
	EI	6.83 d	10.795 e	15.59 cde	8.227 de	9.555 de
Baox	EM	7.566 d	11.38 de	17.994 abcde	9.693 cd	12.602 bc
	LE	7.5 d	10.542 e	14.161 e	10.951 bc	10.132 cd
	LI	7.646 d	10.971 e	15.517 cde	10.318 c	9.768 de
	LM	7.283 d	11.454 de	19.019 abcd	10.364 c	11.968 bcd
	mean	7.217 B	10.877 B	16.73 B	9.598 B	10.445 B
	Control	13.297 a	17.374 a	19.982 ab	13.232 a	17.779 a
	EE	9.962 c	12.688 cde	15.112 de	9.911 cd	10.833 bcd
	EI	10.72 bc	14.112 bcd	16.392 bcde	12.679 ab	13.089 b
Cherry Mom	EM	12.661 ab	16.933 ab	21.186 a	14.259 a	18.216 a
	LE	11.798 abc	14.115 bcd	15.094 de	14.064 a	13.016 b
	LI	13.209 a	15.548 abc	19.268 abc	13.978 a	12.997 b
	LM	13.339 a	17.278 a	19.38 abc	13.408 a	17.432 a
	mean	12.141 A	15.435 A	18.059 A	13.075 A	14.766 A
Water Stress (WS)		<.0001	<.0001	<.0001	<.0001	<.0001
Cultivar (C)	p-value	<.0001	<.0001	0.004	<.0001	<.0001
WS x C		0.003	0.019	0.142	0.115	0.036
Repeated Measures ANOVA						
DAF			<.0001		<.0001	
WS			<.0001		<.0001	
C			<.0001		<.0001	
DAF x WS	p-value		0.0003		<.0001	
DAF x C			<.0001		0.033	
WS x C			0.002		0.036	
DAF x WS x C			0.335		0.068	

Table 4. Total THC Content

		THC (% dry weight)				
			2021		2022	
Cultivar	**Treatment**	**DAF 35**	**DAF 45**	**DAF 55**	**DAF 35**	**DAF 55**
	Control	0.303 d	0.466 c	0.791 ab	0.462 de	0.561 bcd
	EE	0.324 d	0.475 c	0.71 abc	0.362 f	0.410 e
	EI	0.314 d	0.485 c	0.703 abc	0.389 ef	0.502 cde
Baox	EM	0.339 d	0.494 c	0.707 abc	0.442 def	0.571 bcd
	LE	0.344 d	0.502 c	0.618 c	0.502 bcd	0.500 cde
	LI	0.347 d	0.485 c	0.683 bc	0.474 cde	0.490 de
	LM	0.324 d	0.499 c	0.778 ab	0.475 cde	0.568 bcd
	mean	0.327 B	0.486 B	0.712 B	0.443 B	0.513 B
	Control	0.561 ab	0.717 ab	0.784 ab	0.565 abc	0.768 a
	EE	0.465 c	0.624 b	0.697 bc	0.455 def	0.537 bcd
	EI	0.494 bc	0.647 ab	0.699 bc	0.568 abc	0.622 b
Cherry Mom	EM	0.553 abc	0.729 a	0.851 a	0.611 a	0..791 a
	LE	0.527 abc	0.642 ab	0.664 bc	0.616 a	0.608 bc
	LI	0.588 a	0.709 ab	0.809 ab	0.613 a	0.597 bcd
	LM	0.577 ab	0.704 ab	0.812 ab	0.582 ab	0.752 a
	mean	0.537 A	0.682 A	0.759 A	0.572 A	0.668 A
Water Stress (WS)		0.002	0.043	<.0001	<.0001	<.0001
Cultivar(C)	p-value	<.0001	<.0001	0.006	<.0001	<.0001
WS x C		0.02	0.041	0.049	0.179	0.044
Repeated Measures ANOVA						
DAF			<.0001		<.0001	
WS			<.0001		<.0001	
C			<.0001		<.0001	
DAF x WS	p-value		0.002		<.0001	
DAF x C			<.0001		0.138	
WS x C			0.0002		0.07	
DAF x WS x C			0.262		0.076	

Table 5. CBD:THC Ratio

		CBD (% dry weight) : THC (% dry weight)				
			2021		**2022**	
Cultivar	**Treatment**	**DAF 35**	**DAF 45**	**DAF 55**	**DAF 35**	**DAF 55**
	Control	22.774 abc	23.3 abc	24.852 ab	22.306 abcd	21.565 bcd
	EE	21.016 e	21.345 cd	21.338 d	20.420 e	17.472 g
	EI	21.792 cde	21.201 abcd	22.154 bcd	21.186 de	19.074 f
Baox	EM	22.338 bcd	23.104 abc	25.589 a	22.029 bcd	21.97 abc
	LE	21.835 cde	20.981 cd	22.985 abcd	21.842 bcd	20.237 def
	LI	22.05 bcde	22.536 abcd	22.791 abcd	21.864 bcd	19.917 ef
	LM	22.48 bcd	23.049 abc	24.456 abc	21.805 cd	21.097 cde
	mean	22.041 B	22.359	23.452	21.636 B	20.168 B
	Control	23.714 a	24.195 ab	25.441 a	23.414 a	23.179 a
	EE	21.426 de	20.374 d	21.785 cd	21.739 cd	20.149 def
	EI	21.703 cde	21.576 cd	23.368 abcd	22.455 abcd	21.046 cde
Cherry Mom	EM	22.872 abc	23.133 abc	24.887 ab	23.332 a	23.043 ab
	LE	22.345 bcd	21.800 bcd	22.673 abcd	22.801 abc	21.415 cde
	LI	22.503 bcd	21.847 bcd	23.827 abcd	22.812 abc	21.768 abc
	LM	23.157 ab	24.524 a	23.891 abcd	23.029 ab	23.164 a
	mean	22.531 A	22.493	23.696	22.782 A	21.966 A
Water Stress (WS)		<.0001	<.0001	<.0001	<.0001	<.0001
Cultivar (C)	p-value	0.0003	0.64	0.465	<.0001	<.0001
WS x C		0.547	0.17	0.594	0.979	0.165
Repeated Measures ANOVA						
DAF			<.0001		<.0001	
WS			<.0001		<.0001	
C			0.136		<.0001	
DAF x WS	p-value		0.402		<.0001	
DAF x C			0.743		0.004	
WS x C			0.904		0.321	
DAF x WS x C			0.62		0.553	

Figure 1.

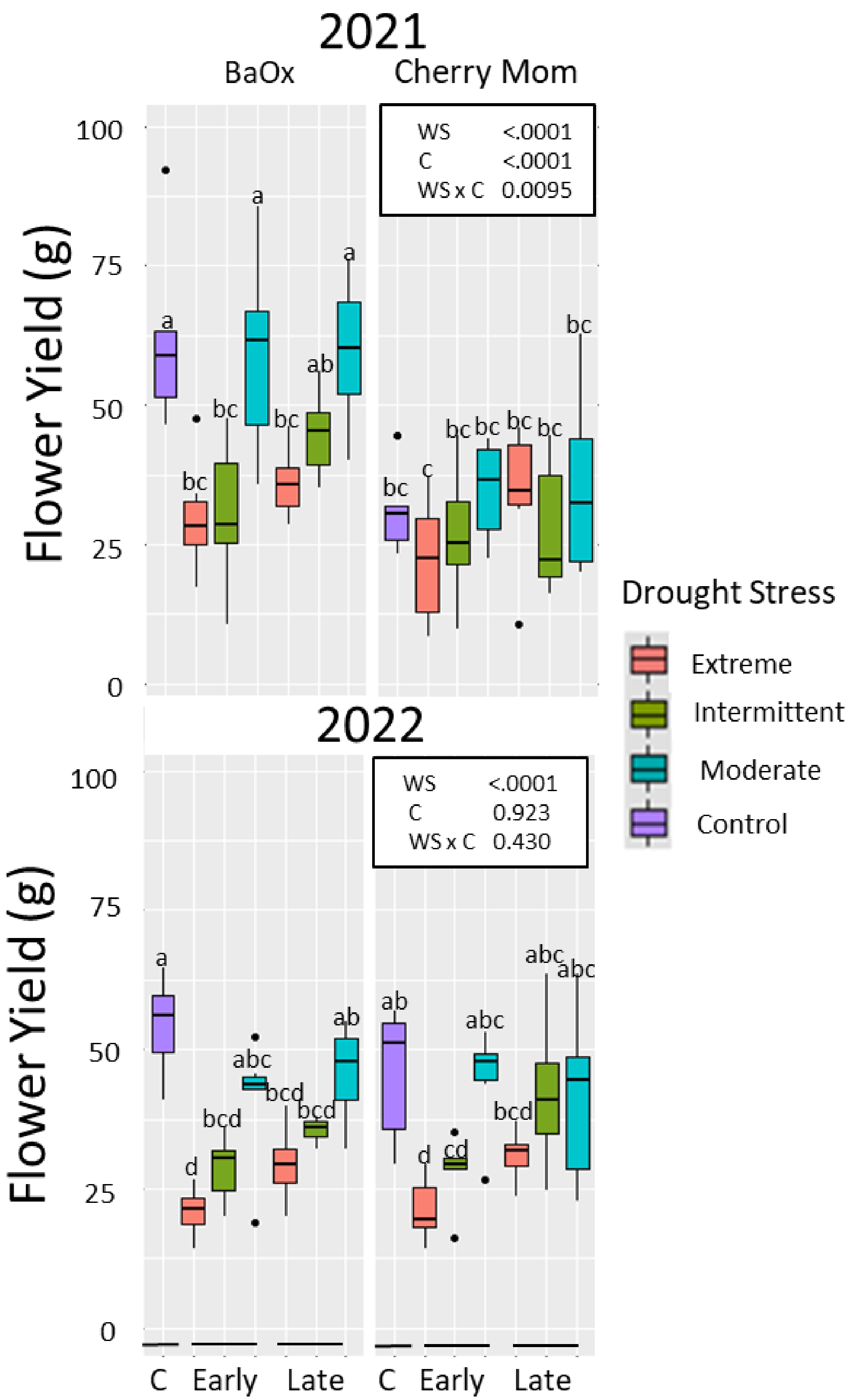
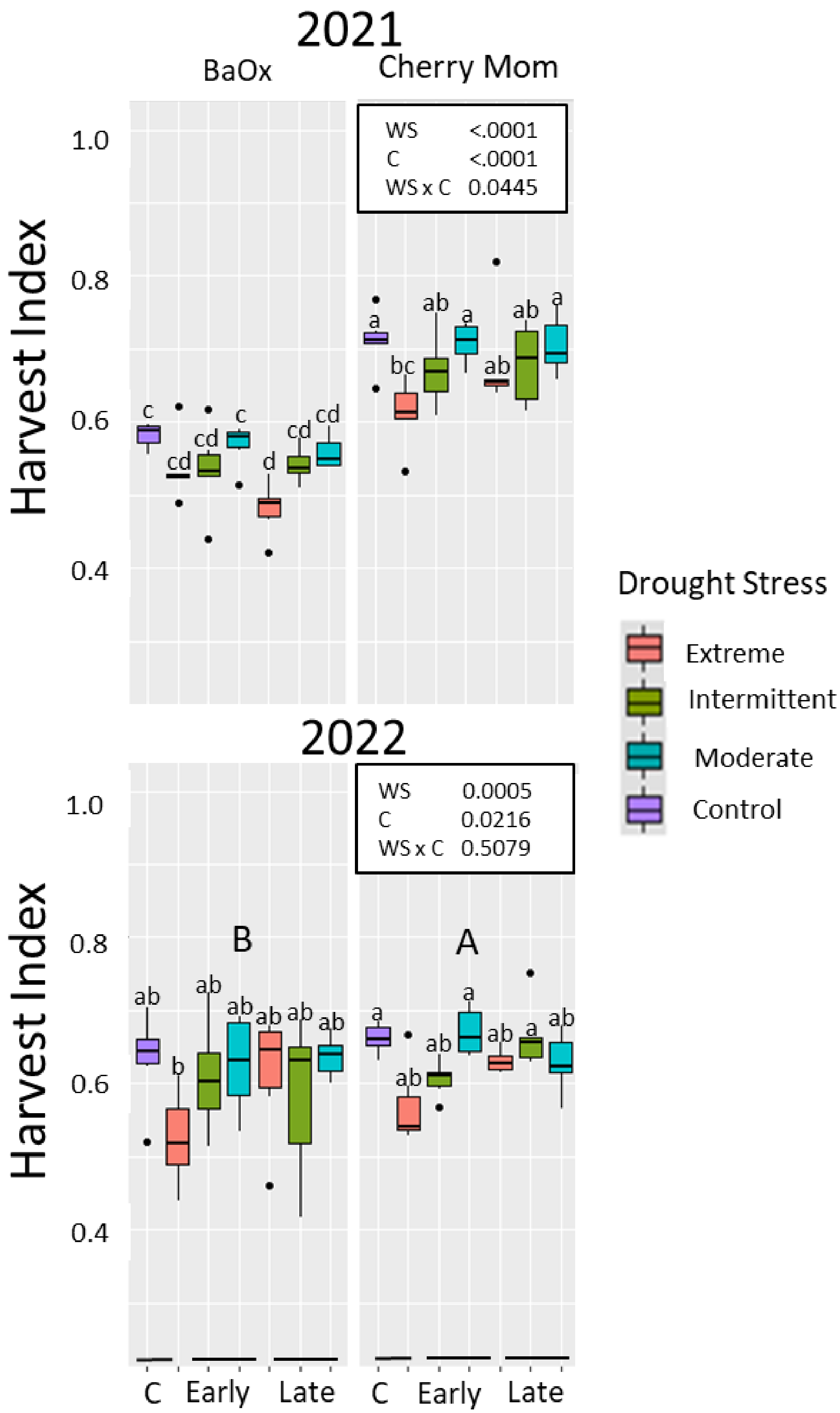

2021
BaOx
Cherry Mom
Flower Yield (g)
WS <.0001
C <.0001
WS x C 0.0095
2022
WS <.0001
C 0.923
WS x C 0.430
C Early Late C Early Late
Drought Stress
Extreme
Intermittent
Moderate
Control

Figure 2.



2021
BaOx
Cherry Mom
WS <.0001
C <.0001
WS x C 0.0445
Harvest Index
1.0
0.8
0.6
0.4
2022
WS 0.0005
C 0.0216
WS x C 0.5079
B
A
C
Early
Late
Drought Stress
Extreme
Intermittent
Moderate
Control

Figure 3.

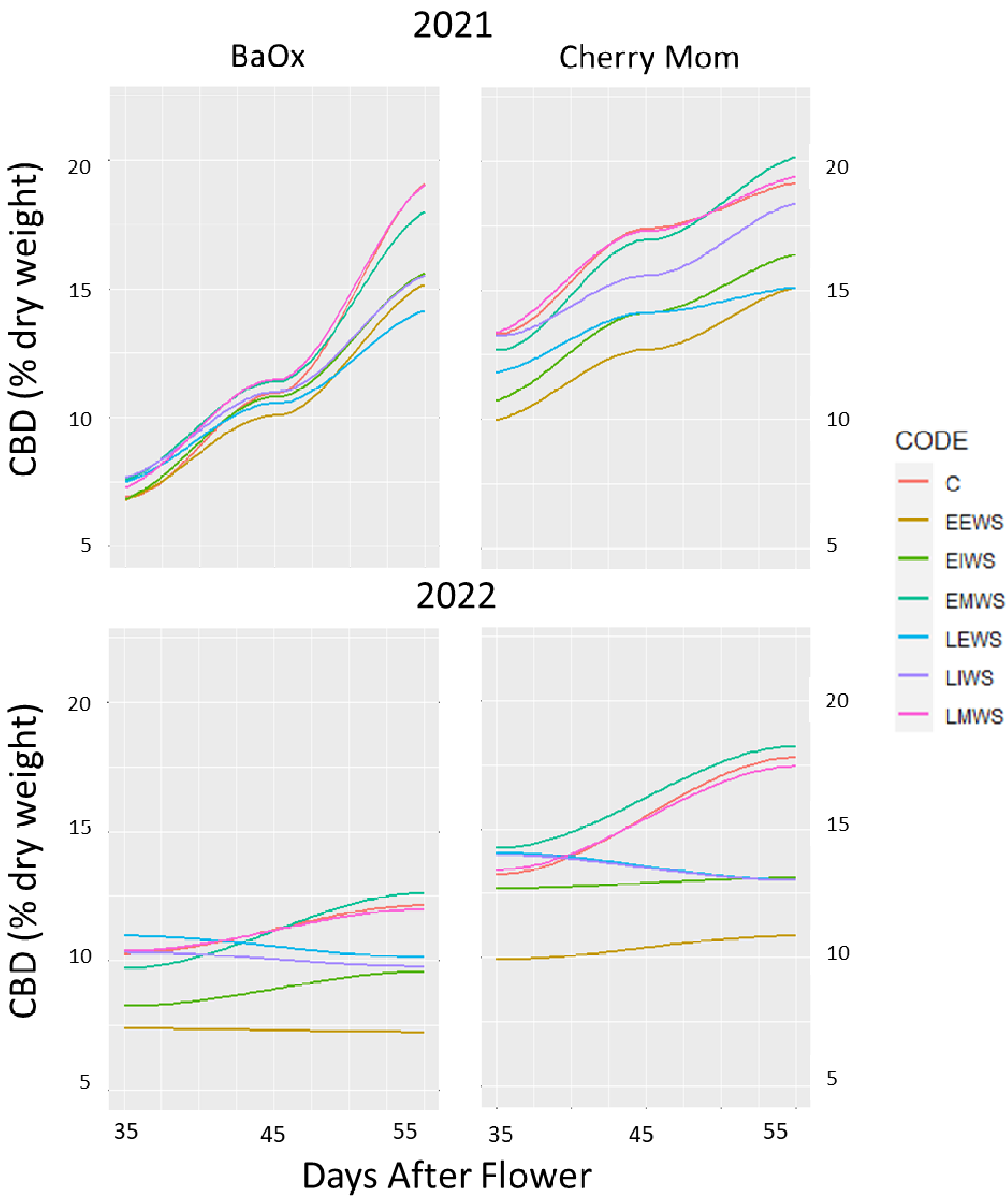

2021
BaOx
Cherry Mom
2022
CBD (% dry weight)
CBD (% dry weight)
20
15
10
5
35
45
55
Days After Flower
CODE
C
EEWS
EIWS
EMWS
LEWS
LIWS
LMWS

Figure 4.

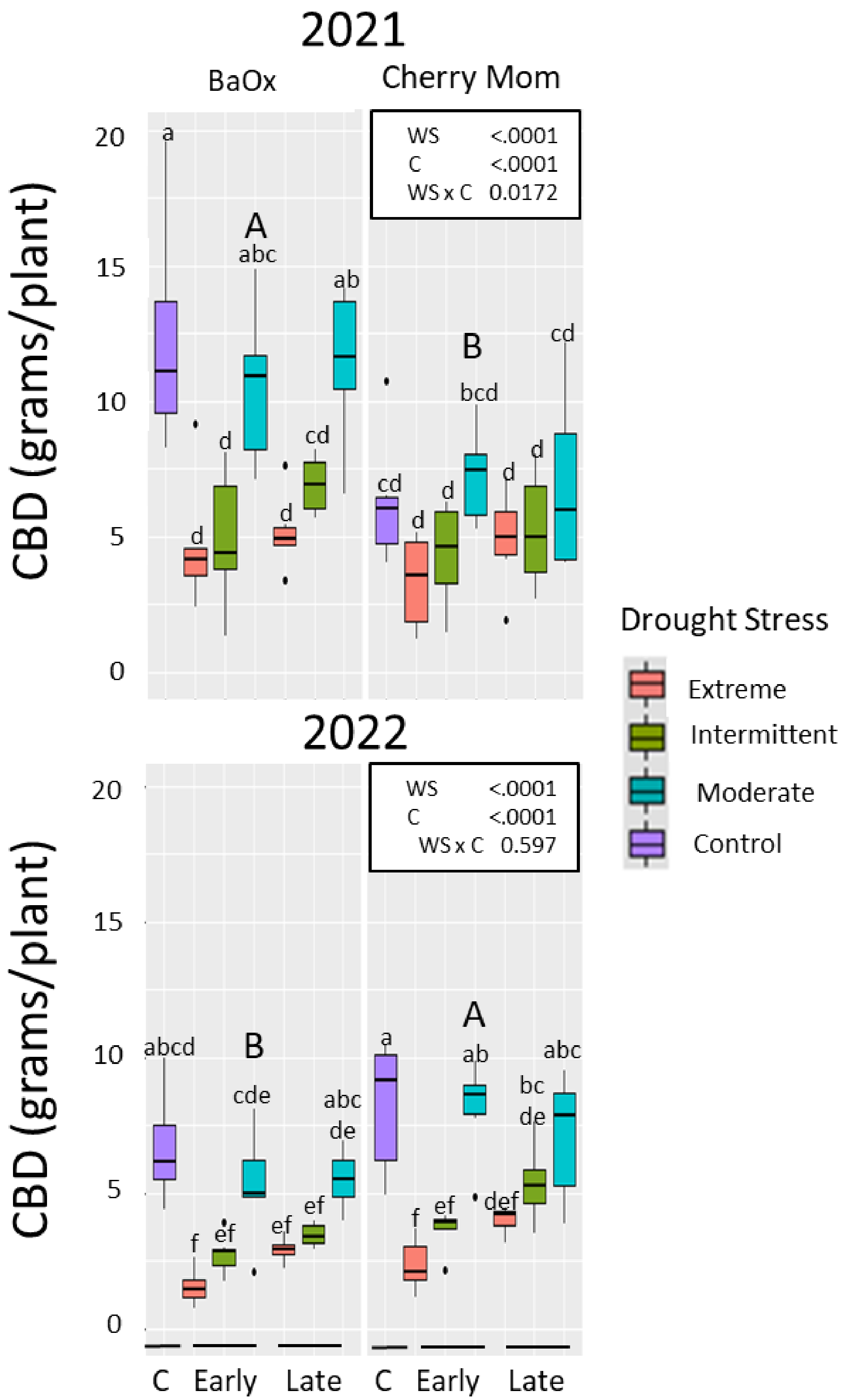

2021
BaOx
Cherry Mom
WS <.0001
C <.0001
WS x C 0.0172
CBD (grams/plant)
20
15
10
5
0
a
A
abc
ab
B
cd
bcd
d
cd
d
cd
d
d
d
d
d
d
2022
WS <.0001
C <.0001
WS x C 0.597
abcd
B
cde
abc
de
ef
ef
ef
f
a
A
ab
abc
bc
de
def
ef
f
C
Early
Late
C
Early
Late
Drought Stress
Extreme
Intermittent
Moderate
Control

Figure 5.

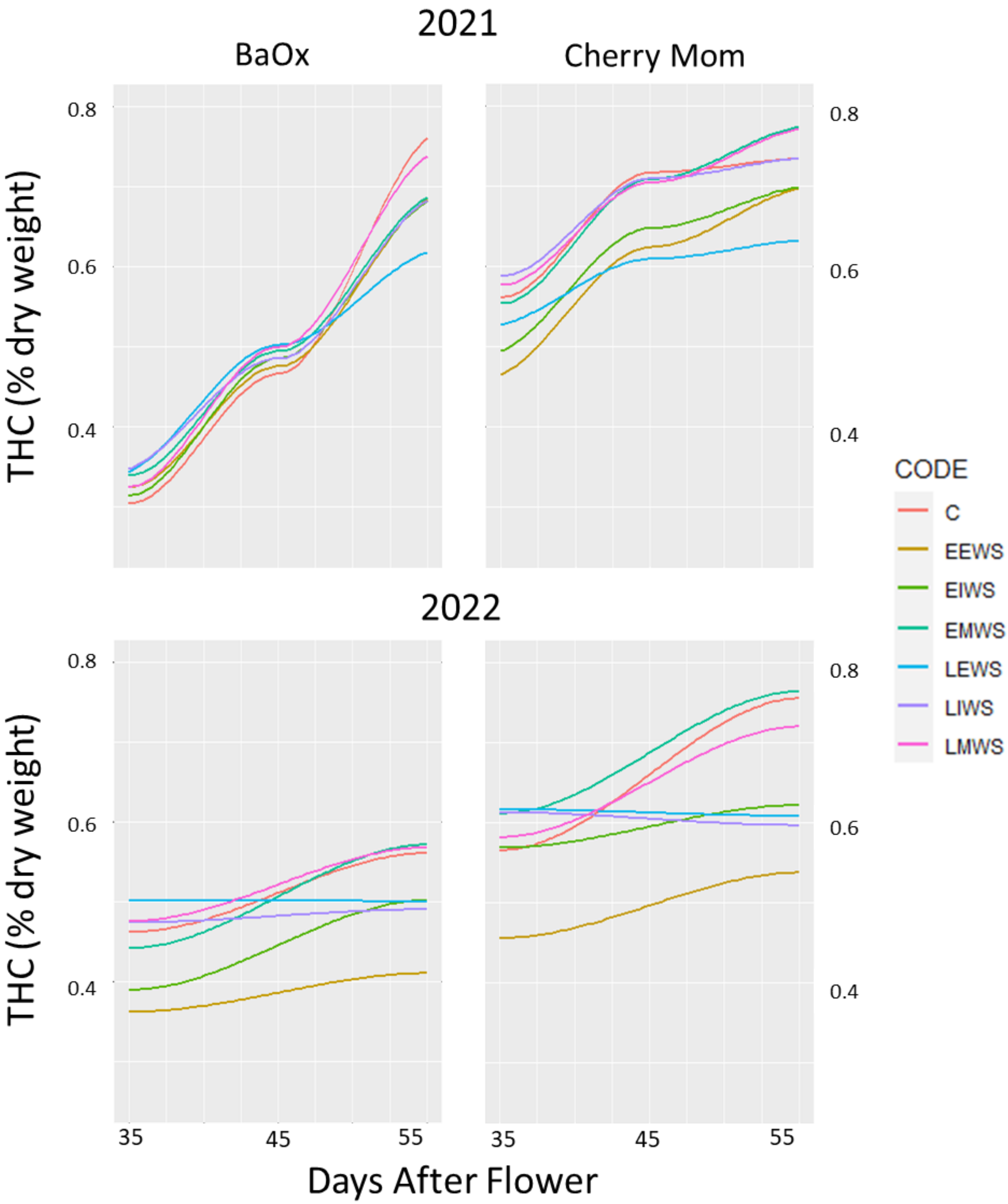
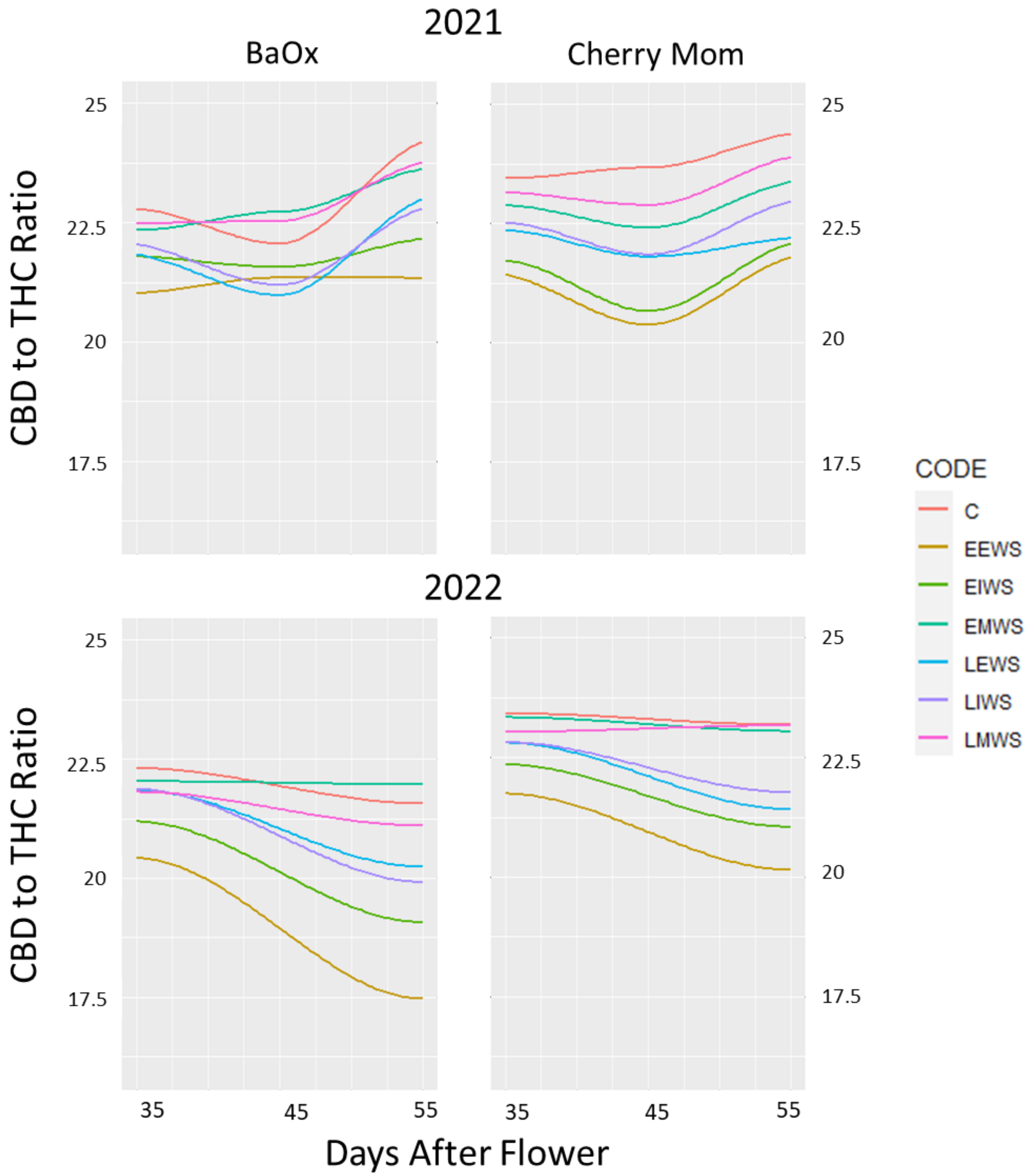

2021
BaOx
Cherry Mom
0.8
0.6
0.4
THC (% dry weight)
2022
CODE
C
EEWS
EIWS
EMWS
LEWS
LIWS
LMWS
35
45
55
Days After Flower

Figure 6.


2021
BaOx
Cherry Mom
25
22.5
20
17.5
CBD to THC Ratio
2022
CODE
C
EEWS
EIWS
EMWS
LEWS
LIWS
LMWS
35
45
55
Days After Flower

CHAPTER 3

Identifying Physiological Traits Related with Water Use Efficiency in Floral Hemp (*Cannabis sativa* L.) for Water Conservation and Cultivar Development

Wayne Morgan[1], Jagdeep Singh[1], Katelyn Kesheimer[2], Jeanine Davis[3], Alvaro Sanz-Saez[1]*

[1] Department of Crop, Soil and Environmental Sciences, 253 Funchess Hall Auburn University, Auburn, AL 36849, USA

[2] Department of Entomology and Plant Pathology, 301 Funchess Hall Auburn University, Auburn, AL 36849 USA

[3] Department of Horticultural Science, North Carolina State University, 455 Research Drive, Mills River, NC 28759

Abstract

Hemp (*Cannabis sativa* L.) is a reemerging crop that was once deeply rooted in many global civilizations. Due to climate change and the increasing risk of water deficit situations, it is important to use water judiciously. Hemp production has interested many producers, however, due to its past illegal status there is a lack of research-based knowledge about the physiological parameters that are affected by drought and which parameters could be responsible for drought tolerance. Therefore, our objective was to study and understand the effects of different drought intensities and timings on physiological parameters of photosynthesis (A), stomatal conductance (g_s), electron transport rate (ETR), photochemical quenching (qP), non-photochemical quenching (qN), and maximum quantum efficiency of photosystem II reaction centers (Fv/Fm) and determine if cultivar variation exists. These findings could then be utilized by producers to make sound irrigation decisions and by breeders to identify high throughput phenotyping techniques for developing more water efficient cultivars. Hemp cultivars BaOx and Cherry Mom were grown in a greenhouse from July to October during 2021 and 2022 under different water stress intensities and timings. It was found that 'BaOx' had a greater water use efficiency (WUE) than 'Cherry Mom'. Moderate water stress (30%-50% soil water content) was found to have the least effect on physiological parameters studied, while intense water stresses significantly reduced many physiological parameters such as A, g_s, and ETR. Strong correlations between physiological parameters and yield under different water stress treatments identify the possibility of using different physiological techniques to select and breed for greater WUE and yield in hemp. This study demonstrates the ability to modify irrigation practices to increase WUE for producers and identify WUE traits both economically and effectively for breeders.

Keywords: drought intensity, floral hemp, photosynthesis, water use efficiency, fluorescence

INTRODUCTION

Hemp (*Cannabis sativa* L.) is a plant with lineage dating back to Central Asia more than 19.6 million years ago (McPartland et al., 2019). Evidence suggests that hemp has been utilized as a crop for approximately 10,000 years (McPartland et al., 2019). In the past, hemp was mainly used for sustenance due to the nutritional value of the seeds, but also for high quality of the fiber (Strzelczyk et al., 2022). The reproductive inflorescences were medicinally, religiously, and recreationally significant to many civilizations due to the many cannabinoids and sometimes psychoactive compounds that it contains (McPartland et al., 2019; Pacher et al., 2019; Strzelczyk et al., 2022). During the early to mid-1900s hemp production decreased globally because of its similarities with marijuana (*Cannabis sativa*) which led to bans on production of all *Cannabis sativa* cultivars (hemp or marijuana) (Schlosser, 1994).

Due to the numerous medicinal uses of cannabinoids, lack of substantial psychoactive tetrahydrocannabinol (THC) in hemp (<0.3%), and elevated cannabidiol (CBD), moderation on the ideology of cannabis led to hemp legalization at the federal level in the United States in 2018 (Campbell et al., 2019; Mattingly, 2020; Pacher et al., 2019). Following the legalization there was a boom in hemp production of approximately 90,000 acres nationally (Mark et al., 2020). Unlike typical crops such as soybean (*Glycine max*), corn (*Zea mays*), cotton (*Gossypium* spp.), or peanuts (*Arachis hypogaea*), with vast amounts of knowledge regarding cultivation practices, hemp, by virtue of being illegal prior to 2018, is relatively unknown to most producers (Mattingly et al., 2020).

Drought stress is the main abiotic stress affecting crop production as it has the ability to significantly reduce crop yields even under irrigated conditions (Araus et al., 2002; Boyer, 1982). In 2012, much of the U.S. experienced a drought so significant that the decrease in yield of row crops resulted in approximately 30 billion dollars lost in the U.S economy (Rippey, 2015). Yield reduction through drought occurs when plants close stomata as a means to reduce transpiration. The result is less carbon that can be utilized and fixed in the photosynthetic cycle, leading to reduced biomass accumulation and yield (Frederick et al., 2001; Gao et al., 2021; Sakoda et al., 2021; Zhang et al., 2022). Additionally, this drought can lead to reduction of electron transport rates (ETR) as drought has already reduced carbon fixation which leads to production of reactive oxygen species (Moustakas et al., 2022) and lowered efficiency of photosystem II (PSII) resulting in loss of energy that could be used in photochemistry (Villalobos-Gonzales et al., 2022). Largely, crop response to drought effects depends on both intensity (Gao et al., 2021) and timing of drought (Frederick et al., 2001).

Due to the prior illegal status of hemp, research on how drought affects hemp has been very limited. Current studies have suggested approximately 40 to 50 centimeters of water per season are required for optimum production; however, upwards of 70 to 75 centimeters of water have been determined elsewhere (Adesina et al., 2020; Cosentino et al., 2013; Garcia-Tejero et al., 2019; Gill et al., 2022). Soybean and peanut appear to be affected greatest with droughts in early flowering and pod filling as the plant is larger at that point, ultimately requiring more water for maintenance and reproduction (Frederick et al., 2001; Zhang et al., 2022). Prior hemp studies have only studied the effects of drought stress starting in the vegetative stage. Garcia-Tejero et al. (2019) showed the effects on yield when water deficit was applied after an establishment period. Gill et al. (2022) showed how yield and physiological parameters (photosynthesis, stomatal conductance, and carbon isotope discrimination) were affected by more consistent drought stresses initiated in early vegetation and continued through harvest. However, there are no studies to our knowledge that thoroughly examine both yield and physiological parameters in hemp using multiple cultivars and drought stress intensities across multiple timings in the reproductive stage of hemp.

When drought stress is intense enough, carbon dioxide uptake is reduced and the energy that was used to fix that carbon ends up damaging the photosystems (Moustakas et al., 2022). This results in a reduction in the amount of PSII receptors and can be observed by a reduction in the electron transport rates (ETR) (Moustakas et al., 2022). This can result in a decrease of photochemical quenching (qP) and an increase of non-photochemical quenching (qN) (Zlatev, 2009) which can be measured by fluorescence detectors used in portable gas exchange analyzers such as LI-6800. Photosystem resistance to drought is a tolerance mechanism utilized by the plant to maintain photosynthetic capabilities in the presence of drought (Buezo et al., 2019). This mechanism depends largely on the increased production of chlorophylls (specifically chlorophyll *a* and antioxidant compounds) to alleviate oxidative stresses that can damage photosystems (Buezo et al., 2019). To date, the effects of drought on the fluorescence parameters of hemp have not been reported and there is no apparent knowledge of fluorescence cultivar variation in this crop.

While drought does negatively affect yield, there are some cultivars of certain crops that have physiological characteristics that make them more tolerant to drought, such as more resistant photosystems in soybean (Buezo et al., 2019), higher water use efficiency cultivars or plants with deeper roots, e.g., peanut (Junjitakarn et al., 2014; Zhang et al., 2022). Tight regulation of the stomata is an efficient method to conserve water and therefore tolerate drought as stomata are able to respond quicker to drought stress, ultimately conserving water in the soil in long term droughts (Zhang et al., 2022). This makes the plant continue growing during the drought periods and as a result, the water use efficiency (WUE) of the crop increases.

Stomatal closure is one of the first reactions to drought stress in which the stomata close to conserve moisture (Zhang et al., 2022). Stomatal conductance measurements using a portable photosynthesis unit (LI-6800, LI-COR Biosciences, Lincoln, NE, USA) is a useful tool to differentiate between drought sensitive and drought tolerant cultivars as Zhang et al. (2022) have found that the peanut stomatal conductance is correlated with yield under drought conditions. In addition, Gill et al. (2022) showed that high hemp stomatal conductance is correlated with plant biomass weights. WUE is calculated as (Total Biomass)/(kg water), however, it can be difficult to quantify the water used by the plant especially in field settings. Carbon Isotope Discrimination ($\Delta^{13}C$) can facilitate WUE estimations as studies have determined that $\Delta^{13}C$ is negatively correlated with WUE (Condon et al., 2004; Farquhar et al., 1989). $\Delta^{13}C$ is negatively correlated because if the WUE of the plant is lower this translates to less ^{13}C being assimilated into the tissues as the stomata are open and allowing for the more abundant ^{12}C to be assimilated (Farquhar et al., 1989). Although drought has been shown to decrease $\Delta^{13}C$ in hemp before (Gill et al. 2022), there is not a report in the literature in which $\Delta^{13}C$ has been used to estimate water use efficiency in this crop. If $\Delta^{13}C$ shows a good correlation with the crop WUE as previously shown in wheat (Condon et al., 2004; Farquhar et al., 1989) this technique could be used to screen big numbers of hemp cultivars as it is less time consuming than mini-lysimeters and/or photosynthetic measurements (Condon et al., 2004; Farquhar et al., 1989; Vadez and Ratnakumar, 2016).

With little previous research on hemp under any type of drought stress, the objective of this research was 1) Study what is the effect of different drought stress timings and intensities on different physiological parameters such as gas exchange, fluorescence, water use efficiency, and carbon isotope discrimination; 2) Find if there is variation in cultivar response to drought of the above mentioned physiological parameters; and 3) Study if there is correlation between crop WUE and other measured physiological parameters such as $\Delta^{13}C$, so it can be used as tool for breeding for WUE in hemp.

MATERIAL AND METHODS

Study design, setup, and management

Hemp was cultivated in a greenhouse in Zirconia, North Carolina, USA (35.213349, -82.416249) during the 2021 and 2022 summer growing season. The trials were planted at the beginning of July each year of the study. More detail on the cultivation parameters can be found in Morgan et al. (2023). Summarizing, the cultivars used in this experiment were BaOx and Cherry Mom, two high cannabidiol (CBD) varieties. Each plant was hand planted in a 19-liter nursery pot filled with a “living soil’ premix supplied by Dirtcraft Organics (Dirtcraft Organics LLC, Marshall, NC, USA).

Each year the plants were grown via typical methods of greenhouse cultivated hemp. During the vegetative stage, the hemp plants were maintained in well-watered, conditions of approximately 70%-100% soil water content (SWC). The plants received 18 hours of light using a Fluence SPYDR series LED lighting system (Fluence USA, Austin, TX, USA) to assure the crop maintained a vegetative status until the predetermined flowering date. Temperatures and thorough airflow were maintained in a suitable range (ideally 24-32°C) with an industrial fan (63.5 cm diameter). Onset HOBO UA-002-64 pendants (Onset Brands, Bourne, MA, USA) were utilized inside and outside of the greenhouse to record environmental data (See Morgan et al.(2023) for details regarding growing conditions).

The weight of each nursery pot was recorded and tared using an Ohaus Range 3000 series scale (Ohaus Corporation, Parsippany, NJ, USA). The pots were then filled with 5,400 grams of the ‘living soil’ mixture and each weight was recorded again. Soil moisture content was calculated according to Morgan et al. (2023).

Following the vegetative period of the crop, the plants were transitioned into the flowering phase by removing the supplemental lighting system. This transition date occurred on August 10, 2021 and August 8, 2022. The crop was maintained under well-watered conditions for one additional week. The drought stress treatments were then initiated. The pots were weighed daily in the morning throughout the duration of the drought treatments. If the pots were at the prescribed threshold (listed below) the pot was watered until the upper limit of the SWC was achieved. Following the 55-day flowering cycle, the plants were harvested, partitioned, and dried.

Forty-two clones per hemp cultivar were hand-planted at random in one of seven possible treatments. Early drought stresses were initiated at seven days post flower initiation and late drought stresses were initiated following 28 days of flowering. The trials were planted using a randomized complete block design (RCBD) that contained six replications per treatment and cultivar. The seven drought treatments were as follows: Control (100%-70% SWC), Early Extreme Drought Stress (EE, 10%-0% SWC), Early Intermittent Drought Stress (EI, 100%-0% SWC), Early Moderate Drought Stress (EM, 50%-30% SWC), Late Extreme Drought Stress (LE, 10%-0% SWC), Late Intermittent Drought Stress (LI, 100%-0% SWC), Late Moderate Drought Stress (LM, 50%-30% SWC). The Extreme Drought Stress maintained a SWC between 0% (extreme wilt) -10% during the whole length of the drought period. The Intermittent Drought stress plants were watered until calculated field capacity (100% SWC) and later let dry until reaching 0% (extreme wilt) SWC, once 0% was achieved the treatment was irrigated to reach 100% SWC repeating this cycle during the entire drought period. Moderate Drought Stress treatments were maintained in a range of 50%-30% SWC for the entire drought period. Mean daily transpiration was calculated as: total water transpired/days of drought.

Physiological Measurements

Photosynthesis, respiration, and fluorescence measurements (ETR, qP, qN) were recorded throughout the reproductive cycle. I021, the first measurements were taken 20 days after flowering (DAF) and the second measurements were taken 46 DAF. In 2022, measurements were taken 12 DAF and 35 DAF. Respiration measurements were performed starting at 10 p.m. leaving at least 1:30 h after sunset for the plants to adapt to darkness. The chamber conditions in the LI-COR LI-6800 portable photosynthesis systems (LI-COR Biosciences, Lincoln, NE, USA) were set to a light of 0 PAR with temperatures and relative humidity set to match atmospheric conditions as recorded by HOBO temperature sensors.

Midday measurements were conducted from 10 a.m. to 2 p.m. to ensure the plants were at their maximum active state. Measurements were taken using two LI-COR LI-6800 portable photosynthesis systems (LI-COR Biosciences, Lincoln, NE, USA). Midday measurements consisted of photosynthetic assimilation rate (A), stomatal conductance (g_s), electron transport rate (ETR), photochemical quenching (qP) and non-photochemical quenching (qN). Environmental conditions such as temperature, light intensity, [CO2] (~410 ppm), and relative humidity were determined before the measurements began using the HOBO data loggers; and the portable gas analyzers were set to meet those conditions. A leaf aperture of 2 cm x 2cm was used so the leaf completely filled the measurement chamber.

A Minolta model SPAD chlorophyll meter (Konica Minolta, Ramsey, NJ, USA) was utilized to determine how drought treatments were affecting leaf nitrogen levels. Soil Plant Analysis Development (SPAD) chlorophyll content measurements were conducted on the same dates as the portable photosynthesis measurements each year. Each plant was sampled at the top, middle, and bottom position of the canopy. Five measurements were taken per leaf and averaged together for each position.

For leaf $\Delta^{13}C$ (‰) each year, leaf samples were collected from the top, middle, and bottom of each plant just before the final harvest, pooled together, dried and prepared to determine $\Delta^{13}C$ values. The samples were ground using a 2010 Geno/Grinder (SPEX. SamplePrep, Metuchen, NJ, USA). Approximately 3 milligrams of each sample were later placed in individual tin capsules, weighed, and sent to the University of California Davis Stable Isotopes Facility (Davis, CA, USA) for carbon isotope analysis (Sanz-Saez et al., 2019; Zhang et al., 2022) using an isotope ratio mass spectrometer (IsoPrime, Elementar, France) connected to an element analyzer (EA3000, EuroVector, Italy).

The ratio (R) of $^{13}C/^{12}C$ was shown as $\delta^{13}C$ (‰), indicating the C isotope composition relative to Vienna Pee Dee Belemnite calcium carbonate (V-PDB):

$\delta^{13}C = (R_{samples}/R_{standard})-1$

$\delta^{13}C$ (‰) values were standardized to C isotope discrimination ($\Delta^{13}C$, ‰) and values were calculated as:

$$\Delta^{13}C\ (‰) = \left(\frac{\partial^{13}C_{atm} - \partial^{13}C_{sample}}{1 + (\frac{\partial^{13}C_{sample}}{1000})}\right)$$

Where $\delta^{13}C_{atm}$ is the C isotope composition of atmospheric CO_2 (-8‰; Farquhar et al., 1989), and $\delta^{13}C_{sample}$ is the C isotope composition of the plant sample.

Harvest

Each year at harvest all remaining leaves were removed from each plant and placed into individual paper bags for drying at 60°C. The remaining portion of plants (flower and stalk) were then hung in the greenhouse to dry. The stem and flower portion of every plant was individually partitioned and weighed separately. Moisture content of each component (except leaves) was also analyzed via a General MMD4E moisture meter (General Tools and Instruments, Secaucus, NJ, USA) so each sample could be corrected to 0% moisture content. Total biomass WUE was calculated as: (Total Aboveground Biomass (g) / Total Water Transpired (Kg)).

Statistical Analysis

RStudio was utilized to conduct statistical analysis (R Foundation for Statistical Computing, Vienna, Austria). A three-way Analysis of Variance (ANOVA) was conducted at the end of all trials to determine if differences existed between the years of study, drought treatment, and cultivars. Based on the results of the three-way ANOVAs it was decided to analyze each year of the data separately. Two-way ANOVA and Tukey's HSD test were utilized to determine if significant differences existed at individual sampling dates for differences in cultivars as well as drought treatments in each year. The AGRICOLAE package in R aided in determining significant Tukey differences among treatments. Visualization of results was completed in both the ggplot2 and corrplot package in RStudio.

RESULTS

Physiological parameters

Harvest

Total biomass showed significant interaction between the cultivar and water treatment in 2021 (Figure 1). Total biomass was 90% greater for the cultivar BaOx than Cherry Mom in 2021 (Figure 1). Total biomass yields were reduced 47% and 44% in the EE and EI treatments compared to the control for 'BaOx', however, there were no significant decreases in total biomass yield for 'Cherry Mom' (Figure 1). In 2022, the cultivars did not have significantly different total biomass (Figure 1). However, 'BaOx' showed 54%, 45%, and 44% reduced biomass weights in the EE, EI, and LE treatments respectively, whereas 'Cherry Mom' only showed a 47% decrease at the EE treatment as compared to the control (Figure 1). These results may indicate that 'Cherry Mom' may be more drought tolerant than the BaOx cultivar probably because of its smaller size. The insignificance of water treatment for 'Cherry Mom' in 2021, may also be related to a slower establishment and smaller plant size noted in the first year.

Water use efficiency related traits

In both years, total biomass WUE was always 22%-28% higher in 'BaOx' than 'Cherry Mom' (Table 1). Additionally, the EE, EI, and EM treatments of both cultivars resulted in significantly higher WUE (Table 1). General trends of the data suggest that for both years the treatments with better water status resulted in the least efficient use of water for biomass production. Daily mean transpiration in 2021 showed that 'BaOx' had 52% greater transpiration than 'Cherry Mom' (Table 1), however 'BaOx', showed a more efficient WUE due to higher biomass accumulation. In 'BaOx' the EE, EI, and LE drought treatments showed a 72%, 54%, and

29% significant reduction in mean daily transpiration in respectively. On the other hand, in 'Cherry Mom' the drought treatments only significantly reduced daily transpiration 61% in the EE treatment (Table 1). These little differences between the transpiration of the drought and control treatments were probably due to the low transpiration observed in the control treatment for 'Cherry Mom' plants probably associated with the small size of these cultivar in 2021. Significance for both water stress and cultivar were found again in 2022, however, 'Cherry Mom' showed a 17% greater daily transpiration than 'BaOx', opposite to 2021 (Table 1). In both cultivars, all treatments showed a significantly lower mean daily transpiration in comparison with the control, however, the EM and LM treatments were similar in WUE, demonstrating similar WUE while using less water (Table 1).

In both years, 'Cherry Mom' showed higher $\Delta^{13}C$ than 'BaOx' (Table 1). Additionally, in both cultivars, the EE and EI treatments displayed lower $\Delta^{13}C$ than the controls while all other treatments were similar (Table 1).

Physiology related traits

'Cherry Mom' showed the highest SPAD in the control for both upper and lower canopy positions at 20 DAF (Table 2). Additionally, all treatments at 20 DAF for 'Cherry Mom' showed significantly lower SPAD than the control (Table 2). In 2021, at all times there was an interaction between cultivars and water stresses. At 20 DAF, there was not an effect of drought in the upper canopy SPAD values compared with the control of any cultivars (Table 2). However, the lower canopy of 'Cherry Mom' showed significantly lower SPAD values in the EE (78%), EI (61%), and EM (43%) treatments while 'BaOx' was not affected (Table 2). In the later measurement (46 DAF), 'BaOx' did not display any reduced SPAD values in the upper or lower canopy, however 'Cherry Mom showed reduced SPAD values in the EI (56%) , LE (75%), and LI (56%) drought treatments in the upper canopy; and EI (54%), LE (81%), and LI (63%) drought treatments in the lower canopy (Table 2). In 2022, there were no effects of SPAD values at 12 DAF for either canopy level (Table 2). At 35 DAF, there was no effect of drought in the upper canopy for either cultivar compared to the control, however, in the lower canopy the EE, EI, and LE treatments showed a 33%, 38%, and 52% reduction in ‘BaOx’ and the EE, EI, and LE drought treatments showed a 53%, 44%, and 34% reduction in SPAD in ‘Cherry Mom’, respectively (Table 2). Drought tends to reduce SPAD values to a greater extent in the lower canopy. When the stress is more notable the upper canopy is also affected. Overall, ‘Cherry Mom’ appeared to have SPAD values more reduced than ‘BaOx’ (Table 2).

In general, in 2021 at 46 DAF and in 2022, the water and cultivars treatments did not affect leaf respiration (Table 3). In 2021 at 20 DAF, the cultivars did not show any significant difference for respiration while water stress showed a significant effect, with the EE treatment showing lower respiration than the water control (Table 3). Maximum quantum efficiency of photosystem II reaction centers (Fv/Fm) did not show any significant differences for water stress in 2021 (Table 3). However, ‘Cherry Mom’ had a significantly lower Fv/Fm at 46 DAF than ‘BaOx’. In 2022, Fv/Fm did not show significant differences for water stress at 12 DAF, but ‘Cherry Mom’ showed a higher Fv/Fm than ‘BaOx’ (Table 3). There was an operational procedure error at 35 DAF in the 2022 season in the fluorescence measurements that did not allow for proper measurement of Fv/Fm, therefore these data were omitted (Table 3).

In both years, water stress did not have a significant impact on qP at the first measurement (20 DAF in 2021; 12 DAF in 2022; Table 4). There was cultivar variation regarding qP, with ‘Cherry Mom’ having a 11% lower qP than ‘Baox’ in 2021 at 20 DAF but not in 2022 (12 DAF) (Table 4). ‘Cherry Mom’ also maintained a 27% lower qP at 46 DAF than ‘BaOx’ (Table 4).

‘Cherry Mom’ displayed a 18% higher qN) than ‘BaOx’ at 20 DAF in 2021 but not at 12 DAF in 2022 (Table 4). In 2021 at 46 DAF, qN was increased significantly in the EI, LE, and LI treatments for both cultivars. This in particular showed that greater drought stresses resulted in the highest qN averages due to the fact qN is energy not used in photochemistry.

In 2021, during the early drought stress (20 DAF) A was 38% higher for ‘BaOx’ in 2021 (Figure 2). In 2021, water stress displayed significant decreases of A in EE (44%) and EI (35%) treatments in comparison with the control for ‘BaOx’ and (55%) and (59%) decreases for EE and EI treatment in ‘Cherry Mom’ at 20 DAF. However, in 2022 (12 DAF), there was a 37% decrease in the EE treatment for ‘Cherry Mom’ and a 26% decrease in the EE treatment for ‘BaOx’ (Figure 2). At 46 DAF in 2021 and 35 DAF in 2022, there were no significant differences between the cultivars, but there was a significant difference in the water stresses for both cultivars at 46 DAF (Figure 2). In 2021 at 46 DAF, the EE, EI, EM, LE, and LI treatments showed a reduction of photosynthesis of 55%, 66%, 31%, 66%, and 65% in comparison with the control in ‘BaOx’ and 60%, 89%, 44%, 92%, and 82%, respectively, lower A in ‘Cherry Mom’ (Figure 2). In 2022, At 35 DAF, there were decreases in A in the EI (37%), LE (63%), and LI (70%) drought treatments for ‘BaOx’ (Figure 2). In ‘BaOx’ plants as water stress increased, the A decreased (Figure 2). In ‘Cherry Mom’, the EI, LE, and LI drought treatments also decreased photosynthesis at 48%, 71 %, and 53% in comparison to the control (Figure 2). In general, late water stresses for both years show lower average A than the early water stress counterpart (Figure 2).

Stomatal conductance (g_s) measurements showed cultivars differences in both years but these were not consistent, as in 2021 ‘BaOx’ showed higher g_s than ‘Cherry Mom’ at 20 DAF but none at 46 DAF; and in 2022, ‘Cherry Mom’ showed higher g_s than Baox at 35 DAF (Figure 3). Water treatments significantly reduced g_s in both years. At 20 DAF in 2021, the EE and EI treatments were significantly lower than the controls in both cultivars (‘BaOx’ 85% and 60%, respectively; ‘Cherry Mom’ 87 and 86%, respectively) while at 46 DAF, the EE, EI, LE and LI treatments were significantly lower than the control (‘BaOx’ 82%, 74%, 52%, 88%, 87%, and 48%, and ‘Cherry Mom’ 77%, 77%, 30%, 95%, 84%, and 37% lower than the control respectively) (Figure 3). In 2022 at 12 DAF, there were significant reductions of 61 and 47 in g_s for EE and EI treatments in ‘BaOx’ and EE and EI (72% and 63% respectively) treatments for ‘Cherry Mom’ (Figure 3). In 2022 at 35 DAF ‘BaOx’ showed a significantly lower g_s and a different response to water stress than ‘Cherry Mom’ (Figure 2). In 2022, ‘BaOx’ was not significantly affected by water stress at 35 DAF, but ‘Cherry Mom’ was 90%, 80%, 94%, and 76% lower in EE, EI, LE, and LI treatments when compared to the control (Figure 2). General trends suggest that on average, g_s was reduced as water stress increased, suggesting the possibility to reduce transpiration.

Electron transfer rate (ETR) was not affected by water stress at the first measurement for either year (Figure 4). However, ETR was between 22%-28% lower in ‘Cherry Mom’ in comparison with Baox in 2021, but no cultivar differences were observed in 2022 (Figure 4). In the last fluorescence measurements, ETR in general decreased as drought stress became more extreme. In 2021, the EI treatment was reduced 41% in ‘BaOx’ and 63% in ‘Cherry Mom’ (Figure 4). Additionally, in 2021, the LE treatment displayed a 34% decrease in ‘BaOx’ and a 74% decrease in ‘Cherry Mom’ while the LI treatments showed a 33% decrease in ‘BaOx; and a 61% decrease in ‘Cherry Mom’ (Figure 4). Similarly, in 2022, the greatest decreases were in LE treatment, with ‘BaOx’ showing a 49% decrease and ‘Cherry Mom’ showing a 54% decrease. The LI treatment was also decreased 38% in ‘BaOx’ and 41% in ‘Cherry Mom’ as these measured the lowest overall overage ETR each year. This shows late drought is potentially more damaging to ETR toward the end of the flowering cycle.

Correlations

In both years, flower yield and total biomass was positively correlated with total and daily transpiration (Figure 5; 6). Although the correlation was lower than with the transpiration parameters, flower yield was correlated with early and late A and g_s in both years. Whole plant WUE was negatively correlated with photosynthesis, stomatal conductance, and $\Delta^{13}C$, with this last one showing the strongest correlation. The stronger correlations between WUE and $\Delta^{13}C$ than with A and g_s shows $\Delta^{13}C$ could be a better tool to screen for large amounts of cultivars. ETR, qP, qN, and respiration did not show any significant or stable correlation with flower yield, total biomass, or WUE in both of the years (Figure 5 and 6).

DISCUSSION

Effects of Drought on Biomass and Water Use Efficiency Parameters

Total biomass of ‘BaOx’ was greater than ‘Cherry Mom’ in 2021 but not in 2022. In 2021 ‘Cherry Mom’ was notably slower to establish, therefore, the cultivar was smaller than ‘BaOx’ in 2021 (Morgan et al., 2023). However, 2022 resulted in quicker establishment of both cultivars, therefore, both grew to finish at similar biomass weights in the second year of the study. ‘BaOx’ showed at least 20% higher WUE than ‘Cherry Mom’ under different water stress conditions demonstrating that hemp cultivars can vary in water use efficiency. To our knowledge, this is the first study in which cultivar variation in WUE is reported. Although the implications of this study are limited as only two cultivars have been tested; it has been determined in peanut that WUE is different among cultivars, and that WUE can be used in cultivar selection and breeding (Zhang et al., 2022). Transpiration efficiency as described by Vadez and Ratnakumar (2016) increases WUE, allowing some cultivars to tolerate drought and produce more pod yield with lower water used. This suggests that there may be genetic component positively correlating WUE to yield (Vadez et al., 2014).

Daily transpiration was also found to be higher in ‘BaOx’ in 2021 but higher in ‘Cherry Mom’ in 2022, thus suggesting that daily transpiration can be linked to plant size as bigger plants transpire more (Alandia et al., 2016; Morgan et al., 2023). However, WUE may be linked to individual cultivars. This ultimately means that when selecting hemp cultivars, high WUE may be a beneficial parameter regardless of final size (Alandia et al., 2016). If water conservation is not necessary because environments where these plants grow do not suffer from terminal drought, cultivars with high water use such as BaOx would be useful as they can produce higher yields under intermittent droughts as found in common bean (*Phaseolus vulgaris*) and peanut (Polania et al., 2016; Zhang et al., 2022). However, if the plants grow in a terminal drought environment, selecting for high WUE is necessary and cultivars with lower transpiration would be the preferred option. In this study, our data show that 'BaOx' may be a high water user cultivar, however, more screening is needed to find other cultivars with high WUE and that can preserve more water.

Effects of Drought on Carbon Assimilation Parameters

In our study, carbon assimilation was often affected by drought stresses and timings. Stomatal control is the first line of defense in drought resistance to prevent water loss and is the first parameter to be reduced by drought (Tatar et al., 2022; Zhang et al., 2022). As photosynthesis depends on stomatal opening to let the CO_2 enter the leaf and get assimilated in the mesophyll, reductions of stomatal conductance results in reduction in photosynthesis (Farquhar et al., 1989; Rascher et al., 2004) and therefore in yield (Hashem et al., 1998). This was observed by the strong correlation observed between photosynthesis and stomatal conductance in both years and at all sampling times. This has been confirmed in other studies, including tropical forests in which there is a correlation between g_s and A under drought (Wu et al., 2020). Overall, our two hemp cultivars responded similarly to water stresses with the greatest reductions in A and g_s occurring in greater intensity drought stresses, with less reductions occurring in the moderate water stresses. This is a similar finding to Gill et al. (2022) in which there are no significant differences in A and g_s in the moderate stress compared to the control, but significant decreases in the intense stress. Our gas exchange values under well-watered and drought conditions were also in line with others obtained under greenhouse and field conditions (Gill et al., 2022; Tang et al., 2017) and corroborate that the growth of the plants was appropriate under well-watered and water stress conditions.

One point of emphasis is the notable decrease in A and g_s as the flowering cycle advanced regardless of watering treatment. This was related to the senescence of the vegetative leaves and apparent remobilization of nutrients to the reproductive tissues (flower). It is shown in soybean that this remobilization, which is accelerated under drought, can cause senescence in leaves as nutrients are mobilized and moved toward reproductive structures (Islam et al., 2017).

The light reactions of photosynthesis are in charge of getting the energy that is necessary to fix the carbon into sugars (Harrison et al., 2020). Fluorescence parameters such as ETR, qP, qN, and Fv/Fm are able to detect if light reactions are affected by any environmental stress (Hura et al., 2011). In our study, we observed that the decrease in carbon assimilation occurred earlier during the drought as a consequence of stomatal closure and that the damage in the light reaction observed by the decrease of ETR and qP only happened when the plants had been under drought for more than 20 days. This decrease in fluorescence traits (ETR and qP) was detected only in the early and late drought stresses and not under moderate drought. These data reinforce the idea that moderate drought does not affect any photosynthetic trait as observed by Gill et al. (2022) with carbon assimilation data.

In addition, we observed that the late water stress (LE and LI) treatments showed lower ETR values in the last sampling time than the early stress ones (EE, EI). This may suggest that the photosystems in the early stress treatments may have adapted to drought, which might explain why they showed higher ETR values than the later drought. In Buffelgrass (*Cenchrus ciliaris*) PSII can adapt to drought in cultivars that have an enhanced ability to scavenge reactive oxygen species (ROS) (Carrizo et al., 2021). Future experiments studying the drought effects and drought tolerance in hemp should focus on measuring ROS and ROS scavengers as they could be a source of drought tolerance in the future.

It is interesting to note that the cultivar Cherry Mom appears to be more sensitive to the water stresses for fluorescence parameters. In 2021 there were significant decreases of ETR and qP in the intense late drought stresses not seen in ‘BaOx’. This coincides with reductions in SPAD values in the upper canopy of ‘Cherry Mom’. Positive correlations between ETR, qP, and SPAD values were probably related to increases in chlorophyll as more chlorophyll will allow for more light energy utilization. These data suggest that ‘Cherry Mom’ is more drought sensitive as the chlorophyll levels tend to decrease under drought affecting the light harvesting apparatus and reducing photosynthesis. Similarly, in faba bean (*Vicia faba*) lower ETR can be associated with lower SPAD values (Khazaei et al., 2019).

Estimation of WUE for Selection Purposes

Measuring real WUE by measuring the crop transpiration and biomass production, although precise, is very time consuming and costly as it requires of a lot of personnel or advanced machinery such as automated mini-lysimeters (Condon et al, 2004; Farquhar et al., 1989; Vadez et al., 2014; Vadez and Ratnakumar, 2016). For that reason, other physiological characteristics can be used to estimate WUE and the crop response to drought such as $\Delta^{13}C$ in rice (*Oryza sativa* L.) (Impa et al., 2005), wheat (Condon et al., 2004, sunflower (*Helianthus annuus* L.) (Adiredjo et al., 2014) and photosynthetic parameters (Farquhar et al., 1989; Zhang et al., 2022). In this experiment we illustrated for the first time in hemp that WUE could be successfully estimated using leaf $\Delta^{13}C$ at the end of the season as in both years the correlation was very high (-0.7 to -0.81) as shown by Condon et al. (2004). This finding could facilitate the screenings of large sets of hemp germplasm for different $\Delta^{13}C$ values which could result in higher drought tolerance as shown in wheat (Condon et al., 2004), common bean (Polania et al., 2016; Sanz-Saez et al., 2019), peanut (Wright et al., 1994), and soybean (Dhanapal et al., 2014). Water Use Efficiency was negatively correlated with A probably due to the fact that to show high A, gs needs to be also high and therefore WUE decreases. However, A and g_s exhibit a moderate positive correlation (r= 0.3-0.5) with yield as plants that show high A, accumulate more biomass resulting in higher yields (Zhang et al., 2022). These two parameters have been used previously on peanut to select for drought tolerant peanuts, as cultivars with high A and low g_s show high WUE and therefore drought tolerance (Zhang et al., 2022). The correlations in our study were lower than the ones observed in Zhang et al. (2022) possibly because we used only two cultivars for this experiment. Therefore, this technique should be useful to select for drought tolerant cultivars when studying a high number of lines.

CONCLUSIONS

This study has shown that drought intensities and timings can have a variety of effects on yield and physiological parameters in hemp. We found that moderate drought stresses minimally affected total biomass production or major physiological parameters (A, g_s, ETR, qP, and qN), whereas more intense stresses significantly impacted these parameters. The findings also indicated that drought timing can induce a plant response at the cellular level as later drought timings often times showed more negative consequences in terms of A, g_s, ETR, and qN. It is likely that these responses are cultivar dependent. Likewise, $\Delta^{13}C$ appears to be an efficient method to estimate WUE as consistent results found differences in cultivar WUE and $\Delta^{13}C$. This multi-year study has indicated the feasibility of utilizing high throughput techniques for economical and effective selection of hemp cultivars for drought tolerance.

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Figure 1. Stacked partitioned biomass separated into leaves, stems, flower, and total biomass. Boxes are color coded based on water stress intensity. Purple boxes identify control treatment, red identifies extreme drought stress, green identifies intermittent drought stress and blue identifies moderate drought stress. P-values are in the upper portion of graphic denoting significance of water stress, cultivar, and interaction. Different capital letters designate significantly different cultivar means. Different letters denote water by cultivar treatment interaction if there was significant interaction. In the absence of interaction letters indicate difference between water treatment for each cultivar. Abbreviations are as follows: C (control), EE (early extreme water stress), EI (early intermittent water stress), EM (early moderate water stress), LE (late extreme water stress), LI (late intermittent water stress), LM (late moderate water stress).

Figure 2. Photosynthetic assimilation rate (µmol m^{-2} s^{-1}) measured at various time points throughout the flowering cycle for each cultivar and water stress treatment each year of the study. Boxes are color coded based on water stress intensity. Purple boxes identifies control treatment, red identifies extreme drought stress, green identifies intermittent drought stress and blue identifies moderate drought stress. P-values are in the upper portion of graphic denoting significance of water stress, cultivar, and interaction. Different capital letter designate significantly different cultivar means. Different letters denote water by cultivar treatment interaction if there was significant interaction. In the absence of interaction letters indicate difference between water treatment for each cultivar. Abbreviations are as follows: C (control), EE (early extreme water stress), EI (early intermittent water stress), EM (early moderate water stress), LE (late extreme water stress), LI (late intermittent water stress), LM (late moderate water stress).

Figure 3. Stomatal conductance (mol m^{-2} s^{-1}) measured at various timepoints throughout the flowing cycle for each cultivar and water stress treatment each year of the study. Boxes are color coded based on water stress intensity. Purple boxes identifies control treatment, red identifies extreme drought stress, green identifies intermittent drought stress and blue identifies moderate drought stress. P-values are in the upper portion of graphic denoting significance of water stress, cultivar, and interaction. Different capital letter designate significantly different cultivar means. Different letters denote water by cultivar treatment interaction if there was significant interaction. In the absence of interaction letters indicate difference between water treatment for each cultivar. Abbreviations are as follows: C (control), EE (early extreme water stress), EI (early intermittent water stress), EM (early moderate water stress), LE (late extreme water stress), LI (late intermittent water stress), LM (late moderate water stress).

Figure 4. Electron transfer rate (µmol s^{-1}) measured at various timepoints throughout the flowering cycle for each cultivar and water stress treatment each year of the study. Boxes are color coded based on water stress intensity. Purple boxes identifies control treatment, red identifies extreme drought stress, green identifies intermittent drought stress and blue identifies moderate drought stress. P-values are in the upper portion of graphic denoting significance of water stress, cultivar, and interaction. Different capital letter designate significantly different cultivar means. Different letters denote water by cultivar treatment interaction if there was significant interaction. In the absence of interaction letters indicate difference between water treatment for each cultivar.

Abbreviations are as follows: C (control), EE (early extreme water stress), EI (early intermittent water stress), EM (early moderate water stress), LE (late extreme water stress), LI (late intermittent water stress), LM (late moderate water stress).

Figure 5. Correlation matrix of all parameters measured in 2021. Blue signifies a positive correlation and red signifies a negative correlation. The greater intensity of the color and size of the circle represents stronger correlations. R-values are also present in each circle to denote calculated R.

Figure 6. Correlation matrix of all parameters measured in 2022. Blue signifies a positive correlation and red signifies a negative correlation. The greater intensity of the color and size of the circle represents stronger correlations. R-values are also present in each circle to denote calculated R.

Table 1. Total Biomass WUE, Daily Transpiration and $\Delta^{13}C$

		Year 2021			Year 2022		
Cultivar	**Treatment**	**WUE (total biomass/kg water)**	**Daily Transpiration (grams)**	**$\Delta^{13}C$**	**WUE (total biomass/kg water)**	**Daily Transpiration (grams)**	**$\Delta^{13}C$**
	Control	1.632 d	1311.398 a	22.418 bcd	1.726 c	1024.061 a	22.682 a
	EE	3.131 a	361.095 g	20.537 e	3.211 a	254.381 e	20.908 c
	EI	1.938 bc	601.905 efg	21.062 e	2.166 b	446.704 d	21.785 b
Baox	EM	2.134 b	976.854 abcd	22.103 cd	1.762 c	753.725 b	22.537 a
	LE	1.651 bcd	929.888 bcde	21.913 d	1.707 c	580.636 cd	22.198 a
	LI	1.578 cd	1070.517 abc	22.17 bcd	1.778 bc	731.49 bc	22.67 a
	LM	1.759 cd	1239.585 ab	22.458 bcd	1.697 c	864.833 b	22.602 a
	mean	1.975 A	927.320 A	21.809 B	2.007 A	665.119 B	22.197 B
	Control	1.185 d	747.929 cdef	23.075 a	1.195 c	1183.35 a	22.963 a
	EE	2.396 a	288.677 g	22.083 cd	2.703 a	278.507 e	21.097 c
	EI	1.642 bc	483.908 fg	22.362 bcd	1.743 b	544.99 d	22.008 b
Cherry Mom	EM	1.642 b	605.357 efg	23.057 a	1.432 c	945.5 b	22.933 a
	LE	1.412 bcd	711.599 def	22.598 abc	1.482 c	678.01 cd	22.73 a
	LI	1.299 cd	619.102 efg	22.742 ab	1.558 bc	821.191 bc	22.848 a
	LM	1.252 cd	808.293 cdef	23.062 a	1.369 c	983.779 b	22.613 a
	mean	1.547 B	609.266 B	22.111 A	1.640 B	776.475 A	22.456 A
Water Stress (WS)		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
Cultivar(C)	p-value	<.0001	<.0001	<.0001	<.0001	0.0006	0.001
WS x C		0.415	0.005	0.0002	0.58	0.859	0.644

Table 2. SPAD Upper and Lower Canopy

		Year 2021				Year 2022			
		20 DAF		46 DAF		12 DAF		35 DAF	
Cultivar	**Treatment**	**SPAD upper**	**SPAD lower**	**SPAD upper**	**SPAD lower**	**SPAD upper**	**SPAD lower**	**SPAD upper**	**SPAD lower**
Baox	Control	39.95 ab	27.65 a	31.317 ab	23.583 abc	48.25	26.867	35.45 ab	30.05 a
	EE	48.517 a	23.333 ab	34.317 ab	23.217 abc	51.483	35.25	39.55 a	20.067 b
	EI	43.833 ab	22.133 ab	21.583 bcd	19.433 abcd	47.75	29.583	27.667 b	18.517 b
	EM	43.9 ab	29.35 a	29.55 abc	25.1 abc	50.417	28.883	33.517 ab	20.617 ab
	LE	-	-	23.033 bc	19.533 abcd	-	-	35.75 ab	14.383 b
	LI	-	-	26.667 abc	21.633 abc	-	-	37.367 ab	23.8 ab
	LM	-	-	26.7 abc	23.667 abc	-	-	35.383 ab	26.367 a
	mean	44.05	25.617 A	27.595 A	22.31	49.475 B	30.146 B	34.267	21.971
Cherry Mom	Control	46.35 b	27.517 a	37.367 a	30.817 a	55.15	37.55	37.45 ab	32.033 a
	EE	42.183 ab	6.033 c	27.417 abc	18.95 abcd	50.867	37.15	41.433 a	15.05 b
	EI	45.767 ab	10.6 c	16.6 cd	14.083 bcd	53.35	34.4	34.633 b	17.917 b
	EM	45.283 ab	15.75 bc	31.233 ab	27.067 ab	50.567	35.1	34.467 ab	25.083 ab
	LE	-	-	9.217 d	5.767 d	-	-	31.533 ab	20.983 b
	LI	-	-	16.533 cd	11.4 cd	-	-	37.65 ab	22.133 ab
	LM	-	-	33.3 ab	26.317 ab	-	-	36.7 ab	28.15 a
	mean	44.896	14.975 B	24.524 B	19.2	52.551 A	35.792 A	34.955	23.05
Water Stress (WS)	p-value	0.494	<.0001	<.0001	<.0001	0.923	0.376	0.016	<.0001
Cultivar(C)	p-value	0.421	<.0001	0.0397	0.054	0.044	0.0021	0.337	0.469
WS x C	p-value	0.0013	0.0089	0.0011	0.0084	0.178	0.381	0.548	0.442

Table 3. Respiration and Fv/Fm of Dark-Adapted Leaves

		* Respiration (µmol m^{-2} s^{-1})							
		Year 2021				Year 2022			
		20 DAF		46 DAF		12 DAF		35 DAF	
Cultivar	**Treatment**	**Respiration**	**Fv/Fm**	**Respiration**	**Fv/Fm**	**Respiration**	**Fv/Fm**	**Respiration**	**Fv/Fm**
	Control	-0.821 b	0.811	-0.355	0.761	-1.202	0.82	-0.818	-
	EE	-0.550 a	0.825	-0.265	0.792	-0.852	0.825	-1.291	-
	EI	-0.615 ab	0.814	-0.382	0.742	-1.021	0.826	-1.217	-
Baox	EM	-0.937 b	0.816	-0.290	0.802	-1.071	0.825	-1.226	-
	LE	-	-	-0.271	0.77	-	-	-0.813	-
	LI	-	-	-0.362	0.803	-	-	-0.843	-
	LM	-	-	-0.399	0.806	-	-	-1.049	-
	mean	-0.731	0.817	-0.332	0.782 B	-1.037	0.834 B	-1.037	
	Control	-0.891 b	0.818	-0.286	0.78	-1.398	0.829	-1.209	-
	EE	-0.633 a	0.81	-0.264	0.691	-1.223	0.83	-0.691	-
	EI	-0.666 ab	0.818	-0.393	0.636	-0.871	0.833	-1.259	-
Cherry Mom	EM	-0.768 b	0.815	-0.296	0.735	-1.283	0.829	-0.863	-
	LE	-	-	-0.183	0.525	-	-	-0.616	-
	LI	-	-	-0.445	0.693	-	-	-0.877	-
	LM	-	-	-0.416	0.798	-	-	-0.879	-
	mean	-0.74	0.815	-0.333	0.697 B	-1.194	0.830 A	-0.914	
Water Stress (WS)		0.0045	0.961	0.211	0.119	0.151	0.298	0.155	-
Cultivar(C)	p-value	0.877	0.758	0.951	0.002	0.175	0.0002	0.201	-
WS x C		0.381	0.359	0.961	0.273	0.435	0.708	0.169	-

Table 4. Non-Photochemical and Photochemical Quenching Coefficients

		Year 2021				Year 2022			
		20 DAF		46 DAF		12 DAF		35 DAF	
Cultivar	**Treatment**	**qN**	**qP**	**qN**	**qP**	**qN**	**qP**	**qN**	**qP**
Baox	Control	0.659	0.614	0.502 b	0.710 a	0.4	0.569	-	-
	EE	0.734	0.673	0.587 ab	0.584 ab	0.534	0.633	-	-
	EI	0.644	0.593	0.707 a	0.490 b	0.468	0.669	-	-
	EM	0.696	0.637	0.629 ab	0.602 ab	0.418	0.627	-	-
	LE	-	-	0.756 a	0.584 ab	-	-	-	-
	LI	-	-	0.750 a	0.586 ab	-	-	-	-
	LM	-	-	0.650 ab	0.624 a	-	-	-	-
	mean	0.681 B	0.627 A	0.653	0.6 A	0.447	0.616	-	-
Cherry Mom	Control	0.741	0.615	0.256 b	0.591 a	0.419	0.656	-	-
	EE	0.838	0.509	0.629 ab	0.484 ab	0.488	0.642	-	-
	EI	0.829	0.496	0.905 a	0.319 b	0.577	0.661	-	-
	EM	0.795	0.61	0.558 ab	0.434 ab	0.421	0.638	-	-
	LE	-	-	0.932 a	0.314 ab	-	-	-	-
	LI	-	-	0.812 a	0.363 ab	-	-	-	-
	LM	-	-	0.419 ab	0.550 a	-	-	-	-
	mean	0.801 A	0.557 B	0.636	0.44 B	0.466	0.65	-	-
Water Stress (WS)	p-value	0.216	0.202	<.0001	0.003	0.096	0.35	-	-
Cultivar (C)		0.0005	0.021	0.751	<.0001	0.603	0.096	-	-
WS x C		0.623	0.163	0.135	0.689	0.64	0.321	-	-

Figure 1.

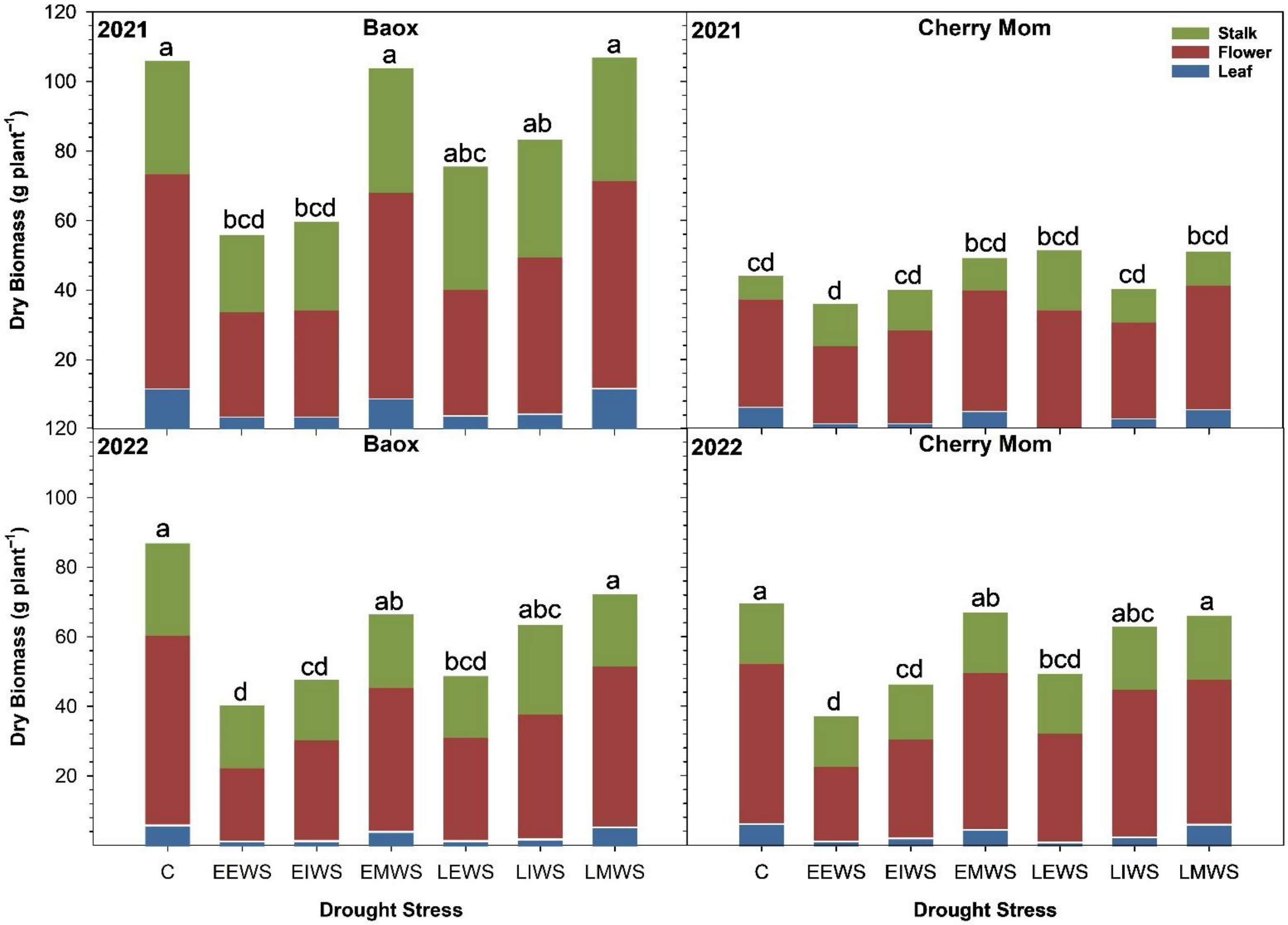

2021
Baox
2021
Cherry Mom
Stalk
Flower
Leaf
2022
Baox
2022
Cherry Mom
Dry Biomass (g plant^{-1})
Dry Biomass (g plant^{-1})
120
100
80
60
40
20
120
100
80
60
40
20
a
bcd
bcd
a
abc
ab
a
cd
d
cd
bcd
bcd
cd
bcd
a
d
cd
ab
bcd
abc
a
a
d
cd
ab
bcd
abc
a
C
EEWS
EIWS
EMWS
LEWS
LIWS
LMWS
C
EEWS
EIWS
EMWS
LEWS
LIWS
LMWS
Drought Stress
Drought Stress

Figure 2.

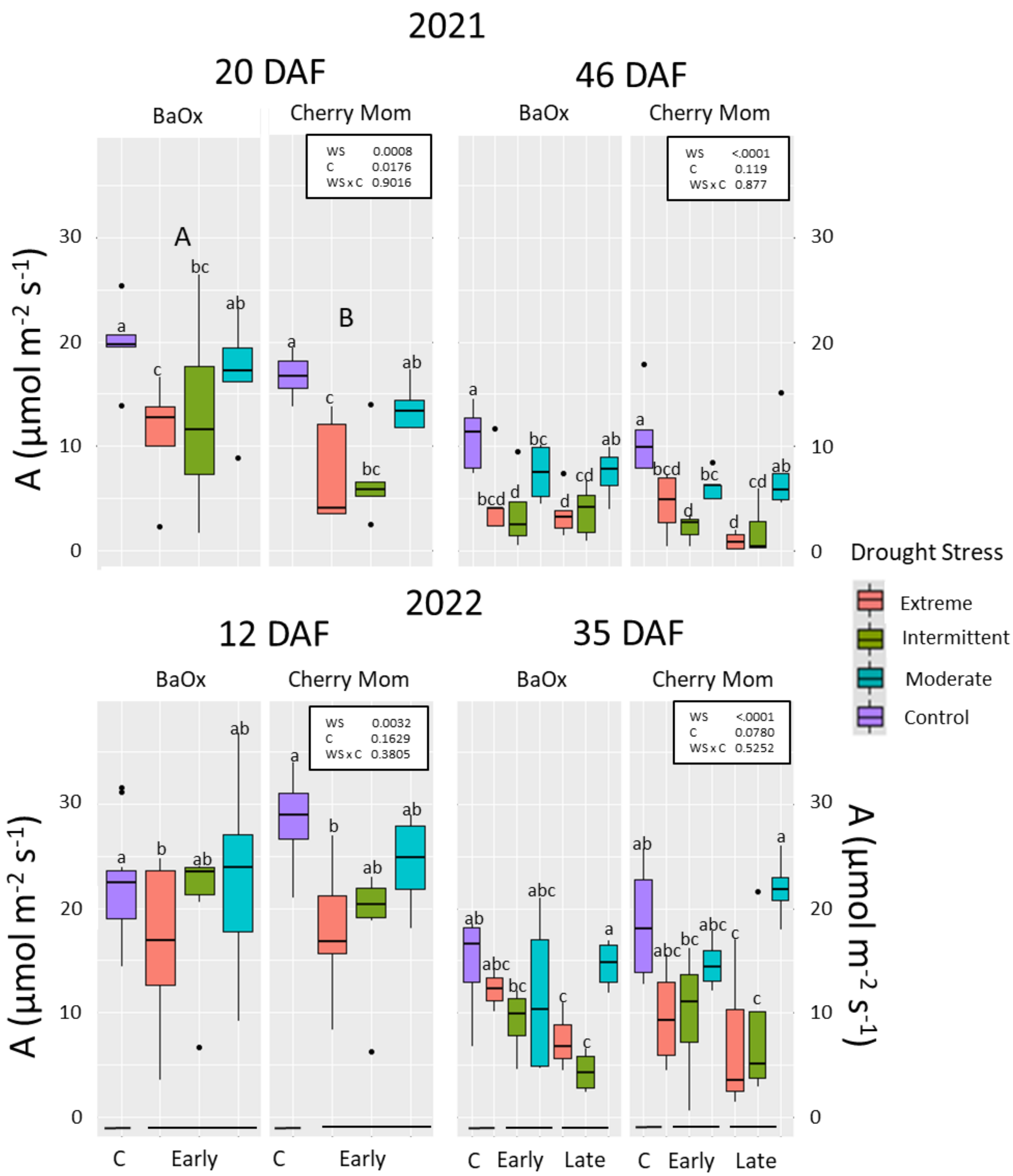

2021
20 DAF
46 DAF
BaOx
Cherry Mom
BaOx
Cherry Mom
WS 0.0008
C 0.0176
WS x C 0.9016
WS <.0001
C 0.119
WS x C 0.877
A
B
A (µmol m-2 s-1)
2022
12 DAF
35 DAF
BaOx
Cherry Mom
BaOx
Cherry Mom
WS 0.0032
C 0.1629
WS x C 0.3805
WS <.0001
C 0.0780
WS x C 0.5252
A (µmol m-2 s-1)
C
Early
C
Early
C
Early
Late
C
Early
Late
Drought Stress
Extreme
Intermittent
Moderate
Control

Figure 3.

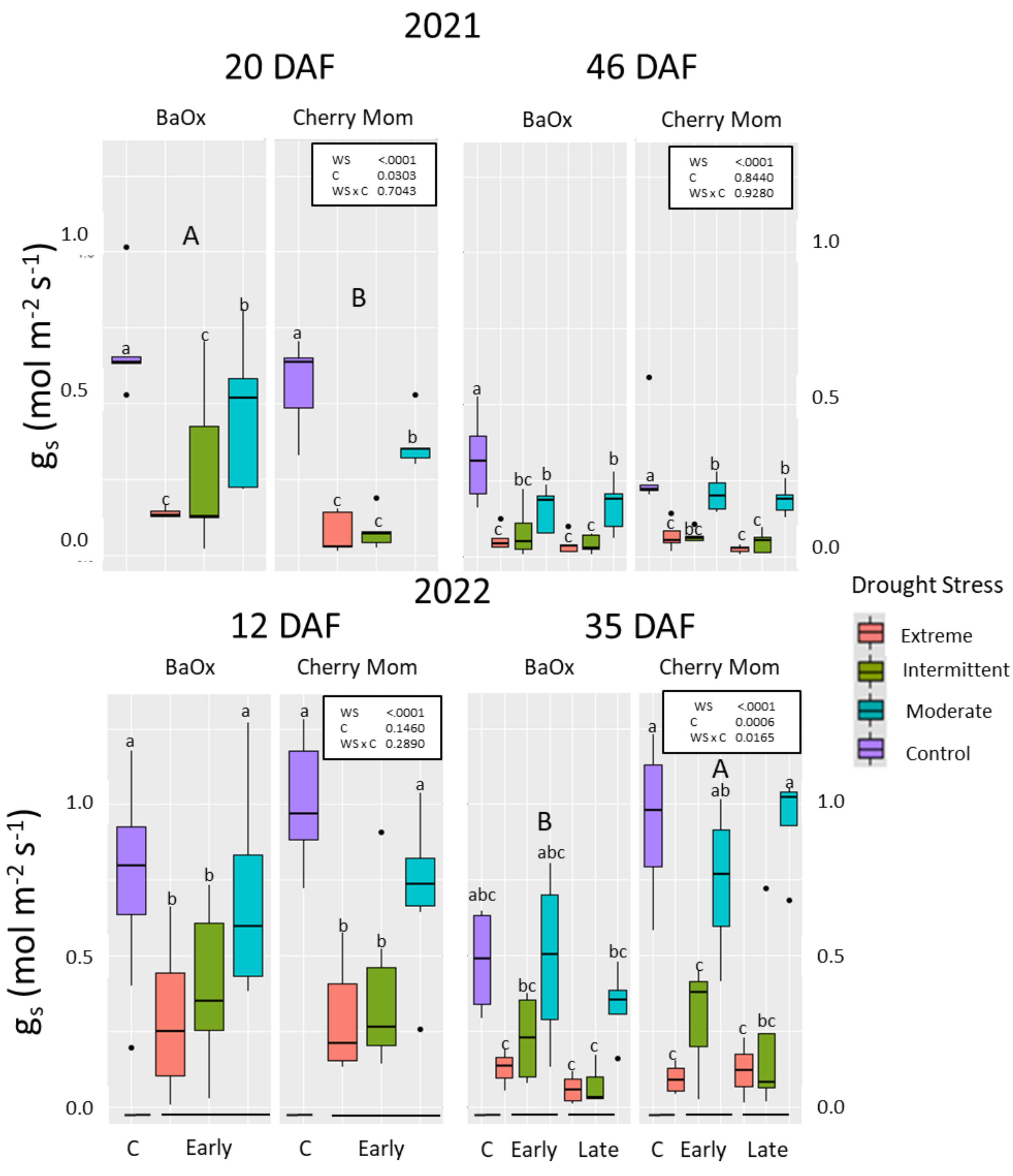

2021
20 DAF
46 DAF
BaOx
Cherry Mom
BaOx
Cherry Mom
WS <.0001
C 0.0303
WS x C 0.7043
WS <.0001
C 0.8440
WS x C 0.9280
A
B
gs (mol m-2 s-1)
1.0
0.5
0.0
2022
12 DAF
35 DAF
BaOx
Cherry Mom
BaOx
Cherry Mom
WS <.0001
C 0.1460
WS x C 0.2890
WS <.0001
C 0.0006
WS x C 0.0165
C
Early
C
Early
C
Early
Late
C
Early
Late
Drought Stress
Extreme
Intermittent
Moderate
Control

Figure 4.

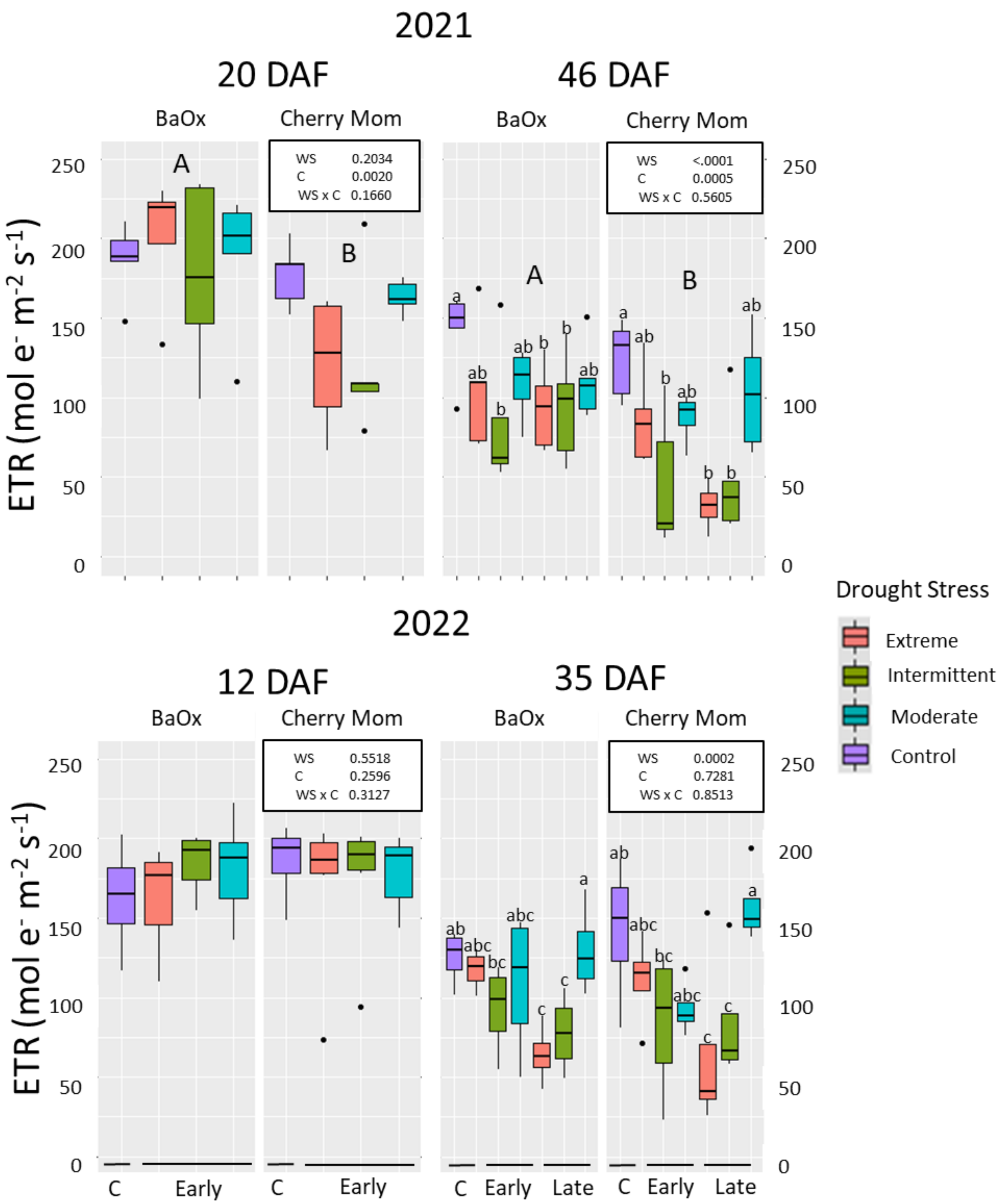

2021
20 DAF
46 DAF
BaOx
Cherry Mom
BaOx
Cherry Mom
WS 0.2034
C 0.0020
WS x C 0.1660
WS <.0001
C 0.0005
WS x C 0.5605
A
B
A
B
ETR (mol e⁻ m⁻² s⁻¹)
2022
12 DAF
35 DAF
BaOx
Cherry Mom
BaOx
Cherry Mom
WS 0.5518
C 0.2596
WS x C 0.3127
WS 0.0002
C 0.7281
WS x C 0.8513
C
Early
C
Early
C
Early
Late
C
Early
Late
Drought Stress
Extreme
Intermittent
Moderate
Control

Figure 5.

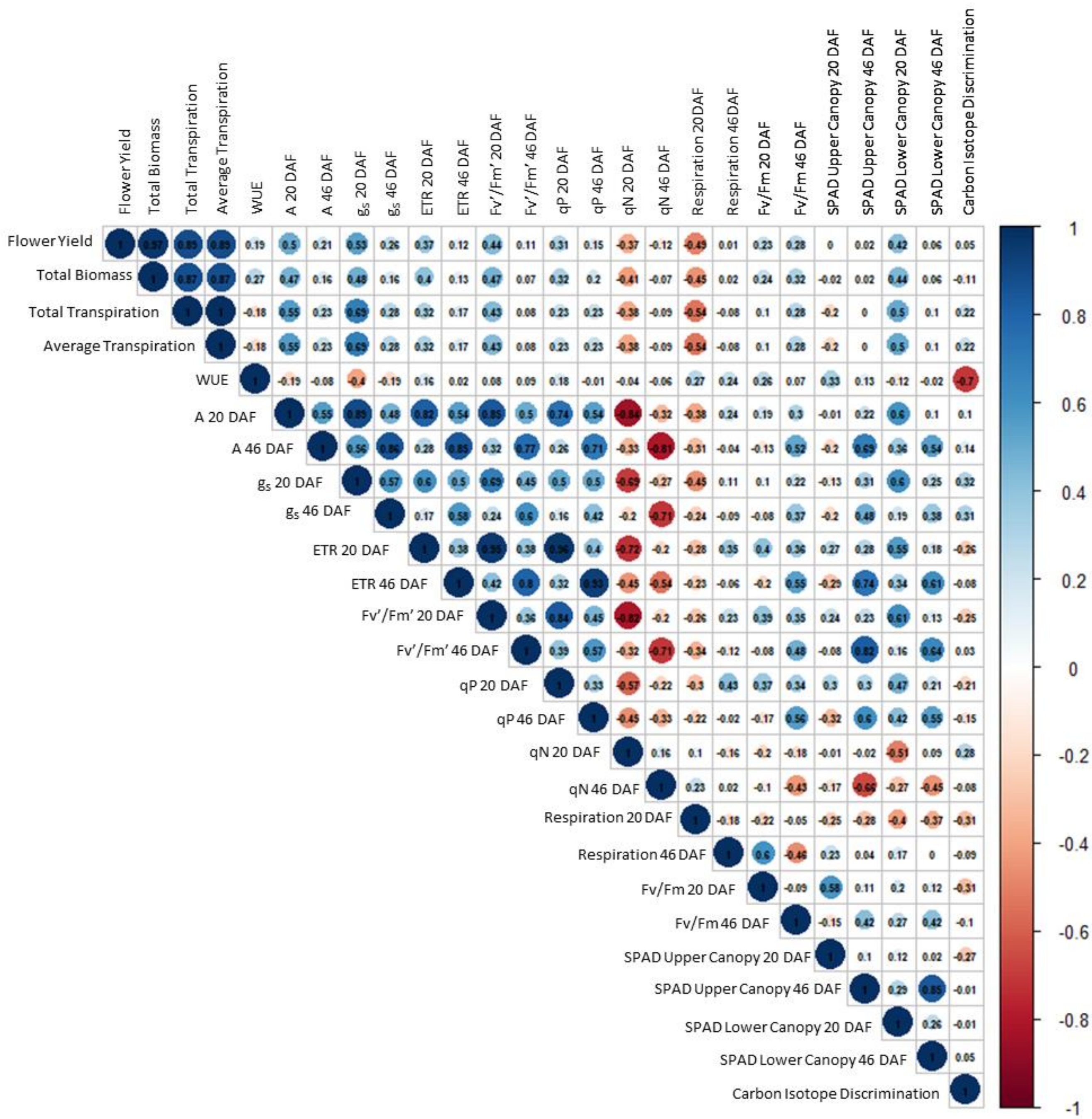
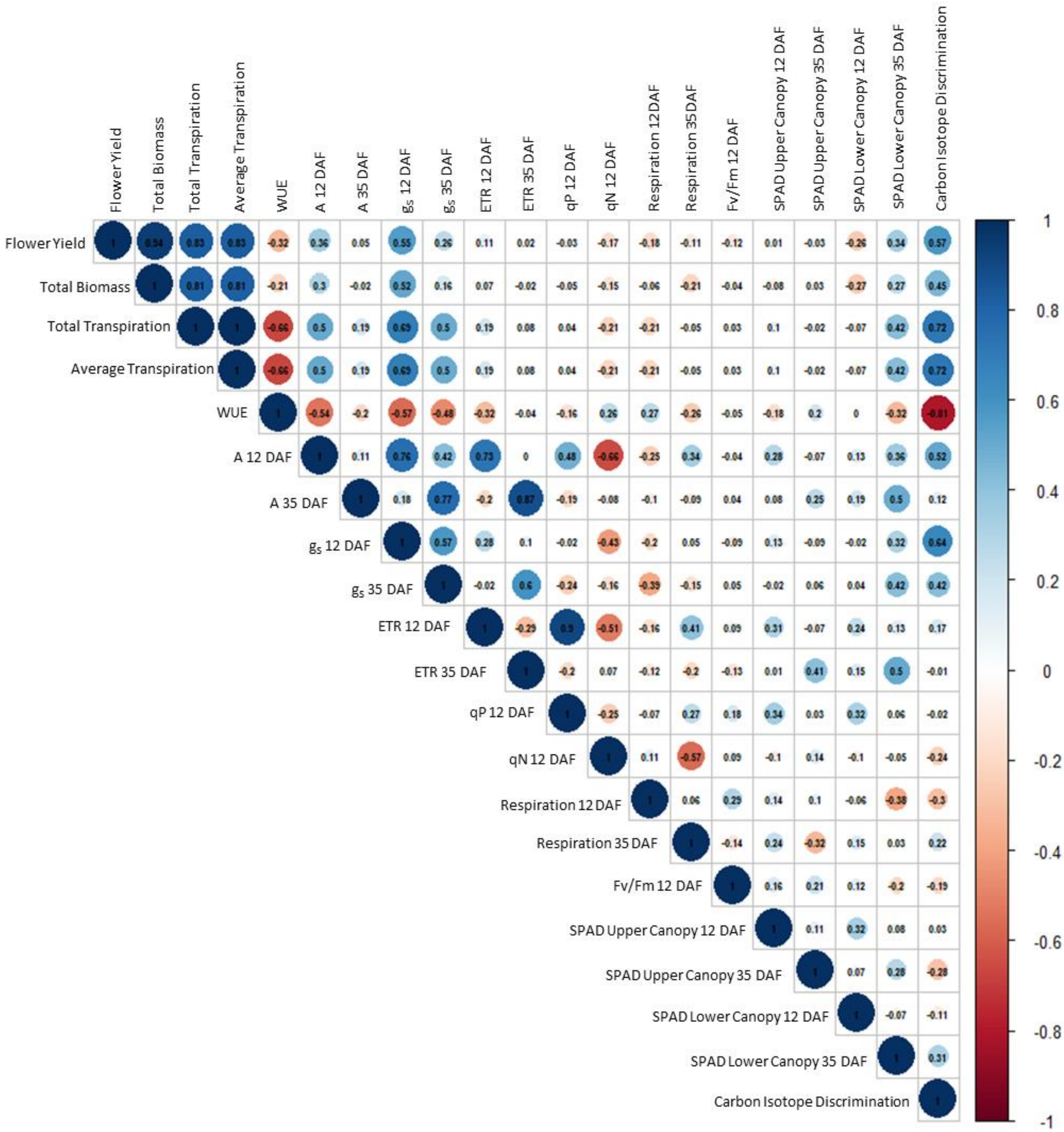

Flower Yield
Total Biomass
Total Transpiration
Average Transpiration
WUE
A 20 DAF
A 46 DAF
gs 20 DAF
gs 46 DAF
ETR 20 DAF
ETR 46 DAF
Fv'/Fm' 20 DAF
Fv'/Fm' 46 DAF
qP 20 DAF
qP 46 DAF
qN 20 DAF
qN 46 DAF
Respiration 20 DAF
Respiration 46 DAF
Fv/Fm 20 DAF
Fv/Fm 46 DAF
SPAD Upper Canopy 20 DAF
SPAD Upper Canopy 46 DAF
SPAD Lower Canopy 20 DAF
SPAD Lower Canopy 46 DAF
Carbon Isotope Discrimination
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1

Figure 6.


Flower Yield
Total Biomass
Total Transpiration
Average Transpiration
WUE
A 12 DAF
A 35 DAF
g_s 12 DAF
g_s 35 DAF
ETR 12 DAF
ETR 35 DAF
qP 12 DAF
qN 12 DAF
Respiration 12 DAF
Respiration 35 DAF
Fv/Fm 12 DAF
SPAD Upper Canopy 12 DAF
SPAD Upper Canopy 35 DAF
SPAD Lower Canopy 12 DAF
SPAD Lower Canopy 35 DAF
Carbon Isotope Discrimination
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1

CHAPTER 4

Investigating Relationships Between Drought Stress and Arthropods in Hemp

Wayne Morgan[1], Katelyn Kesheimer[2], Jeanine Davis[3], Alvaro Sanz-Saez[1]*

[1] Department of Crop, Soil and Environmental Sciences, 253 Funchess Hall Auburn University, Auburn, AL 36849, USA

[2] Department of Entomology and Plant Pathology, 301 Funchess Hall Auburn University, Auburn, AL 36849 USA

[3] Department of Horticultural Science, North Carolina State University, 455 Research Drive, Mills River, NC 28759

Abstract

Hemp (*Cannabis sativa* L.) is an ancient crop that is being reintroduced into modern agriculture. Once grown on a large scale, little is known to modern farmers in terms of production due to decreases in cultivation in the early twentieth century. This is in large part because of the development of easier processed fiber crops and the stigma placed on *Cannabis sativa*. Furthermore, climate change is affecting precipitation patterns in such a way that extreme weather, including prolonged droughts, can negatively affect crop production. The current situation makes it nearly impossible to cultivate crops without having an irrigation plan in place if water stress were to occur. Since the reemergence of hemp cultivation, producers have documented the presence of many arthropod populations cohabitating with hemp. The objective of this study was to discover the relationship of floral hemp cultivars with arthropods in a greenhouse setting under various drought stresses to decipher trends in the arthropod communities and interactions. The ultimate goal of this and future research is to utilize irrigation practices and cultivar selection as crucial tools in an integrated pest management (IPM) program. Floral hemp cultivars BaOx and Cherry Mom were cultivated from early July to early October in 2021 and 2022. Our study found that drought stress did not affect arthropod populations contrary to studies conducted on other crops. However, this study found that there were differences in arthropod population distribution across cultivars, and this may be due to secondary plant metabolites. Furthermore, it was determined that arthropod populations appeared to predictably change over the course of the hemp reproductive season. This indicates the potential for producers to include cultivar selection in their IPM programs and to know which arthropods may cause the most economic damage during the season.

Keywords: Insects, arthropods, Integrated Pest Management (IPM), hemp, greenhouse, drought

INTRODUCTION

Hemp (*Cannabis sativa* L.) was a once common crop that is experiencing a revival in the United States and throughout the world. Tracing its origins to Central Asia, hemp was once utilized by numerous societies for a variety of uses (McPartland et al., 2019). Hemp, while traditionally utilized for clothing and sustenance, was also used as a medicine due to the diverse cannabinoids produced in the flowers (McPartland et al., 2019; Strzelczyk et al., 2022). In addition to the medicinal uses of hemp, the fibers and bast derived from hemp stalks have been used as a construction material comprising building materials like insulation and ropes (McPartland et al., 2019; Strzelczyk et al., 2022). Hemp rivaled other agricultural crops and was often the predominant crop cultivated globally until 1883 (Herer, 1990). However, later hemp production declined as the stigma surrounding the closely related plant, marijuana (*Cannabis sativa*), grew (Schlosser, 1994). Additionally, declines in production were related to alternative fiber crops (i.e., cotton (*Gossypium* spp.)) that became easier and more economical to process, due to inventions like the cotton gin (Fike, 2016).

In recent years, there has been a resurgence in interest in growing and using hemp in the United States, leading to legalization at the federal level in 2018 (Mattingly, 2020). Following legalization, there were over 90,000 acres of hemp cultivated throughout the United States, second only in annual production to the 146,200 acres produced in the United States in 1943 (Mark et al., 2020). Though the crop was once grown extensively, these new producers encountered many hurdles as modern agronomic information is not available.

Hemp is legally distinguished from marijuana based on cannabinoid levels; hemp contains no greater than 0.3% total tetrahydrocannabinol (THC) on a dry weight basis (Skorbiansky et al., 2021). It is hypothesized that both biotic stresses (e.g., pests) and abiotic stresses (e.g., drought) may influence the plant's ability to produce certain cannabinoids (Suzuki et al., 2014). There are multiple types of hemp cultivated including those produced for fiber, seed, and cannabinoids (Carlson et al., 2021) with added interest in cannabinoid rich [e.g., cannabidiol (CBD)] production due to its greater economic potential (Mark et al., 2020).

Arthropod pests pose an economic threat to hemp producers. Recent estimates show nearly $470 billion dollars are lost each year to general crop infestations throughout the globe (Kamatham et al., 2021). Additionally, almost half of the pests that damage these crops are phytophagous (Kamatham et al., 2021). Hemp, due to restrictive laws that discouraged widespread entomological research, is lacking in current, research-based arthropod management strategies. Regardless, there have been recent advances in identifying some of the most troublesome pests of hemp. Corn earworm (*Helicoverpa zea*) has emerged as one of the most injurious pests of outdoor hemp in the U.S. (Britt et al., 2021). Larval corn earworms infest and feed on hemp flowers, which can promote disease within the flower and significantly reduce yield (Ajayi and Samuel-Foo, 2021; Britt et al., 2021; Cranshaw et al., 2019). Common piercing-sucking pests of indoor and outdoor hemp include the two-spotted spider mite (*Tetranychus urticae*) and cannabis aphid (*Phorodon cannabis*) (Ajayi and Samuel-Foo, 2021). Thrips (Order: Thysanoptera) of multiple species have been shown to be associated with hemp, however, depending on the thrips species, damage can be minimal to extensive (Cranshaw et al., 2019). Orthoptera, including grasshoppers (Acrididae) and crickets (Gryllidae), have also been shown to be associated with hemp and can cause heavy damage through defoliation (e.g., grasshoppers) or serious stem damage (e.g., crickets) noted in crops in 2022 in North Carolina and Alabama (Katelyn Kesheimer, Department of Entomology and Plant Pathology, Auburn University, personal communication). Additionally, common greenhouse pests in the order Diptera (e.g., fungus gnats) have been found in multiple greenhouse crops, including hemp, and can be potentially damaging via the transmission of diseases (Jarvis et al., 1993).

Two-spotted spider-mites are well-studied polyphagous pests of indoor and outdoor crops, but their damage has been shown to be greatest in indoor environments (Cranshaw et al., 2019). This pest is incredibly responsive to drought stress (English-Loeb, 1989). In fact, it has been found in common bean (*Phaseolus vulgaris*) that non-drought stressed plants result in the lowest two-spotted spider mite pressure; increasing drought stresses can significantly increase insect populations (English-Loeb, 1989). It must be noted that severe drought stresses can also reduce two-spotted spider mite populations in cases where the plant health is seriously compromised due to drought (English-Loeb, 1989). These relationships with pests are influenced by the health of the plant. Well-watered control plants are generally healthy, but nutrients may not be concentrated enough for pests to thrive. Under extreme stress, the plant is unhealthy and may lack proper nutritional sustenance for the pest population to thrive (English-Loeb, 1989; Mattson and Haack, 1987). However, plants under mild drought stress remain healthy but nutrients are more concentrated for the pests to utilize (English-Loeb, 1989; Mattson and Haack, 1987).

Across many crops, pesticide use is the primary means of controlling pest populations. However, due to the newness of hemp and lack of data, few pesticides have federal labels for hemp. But, individual U.S. states are allowed by the Environmental Protection Agency (EPA) to maintain their own pesticide rules for hemp as long as they comply with state and federal regulations. The lack of available pesticides and research paired with confusing regulations require that growers explore other integrated pest management (IPM) strategies. With the small number of approved and well-researched pesticides (US EPA, OCSPP, 2019) cultivators of cannabis must manage the plant’s environment to minimize detrimental effects of pests on hemp plants.

Biological control through the use of predatory insects can be a successful method to mitigate pest populations and their associated damage (Hayes et al., 2019). Cultural control strategies in an IPM plan include controlling environmental conditions (i.e., temperature and humidity) in addition to providing proper plant nutrition and irrigation. These methods are often effective in the mitigation of pests by maintaining conditions conducive to optimal plant health and may help support beneficial insect populations (Hayes et al., 2019). By promoting an optimal environment for beneficial predators, the possibility of attracting natural predators from the environment such as Hymenopterans, predatory Hemipterans, and spiders (Araneae) (Cranshaw et al., 2019) will further help protect the crop from deleterious pests. These IPM methods can aid producers by maintaining healthy plants in such a way that avoids chemical control.

Drought is a major abiotic stress that significantly reduces plant yield and may lead to economic losses. Further, drought-stressed plants may be more vulnerable to pest infestations, including insects and diseases (Mattson and Haack, 1987; Rippey, 2015). Therefore, it is imperative that producers effectively manage drought stress using techniques such as proper irrigation (Hayes et al., 2019) or cultivar selection (Carlson et al., 2021).

There is also the possibility that insects can affect the levels of cannabinoid production in hemp. Toth et al. (2021) showed that general wounding to mimic insect injury does not increase or decrease THC or CBD in hemp. Some research has shown that the tobacco hornworm (*Manduca sexta*) can decrease CBD content, whereas other research has found that the presence of the corn earworm can increase CBD and THC content (Jackson et al., 2021; Park et al., 2022;).

Given the paucity of current research on the effects of drought stress and insect presence on hemp, we sought to: 1) Conduct an arthropod survey on greenhouse cultivated hemp across two cultivars and different drought stress timings and intensities; 2) Examine temporal variation in arthropod populations at the leaf and soil levels; and 3) Identify the relationship between arthropod populations and drought stress levels.

MATERIAL AND METHODS

Study design, setup, and management

Floral CBD hemp cultivars BaOx and Cherry Mom were planted July 3, 2021 and July 1, 2022 in a greenhouse in Zirconia, North Carolina, USA (35.213349, -82.416249). The clones (42 per cultivar) were planted in 19-liter containers with a "living-soil" media purchased from Dirtcraft Organics (Dirtcraft Organics LLC, Marshall, NC, USA).

The forty-two clones of each cultivar were chosen and planted in a random fashion in one of each of seven drought treatments. The crop was planted in a randomized complete block design (RCBD) with six replications. The seven drought treatments were as follows: Control [100%-70% soil water content (SWC)], Early Extreme Drought Stress (10%-0% SWC), Early Intermittent Drought Stress (10%-0% SWC), Early Moderate Drought Stress (50%-30% SWC), Late Extreme Drought Stress (10%-0% SWC), Late Intermittent Drought Stress (100%-0% SWC), Late Moderate Drought Stress (50%-30% SWC).

During the vegetative period, all clones were supplemented with a Fluence SPYDR series LED lighting system (Fluence USA, Austin, TX, USA) to extend the light period to approximately 18 hours to prevent premature flowering. The plants were irrigated daily to maintain an approximate 70%-100% SWC during the entire vegetative cycle and until each drought timing was commenced during the flowering cycle (7- or 28-days post flower initiation). A drum fan with a diameter of 63.5 centimeters was utilized to maintain sufficient airflow and maintain adequate temperatures. Onset HOBO UA-002-64 pendants (Onset Brands, Bourne, MA, USA) were utilized both inside and outside of the greenhouse to monitor environmental conditions such as light intensity and temperature. Each year the crop was administered a 10-20-20 fertilizer to ensure the crop was supplemented with 68 kilograms of Nitrogen per acre during reproduction.

In order to ensure each drought stress was calculated correctly soil water content (SWC) was calculated as in Morgan et al., (2023)

Reproduction was started by removing the supplemental lighting system so that only natural light was available at a maximum of 14 hours and decreasing daily. Flowering commenced in the first year on August 10, 2021 and the first drought was initiated on August 17, 2021. In the second-year flowering commenced on August 8, 2022 and drought was induced on August 15, 2022. Before the drought treatment started all pots were filled to 100% SWC and weighed to estimate the starting weight of each plant, so that drought stress weight limits could be accurately determined. At that same time, all drainage holes in the pots were plugged so that water could be lost only through evapotranspiration and not leaching. The pots were watered at dawn each morning to determine the SWC. If the SWC was at or below the lower limit the pot was watered until the upper limit was achieved.

In 2021, the crop was treated with Grandevo CG (Marrone Bio Innovations, Inc, Davis, CA, USA) during the vegetative phase using the label recommended rates every seven days from planting through July to prevent major pest outbreaks before the drought started. There was no need to apply Grandevo CG during the second season as there was significantly less pest pressure than year one.

Arthropod Study

Yellow sticky card insect traps (Olson Products, Inc, Medina, OH, USA) with an area of 48 cm^2 were placed near soil level using 30.5 cm metal stakes (Olson Products, Inc, Medina, OH, USA) set in the soil at the beginning of the early drought cycle and replaced every 14 days until harvest each year of study. Sticky traps were collected, placed into sealable plastic bags and then into freezers set at -18° Celsius. On each collection date (21, 35, 49 ,55 days after flowering

(DAF)), three leaves from each plant (top, middle, and bottom) were collected and placed in labeled sealable plastic bags before being transferred to the freezer. Later the sticky traps were removed from the freezer and the arthropods were quantified and identified to order level. Arthropods found on leaves were also identified and quantified to the taxonomic order. The identification and quantification process for both sample types used magnification ranges of 10x-40x depending on the magnification required to properly identify each organism.

Statistical Analysis

All data were analyzed using RStudio (R Foundation for Statistical Computing, Vienna, Austria). The effects of drought and cultivar across all measurements were analyzed with a two-way Analysis of Variance (ANOVA) and a Tukey's HSD post-hoc test. Agricolae package in R was used to identify significant differences between treatments using a Tukey's HSD post-hoc test. Furthermore, a three-way ANOVA was conducted at the end of all trials to identify significant differences between year, drought treatment, and cultivar. Visualization of results was generated using ggplot2 and corrplot packages in RStudio.

RESULTS

Orders Collected

Foliar arthropods encountered each year of the study were two-spotted spider mites (*Tetranychus urticae)* and the cannabis aphid (*Phorodon cannabis*). There were no other mites or signs of mites (russet mite (*Aculops cannabicola*), broad mite (*Polyphagotarsonemus latus*)) present in either year of study. Soil-borne organisms consisted of innocuous, pest, and predatory arthropods. Due to large quantities of organisms, arthropods were identified to the order level in which they belonged. Innocuous organisms tended to be in the orders Coleoptera and Orthoptera, while the majority of Diptera, Thysanoptera, and Lepidoptera had potential to be pests. Hemiptera contained arthropods with individuals such as pest cannabis aphid (*Phorodon cannabis),* as well as predators such as minute pirate bugs (*Orius insidiosus)*. Predators were found mainly in Hymenoptera (parasitoids) and Araneae (spiders).

Foliar Pests

Foliar cannabis aphid populations were not significantly different among water stress treatments at any time point [21, 35, 49, 55 DAF] in either year of study (Table 1). In 2021, the cultivar Cherry Mom did exhibit significantly higher foliar aphid populations at 21 DAF and 35 DAF, however, no significant differences were observed at the later time points (Table 1). In 2022, there was a significantly greater aphid population at 21 DAF for ‘Cherry Mom’, however, the remaining sampling dates were not significantly different (Table 1). It is noted that the populations appeared to have an overall decreasing trend over the course of the flowering cycle in 2021, however, in 2022 populations of foliar aphids did not appear to decrease until after the 35 DAF sampling date for each cultivar (Table 1).

Foliar two-spotted spider mite populations were not significantly different between treatments, regardless of time point or year (Table 2). However, in 2021, ‘Cherry Mom’ had significantly higher spider mite pressure than ‘BaOx’ at 21, 35, and 49 DAF (Table 2). ‘Cherry Mom’ maintained higher total insect pressure at 55 DAF in 2021 as well, however, the difference was not significantly different from ‘BaOx’ (Table 2). In 2022, ‘Cherry Mom’ did not appear to have significantly higher spider mite populations than ‘BaOx’, however, in general, the average was higher than ‘BaOx’ (Table 2). In each year and cultivar, spider mite populations increased through 49 DAF, followed by lower populations at 55 DAF (Table 2).

Soil-borne Arthropods

Hymenoptera showed no significant differences between water treatments at any time points for either year of study (Table 3). There was only one time point (35 DAF, 2021) in which the cultivars had significantly different Hymenoptera populations. ‘Cherry Mom’ had a significantly higher population (Table 3). In 2021, there was a decrease in populations over the course of flowering with 21 DAF demonstrating the highest starting populations for each cultivar (Table 3). In 2022, there was a trend of increasing Hymenoptera for both cultivars maximizing at 35 DAF then decreasing to a complete absence at 55 DAF (Table 3).

Diptera populations showed no significant differences in water treatment or cultivar in 2021 or 2022 (Table 4). However, in 2021 ‘Cherry Mom’ had, while insignificant to an alpha of 0.1, did exhibit generally higher Diptera populations than ‘BaOx’ but in 2022 this trend was reversed. (Table 4). In both years, there was a decrease in Diptera populations over time (Table 4). Coleopterans showed no significant differences between treatments or cultivars in 2021 (Table 5). Similarly, in 2022 there were no significant population differences in ‘BaOx’ and ‘Cherry Mom’. However, there was a significant effect of water stress at 35 DAF (Table 5). This significant difference was determined to be so close to the alpha of 0.1 that the calculation did not show which water stress was significantly different, possibly due to differences in rounding in the Agricolae package in R compared to the initial ANOVA (Table 5). Population numbers suggest low and stable populations were present throughout the entire flowering cycle (Table 5).

In 2021, Lepidopterans did not differ significantly between water stress treatments or cultivars (Table 6). Overall, populations peaked at 21 DAF for both cultivars; however, populations then declined and stayed stable for the remainder of the season. In 2022, there were no significant differences between water stress treatments at any timepoint, however, 'Cherry Mom' had a significantly lower Lepidoptera population at 35 DAF (Table 6). All other dates in 2022 had similar Lepidoptera populations. In 2022, there was no sharp population decline as in 2021; rather there was a consistent low population throughout and no presence of Lepidopterans by 55 DAF (Table 6).

Thysanoptera did not show significant differences between treatments or cultivars during either year of study (Table 7). In 2021, the populations peaked at 21 DAF and decreased as flowering progressed, reaching minimum populations by 55 DAF (Table 7). Similarly, in 2022 the highest average population occurred around 21 DAF ('Cherry Mom') and 35 DAF ('BaOx), before decreasing through 55 DAF (Table 7).

Hemiptera was not significantly different between treatments in 2021 (Table 8). 'BaOx' had an increase at 49 DAF and decrease at 55 DAF, whereas 'Cherry Mom' showed a decrease from 21 DAF to harvest at 55 DAF, however, these differences were not significantly different between 'BaOx' and 'Cherry Mom' (Table 8). In 2022, there were no differences between water stress treatments; however, 'Cherry Mom' had a significantly higher Hemiptera population at 35 DAF and 'BaOx' had a higher population at 49 DAF (Table 8). Populations in 2021 were generally low for Hemiptera with an increase at 35 DAF for each cultivar followed by a decrease before no Hemipterans were collected at 55 DAF (Table 8).

Orthoptera populations were not affected by drought stresses in either year of the study (Table 9). There were also no significant differences between cultivars at any measurement point (Table 9). Insect populations were very low to absent each year. In 2021 ‘BaOx’ only had Orthopterans collected at 49 DAF and ‘Cherry Mom’ only had presence of Orthoptera at 21 and 35 DAF (Table 9). In 2022 ‘BaOx’ showed at presence of Orthoptera only at 35 DAF and ‘Cherry Mom’ had an absence of the order throughout the entire season (Table 9).

There were no differences in Araneae populations between water stress treatments in 2021, however, ‘BaOx’ found a significantly higher Araneae population at 21 DAF than ‘Cherry Mom’ (Table 10). There were no significant differences between cultivars at any other dates in 2021 (Table 10). Populations were also low in 2021 (Table 10). In 2022, there was a significant effect on Araneae populations at 49 DAF in which the LM treatment had the greatest populations of any treatment (Table 10). There was also a significantly greater Araneae population for ‘BaOx’ at 21 DAF in 2021, however, at 55 DAF ‘Cherry Mom’ had a higher population in both years of study (Table 10). In 2022, Araneae populations remained consistently low throughout the season.

Correlations

No significant correlations between predatory Araneae populations and pest spider mite and aphid populations were identified in 2021. Correlation analysis revealed that the presence of aphids at 21 DAF had a positive relationship with Araneae at 35 DAF (r= 0.2). Similarly, the presence of spider mites at 49 DAF had a moderately positive correlation with Araneae at 55 DAF (r= 0.45). No other correlations of interest were identified in 2021.

In 2022, correlation analysis found Araneae presence and two-spotted spider mite presence were positively correlated (Figure 2). A weak positive correlation occurred between the presence of Araneae and two-spotted spider mites at 35 DAF if both Araneae and spider mites were present in the prior sampling date of 21 DAF (r= 0.2) (Figure 2). This trend continued and there as a weak positive relationship maintained at 49 DAF (r= 0.19) and 55 DAF (r= 0.19). Araneae only showed a weak positive relationship with aphids at 55 DAF when aphids were present at 55 DAF and Araneae was present at 35 DAF (r= 0.19) (Figure 2).

DISCUSSION

Arthropod Populations

Trombidiformes (two-spotted spider mites) and Hemipterans (cannabis aphids) were collected from hemp foliage each year. Populations fluctuated throughout the reproductive cycle of the plant. Cannabis aphids decreased during flowering and two-spotted spider mites increased during the same time period.

A larger diversity of arthropods was collected from the soil level of the plant. Hymenoptera, Diptera, Thysanoptera, Coleoptera, Orthoptera, Lepidoptera, Hemiptera, and Araneae were collected, however, population trends were sporadic during reproductive growth.

Foliar Arthropods

Foliar pests such as two-spotted spider mites, *Tetranychus urticae*, and cannabis aphids, *Phorodon cannabis*, displayed opposite population trends in hemp flowering. *T. urticae* numbers increased during flowering and *P. cannabis* decreased over time. Mitchell (1973) found similar patterns with *T. urticae* in which populations on bean seedlings grow in number once populations are established. In fact, the populations grow so large that accurate counts of mites become difficult (Mitchell, 1973). In this study, *P. cannabis* populations showed a decrease over time, with the greatest population decline in the final weeks of flowering. This pattern is not seen in other plants, such as common evening primrose (*Oenothera biennis*) in which populations grow significantly over time, regardless of the growth phase of the plant (Johnson, 2008). This identifies a potentially unique feature of hemp in which the attractiveness of the plant to aphids decreases whereas there is an increase in attractiveness for mites. This difference may lie in the nutritional needs of each pest group.

Soil-borne Arthropods

The most dominant orders present in this study were Hymenoptera, Diptera, and Thysanoptera. Soil-borne arthropods varied in population numbers over the growing season. In this study, Hymenoptera (wasps), Diptera (flies), and Thysanoptera (thrips) populations decreased over the flowering period in both years and across the two varieties, BaOx and Cherry Mom. True bugs, Hemipterans, displayed a different response on the two varieties; in 2021 and 2022, Hemipteran populations remained steady on ‘BaOx’ but decreased during flowering in 2021 on ‘Cherry Mom.’ Genetic predisposition through the expression of various resistance genes is effective in controlling Hemipteran (aphid) populations in soybean (*Glycine max*) (Chirumamilla et al., 2014). Variable genetics in hemp cultivars may contribute to avoidance or preference among pest insects but this has not yet been explored.

In both 2021 and 2022, small but consistent populations of Coleoptera (beetles), larval (caterpillars), adult Lepidoptera, and Orthoptera (grasshoppers and crickets) were seen in the crop. These orders, particularly Coleoptera and Orthoptera, may have been present in the crop due to their association with hemp, however, it may have been primarily driven by the surrounding landscape. The field site was located within a residential area; however, various row crops were present in adjacent fields and may be a source for additional insects. Cranshaw et al. (2019) highlights beetle species that are pests of hemp; no pest beetles were found in this study. The beetles identified from this study included spotted cucumber beetle (*Diabrotica undecimpunctata howardi* Barber) and flea beetles (Chrysomelidae). Orthopterans, primarily crickets, were collected on sticky traps but no feeding damage from crickets was suspected. Crickets are known to be active pests of hemp (Cranshaw et al., 2019).

Larval Lepidopterans (both soil and foliar dwelling) are shown in multiple studies to be a pest of hemp causing heavy destruction of flowers (Britt et al., 2021) and potential biochemical changes (Jackson et al., 2021) within the plant. Specifically, the corn earworm (*Helicoverpa zea*) displays a high propensity of damaging outdoor hemp (Britt et al., 2021), however, minimal larval *H. zea* were present in our study.

Beneficial Arthropods

Spiders, Araneae, were the primary arthropod predator collected in the study. Spider populations had consistent populations throughout the study, with no major changes during flowering. A correlation analysis revealed weak positive correlations with *T. urticae* and *P. cannabis*. Populations of spiders and pest mites and aphids were positively correlated at sampling dates, suggesting that spiders may play a role in biological control of hemp pests. There was also a positive correlation between pest mite and aphids on one sampling date with spiders on a consecutive sampling date. These data suggest that spider populations may have been responding to pest populations and the availability of prey. Spiders (Araneae) are important predators of rosy apple aphids (*Dysaphis plantaginea*) in apple trees (Lefebvre et al., 2017) and blackmargined aphids (*Monellia caryella* (Fitch)) in pecans (Bumroongsook et al., 1992). In hemp, a myriad of spider species are identified as potential predators of many pests of the crop (Cranshaw et al., 2019).

In this study, we collected adult Asian lady beetles (*Harmonia axyridis*) which are generalist predators that feed on a variety of prey. Cranshaw et al. (2019) describes beneficial ladybeetles in hemp including convergent lady beetle (*Hippodamia convergens* Guérin-Méneville) and Asian lady beetles (*Harmonia axyridis*).

Beneficial wasps, Hymenopterans, collected consisted primarily of parasitoids. Parasitoids are identified by Cranshaw et al. (2019), as well. It is also worthy to note that solitary ground wasps (*Scolia dubia*) were also present throughout the greenhouse in mid-August.

Physiological Maturity

Physiological maturity, defined as life stage of the hemp plant in the study (early, middle, and late reproduction), and status of a crop may influence pest and predator populations. Hsu et al., (2021) shows over the reproductive phases in rice (*Oryza sativa*) cultivation, the predatory arthropods increasingly predate on pest arthropods as rice maturity increases. Physiological maturity is important as reproduction progresses nutrient remobilization can occur (Islam et al., 2017). Remobilization of nutrients can determine what part of the plant is most nutritious and can therefore influence the distribution of plant feeding arthropods.

Pests in the orders Lepidoptera, Hemiptera, and Trombidiformes represent the largest threat to the floral portion of hemp. These groups are most often associated with aboveground damage in hemp (Cranshaw et al., 2019). While Lepidopterans were found in low numbers during the study, pests in this group are generally found in the reproductive structures of the plant, specifically corn earworm, *Helicoverpa zea* (Britt et al., 2021). We did not find any caterpillars on the foliage or floral structures during the duration of this study. This was unexpected, as H. *zea* is one of the most common and injurious pests in outdoor hemp. This is an unusual finding and would not be expected from a study replicated over more years.

T. urticae was shown to increase on leaves before moving towards the flowers. *T. urticae* were observed first on leaves then spread to the flowers and as plant reproduction progressed. This shows the potential nutritive value of the flower of the hemp crop, through remobilization of nutrients from leaves to flower as similarly shown in soybean by Islam et al. (2017).

The majority of the true bugs, Hemipterans, identified in this study were *P. cannabis*, which showed a population decrease over time. This decrease in populations of *P. cannabis* may have been influenced by the senescence of the hemp plants. *P. cannabis* were observed primarily on the leaves of the plant and decreases in their populations can be explained via the loss of leaves as flowering progressed. There was a notable absence of aphids within the floral portion of the plants. This is contrary to findings of Cranshaw et al. (2019) in which substantial populations of *P. cannabis* are observed throughout the flowers, continuing through harvest. While no extensive damage was reported on our crop, or plants in Cranshaw et al. (2019), the presence of large populations in flowers remains potentially harmful by excreting honeydew within the flowers (Cranshaw et al., 2019).

Drought

In this study, we did not observe a relationship between drought stress and arthropod populations. This was unexpected as drought has been shown to significantly affect pest populations in multiple crops. For example, *T. urticae* populations on common bean increase as drought stress increases (English-Loeb, 1989). Similarly, it has been determined in corn (*Zea mays*) that drought stressed crops can experience increased mite populations (Ruckert et al., 2021) and increase aphid populations in wheat (*Triticum aestivum*) (Cui et al., 2021).

Cultivar Selection

In this study, we saw significant differences between cultivars in the arthropod populations, especially in the canopy of the plant. Many orders that represented pests were found to be higher in ‘Cherry Mom’ canopies than in ‘BaOx’. The canopy is the site of reproduction within hemp and throughout the vegetative and floral portions of the plant there is production of many secondary metabolites such as cannabinoids (Jackson et al., 2021) and essential oils (Sleiman et al., 2022).

Essential oils are used on many crops as a deterrent for pests, such as the use of linalool, eugenol, and caryophyllene for deterring thrips in basil (*Ocimum basilicum* L.) (Koschier, 2008). In fact, both linalool and caryophyllene have been identified in *Cannabis sativa* among a myriad of other essential oils (Verma et al., 2014). Furthermore, cannabinoids are believed to be an evolutionary adaptation to deter herbivory (Kariñho-Betancourt, 2018). Cannabinoids show an increase under herbivory and negative impacts on corn earworm larva (Jackson et al., 2021).

While this study did not set out to determine cultivar variation in cannabinoid and essential oil concentrations, it is possible that the differences in cultivar susceptibility to *T. urticae* and *P. cannabis* in the canopy of the plant may be influenced by differences in secondary metabolite production. If this was the case, the soil-borne arthropods would be less affected by secondary metabolites produced by the crop due to a further proximity from the source. While not shown in any studies to date, it is possible the distribution of predators may be influenced by pest population and distribution in hemp as well as secondary metabolites. This may explain what we saw in this study as parasitoids and predatory true bugs were found in higher numbers in 'Cherry Mom' compared to 'BaOx.' Araneae (spiders) were consistent as populations were lower, and this may be due to their spatial distribution in the environment. A larger study plot may give more insight into spider and other predator populations. The most prominent spiders identified in this study were crab spiders (Thomisidae) and female black widows (Theridiidae).

CONCLUSIONS

This study illustrated population trends, arthropod-interactions, and plant-arthropod interactions of major insect orders in a multi-cultivar drought stressed hemp crop replicated over two years in Western North Carolina. Arthropod populations were found to vary across time, with individual orders exhibiting varying responses to plant growth and senescence. The pest population trends observed in this study are hypothesized to be related to the maturity stage of the crop, however, plant physiological responses to drought via secondary metabolite production or predetermined cultivar secondary metabolite differences may have been a determining factor in arthropod pressure.

This study did not quantify or qualify the production of secondary metabolites, however, future studies exploring these may provide insight into potential arthropod attraction or deterrence to the plant. From our findings, cultivar selection should be a fundamental part of any integrated pest management program in hemp cultivation. Drought-stricken environments can affect both plant health and surrounding arthropod populations, therefore, continued efforts to maintain crop health through proper irrigation is also suggested.

This unique and novel study involving arthropod and plant interactions in hemp shows the immeasurable potential studies for entomologists, horticulturalists, and physiologists to further tease out the relationships of hemp with biotic and abiotic stresses for optimal production.

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Drought Treatments: C (control), EE (early extreme water stress), EI (early intermittent water stress), EM (early moderate water stress), LE (late extreme water stress), LI (late intermittent water stress), LM (late moderate water stress).

Table 7. Soil-borne Thysanoptera counts for each cultivar with each imposed water stress. Different letters indicate significantly different means. Differences in lowercase letters signify differences between water treatments, while upper case letters denote differences between cultivars. P-values for treatment, cultivar, and interaction are located at the bottom of the table for each year of study. Drought Treatments: C (control), EE (early extreme water stress), EI (early intermittent water stress), EM (early moderate water stress), LE (late extreme water stress), LI (late intermittent water stress), LM (late moderate water stress).

Table 8. Soil-borne Hemiptera counts for each cultivar with each imposed water stress. Different letters indicate significantly different means. Differences in lowercase letters signify differences between water treatments, while upper case letters denote differences between cultivars. P-values for treatment, cultivar, and interaction are located at the bottom of the table for each year of study. Drought Treatments: C (control), EE (early extreme water stress), EI (early intermittent water stress), EM (early moderate water stress), LE (late extreme water stress), LI (late intermittent water stress), LM (late moderate water stress).

Table 9. Soil-borne Orthoptera counts for each cultivar with each imposed water stress. Different letters indicate significantly different means. Differences in lowercase letters signify differences between water treatments, while upper case letters denote differences between cultivars. P-values for treatment, cultivar, and interaction are located at the bottom of the table for each year of study. Drought Treatments: C (control), EE (early extreme water stress), EI (early intermittent water stress), EM (early moderate water stress), LE (late extreme water stress), LI (late intermittent water stress), LM (late moderate water stress).

Table 10. Soil-borne Araneae counts for each cultivar with each imposed water stress. Different letters indicate significantly different means. Differences in lowercase letters signify differences between water treatments, while upper case letters denote differences between cultivars. P-values for treatment, cultivar, and interaction are located at the bottom of the table for each year of study. Drought Treatments: C (control), EE (early extreme water stress), EI (early intermittent water stress), EM (early moderate water stress), LE (late extreme water stress), LI (late intermittent water stress), LM (late moderate water stress).

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Figure 1. Correlation matrix of populations of Araneae (Spider), foliar two-spotted spider mite (*Tetranychus urticae*) and foliar cannabis aphid (*Phorodon cannabis*) measured in 2021. Blue indicates a positive correlation and red indicates a negative correlation. Stronger color intensity and size designates stronger correlations. R-values are also shown to in each matrix cell.

Figure 2. Correlation matrix of populations of Araneae (Spider), foliar two-spotted spider mite (*Tetranychus urticae*) and foliar cannabis aphid (*Phorodon cannabis*) measured in 2022. Blue indicates a positive correlation and red indicates a negative correlation. Stronger color intensity and size designates stronger correlations. R-values are also shown to in each matrix cell.

Supplemental Figures 1-6. Correlation matrix of all soil-borne arthropod populations measured in 2021 or 2022. Data labels designate the dates (DAF) measured in each supplemental figure. Blue indicates a positive correlation and red indicates a negative correlation. Stronger color intensity and size designates stronger correlations. R-values are also shown to in each matrix cell.

Table 1.

		2021 Foliar Aphids			
Cultivar	**Treatment**	**21 DAF**	**35 DAF**	**49 DAF**	**55 DAF**
Baox	Control	2.3333	3	0.5	0
	EE	0.5	0.8333	0.3333	0.8333
	EI	1.6667	1.5	0.1667	0.3333
	EM	2.3333	1.3333	0.6667	0.1667
	LE	2.1667	0.1667	0.6667	2
	LI	1.1667	1.5	1.1667	0
	LM	0.3333	0.8333	0	0
	mean	1.5 B	1.310 B	0.5	0.4762
Cherry Mom	Control	1.6667	0.8333	0.8333	0
	EE	0.1667	2.5	1.3333	0.1667
	EI	2.5	10.5	0.5	0
	EM	1	3.1667	0.3333	0
	LE	7.3333	4.1667	2.3333	0.3333
	LI	9.5	4.6667	0.6667	0
	LM	4.8333	11.8333	2.1667	0.1667
	mean	3.8571 A	5.381 A	1.1667	0.0952

		2022 Foliar Aphids			
Cultivar	**Treatment**	**21 DAF**	**35 DAF**	**49 DAF**	**55 DAF**
Baox	Control	0	1.3333	0	0.3333
	EE	0	0.1667	0	0
	EI	0	0.3333	0	0
	EM	0	0	0.3333	0
	LE	0	0.5	0.3333	0
	LI	0	0	0	0
	LM	0.1667	0.6667	0.1667	0
	mean	0.0238 B	0.4286	0.119	0.0476
Cherry Mom	Control	0.5	1.3333	1.3333	0
	EE	0.1667	0.8333	0.1667	0.5
	EI	0.1667	0.1667	0	0
	EM	0.5	0.1667	0.1667	0
	LE	0	0	0	0
	LI	0	0.1667	2.3333	0
	LM	0.6667	0.1667	0	0
	mean	0.2857 A	0.4048	0.5714	0.0714

			2021		
Water Stress (WS)	p-value	0.3308	0.2872	0.786	0.384
Cultivar (C)		0.0599	0.0032	0.104	0.222
WS x C		0.2856	0.1478	0.524	0.736
			2022		
Water Stress (WS)	p-value	0.5722	0.3157	0.589	0.4929
Cultivar (C)		0.0501	0.9366	0.216	0.7745
WS x C		0.8422	0.9472	0.381	0.2968

Table 2.

2021 Foliar Spider Mites					
Cultivar	**Treatment**	**21 DAF**	**35 DAF**	**49 DAF**	**55 DAF**
Baox	Control	0.1667	0.3333	0.1667	0.5
	EE	0.1667	0.1667	0.8333	6.8333
	EI	0	0	1.8333	3.3333
	EM	0.5	0.5	3.6667	3.1667
	LE	0	0	1.1667	1
	LI	0	0.5	0.5	0.5
	LM	0.1667	0	2.3333	1
	mean	0.1429 B	0.2143 B	1.5 B	2.3333
Cherry Mom	Control	0.1667	0.3333	7.5	9.6667
	EE	1.8333	2.5	8	5.1667
	EI	1.1667	1.3333	1.3333	5.6667
	EM	0.1667	0.6667	0.6667	1.6667
	LE	0.5	1.1667	0	3.1667
	LI	0.8333	8.6667	8	5.8333
	LM	0.3333	1.8333	8.3333	3.8333
	mean	0.7143 A	2.3571 A	4.8333 A	5

2022 Foliar Spider Mites					
Cultivar	**Treatment**	**21 DAF**	**35 DAF**	**49 DAF**	**55 DAF**
Baox	Control	0	1.8333	43.5	13.6667
	EE	0.3333	8.8333	15.8333	8.3333
	EI	0.3333	1.6667	9.3333	16
	EM	0	4.1667	10.3333	5.1667
	LE	0	1.1667	6.5	5
	LI	0	4.8333	3.1667	1.1667
	LM	0	1.3333	1.6667	3.8333
	mean	0.0952	3.4048	12.9048	7.5952
Cherry Mom	Control	0	5.8333	5.1667	14.8333
	EE	23.3333	50	59.6667	57.6667
	EI	0	0	1	3.1667
	EM	0	0	10.5	1.3333
	LE	0.3333	3.1667	37.5	25.3333
	LI	0.3333	0.5	1	1.3333
	LM	0	0.3	9.1667	0.6667
	mean	3.4286	8.5476	17.7143	14.9048

			2021		
Water Stress (WS)	p-value	0.4921	0.5251	0.6545	0.8233
Cultivar (C)		0.0142	0.0811	0.0379	0.1161
WS x C		0.2439	0.6059	0.2914	0.6275
			2022		
Water Stress (WS)	p-value	0.424	0.314	0.43757	0.3034
Cultivar (C)		0.321	0.474	0.62998	0.33978
WS x C		0.454	0.626	0.40668	0.37324

Table 3.

2021 Hymenoptera					
Cultivar	**Treatment**	**21 DAF**	**35 DAF**	**49 DAF**	**55 DAF**
	Control	5	3.8333	0.46667	1.8333
	EE	5.1667	4.1667	3.3333	1.6667
	EI	5.8333	3	2.3333	1.6667
Baox	EM	4	3.1667	4	1.1667
	LE	3.6667	2.1667	4.1667	1.1667
	LI	5.1667	3.6667	2	1.5
	LM	6.5	2.5	2.6667	1.6667
	mean	5.0476	3.2143 B	3.3095	1.5238
	Control	7.8333	5.6667	3.6667	1.5
	EE	4.1667	4.1667	3.3333	2
	EI	3.3333	3.8333	3.1667	1.8333
Cherry Mom	EM	8.1667	4.8333	2.3333	2.1667
	LE	7.1667	4	3.3333	2.1667
	LI	5.5	3.5	2.8333	2.6667
	LM	8	4.1667	3	1.8333
	mean	6.3095	4.3095 A	3.0952	2.0238

2022 Hymenoptera					
Cultivar	**Treatment**	**21 DAF**	**35 DAF**	**49 DAF**	**55 DAF**
	Control	1.3333	1	0.8333	0
	EE	0.8333	1.1667	2.8333	0
	EI	0.8333	2	1.3333	0
Baox	EM	2.1667	1.6667	1.5	0
	LE	2.1667	1.6667	1.3333	0
	LI	1	1.8333	0.8333	0
	LM	0.6667	1	0.5	0
	mean	1.2857	1.4762	1.3095	0
	Control	1.6667	1.8333	1.1667	0
	EE	0.6667	0.8333	1.3333	0
	EI	0.5	1.8333	1.3333	0
Cherry Mom	EM	0.8333	1.3333	1	0
	LE	1.1667	1.3333	1.5	0
	LI	0.8333	1.1667	0.8333	0
	LM	0.8333	0.5	1.1667	0
	mean	0.9286	1.2619	1.1905	0

			2021		
Water Stress (WS)		0.636	0.5906	0.547	0.99736
Cultivar (C)	p-value	0.148	0.0301	0.671	0.1973
WS x C		0.339	0.843	0.78	0.93202
			2022		
Water Stress (WS)		0.3445	0.298	0.218	-
Cultivar (C)	p-value	0.2427	0.411	0.667	-
WS x C		0.754	0.801	0.476	-

Table 4.

2021 Diptera					
Cultivar	**Treatment**	**21 DAF**	**35 DAF**	**49 DAF**	**55 DAF**
Baox	Control	11.5	7.5	12.8333	5.1667
	EE	8.6667	7.3333	10.1667	5.3333
	EI	13	9	7.8333	4.6667
	EM	8.8333	8.3333	7	5.1667
	LE	12.1667	12	10.8333	4.3333
	LI	9.6667	9.1667	10.3333	5.1667
	LM	12.6667	8.6667	6.6667	5
	mean	10.9286	8.8571	9.381	4.9762
Cherry Mom	Control	10.8333	10	10.3333	6.3333
	EE	10.3333	8.8333	6.6667	5.8333
	EI	10.6667	10.5	12.5	3.3333
	EM	14.3333	9.1667	8	4.83333
	LE	11.3333	9.3333	11	6.1667
	LI	11	10.6667	9.8333	5.1667
	LM	18.5	9.5	9.6667	6.8333
	mean	12.4286	9.7143	9.7143	5.5

2022 Diptera					
Cultivar	**Treatment**	**21 DAF**	**35 DAF**	**49 DAF**	**55 DAF**
Baox	Control	9.5	8.3333	8.6667	0.1667
	EE	10	8.6667	11.5	0.3333
	EI	8.6667	11.8333	9.1667	0.8333
	EM	12.8333	7.5	7.5	0.8333
	LE	13.1667	11.8333	8.8333	0.1667
	LI	8.1667	13.8333	6.5	0.1667
	LM	9.3333	9.3333	7.8333	0.5
	mean	10.2381	10.1905	8.5714	0.4286
Cherry Mom	Control	9.3333	8.8333	9.1667	0.3333
	EE	8.6667	10	9.8333	0.3333
	EI	11.3333	8.3333	8.6667	0
	EM	10.3333	10	7.1667	0.8333
	LE	10.5	10.1667	8.3333	0.1667
	LI	8.6667	7.8333	5.1667	0.1667
	LM	8.3333	7.5	10.1667	0.6667
	mean	9.5952	8.9524	8.3571	0.3571

				2021	
Water Stress (WS)	p-value	0.495	0.93322	0.385	0.773
Cultivar (C)		0.328	0.47768	0.766	0.426
WS x C		0.72	0.94884	0.464	0.833
				2022	
Water Stress (WS)	p-value	0.7347	0.7023	0.357	0.311
Cultivar (C)		0.6217	0.2295	0.843	0.671
WS x C		0.9386	0.3242	0.971	0.724

Table 5.

2021 Coleoptera					
Cultivar	**Treatment**	**21 DAF**	**35 DAF**	**49 DAF**	**55 DAF**
Baox	Control	0	0.3333	0	0
	EE	0	0.1667	0	0.1667
	EI	0.5	0	0.1667	0.3333
	EM	0	0.5	0.1667	0.1667
	LE	0.1667	0.1667	0.1667	0
	LI	0.1667	0.1667	0.3333	0
	LM	0.3333	0.1667	0.1667	0
	mean	0.1905	0.2143	0.1429	0.0952
Cherry Mom	Control	0.1667	0.1667	0	0.1667
	EE	0.1667	0.3333	0.1667	0.3333
	EI	0.1667	0	0	0
	EM	0.3333	0.1667	0.3333	0
	LE	0.3333	0.5	0.1667	0
	LI	0	0.6667	0	0
	LM	0.1667	0	0.1667	0
	mean	0.1667	0.2619	0.119	0.0714

2022 Coleoptera					
Cultivar	**Treatment**	**21 DAF**	**35 DAF**	**49 DAF**	**55 DAF**
Baox	Control	0	0.1667	0	0
	EE	0	0.1667	0	0
	EI	0.1667	0	0.3333	0
	EM	0	0	0	0
	LE	0.1667	0	0.1667	0
	LI	0	0.5	0.3333	0
	LM	0	0	0.1667	0
	mean	0.0476	0.119	0.1429	0
Cherry Mom	Control	0	0	0	0
	EE	0	0.3333	0.1667	0
	EI	0.3333	0	0.3333	0
	EM	0.3333	0	0.1667	0
	LE	0	0	0.1667	0
	LI	0	0.1667	0	0
	LM	0	0.1667	0.1667	0.1667
	mean	0.0952	0.0952	0.1429	0.0238

			2021		
Water Stress (WS)	p-value	0.688	0.318	0.789	0.383
Cultivar (C)		0.802	0.642	0.78	0.735
WS x C		0.468	0.3	0.708	0.482
			2022		
Water Stress (WS)	p-value	0.233	0.0948	0.544	0.433
Cultivar (C)		0.466	0.7465	1	0.321
WS x C		0.536	0.5416	0.78	0.433

Table 6.

2021 Lepidoptera					
Cultivar	**Treatment**	**21 DAF**	**35 DAF**	**49 DAF**	**55 DAF**
Baox	Control	0.1667	0	0	0
	EE	0.1667	0	0.3333	0
	EI	0.5	0	0	0
	EM	0	0	0	0.1667
	LE	0.3333	0.1667	0	0
	LI	0.5	0	0.1667	0
	LM	0	0.1667	0	0
	mean	0.2381	0.0476	0.0714	0.0238
Cherry Mom	Control	1	0	0	0
	EE	0.3333	0	0.1667	0
	EI	0	0	0	0
	EM	0.1667	0	0.1667	0
	LE	0	0	0	0.1667
	LI	0.1667	0.1667	0.1667	0
	LM	0.3333	0	0.1667	0
	mean	0.2857	0.0238	0.0952	0.0238

2022 Lepidoptera					
Cultivar	**Treatment**	**21 DAF**	**35 DAF**	**49 DAF**	**55 DAF**
Baox	Control	0.1667	0	0	0
	EE	0	0	0	0
	EI	0	0	0	0
	EM	0	0.3333	0	0
	LE	0.1667	0.1667	0	0
	LI	0	0	0	0
	LM	0	0	0	0
	mean	0.0476	0.0714 A	0	0
Cherry Mom	Control	0.1667	0	0	0
	EE	0	0	0	0
	EI	0.1667	0	0.1667	0
	EM	0	0	0	0
	LE	0.1667	0	0	0
	LI	0	0	0	0
	LM	0.1667	0	0	0
	mean	0.0952	0 B	0.0238	0

		2021			
Water Stress (WS)	p-value	0.6886	0.66	0.228	0.558
Cultivar (C)		0.7556	0.56	0.7	1
WS x C		0.2474	0.346	0.806	0.345
		2022			
Water Stress (WS)	p-value	0.439	0.124	0.433	-
Cultivar (C)		0.406	0.061	0.321	-
WS x C		0.939	0.124	0.433	-

Table 7.

2021 Thysanoptera					
Cultivar	**Treatment**	**21 DAF**	**35 DAF**	**49 DAF**	**55 DAF**
Baox	Control	9.8333	0.8333	1.8333	0.3333
	EE	6	5.1667	1.8333	0.1667
	EI	16.6667	2.6667	2.6667	0.1667
	EM	7	2.3333	1.5	1
	LE	5.6667	1.5	2.6667	0.3333
	LI	5	3	3.5	0.6667
	LM	8.8333	0.6667	1.1667	0.1667
	mean	8.4286	2.3095	2.1667 A	0.4048
Cherry Mom	Control	7.8333	2.6667	1	0
	EE	9.1667	3.1667	1.8333	0.3333
	EI	10.3333	4.3333	1.8333	1.1667
	EM	11.1667	5	0.1667	0.1667
	LE	15.8333	1.6667	2.5	0.1667
	LI	5.1667	3.1667	0.6667	1
	LM	9.1667	1.6667	1	0.8333
	mean	9.8095	3.0952	1.2857 B	0.5238

2022 Thysanoptera					
Cultivar	**Treatment**	**21 DAF**	**35 DAF**	**49 DAF**	**55 DAF**
Baox	Control	5.8333	3.1667	1.6667	0
	EE	6.3333	2.3333	2.5	0.1667
	EI	4.3333	6.3333	3.6667	0
	EM	3.8333	7.6667	0.8333	0
	LE	7	4.6667	3	0
	LI	6.8333	9.5	2.8333	0
	LM	5	7.3333	2	0
	mean	5.5952	5.8571	2.3571	0.0238
Cherry Mom	Control	3.6667	6	1.3333	0
	EE	3.5	7.1667	1.6667	0
	EI	3.3333	7.6667	2.1667	0
	EM	6.6667	6.3333	2.8333	0
	LE	2.3333	6	2	0.1667
	LI	3.5	3.3333	2	0
	LM	5.6667	4.6667	2	0.1667
	mean	4.0952	2.881	2	0.0476

			2021		
Water Stress (WS)	p-value	0.124	0.214	0.241	0.5076
Cultivar (C)		0.356	0.295	0.0369	0.5533
WS x C		0.125	0.729	0.5656	0.2351
			2022		
Water Stress (WS)	p-value	0.9957	0.795	0.798	0.66
Cultivar (C)		0.2163	0.982	0.475	0.56
WS x C		0.6907	0.127	0.606	0.346

Table 8.

2021 Hemiptera					
Cultivar	**Treatment**	**21 DAF**	**35 DAF**	**49 DAF**	**55 DAF**
Baox	Control	0.5	0.5	2.6667	0.3333
	EE	0.3333	1	0.3333	0.1667
	EI	0.6667	0.6667	1.6667	0.3333
	EM	0.5	0.8333	0.5	0.3333
	LE	3	1.6667	0.6667	0.1667
	LI	1.1667	0.3333	2.8333	0.1667
	LM	0	0.3333	0.5	0.3333
	mean	0.881	0.7619	1.3095	0.2619
Cherry Mom	Control	1.1667	0.3333	0.5	0.1667
	EE	0.5	0.5	4.5	0.5
	EI	10.3333	9	0.6667	0.8333
	EM	0.3333	0.3333	0.1667	0.5
	LE	1.6667	0.8333	0.6667	0.3333
	LI	0.6667	2.5	0.6667	0.5
	LM	0.3333	0.3333	1.5	0.6667
	mean	2.1429	1.9762	1.2381	0.5

2022 Hemiptera					
Cultivar	**Treatment**	**21 DAF**	**35 DAF**	**49 DAF**	**55 DAF**
Baox	Control	0	0.6667	0.6667	0
	EE	0	0.5	0.3333	0
	EI	0.1667	0.3333	0.5	0
	EM	0	0	1.5	0
	LE	0	0.8333	1.5	0
	LI	0.1667	0.6667	0.3333	0
	LM	0.3333	0.6667	0.8333	0
	mean	0.0952	0.5238 B	0.8095 A	0
Cherry Mom	Control	0	2	0.5	0
	EE	0.3333	0.8333	0	0
	EI	0.5	1.8333	0.5	0
	EM	0	6.5	0.1667	0
	LE	0	1.5	0.5	0
	LI	0.1667	1.3333	0.3333	0
	LM	0	0.5	0.6667	0
	mean	0.1429	2.0714 A	0.381 B	0

			2021		
Water Stress (WS)	p-value	0.339	0.54	0.726	0.8626
Cultivar (C)		0.347	0.354	0.917	0.1097
WS x C		0.35	0.489	0.204	0.9357
			2022		
Water Stress (WS)	p-value	0.255	0.7048	0.3631	-
Cultivar (C)		0.566	0.0758	0.0432	-
WS x C		0.366	0.4403	0.4904	-

Table 9.

2021 Orthoptera					
Cultivar	Treatment	21 DAF	35 DAF	49 DAF	55 DAF
Baox	Control	0	0	0	0
	EE	0	0	0	0
	EI	0	0	0	0
	EM	0	0	0	0
	LE	0	0	0	0
	LI	0	0	0.1667	0
	LM	0	0	0	0
	mean	0	0	0.0238	0
Cherry Mom	Control	0	0	0	0
	EE	0	0	0	0
	EI	0	0	0	0
	EM	0.1667	0	0	0
	LE	0	0.1667	0	0
	LI	0.1667	0	0	0
	LM	0	0	0	0
	mean	0.0476	0.0238	0	0

2022 Orthoptera					
Cultivar	Treatment	21 DAF	35 DAF	49 DAF	55 DAF
Baox	Control	0	0	0	0
	EE	0	0	0	0
	EI	0	0	0	0
	EM	0	0	0	0
	LE	0	0.1667	0	0
	LI	0	0	0	0
	LM	0	0	0	0
	mean	0	0.0238	0	0
Cherry Mom	Control	0	0	0	0
	EE	0	0	0	0
	EI	0	0	0	0
	EM	0	0	0	0
	LE	0	0	0	0
	LI	0	0	0	0
	LM	0	0	0	0
	mean	0	0	0	0

			2021		
Water Stress (WS)	p-value	0.558	0.433	0.433	-
Cultivar (C)		0.165	0.321	0.321	-
WS x C		0.558	0.433	0.433	-
			2022		
Water Stress (WS)	p-value	-	0.433	-	-
Cultivar (C)		-	0.321	-	-
WS x C		-	0.433	-	-

Table 10.

2021 Araneae					
Cultivar	**Treatment**	**21 DAF**	**35 DAF**	**49 DAF**	**55 DAF**
Baox	Control	0.3333	0	0.3333	0.1667
	EE	0.1667	0	0	0
	EI	0	0.3333	0.5	0.1667
	EM	0.1667	0.3333	0.1667	0
	LE	0.1667	0.1667	0.3333	0
	LI	0.3333	0.1667	0.1667	0.1667
	LM	0.6667	0.1667	0.1667	0
	mean	0.2619	0.1667	0.2381	0.0714
Cherry Mom	Control	0.1667	0	0	0.1667
	EE	0.1667	0.1667	0	0
	EI	0	0.5	0.5	0
	EM	0	0.3333	0.3333	0.1667
	LE	0	0	0	0.1667
	LI	0	0.3333	0.3333	0.1667
	LM	0	0.5	0.6667	0.1667
	mean	0.0476	0.2619	0.2619	0.119

2022 Araneae					
Cultivar	**Treatment**	**21 DAF**	**35 DAF**	**49 DAF**	**55 DAF**
Baox	Control	0.3333	0.1667	0 b	0
	EE	0	0	0 b	0
	EI	0.3333	0	0.1667 ab	0
	EM	0.3333	0.1667	0 b	0
	LE	0	0.3333	0 b	0
	LI	0.3333	0.1667	0 b	0
	LM	0.5	0.1667	0.3333 a	0
	mean	0.2619 A	0.1429	0.0714	0 B
Cherry Mom	Control	0	0	0 b	0
	EE	0.1667	0.5	0 b	0.1667
	EI	0	0.1667	0 ab	0
	EM	0	0.1667	0 b	0.1667
	LE	0.3333	0.1667	0 b	0.1667
	LI	0	0	0 b	0
	LM	0	0.1667	0.3333 a	0
	mean	0.0714 B	0.1667	0.0476	0.0714 A

			2021		
Water Stress (WS)	p-value	0.406	0.188	0.356	0.87
Cultivar (C)		0.012	0.332	0.8415	0.485
WS x C		0.373	0.878	0.51	0.812
			2022		
Water Stress (WS)	p-value	0.9881	0.816	0.00332	0.6924
Cultivar (C)		0.0455	0.77	0.62972	0.0928
WS x C		0.1667	0.275	0.96368	0.6924

Figure 1. 2021 Araneae vs. Leaf Pests

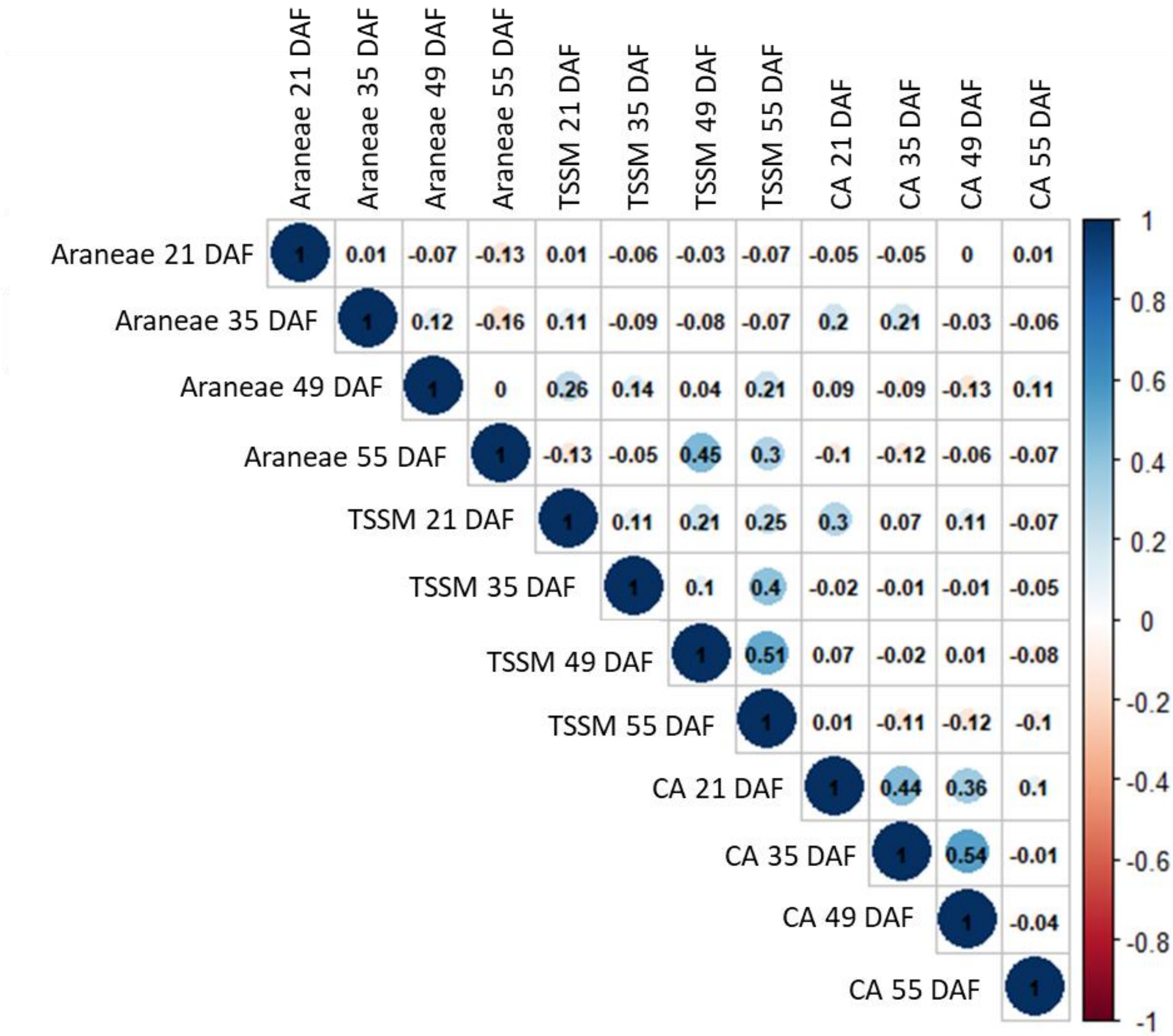
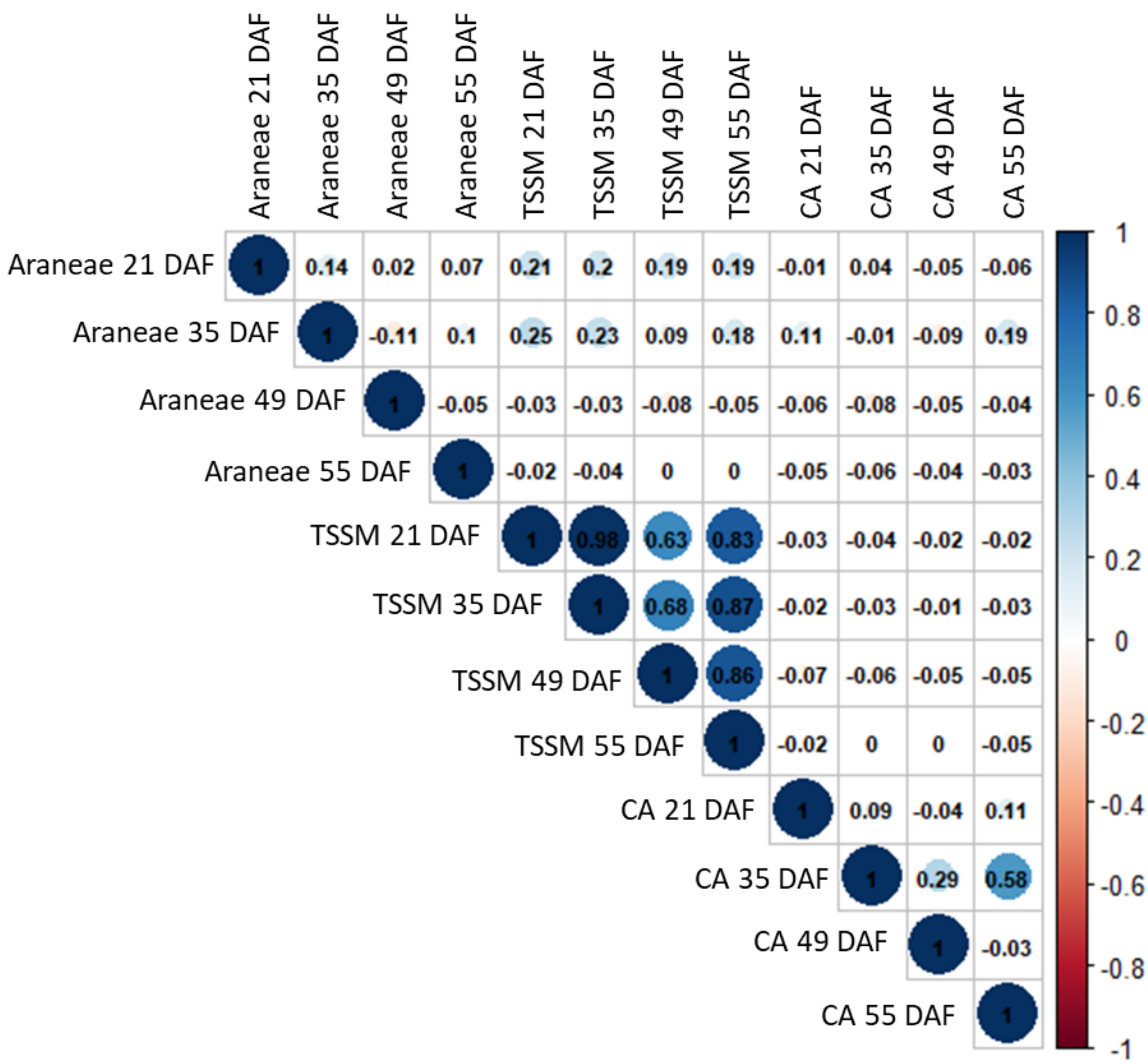
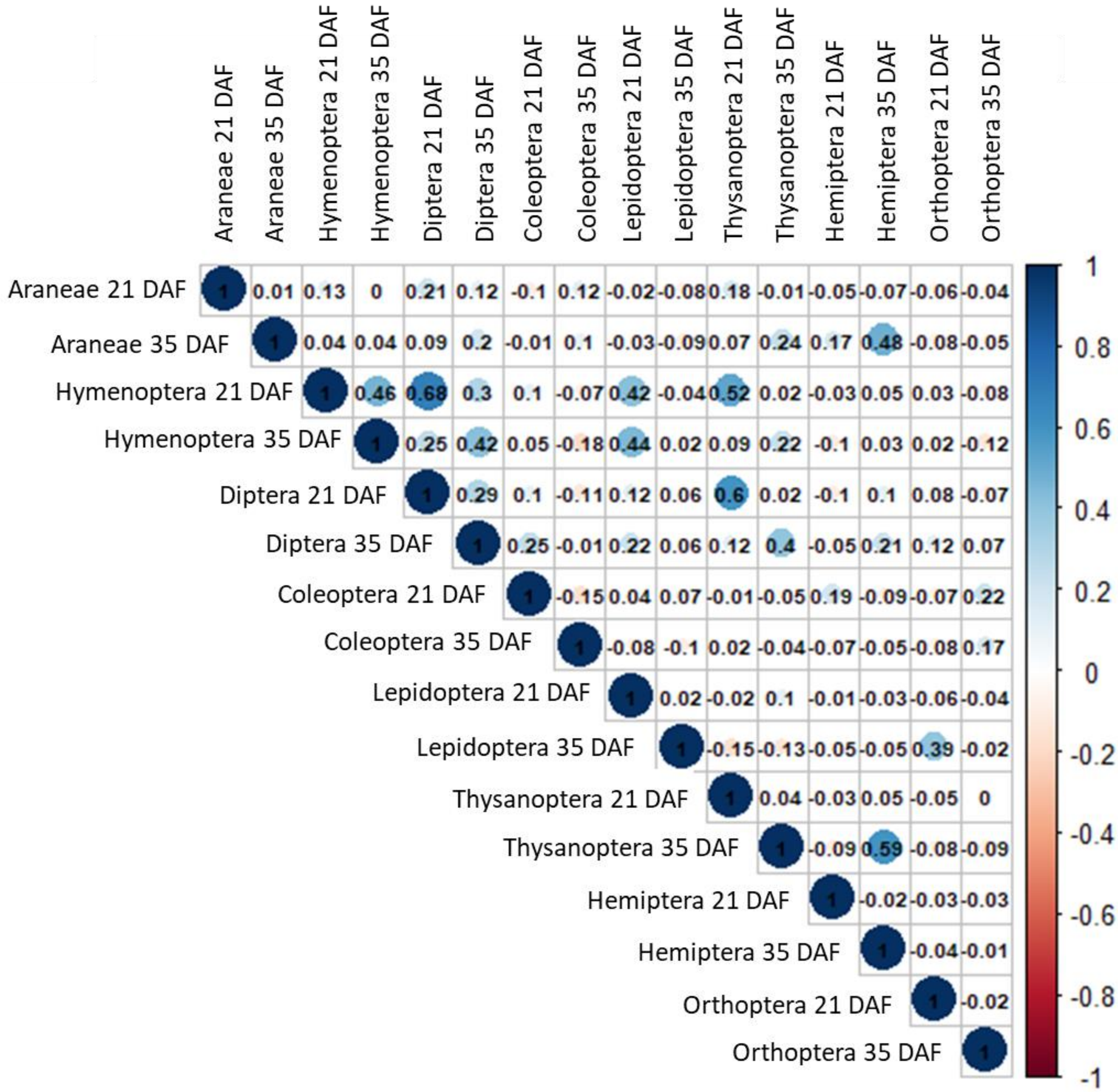


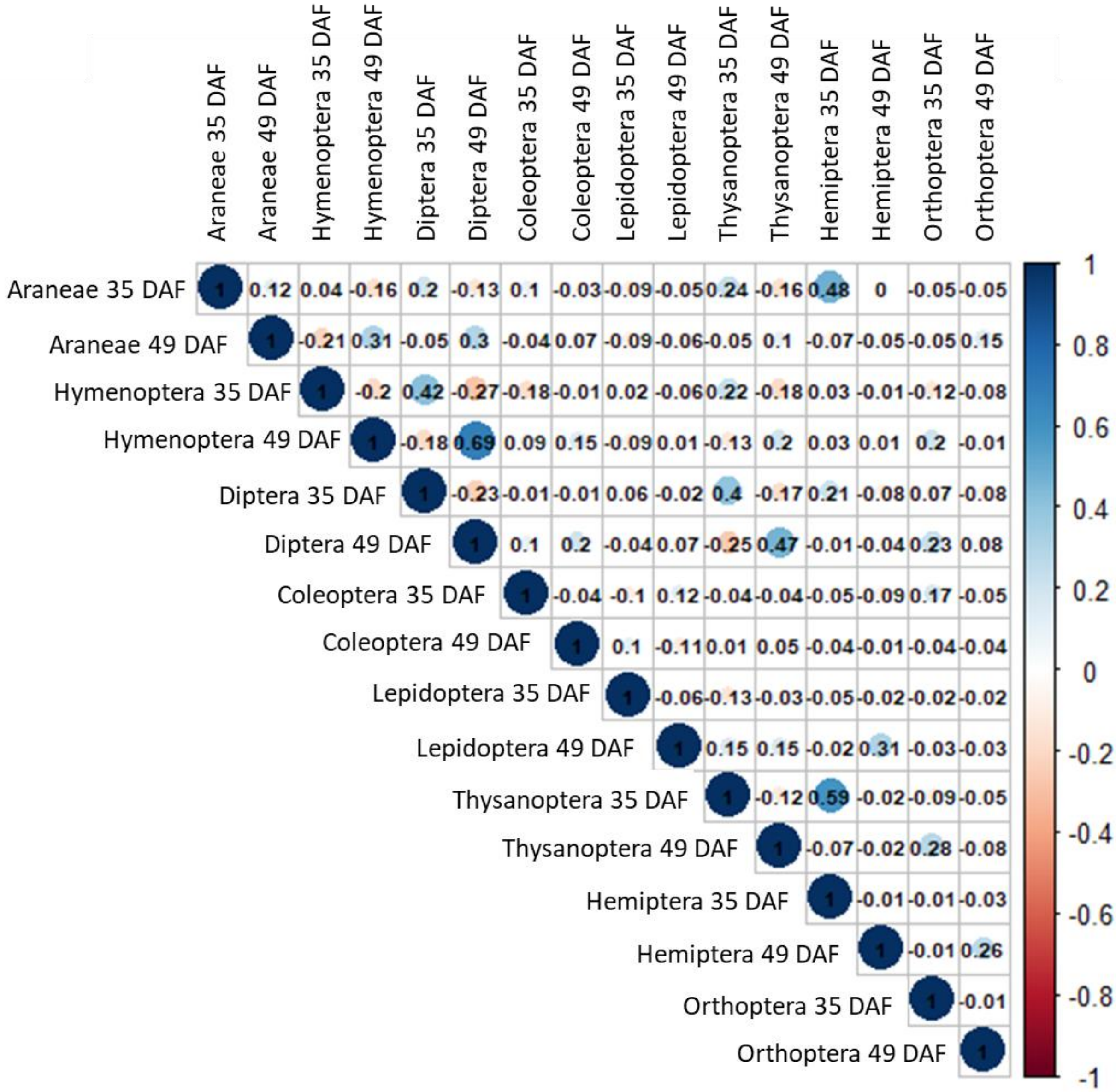
Figure 2. 2022 Araneae vs. Leaf Pests



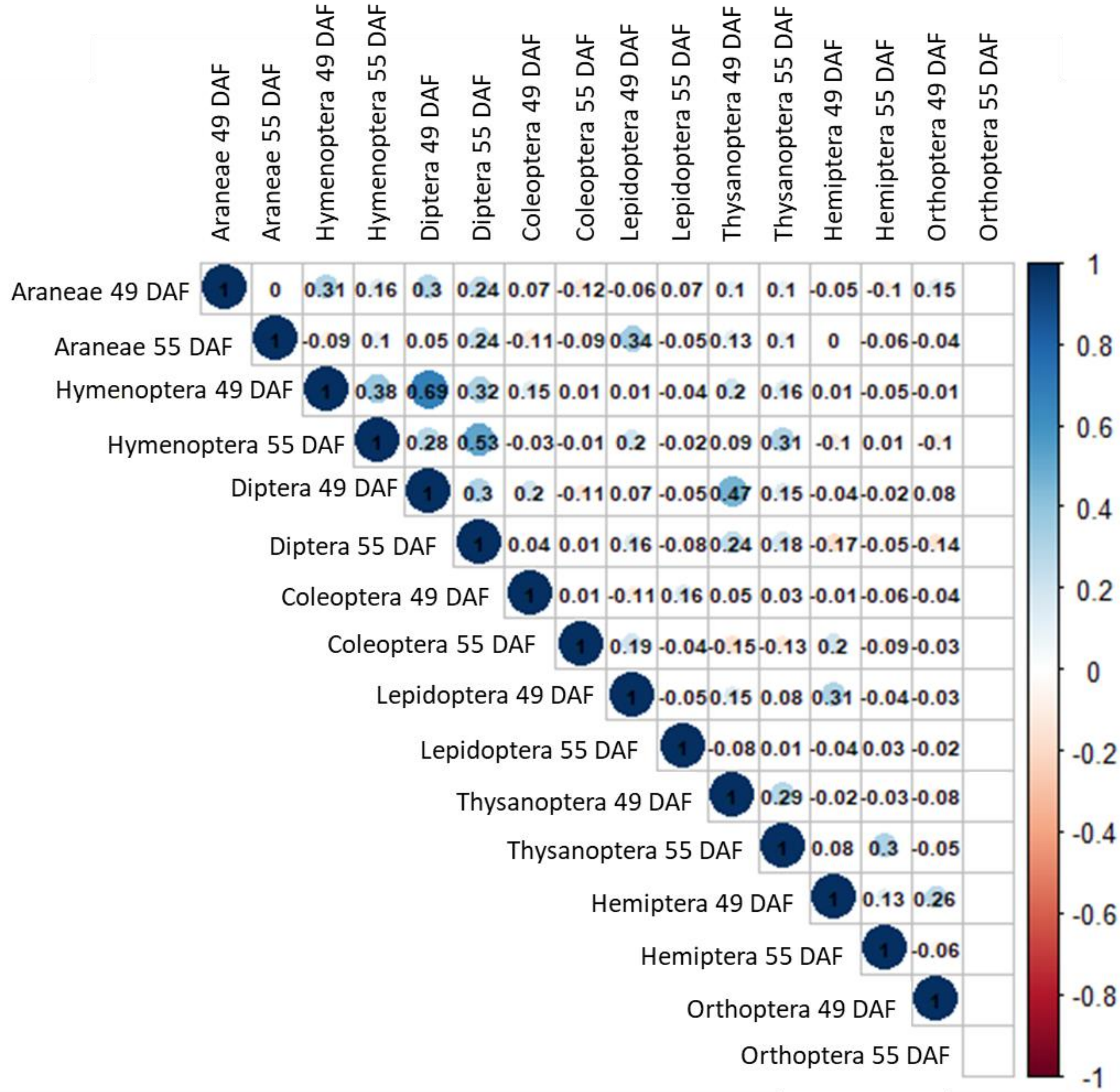
Supplemental Figure 1. 2021 Arthropod Interactions 21 and 35 DAF



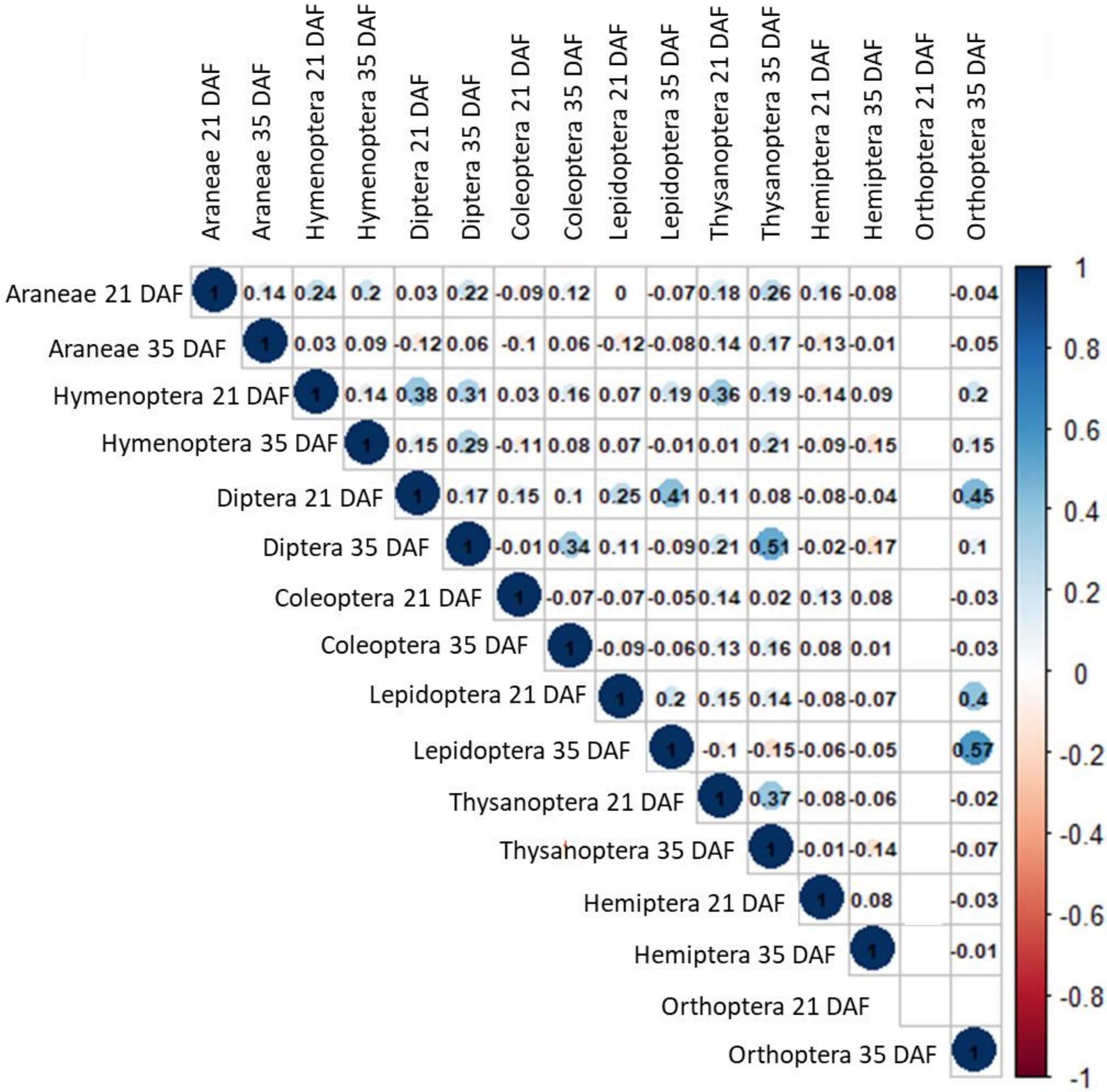
Supplemental Figure 2. 2021 Arthropod Interactions 35 and 49 DAF



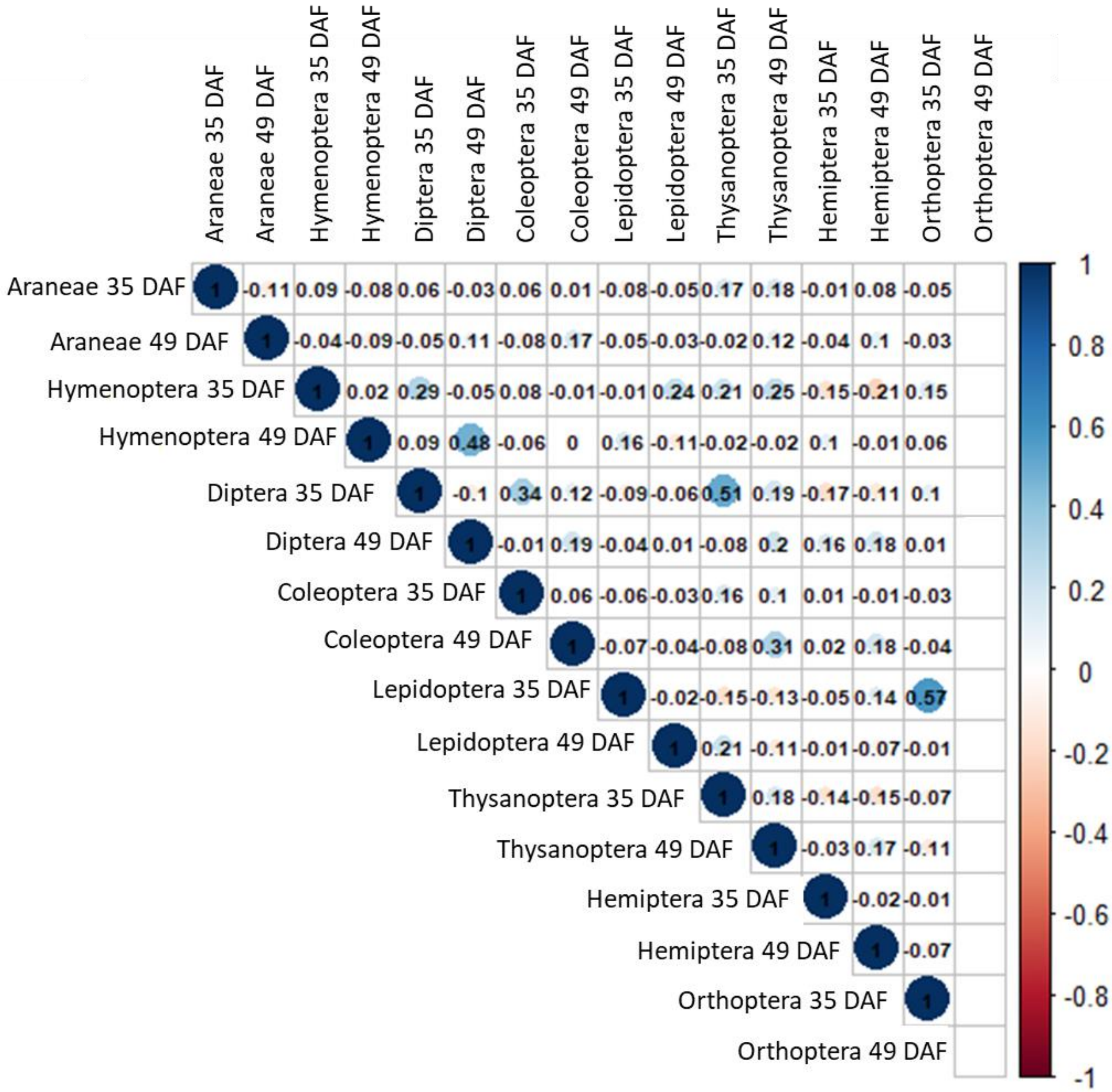
Supplemental Figure 3. 2021 Arthropod Interactions 49 and 55 DAF



Supplemental Figure 4. 2022 Arthropod Interactions 21 and 35 DAF



Supplemental Figure 5. 2022 Arthropod Interactions 35 and 49 DAF



Supplemental Figure 6. 2022 Arthropod Interactions 49 and 55 DAF