Study on Interactions between Endophytes and Hemp for Healthy Plants and Quality Products

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By

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ABSTRACT

Modern *Cannabis sativa L*. cell lines encompass various chromosome variations through ploidy, such as diploid, triploid, and tetraploid, while their associated microbial communities are still underexplored. The lack of knowledge on symbiotic mycobiome-hemp interaction can be considered a bottleneck for sustainable plant and cannabinoids production, which is further exacerbated due to the recent legalization of Cannabis in Western countries, resulting in a surge in demand. Consequently, the imperative to investigate hemp's microbial communities has grown, as it plays a pivotal role in enhancing crop health and ensuring product quality, safety, and security. Hence, understanding the mechanism by which microbiome is acting in protocooperation with ploidy in hemp is a vital step towards microbiome-assisted agriculture and future breeding programs.

The primary objective of this thesis is to elucidate the correlation between diploid (2n) and triploid (3n) hemp plants and their respective microbiome profiles. Specifically, the diploid and triploid Suver haze cultivar varieties and their associated microbial community composition on seeds, flowers and leaves were examined. The research findings shed light on the intricate interplay between the *Cannabis sativa* microbiome and host phenotypic characteristics. Notably, we observed that distinct microbial community structures were linked with shifts in plant growth parameters, hormonal activities, and phenotypic traits. Furthermore, our study underscores the dynamic nature of the hemp microbiome across different plant genotypes and growth stages, resulting in distinctive profiles of secondary metabolites. The variation in endophytic community structures between diploid versus triploid plants coincides with the level of plant plasticity to adapt in response to controlled *in vitro* and phytotron environments. Moreover, we found that differences in microbiome's composition coincides with specific shifts in phenotypic characteristics of each plant host, offering practical applications for optimizing hemp cultivation.

In summary, a combination of microbiology, microscopy, molecular, and phenotypical approaches was applied in addressing the main study's objectives. Tested plants underscore the significance of considering the microbiome as a pivotal factor in shaping physiological (PSII)

and phenotypic attributes in *Cannabis sativa*. A deeper understanding of these complex relationships has the potential to refine cultivation techniques and facilitate the development of hemp varieties with tailored traits, ultimately benefiting both the medical and industrial applications of this versatile plant. Additionally, our research may contribute to identifying biosignature markers of endosymbiosis that enhance the genetic diversity of hemp germplasm during the reproductive seed and flowering stages, potentially improving plant health, agricultural traits, and the quality of products.

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
CBD	Cannabidiol
CBDA	Cannabidiolic Acid
BHT	Butylated hydroxytoluene
DNA	Deoxy Ribonucleic Acid
EG primers	Elongation primers
Fv	Variable Fluorescence
Fm	Maximum Fluorescence
HPLC	High Performance Liquid Chromatography
HPLC-ESI-MS/MS	HPLC-Electrospray Ionization-Mass Spectrometry/Mass Spectrometry
HSD	Honest Significant Difference
ITS	Internal Transcribed Spacer
LSD	Least Significant Difference
NCBI	National Center for Biotechnology Information
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
rRNA	Ribosomal Ribonucleic Acid
SCAR	Sequence Characterized Amplified Regions
SEM	Scanning Electron Microscopy
SPSS	Statistical Product and Service Solutions
TAE	Tris Acetate EDTA
Taq	Thermus aquaticus
TBE	TRIS Borate EDTA
THC	Tetrahydrocannabinol
THCA	Tetrahydrocannabinolic acid

WSM	Whole seed microbiome as an inoculant composed of microbial epiphytes and endophytes
SEp	Seed Epiphytes – plant surface inhabitant of seed coat
SEn	Seed Endophytes – plant intracellular inhabitant of seed embryo and endosperm
WPM	Whole plant microbiome represents diversity of isolated bacteria and fungi from diploid and triploid plant tissues

1 GENERAL INTRODUCTION

1.1 Overview

Cannabis sativa L. has been a part of human culture for centuries, evolving from Central Asia, and is primarily cultivated for food, seed, fiber, textile, bioenergy, recreation, and medicine. Over the past 10 years, it has become increasingly popular in the biochemical and chemical industries due to the production of secondary metabolites, mainly Δ^9 THC and CBD. Several types of beneficial microorganisms can be found as endophytes in plants that reside inside healthy living host tissues and play a significant role in providing resistance to biotic and abiotic stress as well as promoting growth, health, and production of these essential metabolites. Symbiotic associations between endophytes and plants are ecologically important and globally prevalent.

Hemp is a diploid species, meaning that it has two complete sets of chromosomes. Polyploidization, which is the acquisition of one or more additional sets of chromosomes, is considered a valuable tool in the genetic improvement of crop plants (Crawford et al., 2021; Parsons et al., 2019). It can have various advantages, such as increased vigor, size, and quality of leaves, flowers, and fruits (Bagheri and Mansouri, 2015; Suman Chandra et al., 2017); improved tolerance to stress, pests, and diseases; increased production of secondary metabolites which can have medicinal or aromatic properties; protection from harmful mutations due to gene redundancy; and the ability to self-fertilize and overcome reproductive barriers. However, polyploidization does not occur naturally in hemp, but it can be induced by applying antimitotic agents like colchicine, vinblastine, and vincristine to seeds, seedlings, or shoot tips (Trojak-Goluch et al., 2021). Understanding the mechanism by which the microbiome is acting in protocooperation (Powell et al., 2023; Powell and Vujanovic, 2021) with ploidy in hemp is an important step towards microbiome-assisted agriculture and future hemp breeding programs. Protocooperation is a type of ecological interaction in which mutual benefits are shared between species. In this context, we explored the collaborative relationship between beneficial microorganisms and the hemp plant.

The aim of this thesis is to comprehend the relationship between diploid and triploid Suver Haze plants and their microbiomes. Suver Haze is a sativa-dominant hybrid originating from Southern Oregon and is the result of crossing a Neville's Haze male with a Krishna's Special female (Miller, 2022; Park et al., 2023; Stegmeier, 2022). Noteworthy for its high CBD content, mold resistance, and impressive yield, this strain is cherished for its calming effects. It is cultivated in both diploid (2n) and triploid (3n) forms, and these variations are typically 'seedless,' as they do not tend to produce viable seeds. Our research uncovers a complex relationship between the hemp microbiome and phenotypic characteristics. We noted that distinct microbial communities were linked to various plant organs, resulting in unique phenotypic outcomes. Additionally, it emphasized the prevalence of *Penicillium*, *Fusarium*, and *Cladosporium* in diploid cultivars, while triploid varieties exhibited a substantial presence of *Penicillium, Chaetomium, Alternaria*, and *Ascomycota*, which suggests the dynamic nature of the hemp microbiome as it evolves in response to the changing needs of the plant throughout different growth stages. It also showed that altering the composition of the microbiome could indeed lead to desired phenotypic modifications, providing practical applications for optimizing hemp cultivation.

In conclusion, this research underscores the importance of considering the microbiome as a significant factor in the determination of phenotypic characteristics in *C. sativa*. Understanding these intricate relationships can lead to more precise cultivation practices and the development of hemp varieties with tailored traits, ultimately benefiting both the medical and industrial applications of this versatile plant. It will lead to identifying biosignature markers that valorize the genetic diversity of hemp germplasm at the reproductive seed and flowering stages, which can improve plant health and the quality of products.

1.2 Hypothesis

Specific microbial communities associated with hemp will have a notable impact on the changes in the plant’s characteristics during both the germination and flowering stages. Therefore, we hypothesized that:

1. Beneficial microorganisms associated with various plant tissues, such as whole seed microbiome (WSM), seed epiphytes (SEp) and seed endophytes (SEn), would exert distinct effects on the plant's health and vitality.
2. Certain microbes will exhibit a more substantial effect on specific growth stages and on specific organs of the plant.

Figure 1 below represents a schematic relationship between different cell lines in hemp plants and its association with the microbiome for improving the quality of the host traits.

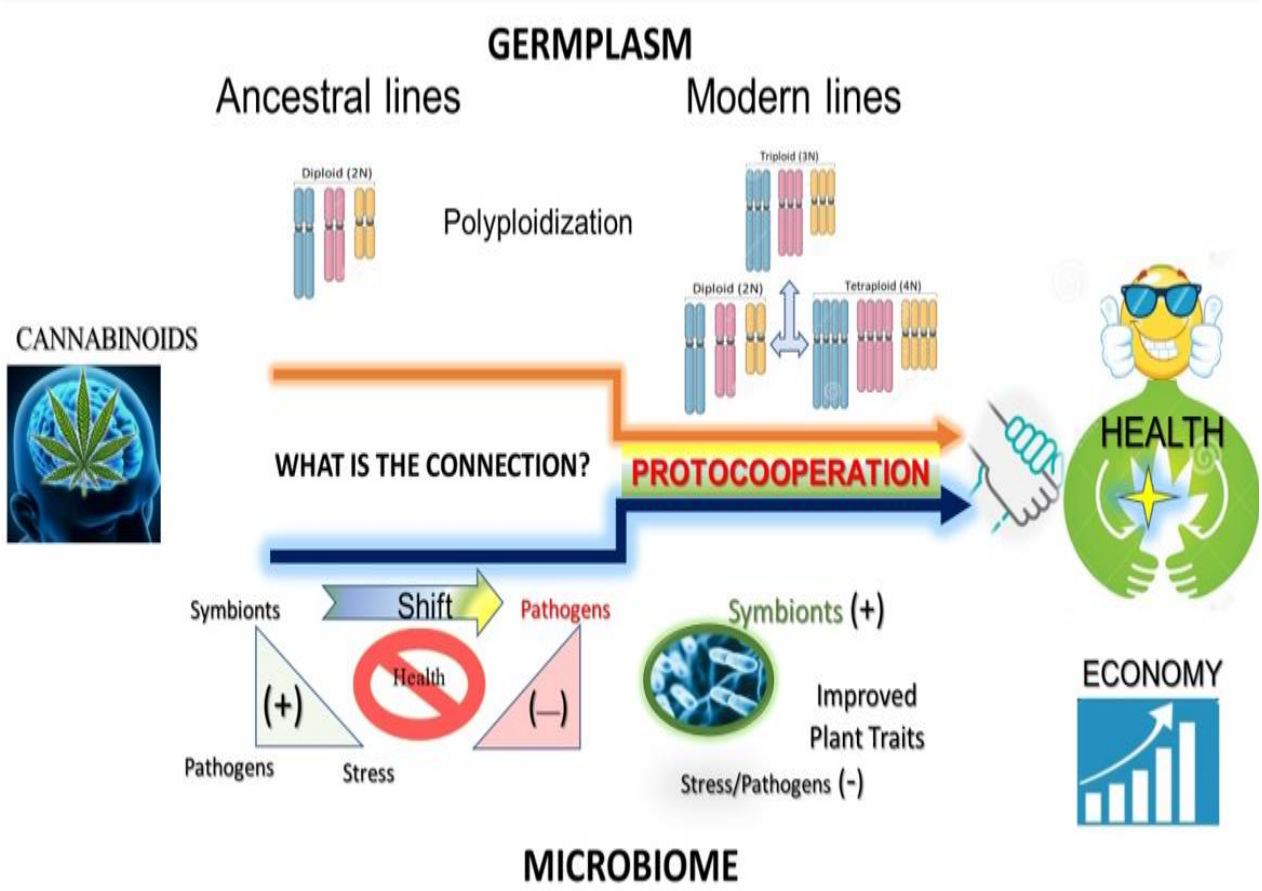


Figure 1: Diagram showing relationship between germplasm and microbiome and how they work in protocooperation for improved plant host traits that are agronomically valuable as well as biochemicals (e.g., cannabinoids, hormones') profile in female plants.

1.3 Objectives

The objectives of this thesis are:

1. Determination of the effect of microbiomes (WSM, SEp and SEn) on the phenotypic characteristics of *Cannabis sativa* seeds and plants.
2. Profiling the bacterial and fungal whole plant microbiome (WPM) associated with the diploid and triploid varieties of Suver haze plants using universal 16SrRNA and ITS primers.
3. Assess the growth-promoting potential of SEn associated with diploid and triploid cultivars under *in vitro* and phytotron conditions.
4. Evaluating the impact of beneficial microbiomes on secondary metabolite production in both diploid and triploid cultivars.

2 LITERATURE REVIEW

2.1 Preface

The aim of this review section is to provide new insight into the intricate relationship between microorganisms and their host plants. It embarks on an exploration of the multifaceted world of hemp microbiomes within diverse cultivars, unraveling the hidden potential of endophytes as nature's shield against abiotic and biotic stressors. This delve into the fascinating role of microbiomes as biological control agents, guardians of plant health, and enhancers of crop resilience. Additionally, it discussed the realm of polyploidy, a genetic phenomenon that can potentially revolutionize the traits and qualities of cultivated hemp, ushering in new horizons for the field of hemp cultivation.

2.2 Cannabis microbiome in different cultivars

Cannabis sativa has originated in the Himalayas and has caught much attention after its legalization in western countries. It represents an interesting model to explore plant-microbiome interactions as it produces numerous secondary metabolic compounds (Winston et al., 2014) that are used for medicinal, recreational, and pharmaceutical purposes. Plant microbiomes include epiphytic and endophytic bacteria, fungi, and viruses within rhizosphere soil, within root tissues (rhizosphere), and aboveground structures such as seeds (semosphere), leaves, and flowers (phyllosphere) (Turner et al., 2013; Vujanovic and Germida, 2017) that perform a plethora of beneficial functions for their hosts. However, the cultivar-specific Cannabis/hemp microbiome is poorly understood and needs further in-depth investigation (Vujanovic et al., 2020). Very few studies have been conducted examining the microbiome associated with *Cannabis sativa* L., which focused on high-THC cultivars grown (Comeau et al., 2020; Winston et al., 2014; Gautam et al., 2013; Kusari et al., 2013; Scott et al., 2018a), or compartment studies of different cultivars with microbiome network assembly from different parts of plants (Barnett et al., 2020; Wei et al., 2021). However, information on the impact of microbiomes associated with polyploid hemp and hemp plants is lacking and needs to be elucidated for higher agronomic traits.

2.3 Endophytes confer resistance to abiotic stress

Various environmental factors like temperature, water deficiency, light intensity, water, salinity, and soil affect secondary metabolite production, especially phytocannabinoids, which are the main compounds used for medicinal purposes (Trancoso et al., 2022). There is evidence that fungal endophytes can protect plants exposed to various abiotic (Dumigan and Deyholos, 2022) and biotic stresses. Endophyte colonization has been shown to enhance plant tolerance for many abiotic stresses and early plant establishment (Vujanovic and Germida, 2017). Indeed, the symbiotic plants showed improved resistance to drought and heat freezing, UV-B radiation (Yao and Liu, 2007), nutrient deprivation, salinity (Shin and Schachtman, 2004) alkaline conditions (Pan et al., 2012), and heavy metal contamination (Carr et al., 2008; Montoya et al., 2020; Seltenrich, 2019). Mycomediated enhancement of performance at the germination stage is known as mycovitalism and seed biostratification (Vujanovic et al., 2016). In addition, it plays multiple roles in plant growth, resulting in an improved interactome (Vujanovic et al., 2019a) and a transgenerational host trait continuum (Vujanovic et al., 2019b). However, no studies have analysed fungal and bacterial endophyte associations with polyploid hemp plants.

2.4 Microbiome as biological control agent in plants

Hemp, similarly, to other plants, is under serious potential threat from diseases, pests, fungi, and bacterial infections (Punja et al., 2019a; Punja, 2021), and finding a sustainable approach towards the cultivation of better-quality plants is the focus of this study. Due to its increasing popularity, especially in countries and states where hemp cultivation is now legal for both medicinal and recreational purposes, growers are more inclined to use plant growth stimulators and pesticides to protect plants and increase crop yield (Taylor and Birkett, 2020). However, it is well known that chemical pesticides and fertilizers used for other plants are affecting the soil, water, air, and other natural resources, and to preserve our environment, we need to think of an alternative source that is less harmful to non-target organisms. There is evidence that beneficial bacteria and fungi can be used as biological control agents that provide protection against plant pathogens (Balthazar et al., 2022) as well as plant growth-promoting inoculants (Comeau et al., 2021; Kusari et al., 2013). However, a better understanding of the

microbial diversity in hemp, in particular beneficial endosymbionts, can assist in the selection of potent biologicals, including biological control agents (Vujanovic et al., 2020).

2.5 Functional role of endophytes in hemp

Beneficial microorganisms present in hemp cultivars have not been studied in detail as compared to other economically important crops like *Triticum* (wheat) and *Glycine* (soybean) or model plants such as *Arabidopsis thaliana*. A study on endophytic bacterial communities in wheat showed that root and leaf communities differed in abundance and composition of endophytes (Robinson et al., 2016). *Proteobacteria* were most prevalent in roots, whereas *Firmicutes* and *Actinobacteria* were mostly found in leaves. Another study found that wheat endophytic bacteria showed specific organ- and growth-stage diversity, which may reflect their adaptations to different plant tissues and seasonal variations with different plant growth promoting abilities. *Bacillus* was the most predominant bacterial taxa isolated, followed by *Pseudomonas* (Pang et al., 2022).

Soybean is also an important legume crop, providing a low-fat and protein-rich diet for both human and animal consumption (Messina, 1999). According to Kim et al. (2022), a total diversity of 87 bacterial and 66 fungal isolates from soybeans contains a spectrum of microbial species with the ability to suppress the growth of seed-borne pathogens. Among the identified biocontrol strains, *Pseudomonas koreensis* exhibited the strongest antagonistic activity against multiple pathogens (Kim et al., 2022). In addition, conventional and transgenic soybeans harbored significantly different microbials with distinct antagonistic capacities for controlling phytopathogens. *Bacillus sp*. and *Burkholderia sp*. were particularly effective in controlling fungal pathogens. The study also identified peptides, bacteriocins, and secondary metabolites as potential antimicrobial compounds produced by these bacteria. These findings contribute to the development of biological control methods for soybean diseases and highlight the influence of management practices and genetics on the diversity of bacterial endophytes in soybean plants (de Almeida Lopes et al., 2018).

A study on the non-mycorrhizal model plant *Arabidopsis thaliana* found that it can form symbioses with dark septate endophytes (DSE) in field, greenhouse, and controlled laboratory environments. However, the host plant's responses to colonization varied and were dependent on the specific ecotype of *Arabidopsis thaliana*. On average, two *Arabidopsis* accessions (Col-0 and Cvi-0) exhibited negative responses to DSE colonization, while one accession (Kin-1) showed no significant response (Mandyam et al., 2013). Healthy wheat tissue-derived endophytic actinobacteria capable of suppressing wheat fungal pathogens were also examined for their impact on key defense gene expression in *Arabidopsis thaliana*. Selected endophytes induced systemic acquired resistance (SAR) and jasmonate/ethylene (JA/ET) gene expression, resulting in enhanced resistance against bacterial and fungal pathogens. Culture filtrates of the endophytes also activated defense pathways influenced by growth conditions and revealed insights into the signaling molecules involved in resistance to different pathogens (Conn et al., 2008).

Endophytes in *Cannabis sativa* L. have been found to play a significant role in plant growth and development, including the production of secondary metabolites. Studies have shown that endophytes can also help modulate the production of these secondary metabolites, mainly consisting of THC and CBD, which are responsible for the therapeutic effects of cannabis (Scott et al., 2018; Taghinasab and Jabaji, 2020). They are also found to control plant pathogens that infect hemp plants, promote disease resistance, and increase hemp and organic compounds, which could cause indoor air quality issues (Vujanovic et al., 2020; Zheng et al., 2021) and cannabinoid yield (Punja et al., 2019b; Vujanovic et al., 2020). In addition, studies have found that both indoor and outdoor hemp growing can influence microbial contaminants. However, literature surveys indicate that only 2% of studies conducted thus far have focused on endophytes in cannabis and hemp plants. It highlights the need for more extensive research in this area to fully understand the diversity, functions, and potential applications of endophytes in plant cultivation and to unlock their potential benefits for plant health, growth, and cannabinoid production. However, since its legalization in western countries, more advancement in research has been made in understanding the microbial communities present inside the plant due to its restricted use of chemical fertilizers and its large production scale.

2.6 Polyploidy for improving traits of cultivated hemp

Cannabis sativa L. produces secondary metabolites called cannabinoids, which are of great interest to growers and researchers due to their medicinal and recreational properties. The synthesis of cannabinoids occurs in basal disk cells and is stored in secretory cavities called glandular trichomes (Livingston et al., 2020), with the highest concentration of trichomes found in female flower tissues that corresponds to the greatest amounts of cannabinoid. Avoiding pollination during the production of cannabinoids is a number one priority for growers, as the energy saved by not making seeds can be used to make more secondary metabolites and essential oils (Vuerich et al., 2019).

Polyploids are organisms that contain more than one set of chromosomes and may be autopolyploids (multiple copies of the same genome) or allopolyploids (two or more different genomes). Polyploidy is a natural evolutionary process that occurs in all major classes of living organisms, including plants, animals, and yeast. The success of a polyploid species depends on its ability to produce viable offspring, i.e., to control meiosis and produce gametes that can combine to form viable embryos. It is difficult to assess the level of polyploidy among plants, but it has been shown that ~70% of 28 angiosperms are polyploid (Soltis et al., 2009; Soltis and Soltis, 1995; Tate et al., 2005; Van de Peer et al., 2021). Avoiding unintentional cross-pollination of *Cannabis sativa* L. plants is one of the most important threats to cannabinoid production and has been shown to reduce cannabinoid yield. Polyploidy as a tool can be "even ploids" (4x, 6x, etc.) that increase genetic diversity and adaptation potential however, it does not typically affect reproduction, while "odd ploids" (3x,5x, etc.) are typically unstable, resulting in the consistent abortion or non-production of seeds, thus leaving the plants seedless (Chen et al., 2019). Ploidy level changes have been used in other crops; however, little is known about the performance of *C. sativa* polyploids (Crawford et al., 2021). Previous biochemical analysis showed that reducing sugars, soluble sugars, total protein, and total flavonoids increased in mixoploid plants of *Cannabis sativa* L. compared with tetraploid and diploid plants, whereas polyploidization increased tetrahydrocannabinol in mixoploid plants (Bagheri

and Mansouri, 2015). No evidence to date has found that correlates microbial communities present in diploid with those of polyploid hemp plants; however, literature suggests that genome duplication affects microbial communities in wheat (Wipf and Coleman-Derr, 2021), Salicornia (Gonçalves et al., 2022), and *Arabidopsis thaliana* (Ponsford et al., 2022).

The relationship between endophytes and hemp plants presents significant research gaps that impede our understanding of crucial aspects with implications for its genetics, breeding, and overall agricultural practices. Firstly, there is a glaring lack of knowledge about hemp genetics and breeding, hindering the exploration of critical genetic variability provided by the microbiome. Another substantial gap lies in the scarcity of information about the cannabis microbiome, particularly concerning sustainability in Hemp production. The need for increased data on Hemp-associated microbiomes is emphasized, urging comprehensive efforts involving enhanced metagenomics and Omics datasets, bioinformatics, and advanced plant phenotyping and imaging techniques. These steps are seen as essential to improve risk management in agriculture, ensuring product security, safety, and quality. Furthermore, the beneficial partnership between this plant and microbes is acknowledged, but the intricacies of this relationship, especially concerning the endogenous microbes associated with various plant tissues, remain poorly understood. This research mainly put through efforts on isolating and identifying hemp endophyte, providing basic information on their diversity and composition. Moreover, the biological effects of these endophyte on plant growth promotion and the modulation of secondary metabolites were also investigated. This study aims in understanding microbial partnerships with hemp which is deemed imperative for improving agricultural practices and enhancing the production yield of cannabinoids. Uncovering the untapped functional traits of endophyte-harboring hemp is highlighted as a critical research direction, emphasizing the need to characterize their role in enriching secondary metabolites and other compounds.

2.7 Summary

The exploration of the interaction between various ploidy hemp plants and their associated microbial communities is of paramount importance, considering its potential impact on plant health and productivity. Within the realm of this research, a notable gap emerges in our current understanding of the intricate relationship between microbial communities associated with diploid as well as triploid varieties of hemp plants. This gap forms a critical knowledge barrier that hinders our ability to harness the full potential of these plants for agricultural and industrial purposes. This study addresses and bridge this research gap, aiming to open the complexities of the interplay between different ploidy hemp plants and their seed microbiome. This study mainly focused on profound implications of seed endophyte treatments on the phenotypic characteristics of hemp plants. By scrutinizing the effects of these treatments, the study seeks not only to elucidate the mechanisms that underlie the observed changes in plant traits but also production of secondary metabolites.

3 Study 1 : Determination of the effect of microbiomes on the phenotypic characteristics of *Cannabis sativa* seeds and plants

3.1 Abstract

Hemp **(***Cannabis sativa* L.*)* is an economically valuable and versatile crop with a wide range of industrial applications. In this study, the effect of whole seed microbiome (WSM), bacterial and fungal seed epiphytes (SEp), and seed endophytes (SEn) inoculation on *Cannabis sativa* traits was assessed. *In vitro* germination along with growth and development in the phytotron chamber were observed for major phenotypic changes like weight of germinant, radicle size, height of plants, etc. It has been reported that maximizing hemp plant growth and germination is of paramount importance for optimizing yields and the quality of products. Additionally, it was observed that phenotypic traits–such as weight and radicle size of germinants, male and female height of the plant, male-female ratio in the first generation and cumulative germination percentage were enhanced by treatments with SEn and WSM. The SEn treatment had particularly positive effect on the overall health of the plant.

3.2 Introduction

Hemp (*Cannabis sativa* L.) stands as a remarkable and versatile crop of immense economic value, boasting a wide array of industrial applications. Its extensive utility encompasses industries as diverse as textiles, medicine, construction, and nutrition, making it a focal point of agricultural interest (Kaur and Kander, 2023; Scott et al., 2018). This multifaceted plant's growth and development have drawn substantial attention, and researchers have increasingly turned their gaze toward the influence of microbiomes in shaping the phenotypic characteristics of *Cannabis sativa*.

The microbiome refers to the collective microbial populations, encompassing both bacteria and fungi, inhabiting the plant's external surfaces (epiphytes) and internal tissues (endophytes) (Afzal et al., 2008; Dong et al., 2018; Rull et al., 2022). Understanding the pivotal role of these microorganisms in the context of hemp cultivation is essential, given their potential to significantly impact crucial phenotypic traits. Traits such as the weight of germinant, radicle

size, plant height, male-to-female ratios, and more come under scrutiny for optimized hemp cultivation.

Maximizing the growth and germination of hemp plants holds paramount importance, not only for bolstering yields but also for enhancing product quality. This research investigates the intricate dynamics between the hemp plant and its associated microbiomes, exploring their potential to enhance growth, germination, and resilience against environmental stressors.

3.3 Hypothesis

The whole seed microbiome, composed of epiphytic and endophytic microorganisms, significantly impacts the seed germination growth of hemp plants. Specifically, when applied to seeds, these microbial communities may enhance plant growth and resistance to environmental stresses.

3.4 Materials and Methods

The materials and methods used for this part of the study are further divided into the following categories:

3.4.1 Experimental Design

Cannabis sativa seeds were packed in aluminium foil sealed bags and kept at 4 ºC for long term storage (Suriyong et al., 2015). Mild treatment with 95% Ethanol for 10 s were performed on these seeds after which five seeds of each Cannabis accession were germinated in sterile environments of Potato dextrose agar (PDA) plate with 2% agar (without antibiotics) in a biosafety cabinet and incubated for 5 days at 23 ºC to allow for germination till 1 cm radicle emergence (Dumigan & Deyholos, 2022; Punja et al., 2019). Typically, for *in vitro* studies, PDA medium provides standard moisture conditions; hence, it is considered optimal for growing seeds. There were three endophytic inoculant treatments: seed epiphytes (SEp from outer seed part or coat), seed endophytes (SEn from inner seed part comprising endosperm and embryo), and whole seed microbiome (WSM from both outer and inner seed parts),

respectively. There was a negative control in which only seeds were grown without any treatments.

3.4.2 Inoculum Preparation

For whole seeds - WSM inoculum preparation, 5 seeds were used to prepare a stock of the inoculum. The pestle and mortar tools were employed to crushing and grinding them into a powder to be submerged in 900 µl of sterile distilled water to obtain a fine paste, as an initial stock., . 100 µl of the sample was taken from the initial stock for serial dilutions to obtain 2x 10^4 cells/ml inoculum. 200µl of 2x 10^4 cells/ml were placed at the centre of the petri plate (Hubbard et al., 2012; Moroenyane et al., 2021) as a seed inoculation method (Figure 3.1).

For seed endophyte (SEn) – 5 cannabis inner part of seeds, after removal of seed coats and germinated till 1 cm of radicle is visible, were taken with the help of forcep and crushed with pestle and mortar in the presence of 900 µl of sterile distilled water. For seed epiphyte (SEp) – 5 cannabis seed coats were taken with the help of forcep and crushed with same quantity as mentioned above in pestle and mortar. Same dilutions were prepared and used for all 3-treatment study (Figure 3.1 A)

Peat, sand, and vermiculite were prepared in the ratio of 3:2:1 and added in the sterile petri plate. 5 fresh cannabis seeds were allowed to germinate at equal distance from each other forming a circle in 1 petri plate after proper sterilization as shown in Table 3.1. After 2nd day of germination, 200 µl of the WSM, SEn, and SEp inoculum prepared were added at the centre of the each petri plate separately (Hubbard et al., 2012). The figure 3.1 B represents here depicts the way it is done.

To ensure stable inoculants, the variability of seeds was reduced to minimum by using same stock of seeds, seeds of the same age from a single source (Company – Oregon, USA.

These seeds were packed in aluminium foil sealed bags and kept at 4 ºC during the full tenure of the experiment (8-10 months).

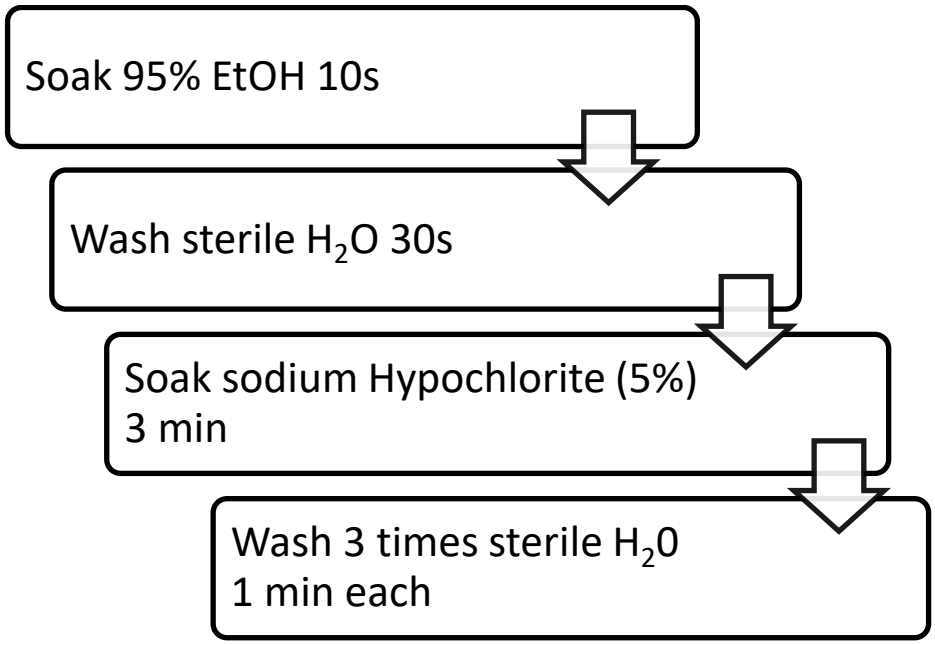
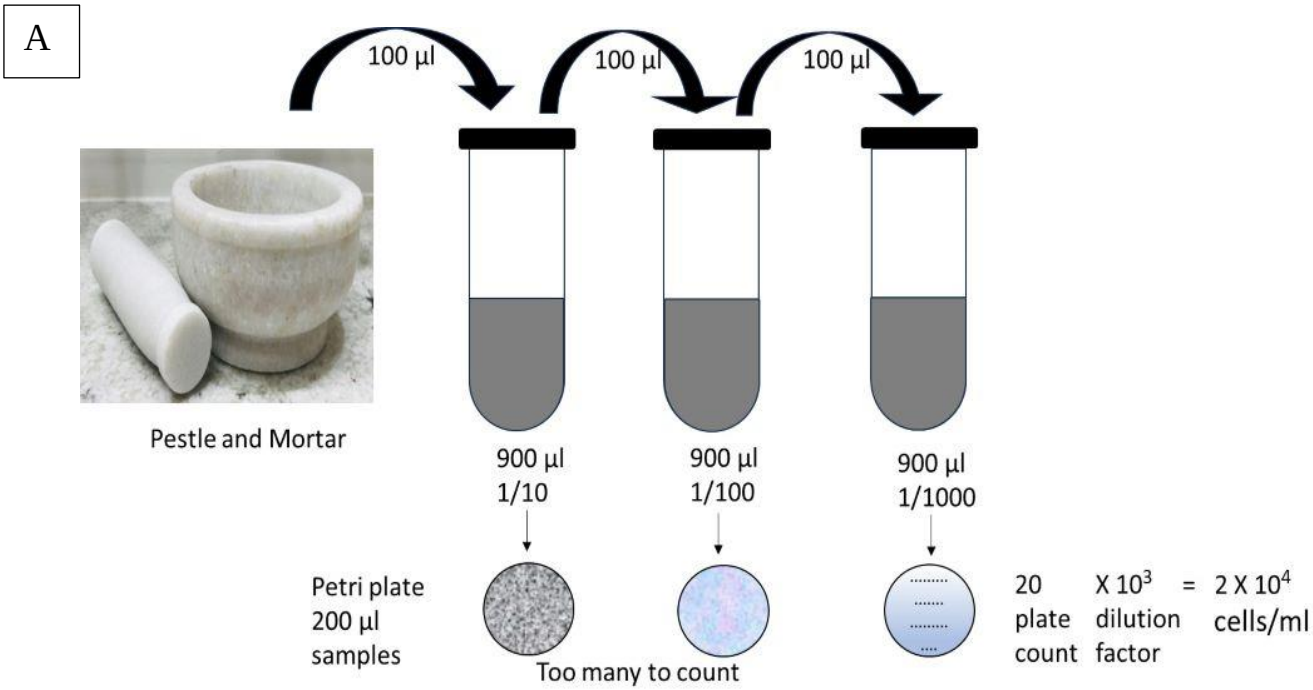


Table 3.1: Surface sterilization procedure for seeds.



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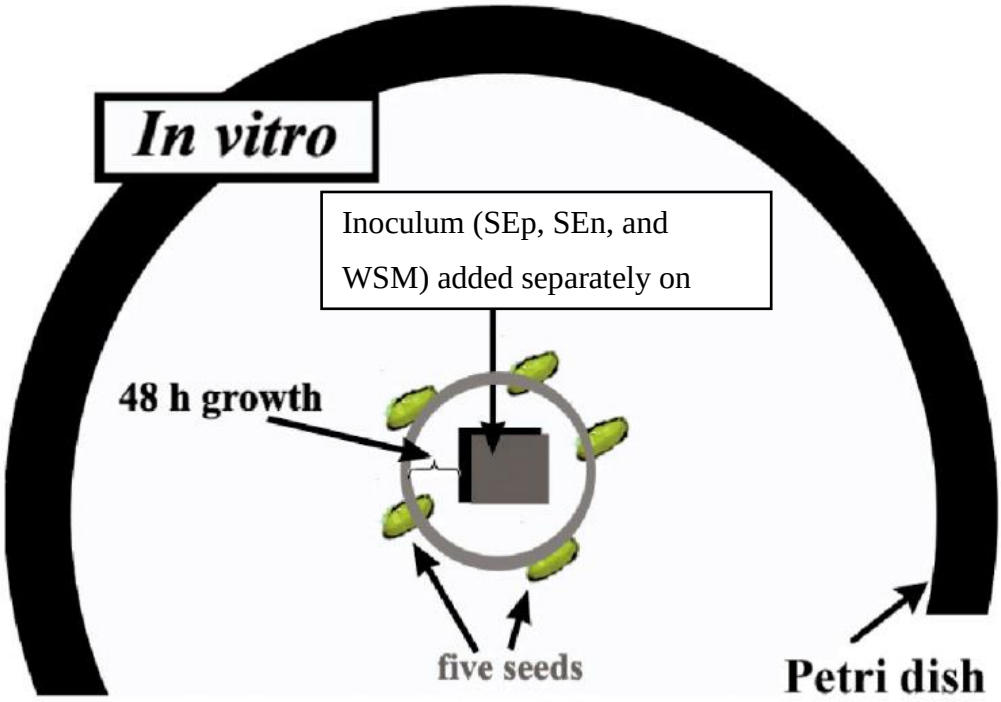


Figure 3.1 A: Serial dilutions prepared for WSM, SEn, SEp inoculant samples. **B**: *In vitro* method of inoculating samples in the *Cannabis sativa* seeds. Taken from *Vujanovic et al. 2012.*

3.4.3 Seed Germination Assay

Seeds were germinated in a 100 mm x 15 mm petri plate, and the germination rate was calculated at days 2, 4, 7, and 10 of each treatment for SEp, SEn, and WSM, with seeds without any treatment as a control (Kumari and Vujanovic, 2020).

3.4.4 Statistical Analysis

In the current study, all experiments were executed in triplicate. The mean and standard error were determined for various experiments, including seed germination, weight of the

germinant, and radicle size. A one-way analysis of variance (ANOVA) technique, followed by post-hoc Tukey honest significant difference (HSD) and least significant difference (LSD) tests, were used to determine the statistical significance of phenotypic characteristics of plant using SPSS (IBM SPSS statistic 22). (Kumari and Vujanovic, 2020).

3.5 Results

The results of this study revealed changes in the physiological traits of *Cannabis sativa* plants influenced by the application of WSM, SEp, and SEn. These alterations encompass significant host characteristics, including germination rates, seed biomass, radicle size, plant height, and male-to-female ratios.

3.5.1 Seed Germination Assay

Percent germination results showed progressive germination on day 2, 4, 7 and 10 where highest germination was seen on day 4 on all treatments and lowest was seen on day 2. Cumulative germination percentage showed that whole seed microbiome (WSM) has the highest percentage of germination as compared with individually applied seed epiphytes and endophytes treatments *in vitro.*

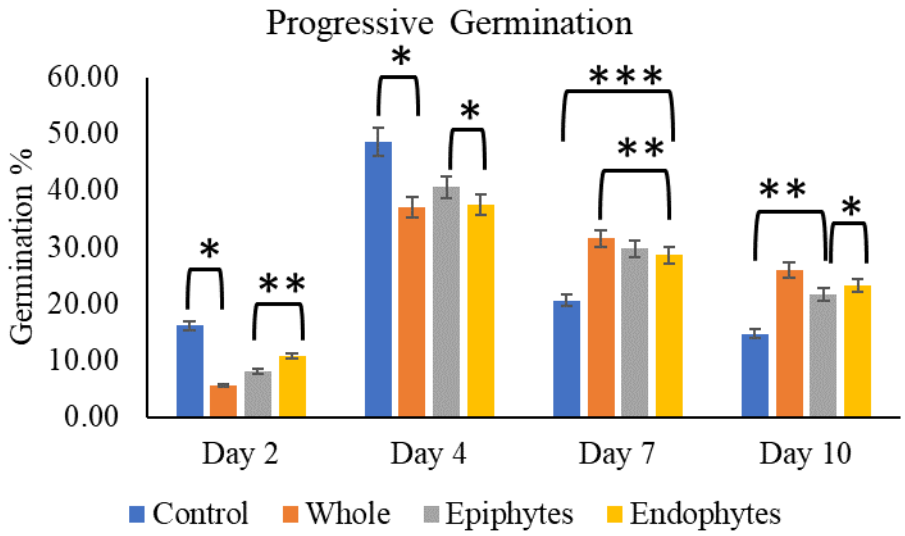


Figure 3.2:Progressive germination of *Cannabis sativa* seeds on days 2, 4, 7 and 10 under different treatments of seed epiphytes, seed endophytes, and whole seed microbiome. Data are means and standard errors of three replicates (Two-way ANOVA with LSD test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

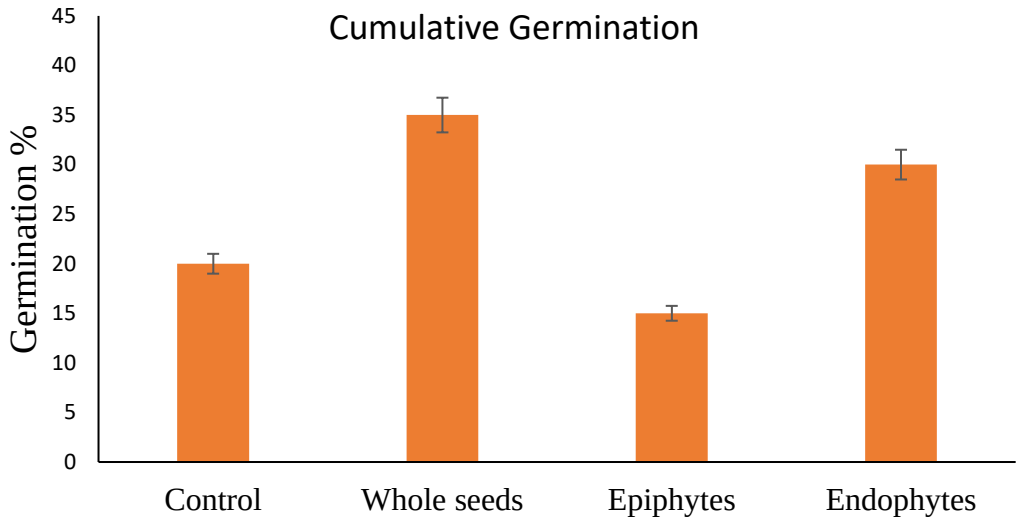


Figure 3.3: Cumulative germination percentage of seeds treated with whole seed microbiome, seed epiphytes, and seed endophytes. Data are means and standard errors of three replicates (One-way ANOVA, $p < 0.05$).

3.5.2 Morphometric Analysis of Seed Germinant Weight and Radicle size

The study revealed a hierarchical trend in germinant weights, with those inoculated with seed endophytes in Figure 3.4 (A) exhibiting the highest weights, followed by seed epiphytes, whole seed microbiome, and the control group. Size of radicle on day 5 in Figure 3.4 (B) was found to be highest in seed endophytes and lowest in whole seed microbiome. 30 seedlings on day 10 in Figure 3.5 showed more hairs present in seed endophytes as compared to other groups (30 in each group).

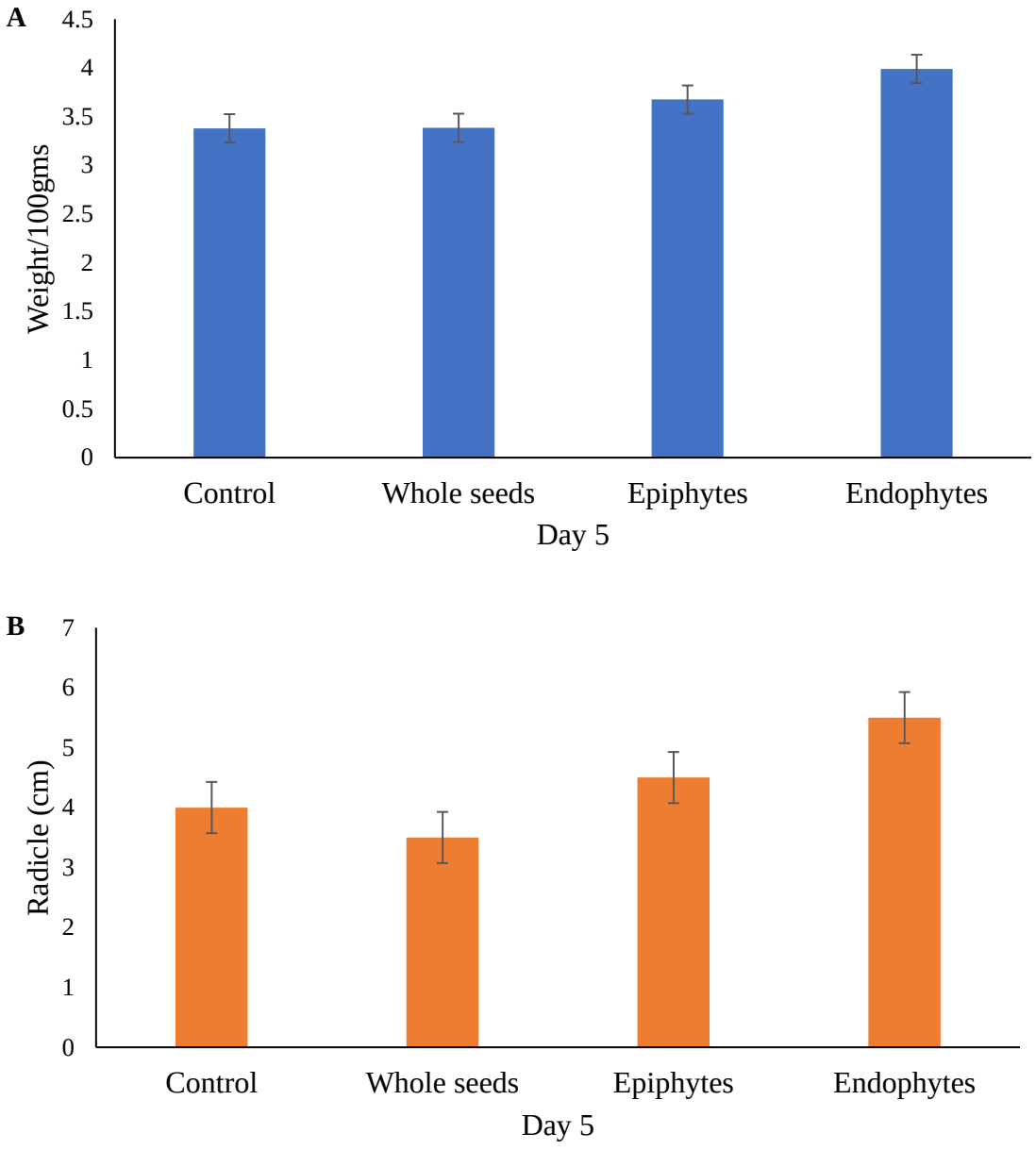


Figure 3.4:Weight of the seeds (A) and size of the radicle (B) on day 5. Data are means and standard errors of three replicates (One-way ANOVA, $p < 0.05$).

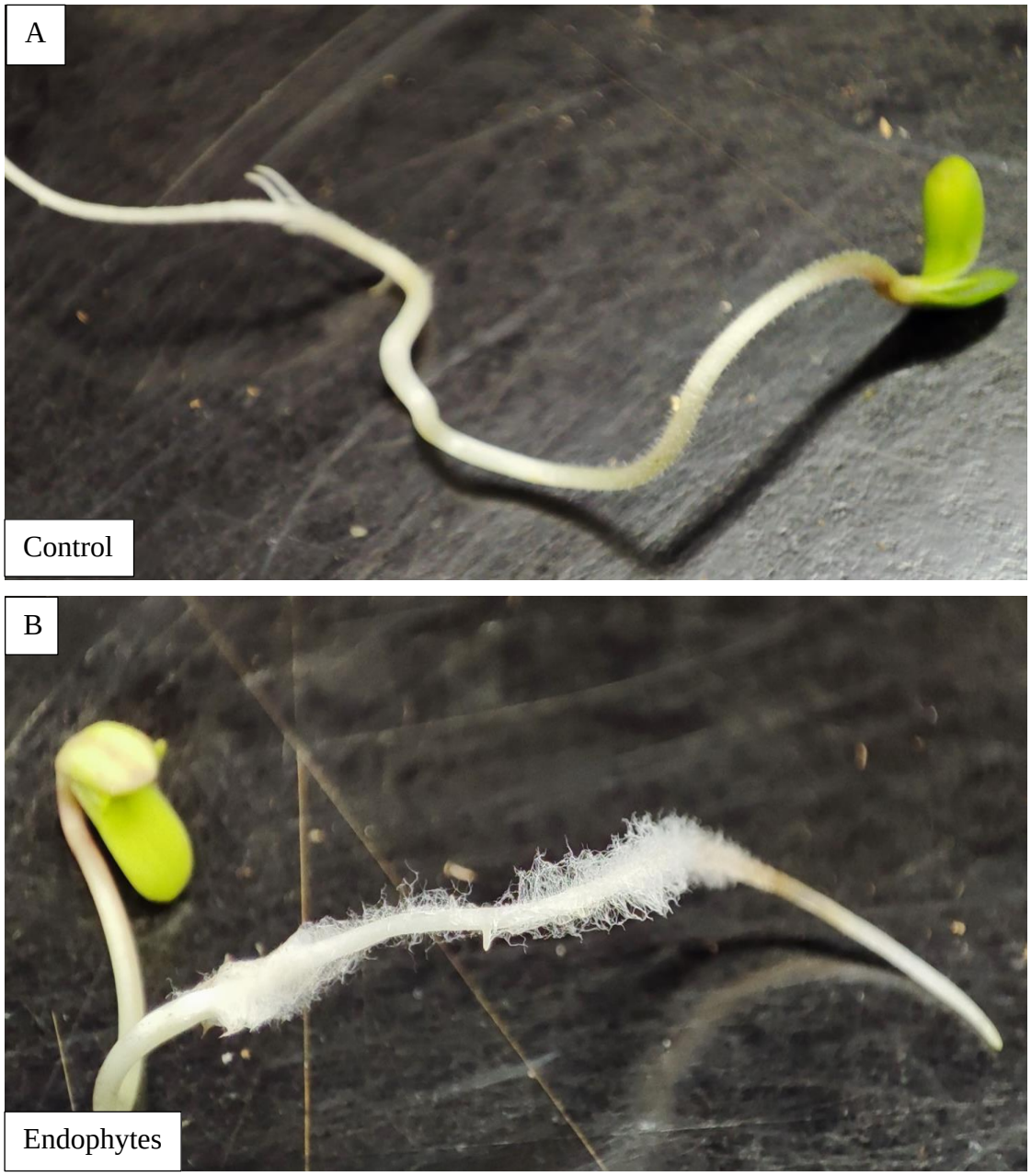


Figure 3.5: Seedlings on day 10 showed different radicle/root architecture and absorbent hairs in germinant without inoculation with SEn (control A) and inoculated with SEn (treatment B).

3.5.3 Quantitative Assessment of Variation in Plant height

Variations in plant height was seen in *Cannabis sativa* L. male and female plants with different treatments. Plants treated with seed endophytes exhibit the greatest height while the plants treated with seed epiphytes showed the least as seen in Figure 3.6 and 3.7.

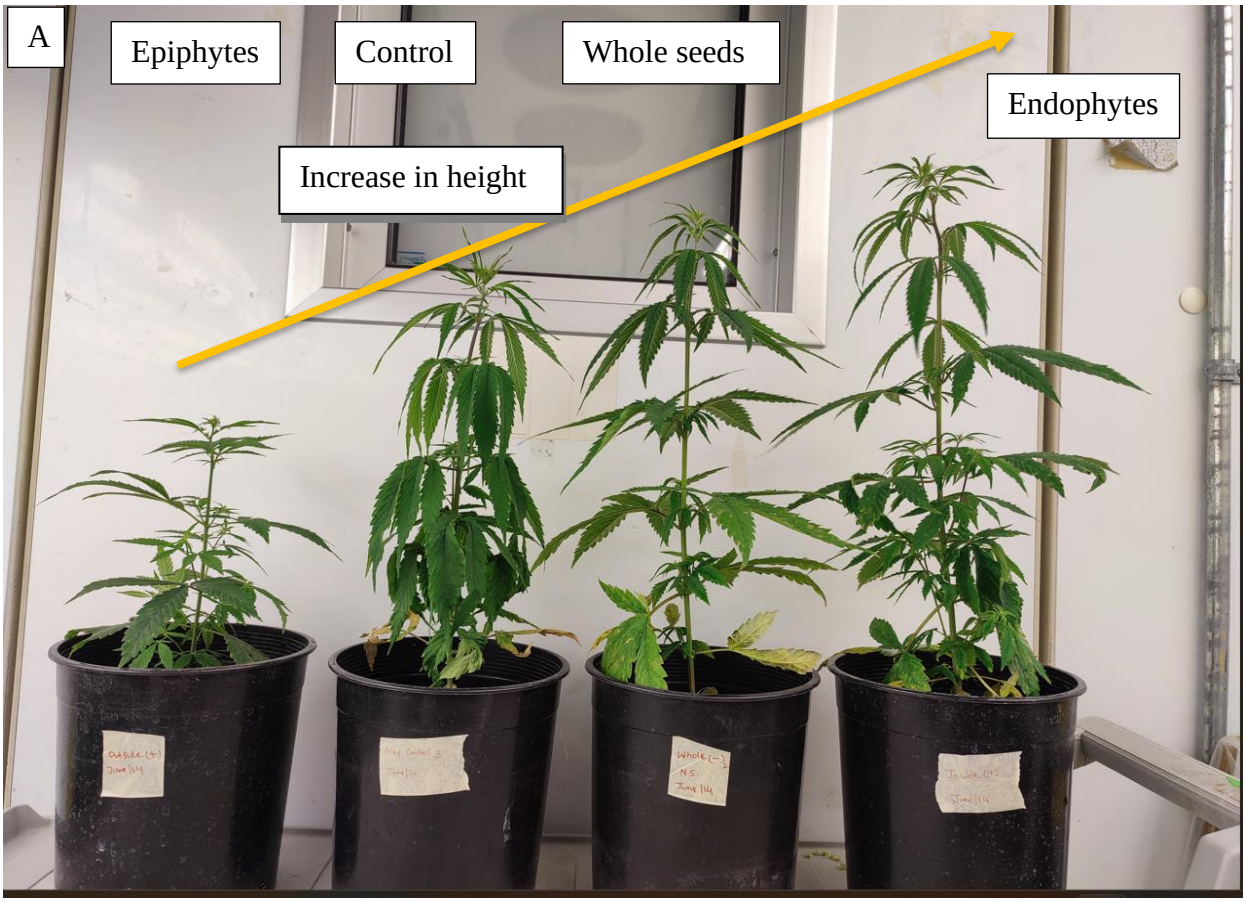


Figure 3.6 (A): Height of the female plant (1.5 months old). Left to right: seed epiphytes-control-whole seeds microbiome-seeds endophytes.(B) Data are means and standard errors of three replicates (One-way ANOVA, $p < 0.01$).

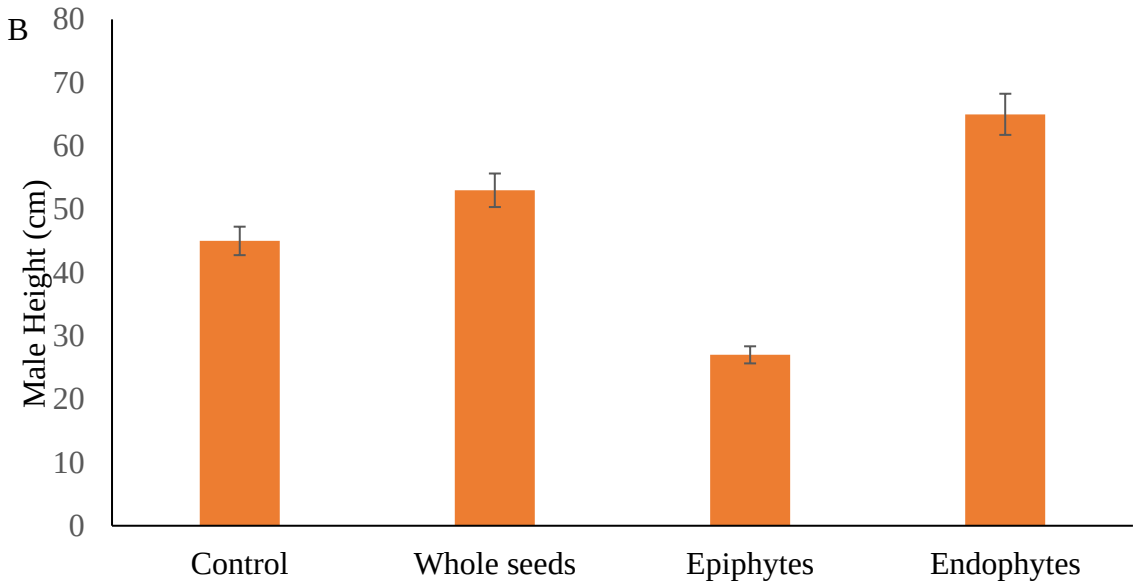


Figure 3.7 (A): Male plant (1.5 months old). Left to right: control-WSM-SEn–SEp. (B) Data are means and standard errors of three replicates (One-way ANOVA, $p < 0.01$).

3.5.4 Male-Female ratio in F1 generation

It was found that plants treated with seed endophytes and whole seed microbiomes had a higher proportion of female individuals (~ 30%) compared to plants treated with seed epiphytes and untreated plants (~15%). However, male plants dominated in control (~30%) and second highest in seed epiphytes plants (~25%) with least in WSM and SEn (~20%).

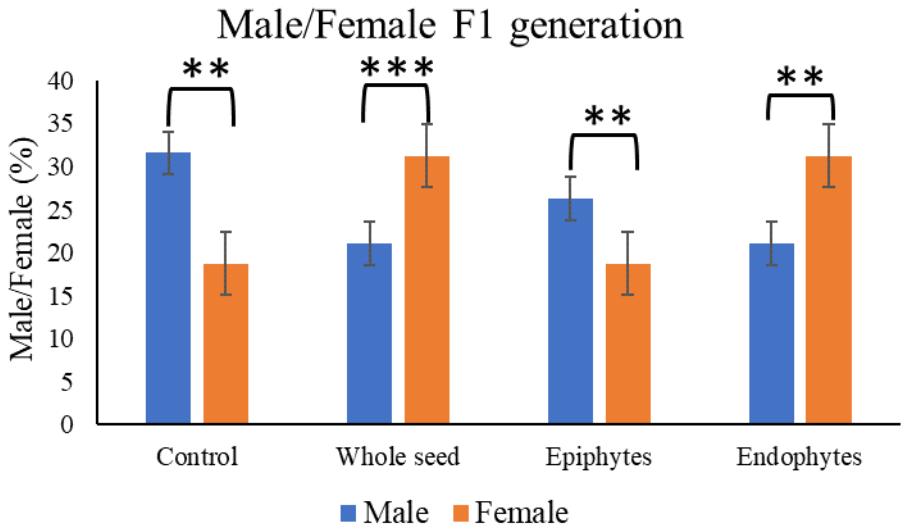


Figure 3.8 :Male-female percentage of different treated plants in the 1st generation. Data are means and standard errors of three replicates (Two-way ANOVA with LSD test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.6 Discussion

Endophytes have been isolated from various species of plants and organs and have mostly been recruited from the generative-seed (Vujanovic and Germida 2017) and vegetative-rhizosphere (Vandana et al. 2021) organs of the host plants. Plants harbors endophytic microbiome with different functional implications, specialization, and competence (Shelake et al. 2019). Seed epiphytes are microbial communities that live on the surface or episphere (outside) and colonize seed coat. Coats is a protective seed layer basically composed of dead cells. It represents a first line of defense against adverse external factors and acts as channel for transmitting environmental cues to the interior of the seed (Rodchuck and Borisjuk, 2014 . It largely consists of microorganisms which are mainly saprophytes that feed on dead organic

matter and are known as decomposers (Martín et al., 2022; Radchuk & Borisjuk, 2014) and antagonists. Their activity is to produce enzymes for decomposition of compounds like phenols which have antimicrobial and antioxidant properties. In this study the findings show that, although epiphyte treatments does increased the weight and radicle size of the germinants, it negatively affect the germination percentage of the *Cannabis sativa* seeds as well as the overall height of female and male plants. This could be because the microbial communities present in epiphytes mainly help germination and early plant establishment compared to mature plants growth and development. other

Seed endophytes on the other hand are those microbes that live inside (the seed endosphere), penetrate and occupy embryo and endosperm. The seed endosphere is composed of living cells often occupied by mutualistic endophytes with symbiotic behaviors (Collinge et al., 2022; Gouda et al., 2016). The endophyte/plant relationship is unique as what endophyte gains from plants are nutrients and shelter and what plants gains from endophytes are numerous- they help in plant growth promotion (by providing essential nutrients), helps in stress mitigation, and acts as biological control agents. They act as reservoirs of novel bioactive secondary metabolites, such as alkaloids, phenolic acids, quinones, steroids, saponins, tannins, and terpenoids that serve as potential candidate for antimicrobial, anticancer and many more properties (Lyu et al., 2019, 2022). This study results vividly showed the advantages of seed endophytes on the phenotypic characteristics of *Cannabis sativa*, germination and plant growth under controlled conditions. The endophyte treatments enhance seed germination , increase plant height, and weight, and improve radicle size and root architecture as important bioindicators of the plant health.

Endophyte favor female plants as they are more likely to be transmitted by female as eggs are larger than pollen (Vannette, 2020). The production of female plants is of great interest for breeding programs. Female plants are preferred because they produce sticky buds from trichome area present on flowers that contain high concentrations of cannabinoids, terpenes, and flavonoids. In contrast, male plants are destroyed in commercial production as seed formation reduces flower quality (Flajšman et al., 2021; Punja and Holmes, 2020). This can

have further potential implications for crop environmental resilience and success in the cultivation of this versatile and economically significant plant.

While the application of these microbial communities to seeds holds promise for augmenting plant vigor and productivity, it is essential to remain vigilant. Certain chemical treatments, such as systemic fungicides, may inadvertently disrupt these beneficial microbial alliances (Glick and Gamalero, 2021; Kharwar et al., 2014; Xiong et al., 2021), potentially resulting in reduced plant vitality and biomass.

3.7 Conclusions

This study showed that seed endophytes and the whole seed microbiome (except seed epiphytes) - all contributed to the growth of the plants and expressed higher growth rates as compared with the untreated control. This result corroborates Hubbard et al. (2014), Vasanthakumari et al. (2019), Vujanovic et al. (2019), and Vujanovic and Germida (2017). In addition to assessing the effects of these microbial communities on plant phenotypes, this study employs changes in phenotypic characteristics of seedlings as well as plants *in vitro* in response to various treatments. The comprehensive evaluation encompasses the whole seed microbiome, including seed epiphytic and seed endophytic microorganisms, revealing their substantial impact on hemp plant growth.

Thus, this study represents a crucial step toward optimizing hemp cultivation practices by unraveling the intricate relationships between microbiomes and the phenotypic characteristics of *Cannabis sativa*. The treatments improved seed germination and biomass growth at germinant and plant levels, as well as the ratio between female and male plants generated from the same lot of seeds. The most positive effect of the treatments was on the weight and radicle size of germinants ($p < 0.05$), as well as on the height ($p < 0.01$) and production of female plants.

3.8 Connection to the next study

The study results showed that treatment with seed endophyte (SEn) had a more positive effect on the overall health of the seedling germinating as well as on the height of the plant; therefore, SEn treatments were used in assessing the growth potential characteristics of diploid and triploid cultivars of Suver haze plant in study 2.

4 Study 2 : Profiling the bacterial and fungal whole plant microbiome associated with the diploid and triploid Suver haze plants using universal 16S rRNA and ITS primers

4.1 Abstract

Suver Haze, a popular strain of hemp (*Cannabis sativa* L.), has been developed in both diploid and triploid forms that originated from the Southern Oregon region by combining Neville's Haze male and Krishna's Special Sauce female. In this part of the study, the evolutionary relationship between diploid and triploid cultivars of Suver Haze was categorized using Mega XI software, and ploidy levels were further confirmed using flow cytometry techniques. In addition, Sanger DNA sequencing analysis was performed to assess fungi and bacteria using universal ITS and 16S rRNA sets of primers. There was a wide variety of microbial communities present in both cannabis cultivars tested with higher diversity in diploids as compared to triploids. These diversified microbial communities are referred to as whole plant microbiome (WPM).

4.2 Introduction

Hemp is a versatile crop with uses in many areas, from industrial parts to health aids (Rupasinghe et al., 2020). Its relationship with helpful microorganisms sets it apart in the farming scene. This study mainly focussed on the beneficial microbes linked to hemp, although the phytopathogens were also analysed and reported.

The reciprocal symbiosis between hemp and its diminutive symbiotic organisms, dwelling within both the soil and the plant, is the subject of extensive scientific research. A variety of bacteria and fungi work together to boost hemp's health, growth, and stamina. This makes them central in efforts to improve how we grow hemp (Compant et al., 2011; Dumigan and Deyholos, 2022; Qadri et al., 2013). The detection of microorganisms in hemp plants through the application of universal primers is a dynamic and evolving field that holds the

promise of enriching our understanding of the intricate partnerships between plants and their microbial allies. It has far-reaching implications for both the scientific community and the broader domains of agriculture, biotechnology, and sustainability.

Over the past decade, both diploid and triploid hemp varieties have gained popularity due to their diverse applications, including in textiles, food products, CBD production, and more (Bagheri and Mansouri, 2015; Parsons et al., 2019; Rutland et al., 2021).

Suver Haze is a popular hemp strain that is known for its high CBD content and low THC levels. It is available in both diploid and triploid varieties. Diploid plants have two sets of chromosomes, while triploid plants have three sets of chromosomes, and both are available in a seedless variety that is known for its solid yields, exotic aromas, and resistance to mold and mildews (Crawford et al., 2021; Darby et al., 2020). In this part of research, profiling the bacterial and fungal microbiomes associated with both cultivars were investigated.

4.3 Hypothesis

It is hypothesized here that diploid and triploid plants are associated with a distinct diversity of whole plant microbiomes (WPM) composed of bacteria and fungi. A greater diversity of beneficial endophytes, as opposed to phytopathogens, is expected to enhance the likelihood of achieving homeostasis (to maintain a stable internal environment despite external factors) and promoting better plant health in these cultivars.

4.4 Materials and Methods

The materials and methods used for this part of the study are further divided into the following categories:

4.4.1 Evolutionary Analysis of Diploid and Triploid hemp plant

Leaves from 4th node were cut during vegetative phase and DNA were extracted using DNeasy Plant Kit # 69104 (Company – Qiagen). After DNA extraction, 2 sets of primers were

selected for Polymerase Chain Reaction. One was SCAR primers (sequence-characterized amplified region) having forward primer SCAR119_F (5'-TCA AAC AAC AAA CCG-3') and reverse primer SCAR_119_R (5'-GAG GCC GAT AAT TGA CTG-3) (Mendel et al., 2016 (Backer et al., 2020)). Another set of EG primers (transcriptional corepressor SEUSS gene) containing forward primer EG160F (5'-TAA TTT CCC GGC TGG TGC TC-3') and reverse primer EG162R (5'-CGG CCA GGC TTT CGA TTG C-3') were used. SCAR primers were used as they differentiates male and female plants with male plants showing 119 bp and no bands with female plants. In addition, EG primers were used for floral development identification and give 127 bp with PCR (Gilchrist et al., 2023). PCRs were performed in a reaction volume of 25 µL consisting of 10ng/µl DNA, 1X buffer with 2mM $MgCl_2$ 0.2mM dNTPs, 0.5µM forward and reverse primers, and 0.1U Taq polymerase.

Amplifications were performed for initial denaturation at 95 ºC for 5 min, followed by 40 cycles of denaturation at 95 ºC for 30 s, annealing at 48 ºC for 45 s and extension at 72 ˚C for 45 s with completion at the final extension of 72 ºC for 5 min. PCR product was run on 1.2% agarose gel made in 0.5X TAE buffer. Gel extraction of desired bands were performed using QIAquick gel extraction kit # 28704 and then the purified gel extracted were sent to NRC (National Research Council – Saskatoon) for Sanger sequencing. Sanger sequencing also known as chain terminating method is a semi-quantitative method where quality of a DNA product is identified using quality score (QS), which is the average for all peaks in the trace with an assigned base. Generally, traces with QS $\geq$ 40 are considered of good quality. The results obtained from Sanger sequencing were further checked on Nucleotide BLAST (Basic Local Alignment Search Tool) which is a widely used bioinformatics tool provided by the National Centre for Biotechnology Information for comparing genome sequences with the closest genus and species. To ensure better quality of sequences, we removed the end of the sequences which were dissimilar and performed Molecular Evolutionary Genetics Analysis using MEGA XI software (version 11.0.13) as shown in Figure 4.1. Phylogenetic tress were generated using Neighbor-joining method. The optimal tree with a sum of branch lengths of 1.60335638 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

4.4.2 Flow Cytometry Analysis

Flow cytometry is a widely used technique to determine genome size and ploidy level in plants. 1 cm^2 of fresh hemp young leaf tip was collected into each well of a 0.5 ml 96-well plate (Bio-One, Cat#82051-472), and a single 5mm Zirkonia-Yttra-coated bead per well was added. Leaf was sealed with a foil (Peel Seal Cat# KBS-0601-001, LGC Genomics) using a heat sealer at 168°C for 3 s (ALPS 50 V, Cat# AB-1443A, Thermo Fisher Scientific) and stored at -80ºC. 100 µl of nuclei extraction buffer (CyStain UV precise P automate nuclei extraction and DNA staining kit, Cat# 05-5002-a, Sysmex) was added to the leaf plate and resealed with foil (Peel Seal Cat# KBS-0601-001, LGC Genomics) using a heat sealer at 168°C for 3 s (ALPS 50 V, Cat# AB-1443A, Thermo Fisher Scientific) and homogenized for 25 s at 1600 rpm (2010 Geno/Grinder, SPEX Sample Prep, Metuchen, NJ, USA). Leaf samples were spun down for 30 s at 1000 rpm, and all of the suspensions were filtered through a 96-well filter plate (AcroPrep Advanced 350, 30-40 µm PP/PE, Cat# 8027, PALL Corporation, New York, USA) into a new 96-well 300 µl elution plate (96-Well Microplates, Greiner Bio-One, Cat# 82050-636, VWR, Radnor, PA, USA) using 200 µl large orifice tips (FisherScientific, Cat.#: 02-707-134) for leaf samples. Centrifuge the suspension through the filter plate for 30 s at 1000 rpm and 4°C. An endogenous control was prepared using fresh Raphanus sativus leaves (8.84 pg/2C genome size). The prepared sample plates were placed at 4°C for at least 10 min (up to max. overnight/12 h) for saturation of the tissue with the stain before measurement. All ploidy and FCSS measurements were performed on a CytoFLEX flow cytometer with plate loader (Beckman Coulter, Indianapolis, IN, USA) equipped with a 405 nm (Violet) laser. Data was measured and analyzed using the CytExpert Software v2.5 (Beckman Coulter). Gain settings for hemp ploidy are: FSC = 92, SSC = 280, and DAPI = 100. Stopping rules are: 200000 nuclei, 90 s. Speed: hemp (~60 µl/min). All positions containing gaps and missing data were eliminated (complete deletion option). There was a total of 28 positions in the final dataset. Evolutionary analyses were conducted in Mega XI (Celniker et al., 2013; Huson, 1998; Tamura et al., 2021).

4.4.3 Identification and diversity of fungal and bacterial taxa

To identify each morphologically unique colony to genus and species level, we combined PDA agar plate and PCR methods utilizing primers for fungi and bacteria, the ITS1-5.8S-ITS2 region of ribosomal deoxyribonucleic acid (DNA) and 16S rDNA, respectively (Patil-Joshi et al. 2021; Punja et al. 2019)

4.4.3.1 ITS Sequencing

Seeds, flowers, and leaves from both diploid and triploid cultivars were allowed to grow on separate Potato Dextrose agar (PDA) for a week and pure cultures were obtained by transferring individual colony type from mixed cultures to the fresh agar plate (dos Reis et al. 2022). Colony forming Units were calculated for each pure culture obtained and their morphology identified and validated - based on DNA sequences (Punja et al. 2019). Genomic DNA from these pure cultures were extracted using DNeasy Plant Kit # 69104 (Company – Qiagen) which were then amplified using forward (CS1_ITS3_KYO2 -5'- ACA CTG ACG ACA TGG TTC TAC AGA TGA AGA ACG YAG TRAA -3') and reverse (CS2_ITS4 - 5'- TAC GGT AGC AGA GAC TTG GTC TTC CTC CGC TTA TTG ATA TGC -3') universal ITS primers (Petit et al., 2020; Wei et al., 2021). PCRs were performed in a reaction volume of 25 µL consisting of 10ng/µl DNA, 1X buffer with 2mM $MgCl_2$ 0.2mM dNTPs, 0.5µM forward and reverse primers, and 0.1U Taq polymerase.

Amplifications were performed for initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 30 s annealing at 58 °C for 45 s and extension at 72 °C for 45 s with completion at the final extension of 72 °C for 5 min. PCR product of 400 bp was run on 1.2% agarose gel made in 0.5X TAE buffer, and then gel extraction was performed with Qiagen gel extraction kit Catalog # 28704, which was then sent for sequence analysis by Sanger method to the NRC (National Research Council -Saskatoon).

Universal ITS sequences are particularly suitable to study the Kingdom Fungi (Nilsson et al., 2015; Schoch et al., 2012). The laboratory steps involved a) the fungal isolation and purification on the PDA plate to determine the composition of fungal communities), 2) the species identification - based on its morphology and calculating number of colonies (CFUs), 3)

the isolation of DNA and final sequencing analysis for the confirmation of a particular fungal taxon by Sanger method. Sequence similarity analyses was performed using NCBI-Nucleotide BLAST (National Center for Biotechnology Information – Basic Local Alignment Search Tool) using mega BLAST as created specifically for the task of efficiently looking for very similar sequences(McGinnis and Madden , 2004).

4.4.3.2 16S rRNA sequencing

Seeds, flowers, and leaves from both diploid and triploid hemp cultivars were allowed to grow on separate Luria Bertani (LB) plate for bacterial isolation and incubation at 21 ºC over 3 days. The dilution series was applied (Tao et al., 2022) to isolate bacteria (Figure 3A) and purified colonies from mixed bacterial cultures. Colony forming units were calculated for each pure culture obtained and their morphology identified and validated using PCR technique. Genomic DNA from each purified cultures was extracted using DNeasy Plant Kit # 69104 (Company – Qiagen) which were then amplified using forward (CS1_341- 5'- ACA CTG ACG ACA TGG TTC TAC ACC TAC GGG NGG CWG CAG- 3') and reverse (CS2_806 - 5'- TAC GGT AGC AGA GAC TTG GTC TGA CTA CHV GGG TAT CTA ATC C- 3') universal 16S rRNA primers (Pal et al., 2022). PCRs were performed in a reaction volume of 25 µL consisting of 10ng/µl DNA, 1X buffer with 2mM $MgCl_2$, 0.2mM dNTPs, 0.5µM forward and reverse primers, and 0.1U Taq polymerase.

Amplifications were performed for initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 30 s annealing at 60 °C for 45 s and extension at 72 °C for 45 s with completion at the final extension of 72 °C for 5 min. PCR product of 450 bp was run on 1.2% agarose gel made in 0.5X TAE buffer, and then gel extraction was performed with Qiagen gel extraction kit Catalog # 28704, which was then sent for sequence analysis by Sanger method to the NRC (National Research Council -Saskatoon).

Universal 16S rRNA gene is a component of the prokaryotic ribosome that exhibits regions of conserved sequences interspersed with variable regions, making it a valuable target for The laboratory steps involved a) the bacterial isolation and purification on the LB plate to determine the composition of bacterial communities), 2) the species identification - based on

its morphology and calculating number of colonies (CFUs), 3) the isolation of DNA and final sequencing analysis for the confirmation of a particular bacterial taxon by Sanger method. Further investigation into sequence similarities was carried out employing NCBI-Nucleotide BLAST, a resource provided by the National Center for Biotechnology Information (NCBI) known as the Basic Local Alignment Search Tool. Within this framework, emphasis was placed on utilizing mega BLAST, a tool tailored for the efficient identification of closely related sequences (Paul, 2023; Thein & Maung, 2017.).

4.4.4 Shannon-Weiner Diversity Index

The Shannon-Wiener Diversity Index, also known as Shannon's Diversity Index or Shannon-Weaver Index, is a commonly used metric to quantify the diversity of species in a community (Spellerberg and Fedor, 2003). It considers both the number of species present and their relative abundances and is calculated as the negative sum of the product of the proportion (p_i) and the natural logarithm of the proportion ($\ln p_i$) for each species. In mathematical terms, this is represented as:

$$H = -\Sigma (p_i * \ln p_i)$$

Since this is a negative number, we take the negative of the negative of this sum as reported in (Nolan and Callahan, 2006).

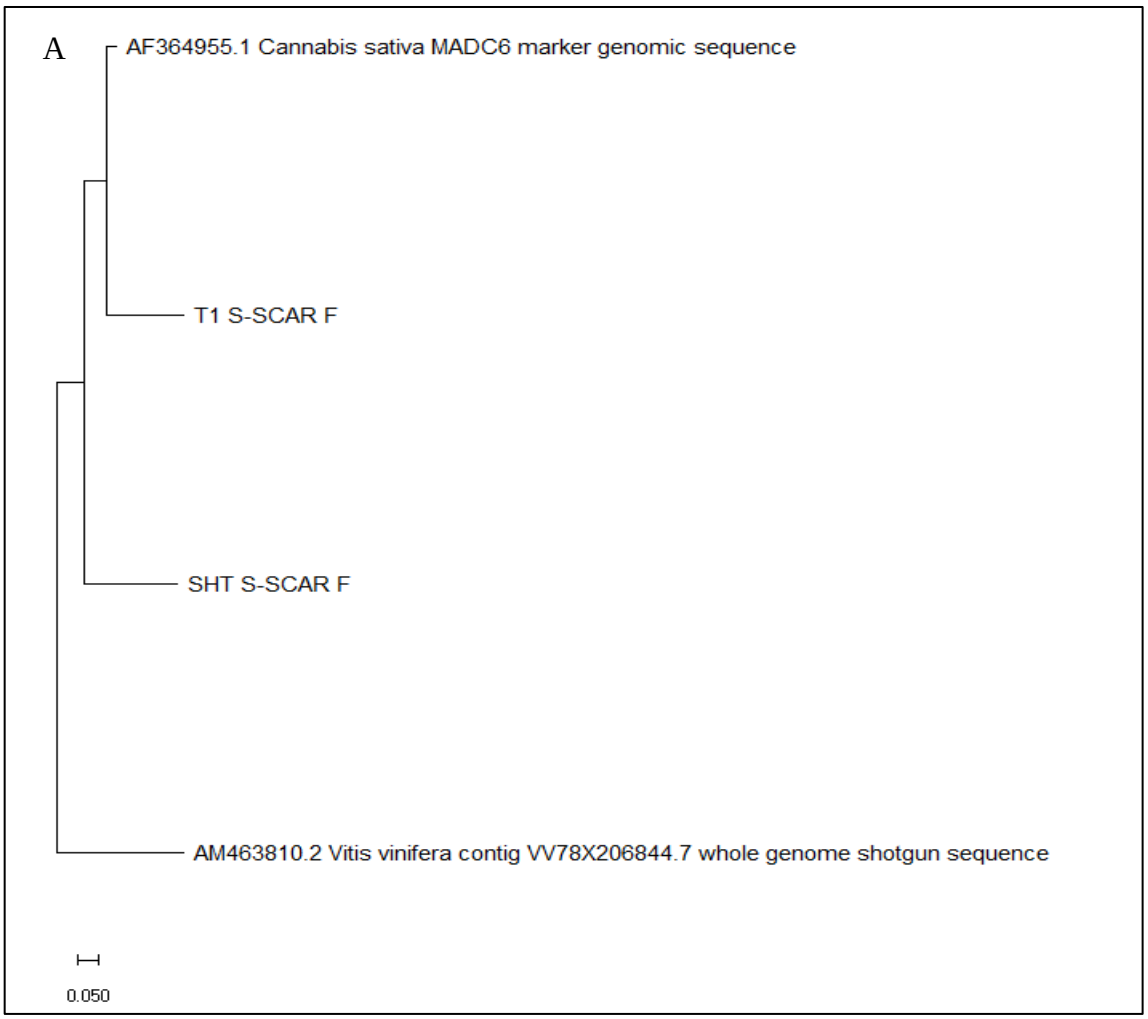
Relative abundance of fungal and bacterial communities were calculated by counting the number of colonies found on the PDA and LB plate, its frequency by richness and evenness (Gauthier & Derome, 2021; Strong, 2016). This composition of fungal (Boniek et al., 2019; Nanjundiah et al., 2007) and bacterial (Deepika & Laxmi Sowmya, 2016; Hill et al., 2003; Martin, 2002) communities identified were evaluated by using the diversity index which was calculated and presented in Figure 4.5 and Figure 4.6 respectively.

4.5 Results

The study results revealed the phylogenetic profiles of diploid and triploid Cannabis (hemp) Suver-Haze hosts and diversity of associated microbiome composed of fungal and bacterial communities. Findings further unravel the genetic relationships and microbiological ecosystems inherent to both diploid and triploid hemp varieties.

4.5.1 Phylogenetic tree comparing diploid and triploid Suver Haze

The phylogenetic tree rooted in *Vitis vinifera,* often used as a model plant for rRNA sequence analyses and floral development (Barcaccia et al., 2020), was created in *Cannabis sativa.* It showed that the two clusters for MADC6 (sex) and SEUSS (floral development) genes separated diploid from triploid plants. This concords with the results presented below under flow cytometry.


A
AF364955.1 Cannabis sativa MADC6 marker genomic sequence
T1 S-SCAR F
SHT S-SCAR F
AM463810.2 Vitis vinifera contig VV78X206844.7 whole genome shotgun sequence
0.050

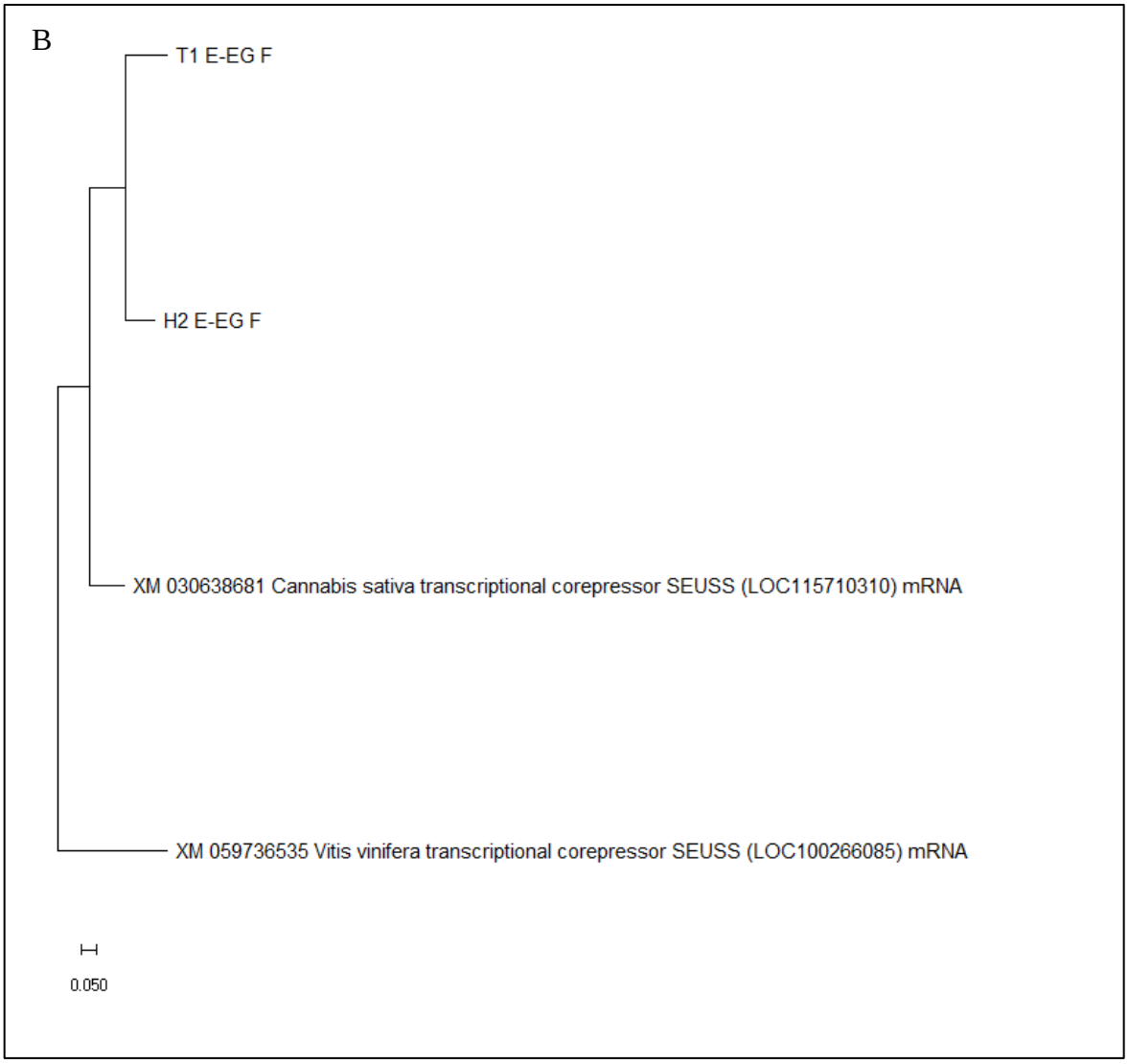


Figure 4.1(A) and (B): A phylogenetic trees were inferred using the neighbor-joining method using two cluster of genes - MADC6 gene in (A) and SEUSS gene in (B).

T_S-SCAR_F: *Cannabis sativa* sequenced with MADC6 forward primer
SHT_S-SCAR_F: Suver haze triploid plant sequenced with MADC6 forward primer
T1_E-EF_F: *Cannabis sativa* sequenced with SEUSS forward primer
H2_E-EG_F: Suver Haze diploid plant sequenced with SEUSS forward primer

4.5.2 Flow Cytometry Analysis

In flow cytometry, a shift in the peak represents a change in the DNA content of the cells being analyzed. This can be indicative of changes in ploidy, which is the number of sets of chromosomes in a cell. A shift to the left of the peak indicates a decrease in DNA content which is diploid and a shift to the right of the peak indicates an increase in DNA content which is triploid plant. This shift in the peak represents change in the sample from diploid to triploid, which corresponds to the confirmation of two types of cultivars in the sample. The control used here is *Raphanus sativus.*

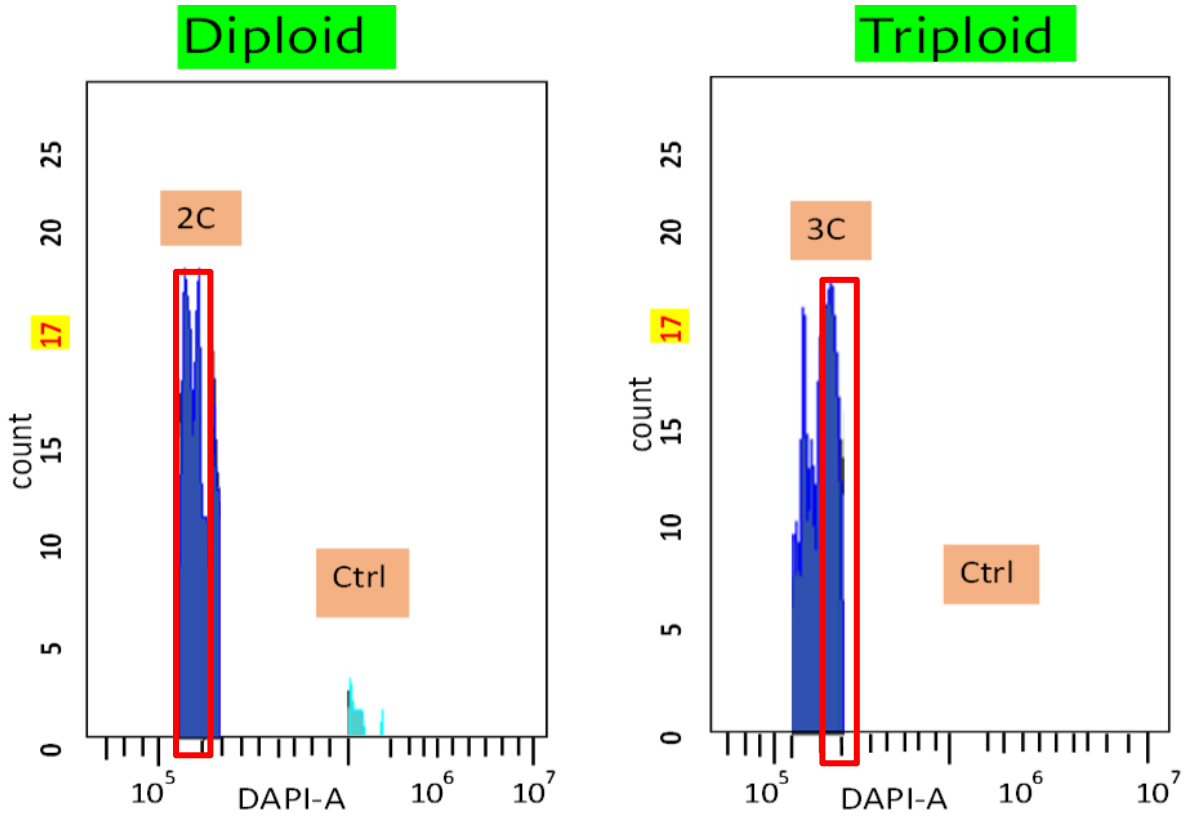


Figure 4.2: Flow cytometry results show changes in ploidy levels in diploid and triploid varieties of the Suver Haze cultivar.

4.5.3 Table 4.1:Identification of fungi (ITS rRNA) and bacteria (16S rRNA) microbiome associated with Suver haze diploid and triploid cultivars.

Sequence similarity was performed using NCBI-BLAST (National Center for Biotechnology Information – Basic Local Alignment Search Tool) which refers to the degree of resemblance or likeness between two nucleotide sequences. The sequence identity was assessed using the pre-established criteria to examining the BLAST results (FDS, 2023). Also, the genus name is kept only for the similarity between <90% and >70%.

1	Suver Haze Diploid	*Fusarium proliferatum*	100	ITS
2	Suver Haze Diploid	*Trichoderma pubescens*	100	ITS
3	Suver Haze Diploid	*Fusarium fujikuroi*	100	ITS
4	Suver Haze Diploid	*Fusarium annulatum*	100	ITS
5	Suver Haze Diploid	*Fusarium* sp.	87	ITS
6	Suver Haze Diploid	*Fusarium oxysporum*	99	ITS
7	Suver Haze Diploid	*Sordariomycetes* sp.	100	ITS
8	Suver Haze Diploid	*Fusarium graminearum*	99	ITS
9	Suver Haze Diploid	*Penicillium olsonii*	97	ITS
10	Suver Haze Diploid	*Penicillium brevicompactum*	97	ITS
11	Suver Haze Diploid	*Penicillium astrolabium*	97	ITS
12	Suver Haze Diploid	*Alternaria alternata*	97	ITS
13	Suver Haze Diploid	*Ustilago* sp.1	73	ITS

14	Suver Haze Diploid	*Hymenopellis* sp.1	75	ITS
15	Suver Haze Diploid	*Penicillium chrysogenum Wisconsin 54-1255*	91	ITS
16	Suver Haze Diploid	*Rhizopus* sp.1	79	ITS
17	Suver Haze Diploid	*Uromyces* sp.1	75	ITS
18	Suver Haze Diploid	*Talaromyces liani*	100	ITS
19	Suver Haze Diploid	*Alternaria alternata*	100	ITS
20	Suver Haze Diploid	*Penicillium coffeae DTO 273-A7*	99	ITS
21	Suver Haze Diploid	*Penicillium olsonii UBOCC-A-118177*	99	ITS
22	Suver Haze Diploid	*Penicillium astrolabium AUMC 11703*	99	ITS
23	Suver Haze Diploid	*Penicillium olsonii HF15181*	99	ITS
24	Suver Haze Diploid	*Fungal sp. Isolate ARIZ:C231*	98	ITS
25	Suver Haze Diploid	*Didymella pinodella*	100	ITS
26	Suver Haze Diploid	*Penicillium brevicompactum*	100	ITS
27	Suver Haze Diploid	*Penicillin astrolabium*	100	ITS
29	Suver Haze Diploid	*Sordariomycetes* sp.	97	ITS
31	Suver Haze Diploid	*Penicillium brevicompactum CBS626.72*	99	ITS
32	Suver Haze Diploid	*Alternaria alternata M18*	99	ITS
33	Suver Haze Diploid	*Fungal endophyte EUSF00841*	99	ITS
34	Suver Haze Diploid	*Cladosporium tenuissimum*	99	ITS
35	Suver Haze Diploid	*Cladosporium cladosporioides*	99	ITS
36	Suver Haze Diploid	*Cladosporium benschii*	99	ITS

37	Suver Haze Diploid	*Cladosporium colombiae*	99	ITS
38	Suver Haze Diploid	*Ectophoma multirostrata*	99	ITS
39	Suver Haze Diploid	*Cladosporium oxysporum*	99	ITS
40	Suver Haze Diploid	*Cladosporium kenpeggii*	99	ITS
41	Suver Haze Diploid	*Dothideomycetes sp.isolates*	99	ITS
42	Suver Haze Diploid	*Colletotrichum* sp.	99	ITS
43	Suver Haze Diploid	*Cladosporium allicinum*	99	ITS
44	Suver Haze Diploid	*Aspergillus* sp.1	76	ITS
45	Suver Haze Diploid	*Didymella pinodella*	97	ITS
46	Suver Haze Diploid	*Talaromyces liani*	89	ITS
47	Suver Haze Diploid	*Bacillus aryabhattai strain A6*	96	16S
48	Suver Haze Diploid	*Bacillus sp. (in:firmicutes) strain K6*	96	16S
49	Suver Haze Diploid	*Priestia flexa strain 25214*	95	16S
50	Suver Haze Diploid	*Bacterium strain BB24*	95	16S
51	Suver Haze Diploid	*Geobacillus stearothermophilus strain SCSGAB0115*	99	16S
52	Suver Haze Diploid	*Bacillus flexus strain F29*	98	16S
53	Suver Haze Diploid	*Fictibacillus barbaricus*	98	16S
54	Suver Haze Diploid	*Streptomyces fodineus*	99	16S
55	Suver Haze Diploid	*Actinomycetes* sp.	99	16S
56	Suver Haze Diploid	*Streptomyces cerasinus*	99	16S

57	Suver Haze Diploid	*Streptomyces spinosirectus*	99	16S
58	Suver Haze Diploid	*Streptomyces graminisoli*	99	16S
59	Suver Haze Diploid	*Bacillus megaterium*	96	16S
60	Suver Haze Diploid	*Streptomyces murinus*	99	16S
61	Suver Haze Diploid	*Streptomyces plumbidurans*	99	16S
62	Suver Haze Diploid	*Streptomyces puniciscabiei*	99	16S
63	Suver Haze Diploid	*Streptomyces similanensis*	99	16S
64	Suver Haze Diploid	*Streptomyces shenzhenensis*	99	16S
65	Suver Haze Diploid	*Streptomyces* sp.	99	16S
66	Suver Haze Diploid	*Bacillus licheniformis strain SR2EB5*	98	16S
68	Suver Haze Diploid	*Bacterium strain BBA7*	95	16S
69	Suver Haze Diploid	*Priestia megaterium strain NRPC-CRM-37*	95	16S
70	Suver Haze Diploid	*Bacillus thuringiensis strain GTG-5*	96	16S
71	Suver Haze Diploid	*Corynebacterium* sp.1	82	16S
72	Suver Haze Diploid	*Mycoplasma* sp.1	82	16S
73	Suver Haze Diploid	*Bacillus* sp.1	78	16S
74	Suver Haze Diploid	*E.coli plasmid p5 DNA strain*	87	16S
75	Suver Haze Diploid	*Mucilaginibacter* sp. *PAMC 26640*	87	16S
76	Suver Haze Diploid	*Ruficoccus* sp. *ZRK36*	87	16S
77	Suver Haze Diploid	*Burkholderia glumae strain YH67*	89	16S
78	Suver Haze Diploid	*Erwinia amylovora ATCC BAA-2158*	93	16S

79	Suver Haze Diploid	*Streptococcus* sp.1	76	16S
80	Suver Haze Diploid	*Agrobacterium* sp.1	76	16S
81	Suver Haze Diploid	*Bacillus megaterium strain Y18-01*	99	16S
82	Suver Haze Diploid	*Bacillus sp.(in:firmicutes)*	98	16S
83	Suver Haze Diploid	*Pseudomonas* sp.1	82	16S
84	Suver Haze Diploid	*Sphingomonas* sp.1	81	16S
85	Suver Haze Diploid	*Aeromonas* sp.	87	16S
86	Suver Haze Diploid	*Micromonospora* sp.	87	16S
87	Suver Haze Diploid	*Pseudomonas putida*	89	16S
88	Suver Haze Diploid	*Staphylococcus* sp.	87	16S
89	Suver Haze Diploid	*Mycolicibacterium* sp.	87	16S
90	Suver Haze Diploid	*Bilophila* sp.1	76	16S
91	Suver Haze Diploid	*Klebsiella* sp.1	76	16S
92	Suver Haze Diploid	*Alteromonas* sp.1	74	16S
93	Suver Haze Diploid	*Bacillus flexus strain T9-27*	99	16S
94	Suver Haze Diploid	*Pantoea agglomerans*	95	16S
95	Suver Haze Diploid	*Enterobacte ludwigii strain XF1*	95	16S
96	Suver Haze Diploid	*Enterobacter cloacae*	95	16S
97	Suver Haze Diploid	*Leclercia adecarboxylata strain*	95	16S
98	Suver Haze Diploid	*Pseudomonadota bacterium strain 8*	95	16S
99	Suver Haze Diploid	*Enterobacter roggenkampii*	95	16S

100	Suver Haze Diploid	*Enterobacter mori strain HBUAS71149*	95	16S
101	Suver Haze Diploid	*Erwinia* sp.	95	16S
102	Suver Haze Diploid	*Enterobacter* sp.	95	16S
103	Suver Haze Diploid	*Pantoea* sp.	95	16S
104	Suver Haze Diploid	*Enterobacter asburiae*	95	16S
105	Suver Haze Diploid	*Leclercia sp.*	95	16S
106	Suver Haze Diploid	*Enterobacter kobei*	95	16S
107	Suver Haze Diploid	*Enterobacter kobei*	95	16S
108	Suver Haze Diploid	*Cedecea* sp.	95	16S
109	Suver Haze Diploid	*Klebsiella quasipneumoniae*	95	16S
110	Suver Haze Triploid	*Chaetomium globosum SVUC2*	95	ITS
111	Suver Haze Triploid	*Ascomycota* sp. *LTS297*	94	ITS
112	Suver Haze Triploid	*Chaetomium tenue strain CN017C6*	94	ITS
113	Suver Haze Triploid	*Ascomycota* sp. *LTS317*	93	ITS
114	Suver Haze Triploid	*Chaetomium cruentum*	95	ITS
115	Suver Haze Triploid	*Chaetomium meridionalense strain COAD 3451*	94	ITS
116	Suver Haze Triploid	*Colletotrichum* sp. *PT1794*	94	ITS
117	Suver Haze Triploid	*Didymella pinodella*	99	ITS
118	Suver Haze Triploid	*P.coffeae strain DTO 273-A7*	99	ITS
120	Suver Haze Triploid	*Fungal sp. DT43 ITS*	99	ITS

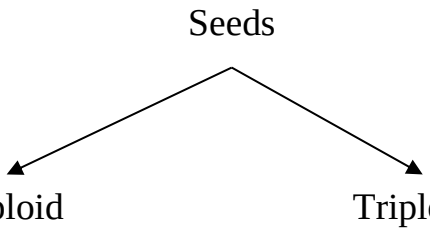
121	Suver Haze Triploid	*Penicillium olsonii DUCC5743*	99	ITS
122	Suver Haze Triploid	*Penicillium olsonii CBS 232.6*	99	ITS
123	Suver Haze Triploid	*Alternaria alternata M17*	99	ITS
124	Suver Haze Triploid	*Penicillium astrolabium strain AUMC 11703*	100	ITS
125	Suver Haze Triploid	*Priestia* sp.1	76	16S
126	Suver Haze Triploid	*Priestia* sp.2	76	16S
127	Suver Haze Triploid	*Neobacillus* sp.1	76	16S
128	Suver Haze Triploid	*Priestia* sp.3	76	16S
129	Suver Haze Triploid	*Bacillus* sp.1	77	16S
130	Suver Haze Triploid	*Bacillus* sp.2	77	16S
131	Suver Haze Triploid	*Niallia* sp.1	74	16S
132	Suver Haze Triploid	*Bacillus* sp.3	76	16S
133	Suver Haze Triploid	*Bacillus* sp.4	76	16S
134	Suver Haze Triploid	*Bacillus* sp.5	77	16S
135	Suver Haze Triploid	*Priestia* sp.4	76	16S
136	Suver Haze Triploid	*Schinkia* sp.1	77	16S
137	Suver Haze Triploid	*Bacillus* sp.6	77	16S
138	Suver Haze Triploid	*Bacillus cereus*	97	16S
139	Suver Haze Triploid	*Bacillus albus*	97	16S
140	Suver Haze Triploid	*Bacillus mycoides*	97	16S

141	Suver Haze Triploid	*Bacillus thuringiensis*	97	16S
142	Suver Haze Triploid	*Bacillus wiedmannii*	97	16S
143	Suver Haze Triploid	*Bacillus anthracis*	97	16S
144	Suver Haze Triploid	*Enterobacter cloacae*	97	16S
145	Suver Haze Triploid	*Enterobacter ludwigii*	99	16S
146	Suver Haze Triploid	*Bacillus proteolyticus*	97	16S
147	Suver Haze Triploid	*Bacillus mobilis*	97	16S
148	Suver Haze Triploid	*Bacillus* sp. *KAL*	97	16S
149	Suver Haze Triploid	*Bacillus bingmayongensis*	97	16S
150	Suver Haze Triploid	*Bacillus pseudomycoides*	97	16S
151	Suver Haze Triploid	*Bacillus paramycoides*	97	16S
152	Suver Haze Triploid	*Bacillus nitratireducens*	99	16S
153	Suver Haze Triploid	*Bacillus tropicus*	99	16S

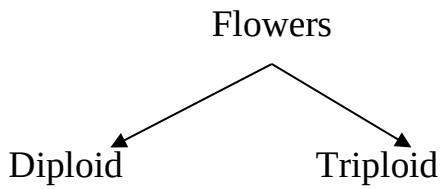
4.5.4 Identification of fungi microbial communities using Universal ITS primers

The identification was done based on different organs – seeds, flowers, and leaves (One-way ANOVA were performed with three replicates; $p < 0.05$). It was observed that diversity is unchanged in seeds of diploid and triploid whereas in flowers and leaves - diploid plants were more diverse than triploids. It also showed that as plants progress through their growth stages, an increase in the presence of pathogenic organisms like *Fusarium* and *Cladosporium* were observed in the leaves and flowers of diploids, as opposed to the seeds.

The reduction in diversity among triploid plants implies a decrease in the overall stability of their ecological community. A decrease in stability means that the community may be more vulnerable to disruptions, such as disease outbreaks or environmental changes, as it has fewer species to compensate for potential losses or changes in the ecosystem.



Penicillium brevicompactum	*Penicillium brevicompactum*
Penicillium coffeae	*Penicillium coffeae*
Penicillium olsonii	*Penicillium olsonii*
Penicillium astrolabium	*Penicillium astrolabium*
Alternaria alternata	*Alternaria alternata*
Didymella pinodella	*Didymella pinodella*
Talaromyces liani	
Sordariomycetes sp.	



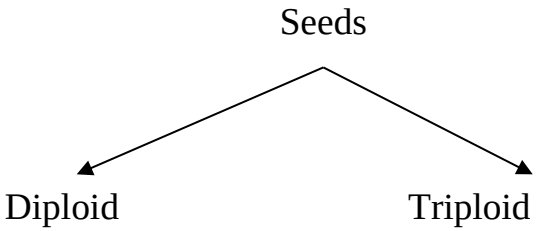
Fusarium proliferatum	*Chaetomium globosum*
Fusarium fujikuroi	*Chaetomium cruentum*
Fusarium annulatum	*Chaetomium meridionalense*
Fusarium hechiense	*Chaetomium tenue*
Penicillium olsonii	*Colletotrichum* sp.
Penicillium brevicompactum	*Ascomycota* sp.
Penicillium chrysogenum	
Penicillium astrolabium	
Alternaria alternata	
Didymella pinodella	
Talaromyces liani	
Hymenopellis sp.1	
Uromyces sp**.**	
Rhizopus sp.1	
Trichoderma pubescens	
Sordariomycetes sp.	
Ustilago sp. 1	

Diploid	Triploid
Cladosporium tenuissimum	*Aspergillus* sp. 1
Cladosporium oxysporum	*Ascomycota*
Cladosporium ladosporioides	*Alternaria alternata*
Cladosporium kenpeggii	*Leotimycetes*
Cladosporium benschii	
Cladosporium colombiae	
Cladosporium allicinum	
Colletotrichum sp.	
Ectophoma multirostrata	
Dothideomycetes sp. *isolates*	
Fusarium proliferatum	
Fusarium fujikuroi	
Fusarium annulatum	
Fusarium oxysporum	
Fusarium graminearum	

Figure 4.3 2:Diversity of fungal microbial communities isolated from universal ITS primers.

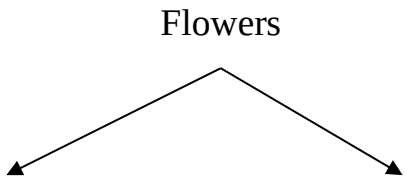
4.5.5 Identification of culturable bacteria using 16S rRNA primers

The identification was done based on different organs – seeds, flowers, and leaves. It was observed that bacterial diversity was more prominent than fungal diversity in both diploid and triploid cultivars.

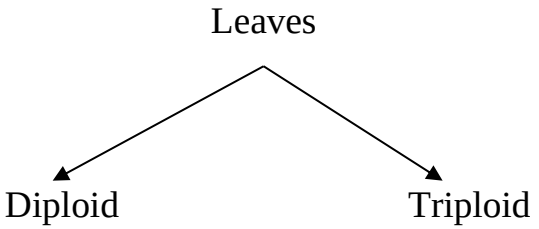


Bacillus sp. *(in: firmicutes)*	*Bacillus* sp. *(in: firmicutes)*
Bacillus megaterium	*Bacillus cereus*
Bacillus flexus	*Bacillus albus*
Bacillus aryabhattai	*Bacillus proteolyticus*
Bacillus thuringiensis	*Bacillus mycoides*
Aeromonas veronii	*Bacillus mobilis*
Mycoplasma sp.1	*Bacillus thuringiensis*
Micromonospora sp.	*Bacillus*.sp,
Mucilaginibacter sp.	*Bacillus wiedmannii*
Ruficoccus sp.	*Bacillus bingmayongensis*
Staphylococcus haemolyticus	*Bacillus anthracis*
Burkholderia glumae	*Bacillus pseudomycoides*
Mycolicibacterium smegmatis	*Enterobacter cloacae*
Erwinia amylovora	*Bacillus paramycoides*
Bilophila sp.	*Enterobacter ludwigii*
Streptococcus sp. 1	*Bacillus nitratireducens*
Klebsiella sp.1	*Bacillus tropicus*
Agrobacterium sp. 1	*Bacillus* sp.3
Alteromonas sp. 1	
Sphingomonas sp.	

Corynebacterium sp. 1	
Pseudomonas sp.	
Pseudomonas putida	



Diploid	Triploid
Bacillus aryabhattai	*Bacillus aryabhattai*
Bacillus megaterium	*Bacillus megaterium*
Bacillus sp. *(in: firmicutes)*	*Bacillus* sp. *(in: firmicutes)*
Bacillus flexus	*Bacillus flexus*
Bacillus thuringiensis	*Neobacillus* sp. 1
Geobacillus stearothermophilus	*Priestia* sp. 4
Bacillus licheniformis	*Bacillus* sp. 1
Fictibacillus barbaricus	*Schinkia* sp. 1
Streptomyces sp.	*Bacillus* sp. 2
Streptomyces fodineus	*Bacillus* sp. 3
Streptomyces shenzhenensis	*Niallia* sp. 1
Actinomycetes sp.	*Bacillus* sp. 3
Streptomyces similanensis	
Streptomyces cerasinus	
Streptomyces puniciscabiei	
Streptomyces spinosirectus	
Streptomyces plumbidurans	
Streptomyces graminisoli	
Streptomyces murinus	



Pantoea agglomerans	*Bacillus* sp. 2
Enterobacter sp.	*Bacillus* sp. 3
Enterobacter ludwigii	*Bacillus cereus*
Pantoea sp.	*Bacillus albus*
Enterobacter cloacae	*Bacillus proteolyticus*
Enterobacter asburiae	*Niallia* sp. 1
Leclercia adecarboxylata	
Leclercia sp.	
Pseudomonadota bacterium	
Enterobacter kobei	
Enterobacter roggenkampii	
Enterobacter mori	
Cedecea sp.	
Erwinia sp.	
Klebsiella quasipneumoniae	

Figure 4.4 3:Number of bacterial microbial communities isolated from Universal 16S rRNA primers.

4.5.6 Analyzing Shannon Weiner Diversity Index

It was shown that regarding relative abundance in fungal colonies, *Penicillium, Fusarium,* and *Cladosporium* genera dominated diploid cultivars, whereas *Penicillium* and *Chaetomium* were dominant in triploid cultivars, with *Alternaria* and *Ascomycota* as codominant taxa. The Shannon Weiner diversity index was 2.29 for diploids and 1.9 for triploids.

In terms of the relative abundance in bacterial colonies, *Bacillus*, *Streptomyces,* and *Enterobacter* were the dominant genera in diploid cultivars, whereas *Bacillus* was dominant with 85% abundance in the triploid cultivar of Suver Haze. The index was found to be 2.65 and 0.53 for diploids and triploids respectively.

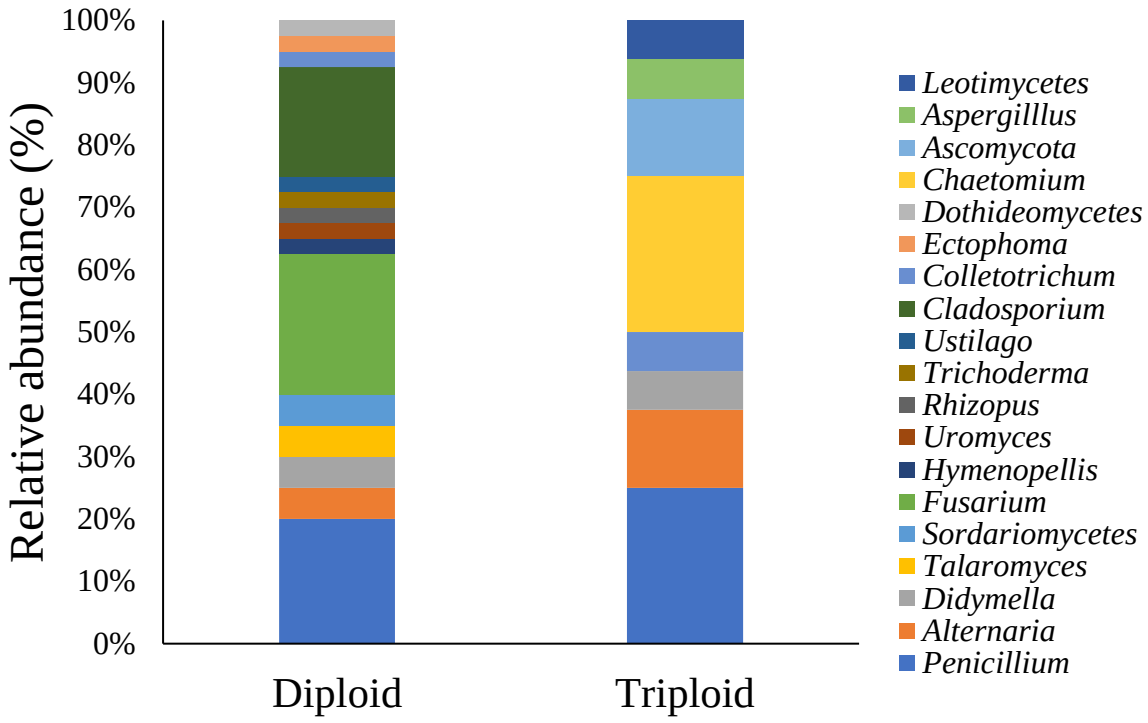


Figure 4.5 : Relative abundance of fungal genera present in diploid and triploid plants and their Shannon Weiner Diversity Index: H = 2.29 for diploids and H = 1.9 for triploids.

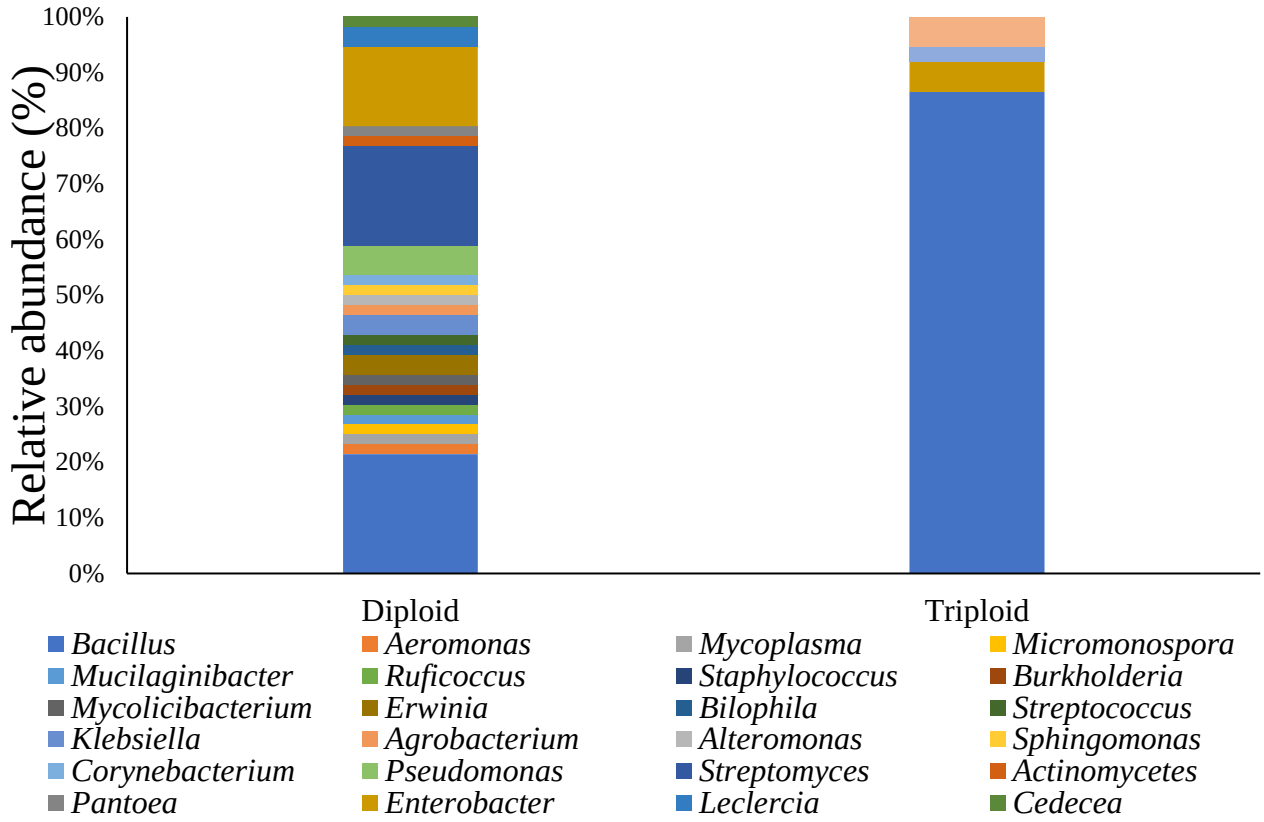


Figure 4.6: The relative abundance of bacterial genera present in diploid and triploid plants is H = 2.65 for diploid and H = 0.53 for triploid.

4.6 Discussion

The development and applications of molecular markers to hemp breeding dates to mid-1990s (Mandolino & Carboni, 2004). Active research is carried out in the field of identification and characterization of genes that help in understanding the phylogenetic relationships between different traits of hemp. MADC6 (Male-associated DNA from *Cannabis sativa*) is a SCAR marker that stands for Sequence Characterized Amplified Region used in molecular genetics for sex determination in hemp which is monomorphic in functionality (Borin et al., 2021; Mendel et al., 2016; Törjek et al., n.d.). SEUSS (SEU transcription regulator) markers are chemotype markers used in hemp for determining the composition of different cannabinoids and hence is used as identification of floral development genes (Gilchrist et al., 2023). Both markers are unique and were used in this study to characterize phylogenetically the cannabis hosts. The phylogenetic tree helped to distinguish between diploid and triploid varieties of the same Haze cultivars.

Sex determination in hemp is a crucial aspect of its reproductive biology. Hemp plants are mainly dioecious and the sex of the plant determines its reproductive role in plant fertility and capacity to producing seeds However, modern cannabis science's interest in the production of feminized (seedless) plants is linked to the growing interest in the production and consumption of cannabinoids (Flajšman et al., 2021). The primary compounds of interest in hemp, cannabinoids like THC and CBD, are mainly found on the trichomes of the female flowers, making female plants more desirable for cultivation in medicinal and recreational uses (Flajšman et al., 2021; Spitzer-Rimon et al., 2022; Vannette, 2020). Floral developmental genes play a vital role in regulating the growth and development of flowers in hemp. These genes control the formation of reproductive organs and influence the overall structure of the flowers (Apicella et al., 2021). Understanding these genes is crucial for manipulating the plant's traits and optimizing the production of desired compounds.

Polyploidy can be traced back to the origin of major plant lineages, including angiosperms, core eudicots, monocots, orchids, grasses, composites, and legumes that suggests an important role in promoting phenotypic diversity (Van De Peer et al., 2017; Van de Peer et al., 2021). *Cannabis sativa* L. is basically a diploid plant with many different traits and varieties. Triploid plants are basically formed by crossing one haploid chromosome with one diploid

chromosomes, which in this case was done by autoploidy. Different ploidy plants have shown to affect differently under varying conditions of physical, chemical, and biological stress and seems to perform better than diploids under different environmental conditions. Two genotypes – one normal and other triploid hemp were used to understand the genetic complexity involved in host-microbe interactions and in their evolutionary processes. This is referred to as "Hologenome concept" which refers to the collective genetic material of a host plant along with diverse array of microorganisms associated with it (Bordenstein and Theis, 2015). Here two hologenomes are studies which helped us to understand the microbial diversity found in different plants and how these interactions affect other phenotypic and secondary metabolite characteristics together (Taghinasab and Jabaji 2020).

There was found to be loss of diversity in triploid plants as compared to diploid plants under optimal *in vitro* and phytotron conditions. Diversity is expected to reduce under any kind of biotic and abiotic stresses (Vujanovic & Brisson, 2002) where plants microbiome modifies dynamics and modulate diversity vs. abundance for the benefit of host adaptability vs. survival, respectively (Vujanovic et al. 2019). However, in this study plants were nor exposed to any particular no physical, chemical, or biological stress. However, the changes in Suha Haze genome from diploid to triploid coincides with lowering microbial diversity, In triploid, the low microbial diversity might signify reduced plant adaptability. It might not be able to survive or produce secondary metabolites in higher amounts. They are expected to be more vulnerable to environmental factors and might not survive in changing conditions of temperatures, water, humidity etc. It is interesting that wheat polyploidization towards hexaploid cultivars resulted in an increasing reduction in taxonomical diversity of microbiome associated with reduced plant resistance (Gruet et al. 2022; Vujanovic and Germida 2017). More research is needed to further authenticate this concept of plant resistance including adaptability and survivability of triploid hemp plants and associated microbiome under field settings.

4.7 Conclusions

The presence of a wide variety of microbiomes in both diploid and triploid cultivars of the hemp plant has significant implications for the plant's health, growth, and productivity (Barnett et al., 2020; Sattler et al., 2016; Wipf and Coleman-Derr, 2021). In this study, the

microbial communities, including bacteria (*Bacillus*, *Enterobacter*, and *Streptomyces)*, *were* dominant in diploid and *Bacillus* dominant in triploid, whereas in fungi (*Fusarium*, *Penicillium*, and *Cladosporium)* were dominant in diploid and *Chaetomium* and *Penicillium* dominant in triploid plants. While the presence of *Fusarium* spp. in diploid hosts increases the risk for plant health, the low microbial diversity index of the triploid variety could negatively affect the plant. (Kim et al., 2022; Wang et al., 2022; Zverev et al., 2021). The core microbiome communities identified in this study can be further investigated to improve the cultivation of this crop. Mycotoxigenic fungi like *Fusarium* and *Cladosporium* pose safety and quality concerns for hemp products, whereas bacteria like *Bacillus* and *Streptomyces* have concerns related to the safety of human health (Vujanovic et al., 2020).

This research has the potential to shed light on the distinctive relationships that the diploid and triploid Suver Haze plants share with their microbial residents. By focusing on their microbiomes, we can anticipate discovering unique patterns and interactions that may influence homeostasis versus dysbiosis, to the benefit of the phenotypic traits and overall adaptability of these plants. These results may provide valuable insights not only to the field of plant biology and microbiology but also to the broader contexts of agriculture, breeding efforts, and sustainability. The knowledge gained from profiling these microbiomes may serve as a cornerstone for refining cultivation practices, optimizing yields, and unlocking the full potential of Suver Haze hemp plants in various applications.

4.8 Connection to the next study

The microbial communities identified in this study hold great potential for applications across diploid and triploid varieties of Suver Haze cultivars to significantly influence both the phenotypic traits of the plants and the production of secondary metabolites, which are covered in studies 3 and 4, respectively.

5 Study 3 : Assess the growth promoting potential of seed endophyte associated with diploid and triploid cultivars under *in vitro* and phytotron conditions

5.1 Abstract

Endophytes offer a promising avenue for sustainable agriculture by enhancing crop productivity, reducing the use of synthetic fertilizers and pesticide inputs, and promoting environmental stewardship. In this study, the effect of seed endophytes on diploid and triploid cultivars of Suver haze was assessed. *In vitro* germination studies, along with vegetative growth and flowering studies in phytotron, were performed. The major phenotypic characteristics were assessed, including plant height, length and width of leaves, photosynthesis, days of flowering and senescence, the number of inflorescences, and internodal distances. Statistical analyses were performed using the obtained data and found that SEn played a significant role in influencing the phenotypic characteristics of this cultivar varieties.

5.2 Introduction

The cultivation of hemp varieties, specifically the Suver Haze cultivar, has gained considerable attention due to its diverse characteristic properties (Park et al., 2023; Veazie et al., 2021) after its legalisation. In recent years, there has been a growing interest in exploring the influence of endophytic microorganisms on plant growth and development (Compant et al., 2011; Kusari et al., 2013; Qadri et al., 2013). Endophyte, microorganisms residing within plant tissues without causing harm, have demonstrated their potential to enhance plant growth and stress tolerance (Barnett et al., 2020; Compant et al., 2021; Scott et al., 2018; Taghinasab and Jabaji, 2020).

This study focuses on assessing the growth-promoting potential of seed endophytes (SEn) associated with two distinct ploidy levels: diploid and triploid varieties of the Suver Haze cultivar. Diploid and triploid hemp plants exhibit differences in their chromosomes that may influence the composition and functioning of their associated endophytic communities.

The investigation takes place in controlled environments, combining *in vitro* and phytotron conditions. The use of *in vitro* techniques allows for a controlled and sterile setting to explore the direct effects of seed endophytes on plant growth parameters. Meanwhile, phytotron conditions provide a more ecologically relevant environment, facilitating the examination of SEn-plant interactions under conditions that mimic the natural growth environment of the Suver Haze cultivar (Darby et al., n.d.).

5.3 Hypothesis

In this study, we put forth the hypothesis that endophytic microbial communities inhabiting diploid and triploid cultivars possess the capacity to exert distinct influences on growth promotion and the modification of phenotypic traits. These effects may arise from alterations in the organization of their respective microbiomes. We anticipate, and as found in study 1, that seed endophyte treatments had more beneficial effect on the overall health of a plant. Therefore, these diploid and triploid cannabis hosts, due to their genetic differences, might respond differently in terms of protocooperation with their respective endophytic communities, which in turn will lead to differential plant growth promotion and shifts in phenotypic traits. This investigation seeks to uncover the relationship between microbial composition, genetic ploidy, and their combined impact on plant development and traits.

5.4 Materials and Methods

The experimental design used in this study was same as that used for study 1 (refer section 3.4.1) as well as inoculum preparation were performed for only seed endophyte here (refer section 3.4.2). To ensure stable inoculants, the variability of seeds was reduced to minimum by using same stock of seeds which are from the same source (Company – Oregon, USA), harvested in the same year and at the same time and from same cultivar of hemp. These seeds were packed in aluminium foil sealed bags and kept at 4 ºC during the full tenure of the experiment (8-10 months). However few more parameters were taken, which were further divided into the following categories:

5.4.1 Plant Height

The plant's height was measured using a ruler on three randomly selected replicates every week for 10 weeks, and a one-way analysis of variance (ANOVA) technique followed by post hoc Tukey honest significant difference (HSD) and least significant difference (LSD) tests were applied to determine the statistical significance at $P \leq 0.05$ using SPSS (IBM SPSS statistic 22) (Burgel et al., 2020; Kumari and Vujanovic, 2020).

5.4.2 Leaves

The length and width of leaves were calculated using a ruler on 3 randomly selected replicates at nodes 2, 4, 6, and 8 and the one-way analysis of variance (ANOVA) technique, followed by post hoc Tukey honest significant difference (HSD) and least significant difference (LSD) tests, to determine the statistical significance at $P \leq 0.05$ using SPSS (IBM SPSS statistic 22) (Ding et al., 2020; Raj et al., 2021). Measurements of nodes 2, 4, 6, and 8 were done in weeks 2, 5, 7, and 10, respectively, and a graph was plotted accordingly.

5.4.3 Flowering

Other than the above parameters, the days of flowering and senescence were calculated, by assessing the number of days before plant flowering, maturation, and senescence. The indoor results under controlled conditions are often good indicators of the cultivar's effectiveness when grown outdoors or under field conditions. The estimated marginal means of days flowering and senescence were calculated on three randomly selected replicates, and the one-way analysis of variance (ANOVA) technique was followed by post hoc Tukey honest significant difference (HSD) and least significant difference (LSD) tests to determine the statistical significance at $P \leq 0.05$ using SPSS (IBM SPSS statistic 22). Also, the estimated means of the number of axillary branches (n=3; taken every week when flowering started until plant reached senescence), the number of inflorescences (calculated manually at a matured

flower), and the internodal distances were also calculated (every week when flowering started and reached senescence) (Punja & Holmes, 2020; Raj et al., 2021; Schrader et al., 2021).

5.4.4 Photosynthesis and Stomatal Imprints

Measurement of photosynthesis by chlorophyll- fluorometer is a common method used to assess the health of every plant, including hemp. It involves measuring the maximum efficiency of photosystem II (PSII) photochemistry in dark-adapted leaves. To determine this ratio, a weak modulated measuring beam is applied to determine the minimal fluorescence yield in a dark-adapted leaf (F0), and a saturating flash is then superimposed to induce the maximal yield of chlorophyll fluorescence (Fm) (Banks, 2018; Hubbard et al., 2014 (*Opti-Sciences, Inc*, n.d.)). An Fv/Fm value in the range of 0.79 to 0.84 is considered optimal for many plant species, with lowered or higher values indicating plant stress (3 replicates of each treatment were used).

Transparent tape is a traditional method to measure stomatal size and density. The tape allowed for making the impression and viewing stomata under a microscope for completing the analyses (Millstead et al., 2020).

5.4.5 Shoot and Root Biomass

When plants reached senescence, fresh shoot weights were measured for randomly selected plants (n=3) and then those plants were kept in a desiccator for 3-4 days until shoots were fully dried and free water is fully removed from it. Dry shoot weights were then measured for those plants, and regression analysis was set up to establish a relationship between the weight of the shoot and the diameter of the stem. The same was repeated for fresh and dry roots (S. H. Kim et al., 2021; Kumari and Vujanovic, 2020). A desiccator is a sealable enclosure containing Silica gel desiccants used for preserving moisture-sensitive items. These items can include chemicals that are hygroscopic or react with water. Desiccators come in various materials, including glass, polypropylene, polycarbonate, and acrylic (Shaw, 1959).

5.5 Results

The study results illustrate pronounced alterations in Suver Haze diploid and triploid host phenotypic characteristics induced by the introduction of whole seed endophytes. These modifications encompass significant host attributes, including plant height, leaf dimensions (length and width), flowering and senescence duration, inflorescence and axillary branch count, stomatal density, root and shoot biomass, and more.

5.5.1 Vertical Growth Patterns of Plants

It was observed in Figure 5.1 (A) that untreated diploid plants were taller than seed endophyte treated diploid plants in all weeks from 3 till 10. However, opposite characteristics were observed in the triploid host in Figure 5.1 (B), where untreated triploid plants were shorter as compared to SEn treated plants. It was also seen that the average triploid plants were taller (~120 cm) than diploid plants (~90-100 cm). These changes are due to the genetic differences in the plant that influence their response to treatments (Darby et al., n.d.; Stegmeier, n.d.).

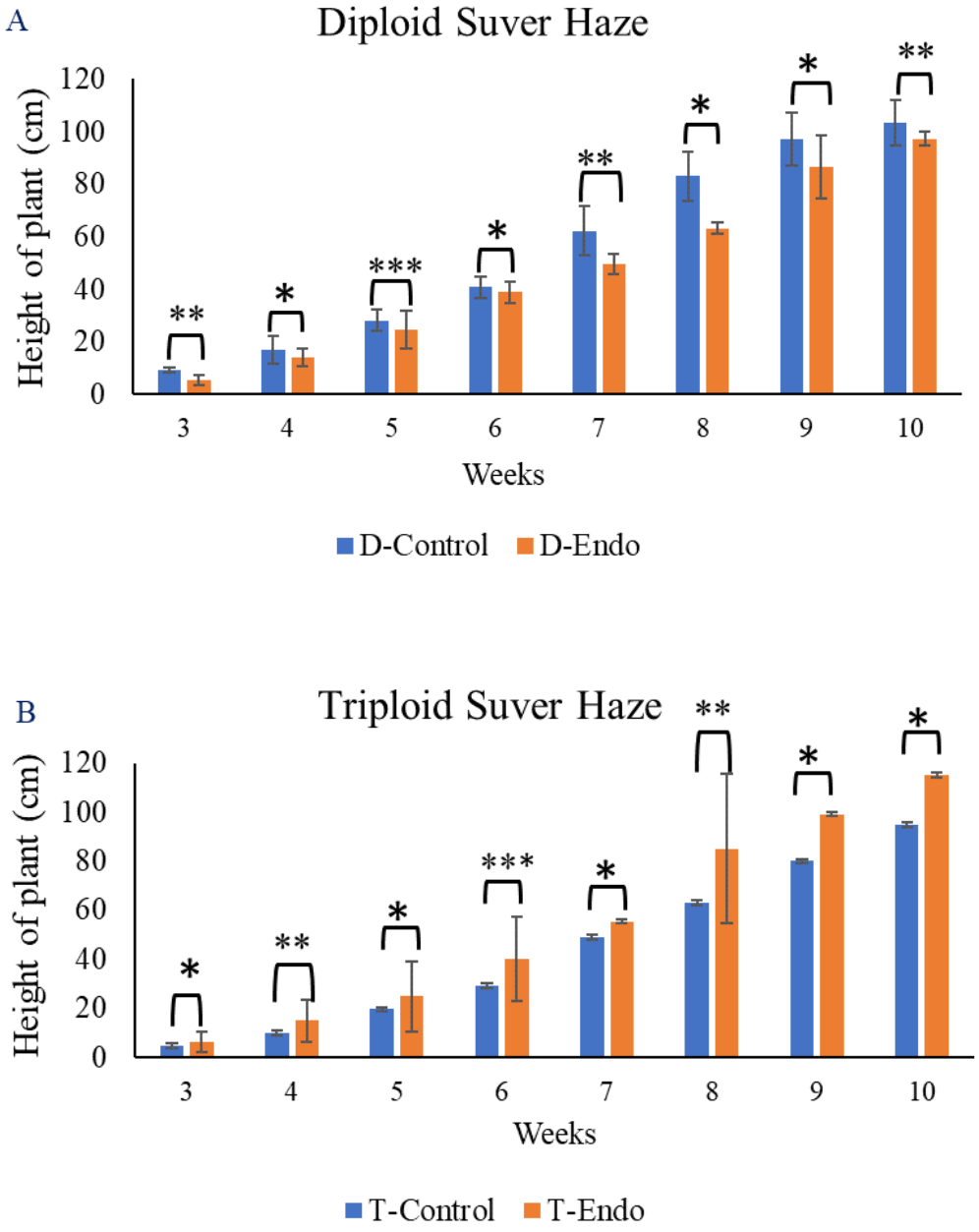


Figure 5.1: The height of the plant is taken every week until 10 weeks in untreated diploid and SEn-treated diploid (A), as well as untreated triploid and SEn-treated triploid (B). D-Control: diploid control; D-Endo: diploid SEn; T-Control: triploid control; T-Endo: triploid SEn. Data are means and standard errors of three replicates (Two-way ANOVA with LSD test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

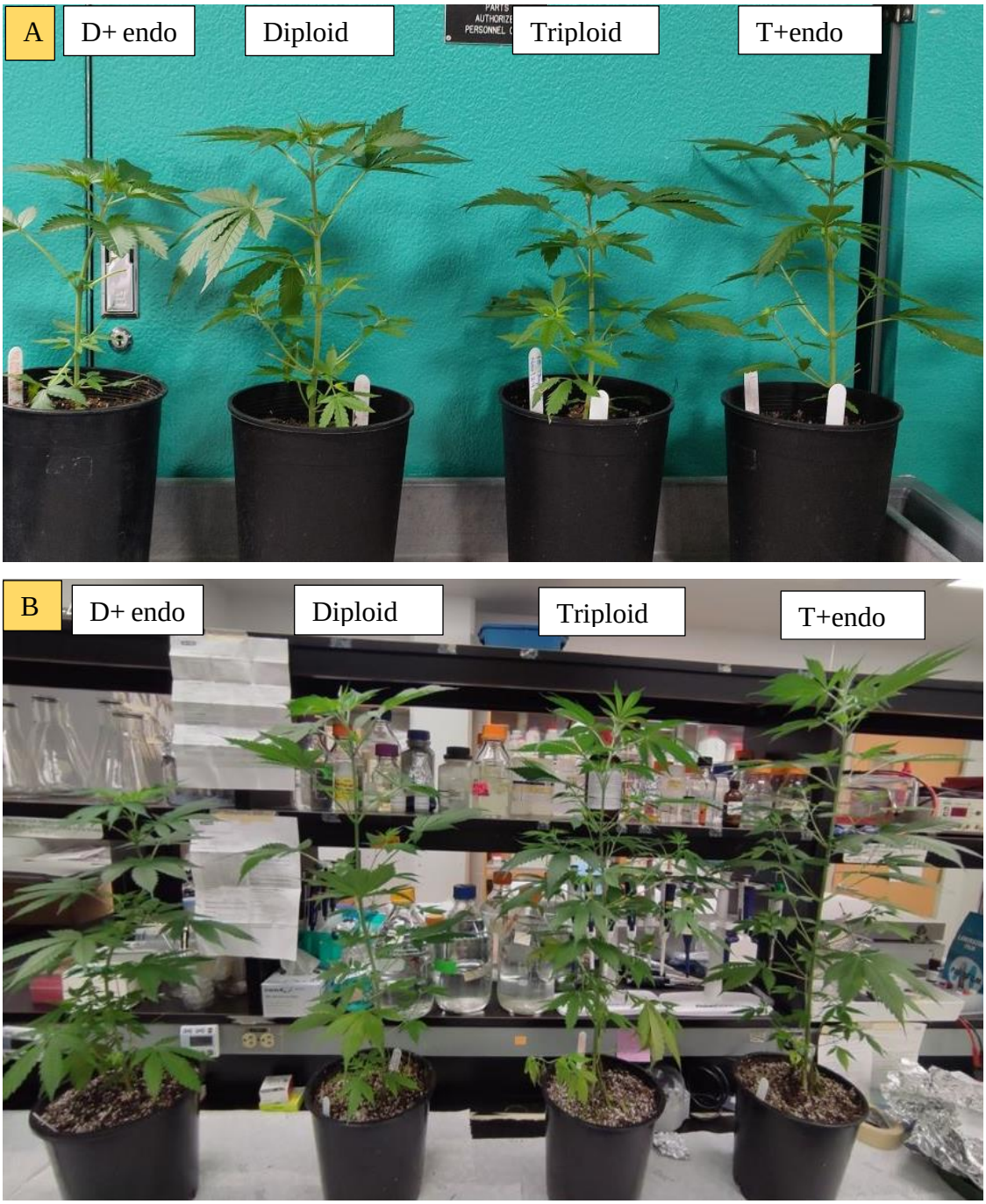


Figure 5.2: Picture showing plants growing at days 35 (A) and 50 (B): from left to right: diploid seed endophytes; diploid control-triploid seed endophytes.

5.5.2 Leaf Dimensions

The length and width of leaves were measured in diploid and triploid traits of Suver haze cultivar on nodes 2, 4, 6 and 8. It was found that node 2 of the untreated diploid plant showed increased length of leaves as compared to the SEn -treated plant, whereas at nodes 4, 6, and 8, SEn-treated diploid plants have increased length compared to the untreated control. In the case of triploids, at nodes 2, 6, and 8, seed endophyte-treated plant showed increased length as compared to the untreated plant; however, at node 4, it was exactly opposite. Overall, the triploid plants showed longer leaves (~15 cm) compared with diploids (~10 cm).

In diploid plants treated with seed endophytes, leaf width is greater compared to untreated diploid plants. Conversely, in triploid plants at the 4th, 6th, and 8th nodes, untreated triploid leaves exhibit a broader size in comparison to seed endophyte-treated triploids. The exception was registered for the second node. A broader expanse of plant leaves is anticipated to provide a larger surface area conducive to increased photosynthetic activity.

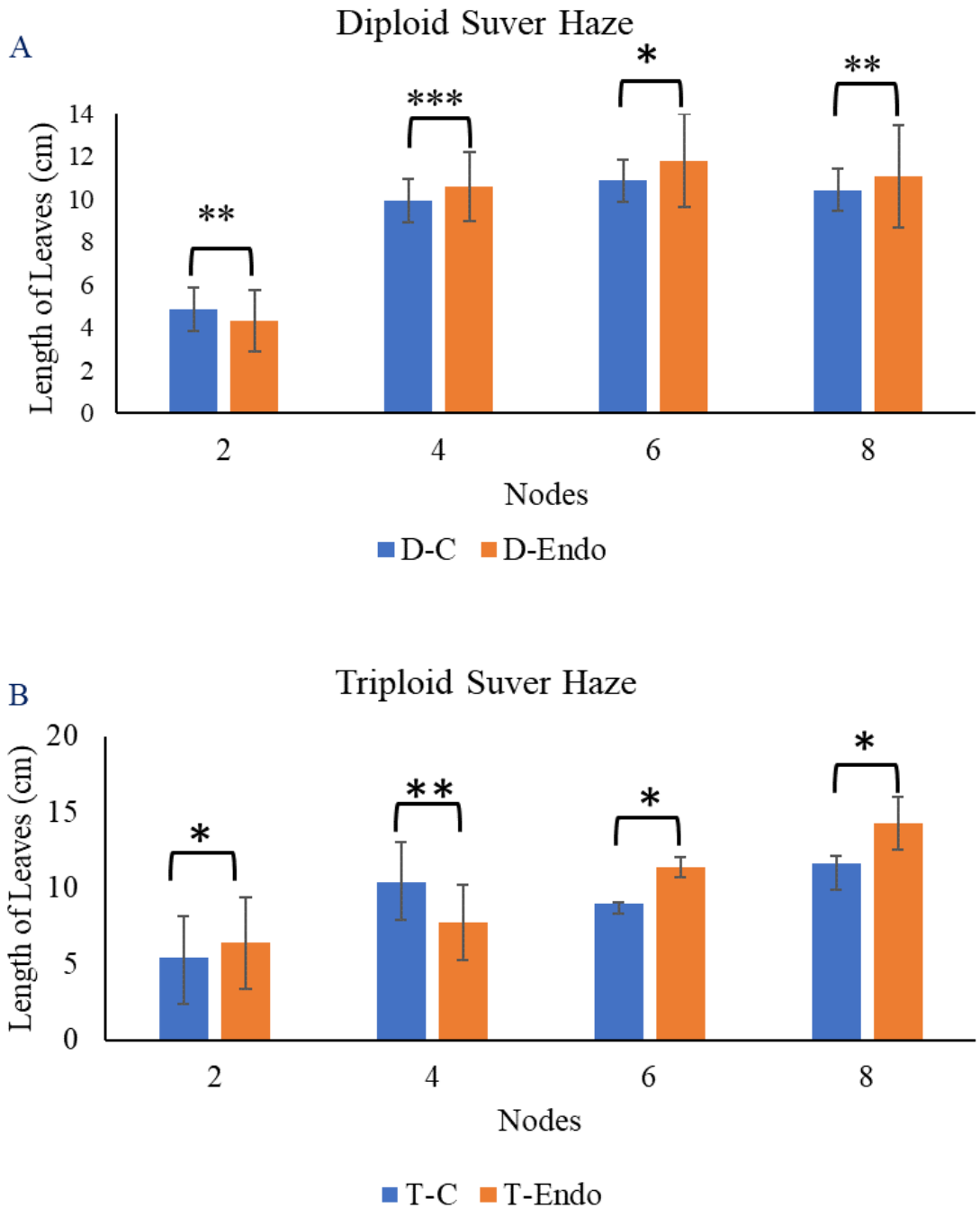


Figure 5.3: Length of leaves at different nodes in diploid (A) and triploid (B) plants during the vegetative phase of their life cycle. Measurements were done on weeks 2, 5, 7, and 10 for nodes 2, 4, 6, and 8. D-C-diploid control; D-Endo-diploid SEn; T-C-triploid control; T-Endo-triploid SEn. Data are means and standard errors of three replicates (Two-way ANOVA with LSD test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

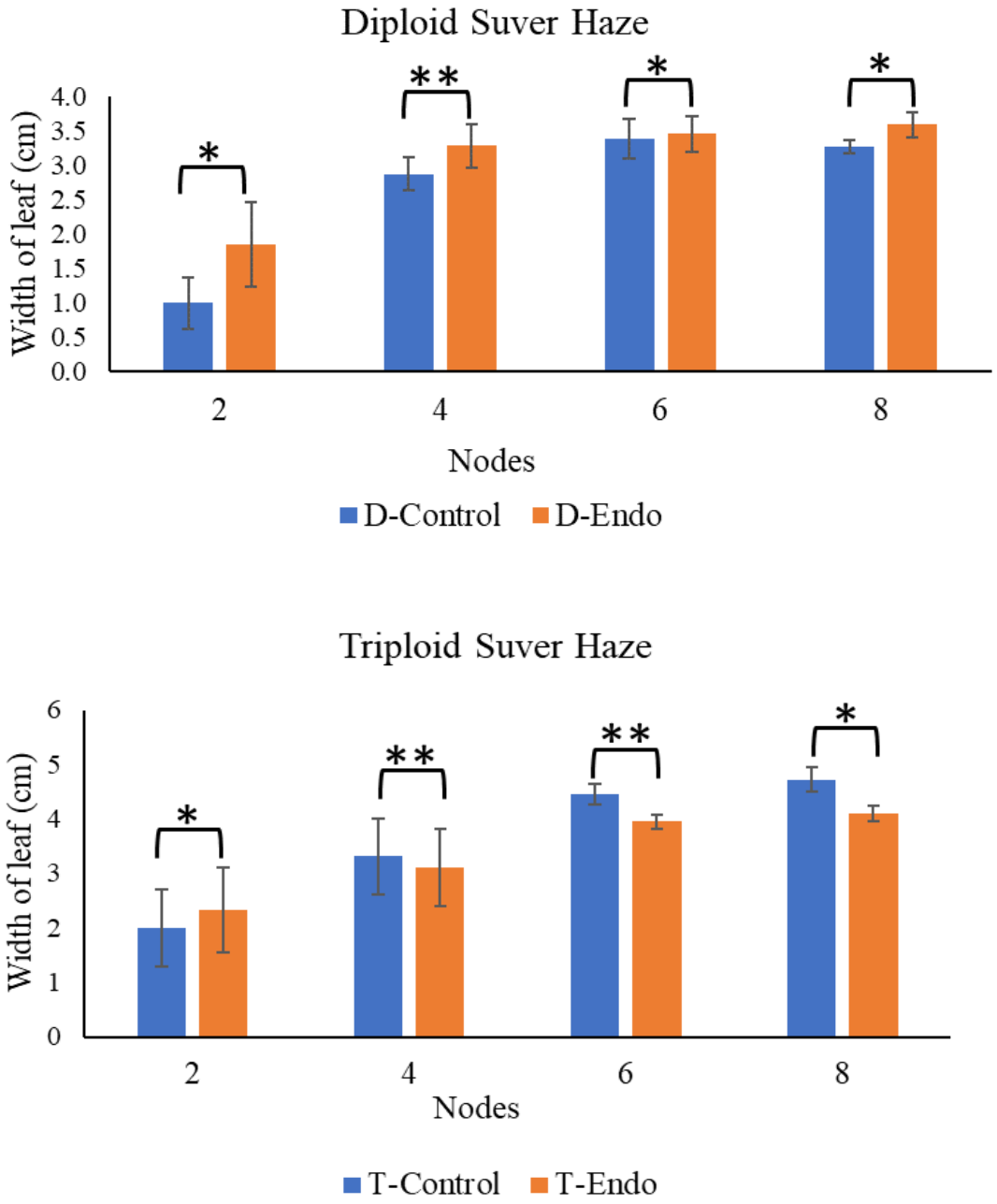


Figure 5.4: The width of leaves at different nodes in diploid and triploid plants during the vegetative phase of their life cycle. Measurements were done on weeks 2, 5, 7, and 10 for nodes 2, 4, 6, and 8. D-C: diploid control; D-Endo: diploid SEn; T-C: triploid control; T-Endo: triploid SEn. Data are means and standard errors of three replicates (Two-way ANOVA with LSD test, * $p < 0.05$, ** $p < 0.01$).

Figure 5.54: A pictorial representation of the leaf shows different widths with the help of a ruler at the 4th node of the plant.

5.5.3 Timelines of Flowering and Senescence

The findings revealed negative disparity between flowering durations between control (~100 days) and seed endophytes-treated diploid plants (~ 96 days); however, in triploids, the endophytes-treated plants showed reduced flowering time (~ 85 days) when compared to their untreated counterparts (~100 days).

Days of senescence were increased in seed endophytes-treated diploids (~155 days) and triploids (~180 days) as compared to their control counterparts (~150 days). Overall, both diploid and triploid plants treated with seed endophytes experienced longer growth phenophases as compared to untreated ones.

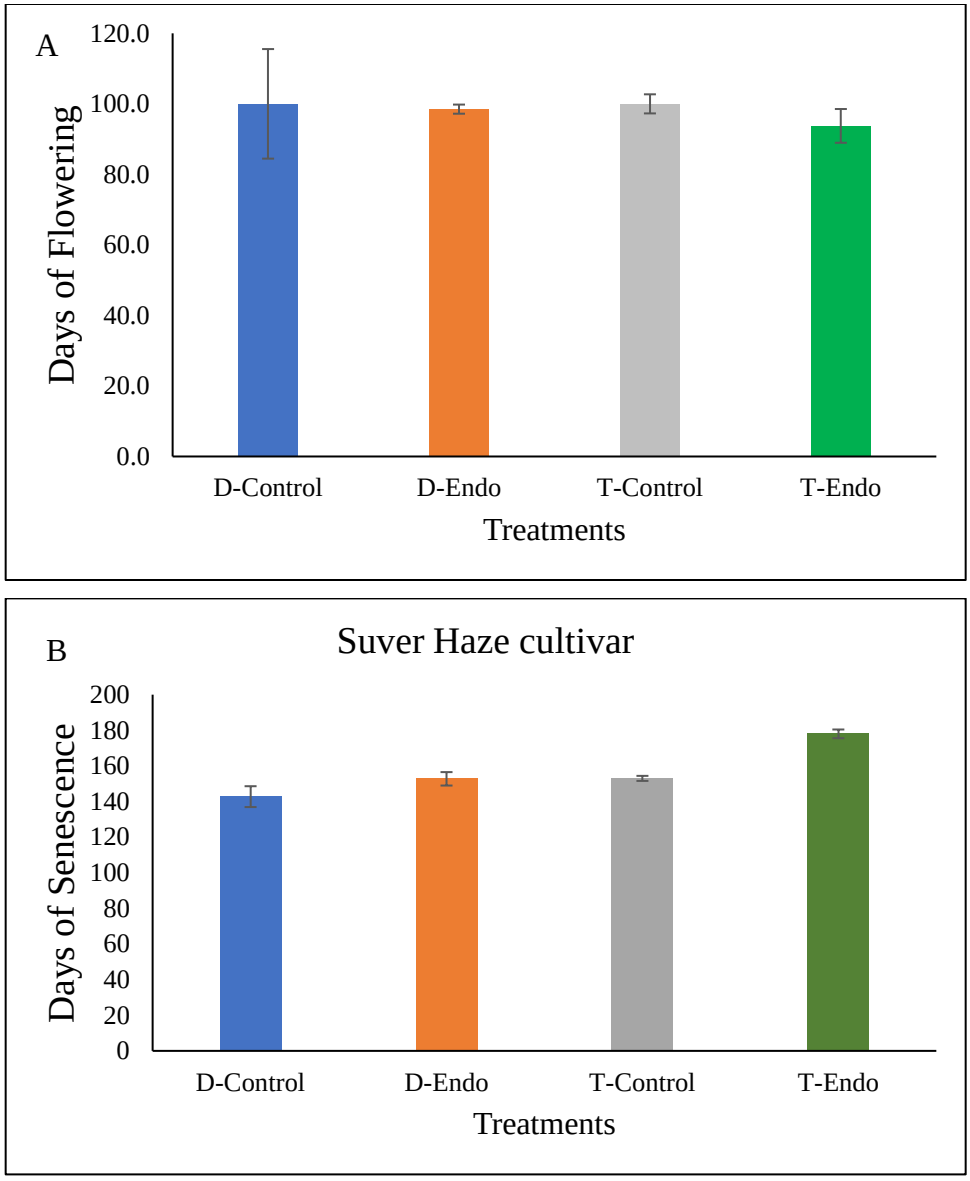


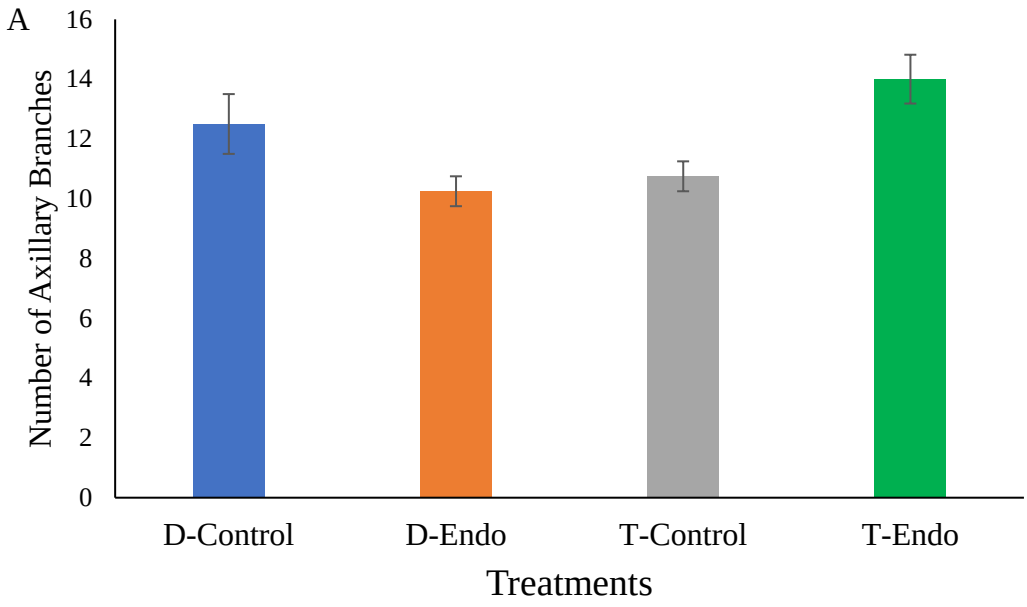
Figure 5.6: Days of flowering (A) and senescence (B) were calculated for different treatments of plants. D-Control: diploid control; D-Endo: diploid SEn; T-Control: triploid control; T-Endo: triploid SEn. Data are means and standard errors of three replicates (One-way ANOVA, $p < 0.05$).

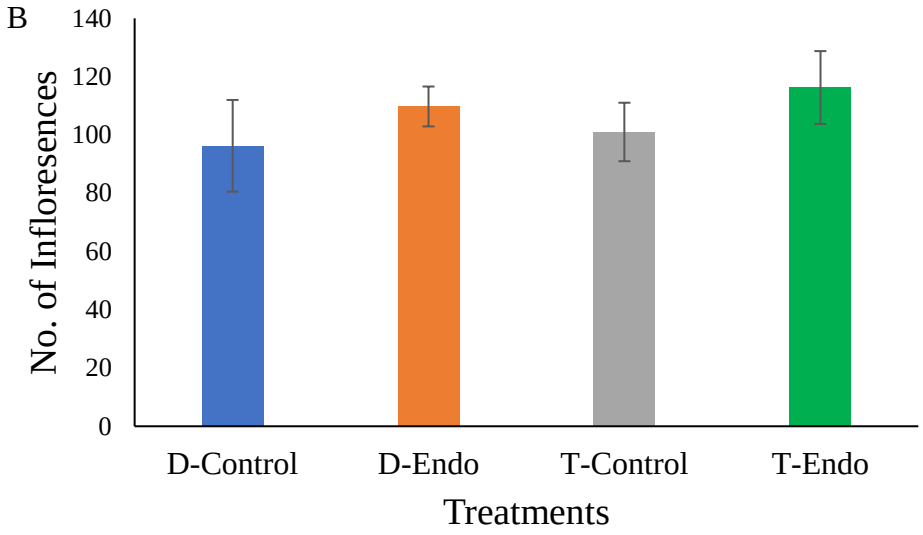
5.5.4 Quantitative analysis of Branching Architecture

The results presented here showed that the number of axillary branches was highest in triploids treated with SEn and lowest in diploids treated with SEn compared to the untreated control (A).

The number of inflorescences was higher in SEn -treated diploids (~105) and triploids (~118) as compared to their non-treated counterparts (~ 90). The greater number of inflorescences is directly proportional to more flowers (B).

Figure C showed that the internodal distance is highest in the SEn-treated triploid plants (7.5cm) and lowest in the SEn-treated diploid plants (5 cm), indicating that the values in untreated plants in both hemp genotypes lie in between these ranges.





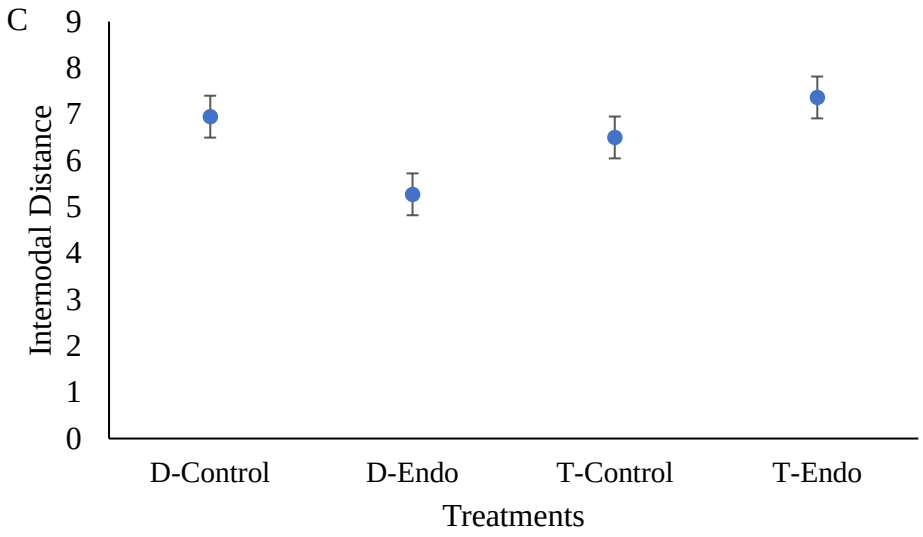
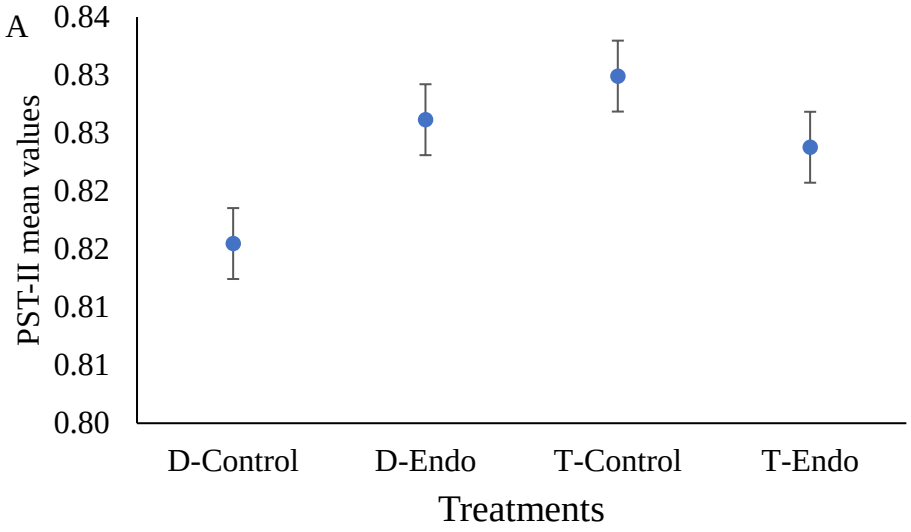


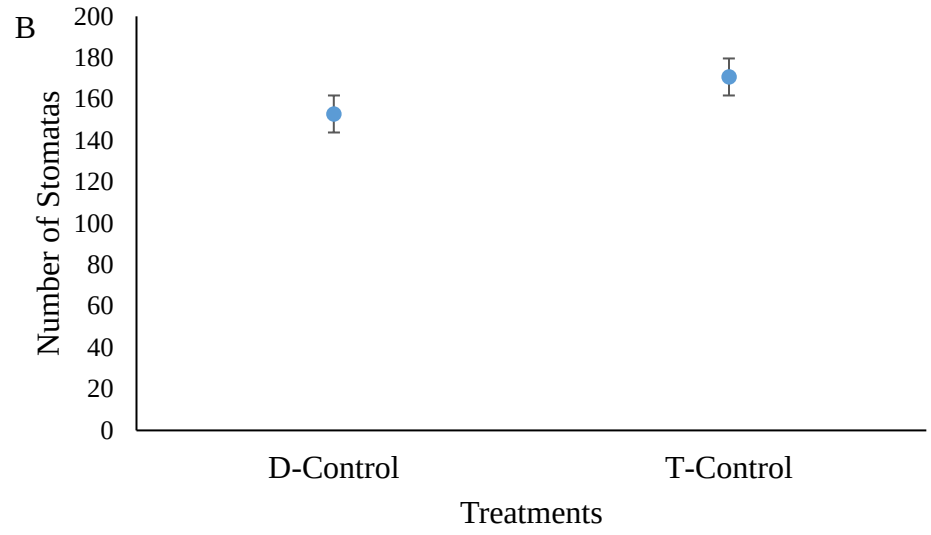
Figure 5.7: Number of axillary branches (A), inflorescence (B), and means of internodal distance (C) calculated for different treatments of plants. D-Contro: diploid control; D-Endo: diploid SEn; T-Contro: triploid control; T-Endo: triploid SEn. Data are means and standard errors of three replicates (Two-way ANOVA, $p < 0.05$).

5.5.5 Correlation between Photosynthetic rates and Stomatal Impressions

Figure 5.8 (A) shows the photosynthesis (PSII) Fv/Fm ratio which was in the range of 0.79–0.83 representing that all four cultivars were not under stress and all conditions were appropriate in the phytotron chambers for them to grow (Bauerle et al., 2020; Römermann et al., 2016).

The stomatal imprints in Figure 5.8 (B) showed that the number of stomata was higher in triploid (~170) as compared to diploid (~150). Stomata are responsible for the leaf water economy and gas exchanges, thus allowing better physiological functions to perform photosynthesis.





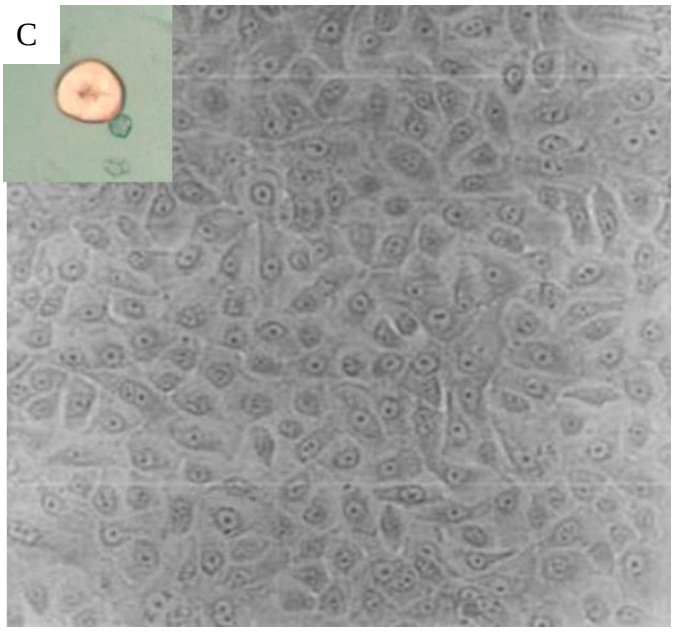


Figure 5.8: Fv/Fm ratio calculated for different treatments on 2 cultivars of hemp (A). Figure (B) represents the number of stomata present on diploid and triploid plants. Photomicrograph of the abaxial leaf surface, taken from transparent tape imprints (C). Stomata viewed under a microscope at 100X magnification. D-Control: diploid control; D-Endo: diploid SEn; T-Control: triploid control; T-Endo: triploid SEn.

5.5.6 Shoot and Root Weight

Figure 5.9 (A) shows that fresh shoot weight increased in seed endophyte-treated triploid plants (~ 65 g), whereas it was lowest in SEn-treated diploid plants (~ 50 g). In terms of the dry shoot weight Figure 5.9 (B), the highest values were recorded in untreated diploid plants (~ 25 g) and the lowest in untreated triploid cultivars (~ 15 g). Linear regression analysis of diploid and triploid plants in Figure 5.10 (A) and (B) respectively showed the effect of fresh and dry shoot weight compared with the diameter of the stem. As the diameter of the stem increases, so does the biomass of the shoots. Slope rate is increased in diploids as compared with triploids and a greater rate of change in fresh shoot and dry shoot diploids were seen compared with triploids. The slope angle in diploid fresh shoot and triploid fresh shoot was found to be 13º and 9º respectively whereas 7º and 4º in dry shoot diploids and triploids respectively.

It showed that fresh root weight in Figure 5.11 (A) was highest and same in seed endophyte-treated and untreated triploid cultivars (~15 g), whereas it was lowest in seed endophytes-treated diploid cultivars (~7 g). Regarding dry root weight in Figure 5.11 (B), it was increased from diploid untreated (~ 1.8 g) to SEn - treated diploid (2.75 g), being highest at untreated triploid (3.1 g) plants with a slight decrease in SEn - treated triploids (2.5 grams). Linear regression analysis of diploid and triploid plants in Figure 5.12 (A) and (B) respectively showed the rate of change in fresh root diploids and triploids were greater than dry root diploids and triploids. The weight of root in diploids ranged from 7-12 g whereas in triploids it ranged from 12-18 g. The slope angle for diploid fresh roots and triploid fresh roots was consistent at 10º, indicating a similarity in their profiles. In contrast, both diploid and triploid dry shoots exhibited a slope angle of 4º.

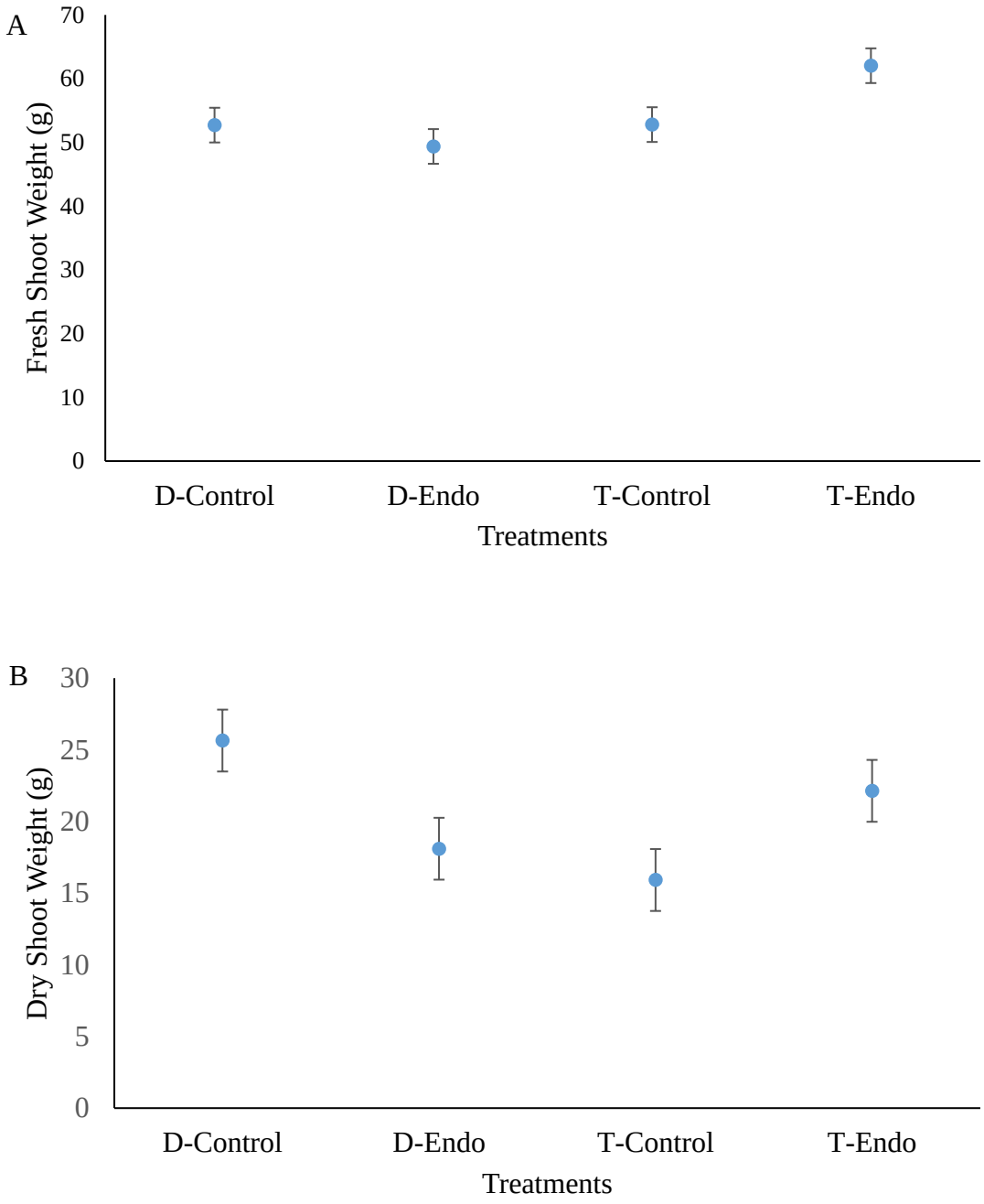
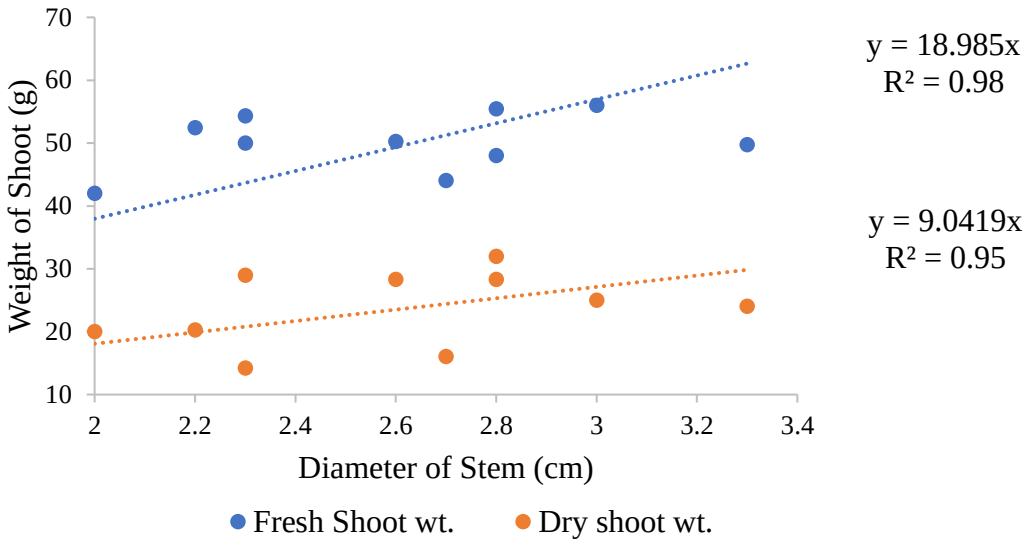


Figure 5.9: Means of fresh (A) and dry shoot (B) weight under different treatments of plants. D-Control: diploid control; D-Endo: diploid SEn; T-Control: triploid control; T-Endo: triploid SEn. (Two-way ANOVA , $p < 0.05$)

A



B

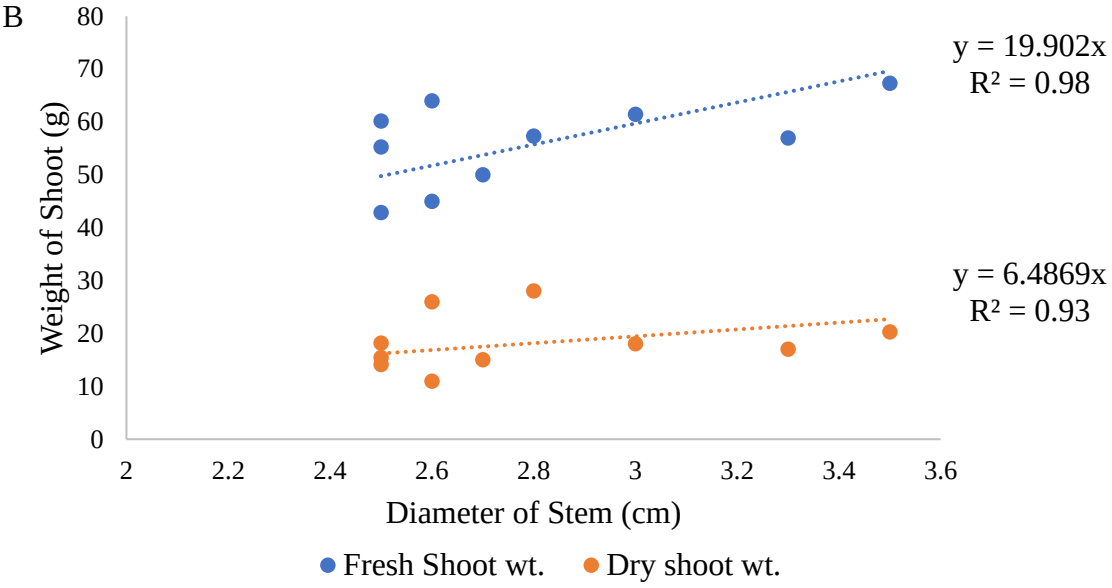


Figure 5.10: Regression analysis of fresh and dry shoot weight in relation to the diameter of the stem in diploid (A) and triploid (B) plants.

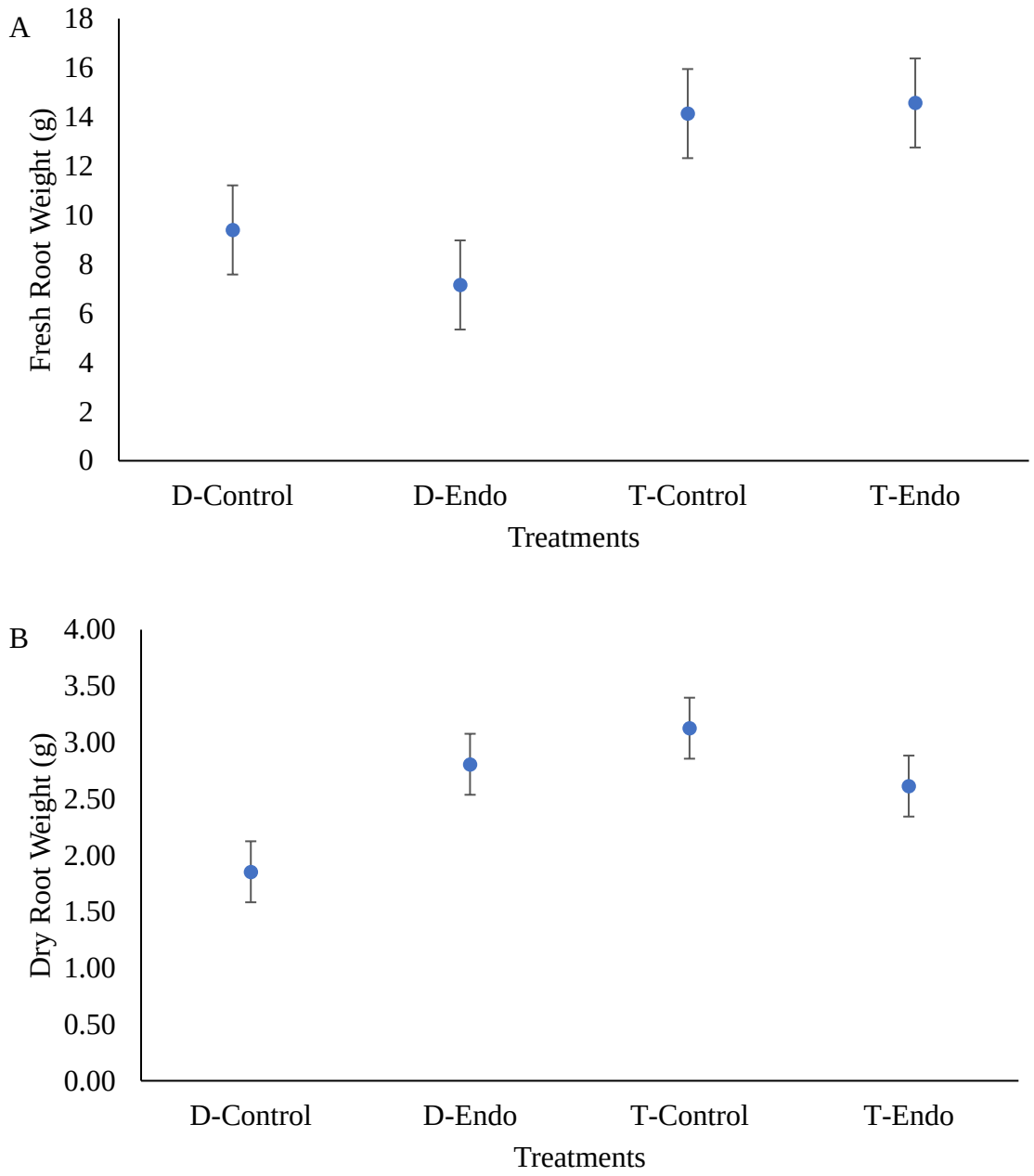
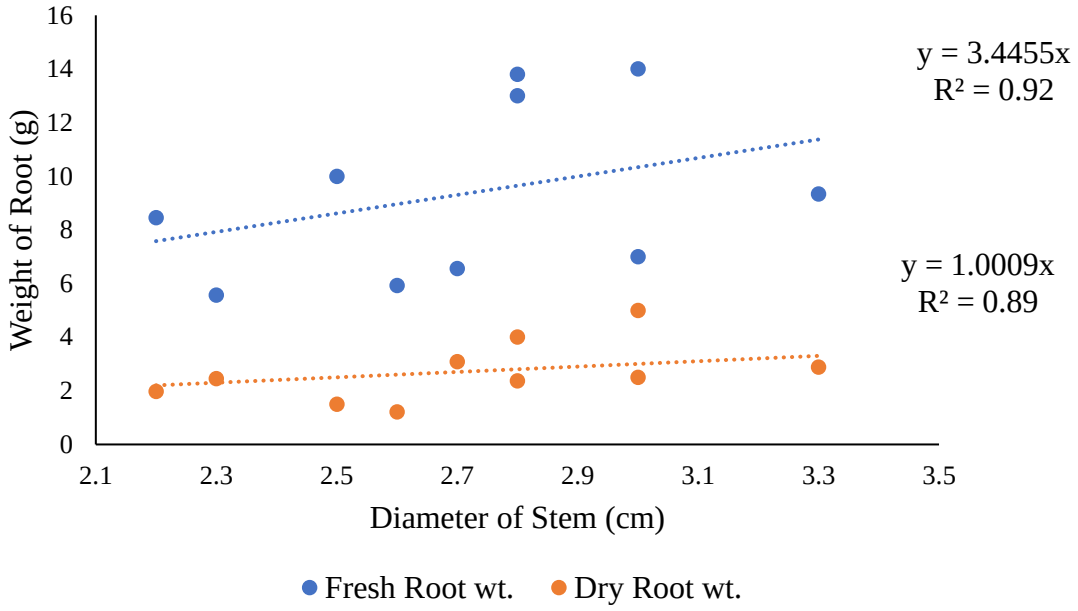


Figure 5.11: Means of fresh and dry root weight under different treatments of plants. D-Control: diploid control; D-Endo: diploid SEn; T-Control: triploid control; T-Endo: triploid SEn. (Two Way ANOVA, $p < 0.05$)

A



B

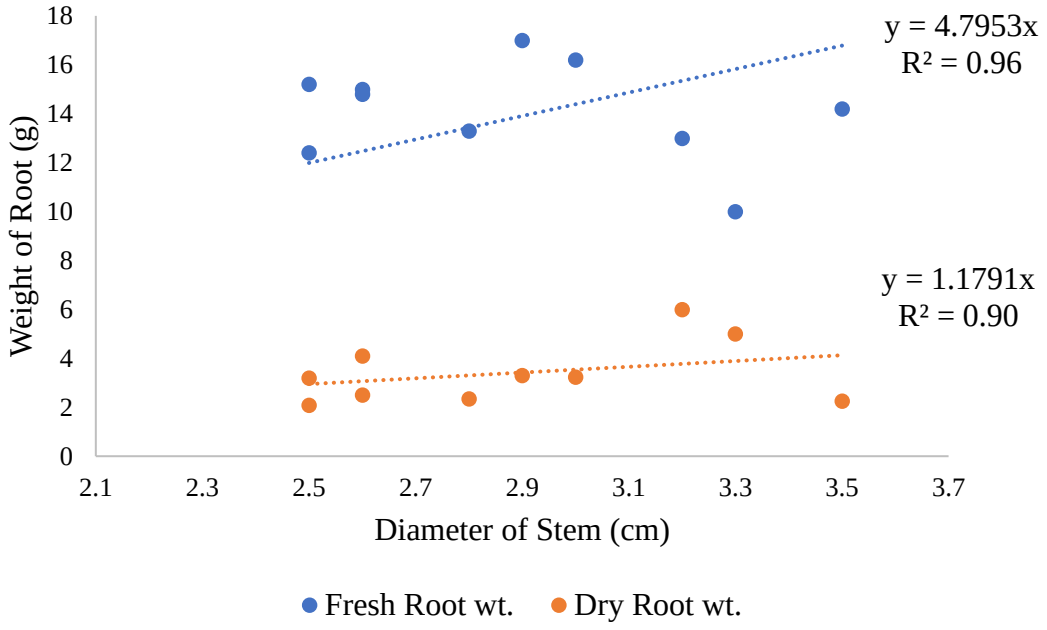


Figure 5.12: Regression analysis of fresh and dry root weight in relation to the diameter of the stem in diploid (A) and triploid (B) plants.

5.5.7 Principal Component Analysis

Principal Component Analysis (PCA) was used as dimensionality reduction technique to analyze patterns in multivariate data (Ivosev et al., 2008; Mishra et al., 2017). PCA is based on the covariance matrix of the original data, but when dealing with compositional data, which is often in the form of relative abundances, Bray-Curtis dissimilarity is frequently employed as a measure of dissimilarity between samples. Bray-Curtis dissimilarity matrix was calculated between all pairs of samples based on the relative abundances of different parameters considered here using Origin Pro software (Abdullah & Khairurrijal, 2016). The dissimilarity matrix will represent the pairwise differences in composition between samples.

A very clear separation of triploid treated with endophytes were seen with PC1 and PC2 that accounted for 61.46% and 29.76% of the total variation respectively was made using Origin Pro 8 software In this software, data was normalized using Min-Max Scaling (Cao et al., 2016; Seleþchi & Duliu, 2006) where scaling data were selected accordingly. 10 parameters were selected for all four different cultivars with 6 samples in each parameter for the analysis. The results showed that flowering days and phytohormone levels characterize both diploid and triploid untreated cultivars, whereas phytocannabinoid and inflorescence counts, and senescence days are closely related to triploid endophytes.

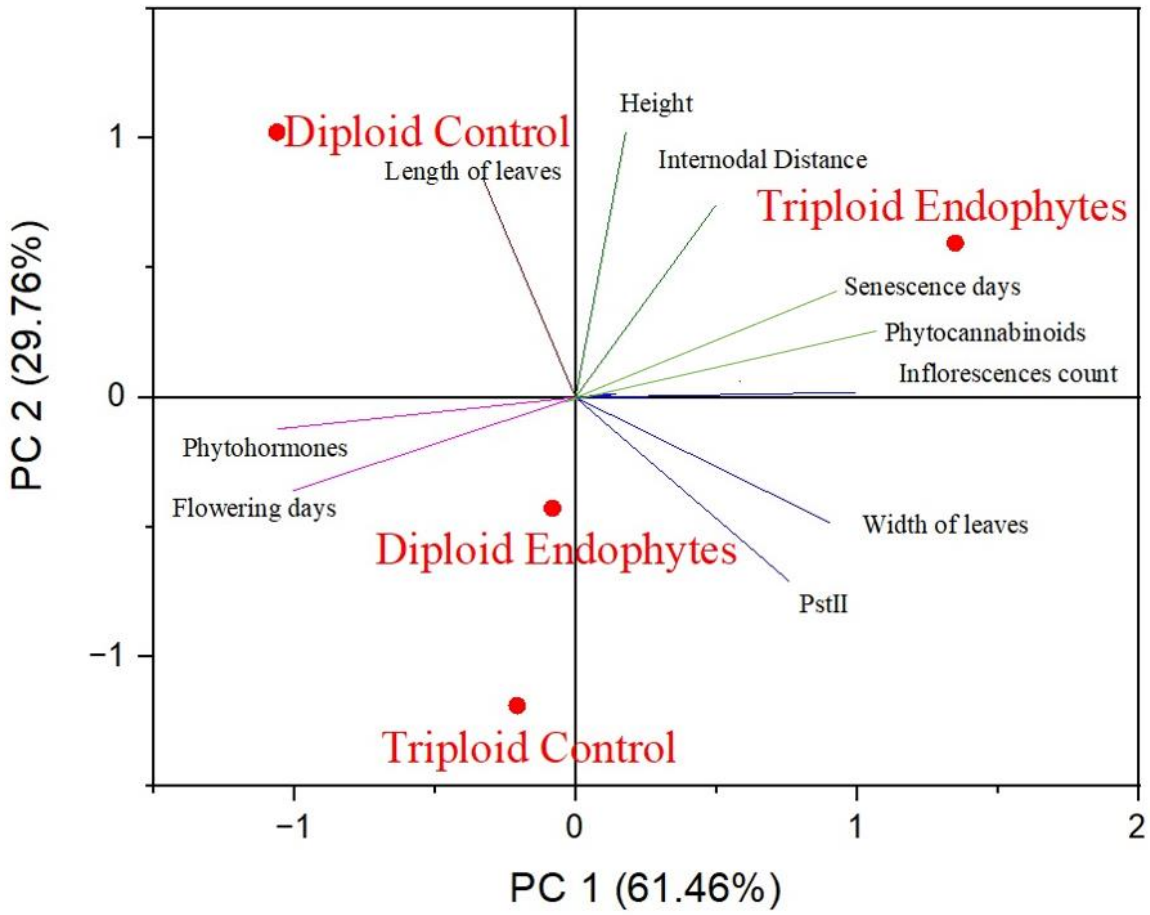


Figure 5.135: Principal component analysis based on Bray-Curtis dissimilarity between different phenotypic parameters associated with Suver Haze plants grown in a phytotron chamber.

5.6 Discussion

The observed changes in plant characteristics between untreated diploid and triploid plants and those treated with seed endophytes highlighted the multifaceted impact of endophyte associations on plant growth and development. Notably, diploid plants without treatment displayed greater height (weeks 4,6,8,9: $p < 0.05$; weeks 3,7,10 : $p < 0.01$; week 5: $p < 0.001$), whereas the opposite was observed in triploids, where untreated plants were shorter than their endophyte-treated counterparts (weeks 3,5,7,9,10 : $p < 0.05$; week 4,8: $p < 0.01$; week 6: $p < 0.001$). This can be mainly driven by differences in cannabis genotype phototropism or a growth

response to light under defined indoor environment. According to (Benková & Hejátko, 2009; Kerstetter & Hake, 1997; Pfeiffer et al., 2016), the apical meristem, located at the tips of shoots and roots, is particularly sensitive to light. When exposed to light, the plant hormone auxin moves away from the illuminated side, causing the cells on the shaded side to elongate more rapidly. This differential cell elongation results in the bending of the plant toward the light source. In the case of shoots, this often leads to upward growth (Lund et al., 2007; Munir & Alhajhoj, 2017; Ying et al., 2020). This height disparity is influenced by a complex interplay of genetics and endophyte interactions, as shown by Mattoo and Nonzom (2021), Mushtaq et al. (2023), Pathak et al. (2022) and Rani et al. (2022). In this study, triploid plants exhibited an overall greater height than diploid stating that triploids are made taller than diploids. According to Fernandes et al., 2023) hemp plant morphology revealed a significant increase in plant height and leaf size with increasing ploidy levels in a cultivar-dependent manner. In contrast, cannabinoids were negatively affected by polyploidization, with the concentration of total cannabinoids, As based on this study results, the influence of light on leaf length and width, varied between cultivars. Leaves exposed to sufficient light may exhibit increased vigor, cell expansion and elongation, resulting in longer and wider leaves (Falster & Westoby, 2003; Humphries & Wheeler, 1963; Saebo et al., 1995). Although, this is a complex biological process, our study demonstrated an important influence of endophytes on this particular trait controlled by various physiological and developmental mechanisms. The adequate spectrum of light effect - optimised for balanced photosynthesis and energy production in cannabis – shoved the interference or influence by symbiotic host-endophyte interactions as drivers of trade- offs in plants (Santangelo et al. 2016). The variations in leaf length at specific nodes further emphasize the intricate relationship between endophytes and plant growth. In diploids, untreated plants showed longer leaves at node 2, while seed endophyte-treated diploids displayed extended leaf lengths at nodes 4, 6, and 8. A similar trend was observed in triploids, with treated plants having longer leaves at nodes 2, 6, and 8, except at node 4, from which it could be concluded that there is a switch from vegetative to flowering stage after 4th node production, This could be influenced by the microbial dynamics and shifts in communities (Chaparro et al., 2014; Hesami et al., 2023) and hormonal profiles over the plant growth stages (Backer et al., 2019). Spitzer-Rimon et al., 2022 reported that the hemp plant takes a leap into

the reproductive stage when a pair of solitary flowers emerges at the 7th node. Examination at the histological level revealed that this transition involves the fresh formation of flower meristems that are absent during the vegetative stage or lying dormant at nodes 4 and 6. Additionally, a significant shift occurs in the transcriptomic profile of genes related to flowering across nodes 4, 6, and 7.

We found that overall cannabis plant biomass formation, dynamics and productivity can be accurately and simply evaluated by the stem diameter assessment, The higher the diameter of the stem, the higher is the biomass of the shoots and roots. It seems that the diameter size is directly proportionate to plant physiological potential for absorbing water and nutrients as well as more physical stability for higher biomass. This study for the first time represented the correlation of diameter of the stem directly with the biomass in hemp plants (Bektas et al., 2023; Gichangi et al., 2017). Polyploid plants have larger cells, which contributes to more photosynthetic area as well as also more stomata. Additionally, the flowering duration highlighted that endophyte-treated triploids flowered earlier than their untreated counterparts, suggesting that plants remain more physiologically active, particularly triploid plants. The extended days of senescence in both endophytes-treated diploids and triploids suggest that endophyte treatment may prolong plant lifespans in both cultivars by producing primary and secondary metabolites which contributes to the overall health and longevity of the plant (Ali et al., 2012; Zhang et al., 2022). The number of axillary branches and inflorescences revealed significant differences. Triploids treated with endophytes had the highest number of axillary branches, while endophyte-treated diploids had the fewest, with untreated cultivars falling in between. Moreover, endophyte-treated plants in both cultivars displayed a higher number of inflorescences, implying the potential for increased phytocannabinoid production. Internodal distances were longest in seed endophytes-treated triploids and shortest in seed endophytes-treated diploids, with untreated cultivars falling in between. These results indicated that endophytes could affect the spatial arrangement of nodes (Naveed et al., 2014; Purushotham et al., 2018). The Fv/Fm ratio, within an optimal range for all four cultivars, confirms suitable growth conditions in the phytotron chambers. Stomatal imprints revealed a higher stomatal density in triploids compared to diploids, implying a positive impact on leaf water economy and gas exchange, ultimately enhancing photosynthetic functions. The dynamic change in

diameter is a good indicator of changes of biomass of plants for both fresh and dry that allow us to establish one more easy and simple method to estimate the biomass of hemp plant during the growth stages of development and maturity.

5.7 Conclusions

In conclusion, the intricate interplay between ploidy levels and endophyte treatment influences various aspects of plant development and performance. These findings provide valuable insights into the potential of endophytes to modulate plant characteristics, flowering times, and overall performance, with implications for the hemp industry and agriculture. Further research is needed to unravel the underlying mechanisms behind these observations.

5.8 Connection to the next study

These observations suggested the pivotal role of SEn in shaping plant traits in diploid and triploid hemp lines. To delve deeper into this phenomenon and improve our understanding, my upcoming study 4 involved comprehensive research that centers on investigating specific parameters, such as the production of secondary metabolites, hormones, and proteins by these plants.

6 Study 4 : Evaluating the impact of beneficial microbiomes on secondary metabolite production in both diploid and triploid cultivars

6.1 Abstract

This part of the study focuses on the effect of seed endophytes (SEn) on secondary metabolite production in diploid and triploid cultivars of Suver haze. Phytocannabinoid analysis and hormone analysis were performed by high-performance liquid chromatography (HPLC) and a modified version of HPLC, including electron ionization mass spectrometry, respectively. Terpene analyses were performed by gas chromatography, with protein quantification by the LECO method. Scanning electron microscopy was done to check the interaction between endophytes and Suver haze seeds. Phytocannabinoid concentrations were increased in SEn treated plants whereas protein and terpene ratios decreased in those samples, thereby showing complex relationship between these compounds.

6.2 Introduction

Phytocannabinoids are a group of natural bioactive compounds containing isopropenyl residues at the terpene moiety, resorcinol nucleus, and alkyl side chain. They are primarily produced by the cannabis and hemp plants but can also be found in other plant species like *Rhododendron* (Ericaceae), *Helichrysum umbraculigerum* Less. (Asteraceae), *Glycyrrhiza foetida* Desf. (licorice; Fabaceae), *Amorpha fruticose* L. (bastard indigo; Fabaceae) (Fuhr et al., 2015; Pollastro et al., 2017), and some liverworts. Phytocannabinoids are vital compounds in the plant kingdom, with immense implications for medicinal and industrial applications.

Hormones play a pivotal role in the regulation of various developmental processes in hemp plants. Among these, auxins, such as indole-3-acetic acid (IAA), are crucial for cell elongation and apical dominance, influencing the plant's overall growth and shape. Gibberellins, like gibberellic acid (GA), stimulate stem elongation, germination, and flowering. Cytokinins, including zeatin, are associated with cell division and differentiation, impacting

leaf and shoot development. Abscisic acid (ABA) influences seed dormancy, stomatal closure, and stress response (Burgel et al., 2020; Fenn and Giovannoni, 2021; Miransari and Smith, 2014; Yu et al., 2020). Additionally, ethylene is involved in fruit ripening and senescence. The balance and interaction of these hormones are essential for orchestrating the growth, development, and response to environmental cues in hemp plants.

The hemp flower is not only of interest for its chemical constituents, such as cannabinoids and terpenes, but also for its protein content. Proteins play an essential role in the growth and development of hemp plants, and the flower serves as a significant site for their production. Proteins within the hemp flower are not only involved in plant development but also have intriguing implications for the synthesis of valuable compounds like cannabinoids (Gorelick et al., 2022; Kinghorn et al., 2017; S. U. Sirikantaramas et al., 2005). Enzymes, which are specialized proteins, participate in key biochemical reactions within the flower, including the biosynthesis of cannabinoids like cannabidiol (CBD) (Riquelme-Sandoval et al., 2020; Sun et al., 2021; Tahir et al., 2021; Xu et al., 2022a). Understanding the role of proteins in the hemp flower is essential not only for advancing our knowledge of plant biology but also for optimizing the cultivation and utilization of hemp, with applications spanning from medicine to industry. This part of study dug into the intriguing interplay between SEn and the biochemical composition of diploid and triploid plants, with a particular focus on phytocannabinoids, hormones, proteins, and secondary metabolite production.

6.3 Hypothesis

Firstly, it was anticipated that the seed endophytes residing within the plant tissues may contribute to alterations in the phytocannabinoid profile. The microbiome's potential to influence the types and quantities of phytocannabinoids synthesized by diploid and triploid plants is a central facet of this study.

Secondly, it was proposed that these microbiome plays a pivotal role in shaping the hormonal dynamics of these plants, and the microbiome could impact the production, regulation, and response to hormones, thus influencing growth and development.

Thirdly, it was hypothesized that the microbiome has the capacity to modify protein synthesis within plant cells, and the microbiome's influence on the types and abundance of proteins could have far-reaching consequences for plant growth and adaptation.

Lastly, it was anticipated that the microbiome may play a role in modulating the production of secondary metabolites, which encompass a diverse array of compounds with ecological and physiological significance. Changes in the secondary metabolite profile could impact the plant's interactions with its environment and potential applications in various industries.

6.4 Materials and Methods

The materials and methods used for this part of the study are further divided into the following categories:

6.4.1 Phytocannabinoid Potency Measurement by High Performance Liquid Chromatography

Matured fresh hemp flower was cut from the top and sealed in a zip lock bag and send to Saskatchewan Research Council (SRC, Saskatoon, SK) for HPLC analysis at room temperature on the same day. Hemp flower samples were ground and then 0.5 g was weighed into a tube and extracted with methanol. The resulting extract was diluted and placed into vials. Samples were analyzed by liquid chromatography with a photodiode array detector using a polar C18 column to detect the cannabinoids in the sample (Burgel et al., 2020).

6.4.2 Hormone Analysis by HPLC-ESI-MS/MS

The leaves at the 4th node of the hemp plant were cut and freeze dried at -20 ^{o}C for 72 hours before they were sent to National Research Council (NRC, Saskatoon, SK) for hormone analysis. The samples were ground in a mortar (on ice) with 5 mL of 80% (v/v) methanol as the extraction medium, which contained 1 mmol/L of butylated hydroxytoluene (BHT) as an antioxidant. The methanolic extracts were incubated at 4°C for 4 h and then centrifuged at

10,000 × g for 15 min at the same temperature. The supernatants were passed through Chromosep C18 columns (C18 Sep-Park Cartridge; Waters Corp., Millford, MA). The columns were prewashed with 10 mL of 100% and 5 mL of 80% methanol. The hormone fractions were dried with N2 and dissolved in 1 mL phosphate-buffered saline (PBS), containing 0.1% (v/v) Tween 20 and 0.1% (w/v) gelatin (pH 7.5), for the enzyme-linked immunosorbent assay (ELISA) (Miransari and Smith, 2014; Müller and Munné-Bosch, 2011).

The quantitative analysis utilizes the Multiple Reaction Monitoring (MRM) function of the Mass Lynx v4.1 (Waters Inc.) control software. The resulting chromatographic traces for each analyte (endogenous phytohormone) and their respective deuterium-labeled internal standard are quantified off-line using the Quan Lynx v4.1 software (Waters Inc.), wherein each trace is integrated, and the resulting ratio of signals (analyte/internal standard) is compared with a previously constructed calibration curve to yield the amount of analyte present (ng per sample). Calibration curves were generated from the MRM signals obtained from standard solutions based on the ratio of the chromatographic peak area for each analyte to that of the corresponding internal standard. The QC samples, internal standard blanks, and solvent blanks were also prepared and analyzed along with each batch of tissue samples.

6.4.3 Protein Quantification

Protein was measured in the seeds and mature flowers of diploid and triploid cultivars using the Leco test (Lowry and Evans Copper Oxidase) provided by the Crop Science facility at the University of Saskatchewan. It is a biochemical assay used to determine the concentration of proteins in a sample (Etheridge et al., 1998; Pérez-Vila et al., 2024), specifically proteins that contain tyrosine and tryptophan residues. This test relies on the reaction between these amino acids and copper ions in an alkaline solution.

The CHN628 Series Elemental Determinator was used to determine nitrogen, carbon, and nitrogen. And carbon, hydrogen, and nitrogen in organic matrices. The instrument utilizes a combustion technique and provides a result within 4.5 min for all elements being determined. The instrument is connected to an external PC to control system operation and data

management. One gram sample weighed and placed in the instrument's loader, where the sample will be transferred to its purge chamber directly above the furnace, eliminating the atmospheric gases from the transfer process. The sample is then introduced to the primary furnace, which contains only pure oxygen, resulting in rapid and complete combustion (oxidation) of the sample. Carbon, hydrogen, and nitrogen present in the sample are oxidized to carbon dioxide, water, and NOx, respectively, and are swept by the oxygen carrier through a secondary furnace for further oxidation and particulate removal. The results are typically displayed in weight percent or parts per million (ppm), but they can also be displayed in percent total protein.

6.4.4 Terpenes Analysis by Gas Chromatography

Terpenes are a class of natural products that are synthesized by plants. They are known for their diverse biological activities and are used in various industrial applications. The biosynthesis of terpenes in plants involves different pathways, such as the acetate-mevalonic acid pathway and the non-mevalonic acid pathway (Singh and Sharma, 2015).

Matured fresh hemp flower was cut from the top and sealed in a ziplock bag and send to Saskatchewan Research Council (SRC, Saskatoon, SK) for Gas Chromatography. Samples were ground while frozen and then 0.25 g weighed into a 15 ml plastic tubes and extracted with methanol on SPEX sample Prep 2010 Geno/Grinder. The extract was analyzed on a GC-MS, via direct liquid injection into the GC.

The synthesis of plant terpenes is known to be regulated by many transcription factors (TFs), including members of the APETALA2/ethylene-responsive factor (AP2/ERF), basic-helix-loop-helix (bHLH), v-myb avian myeloblastosis viral oncogene homolog (MYB), NAM, ATAF1/2, CUC1/2 (NAC), WRKY, and basic leucine zipper (bZIP) families. The regulation of terpene biosynthesis in hemp involves various transcription factors (TFs), like three novel TFs, CsAP2L1, CsWRKY1, and CsMYB1 (Liu et al., 2021), that interact with the trichome-specific THCAS promoter regulatory region. These TFs were shown to regulate the THCAS promoter in plants. The O-Box element within the proximal region of the THCAS promoter is

necessary for CsAP2L1-induced transcriptional activation of the THCAS promoter. CsAP2L1 plays a positive role in the regulation of THCAS expression, while CsWRKY1 and CsMYB1 may serve as negative regulators of THCAS expression. In this study, the untreated cultivars were subjected to terpene analysis by gas chromatography to check the changes in their levels due to ploidy.

6.4.5 Scanning Electron Microscopy

Scanning electron microscopy (SEM) is a characterization technique that images and analyzes a specimen by scanning an accelerated electron beam, followed by selectively collecting and recording secondary electrons, back-scattered electrons, and other signals arising from the beam and specimen interactions. SEM can be used to study the morphology and structure of seeds and their interactions with the seed microbiome. SEM can provide high-resolution images of seed surfaces and internal structures, which can help identify the microbial communities associated with seeds (Kim et al., 2021).

The methodology involved the germinated hemp seeds with radicle coming out 1cm around were allowed to interact with endophytic bacterial and fungal microbial communities for 2 days. The germinating hemp seeds along with endophytes were dissected and then fixed with 2% glutaraldehyde (GA) in 0.1 M sodium cacodylate (NaCAC), at pH 7.2 for 3 hrs at room temperature and then stored overnight at 4 ºC before osmium fixation. The pre-fixed samples were further fixed in 1% osmium in 0.1 M NaCAC for 1 hr and three times rinsed with 0.1 M NaCAC and sterile distilled water. The fixed samples were dehydrated with ethanol gradually (30%, 50%, 70%, 80%, 90%, 95%, and absolute ethanol) for 15 min. The last step was repeated three times. The dehydrated samples were placed on a critical point dryer (Polaron E3000).

6.5 Results

The results of this study revealed changes in the primary and secondary metabolite production of diploid and triploid cultivars of Suver haze influenced by the application of seed endophytes. These alterations encompass significant host characteristics, including total protein, phytocannabinoids, hormones, and terpene production.

6.5.1 Phytocannabinoid analysis

It was found that phytocannabinoid (THCA/CBDA) increased in seed endophytes treated plants in both the cultivars (diploid and triploid) as compared to their untreated cultivars. The results provided evidence that addition of endophytic microbiome can play a role, directly or indirectly, in increasing the THCA/CBDA ratio.

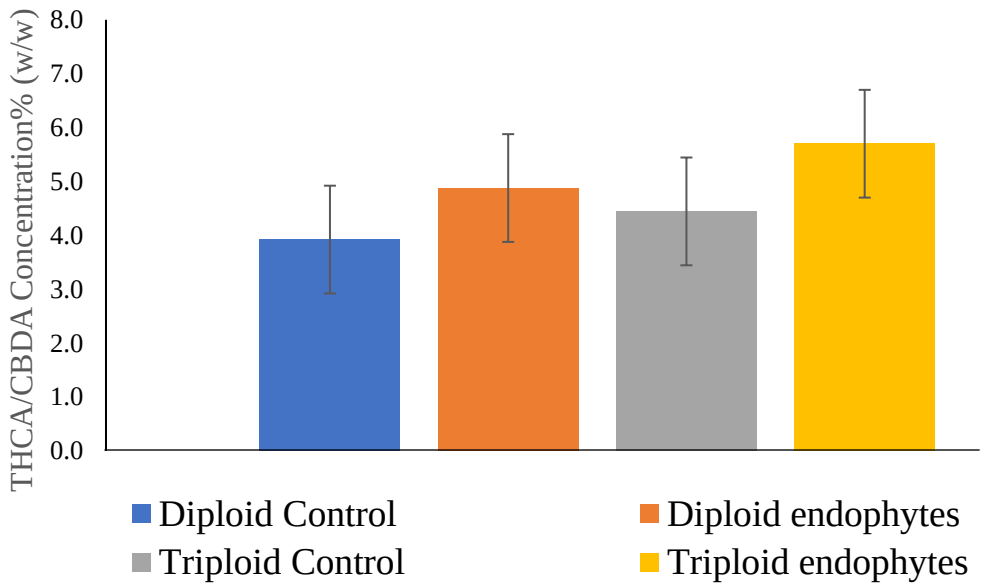


Figure 6.1: Phytocannabinoid concentrations isolated from flowers of different cultivars under treatments at day 98. Data are means and standard errors of three replicates (One-way ANOVA, $p < 0.01$)

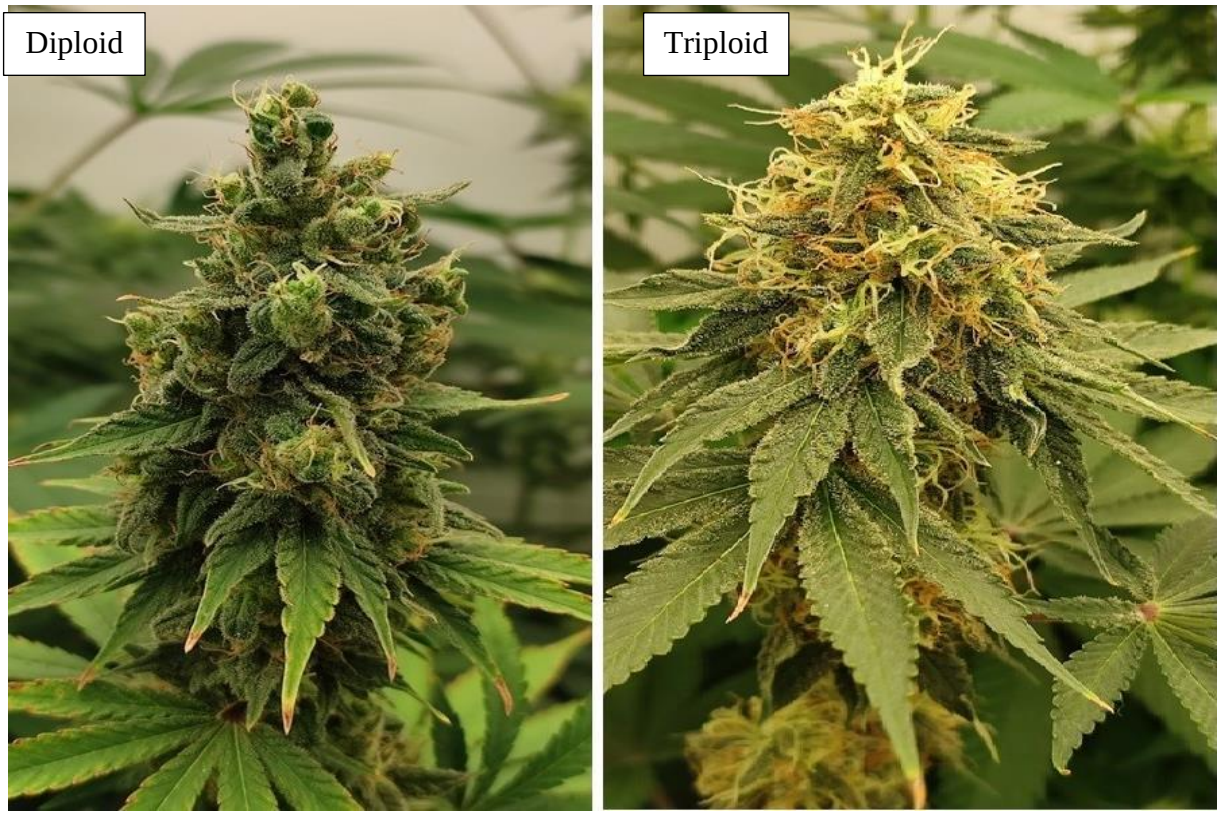


Figure 6.2: Pictorial representation of diploid (left) and triploid (right) flower measured for phytocannabinoid concentrations at day 98.

6.5.2 Growth Promoting and Growth Suppressing hormones

It showed that the number of accumulated hormones in hemp leaves. The highest quantity of auxins in figure 33 (A) was registered in the untreated diploid cultivar (250 ng/µl) and the minimum (50 ng/µl) in seed endophytes-treated diploid and triploid plants, while 100 ng/µl was measured in untreated triploid. Abscisic acid in figure 33 (B), which was again highest in untreated diploid control (3000 ng/µl), while a reduced amount (700–1000 ng/µl) was associated with all treatments as well as with triploid control. Cytokinin, which was the same in untreated diploid, triploid, and seed endophyte treated triploid (1 ng/µl) in figure 33 (C), with the lowest values in endophyte-treated diploid plants (0.5 ng/µl). Gibberellin in figure 33 (D) showed the highest values in diploid plants, with untreated diploids having 4 ng/µl and endophyte-treated having 3.5 ng/µl, while the lowest values are seen in triploid plants at 1 ng/µl.

This study also displayed consolidating growth-promoting and growth-suppressing hormones. The untreated diploid plants exhibited the highest values, with 300 ng/µl in growth-promoting hormones (figure 34 (A) and 3000 ng/µl in growth-suppressing hormones (figure 34 (B). The second-highest values were observed in the untreated triploid plants, with 150 ng/µl in growth-promoting hormones and 1300 ng/µl in growth-suppressing hormones. The fact that the plants treated with seed endophytes exhibited the lowest hormonal values implies that the plant microbiome is aiding in the promotion of plant growth in treated hemp plants without causing an undue buildup of hormones.

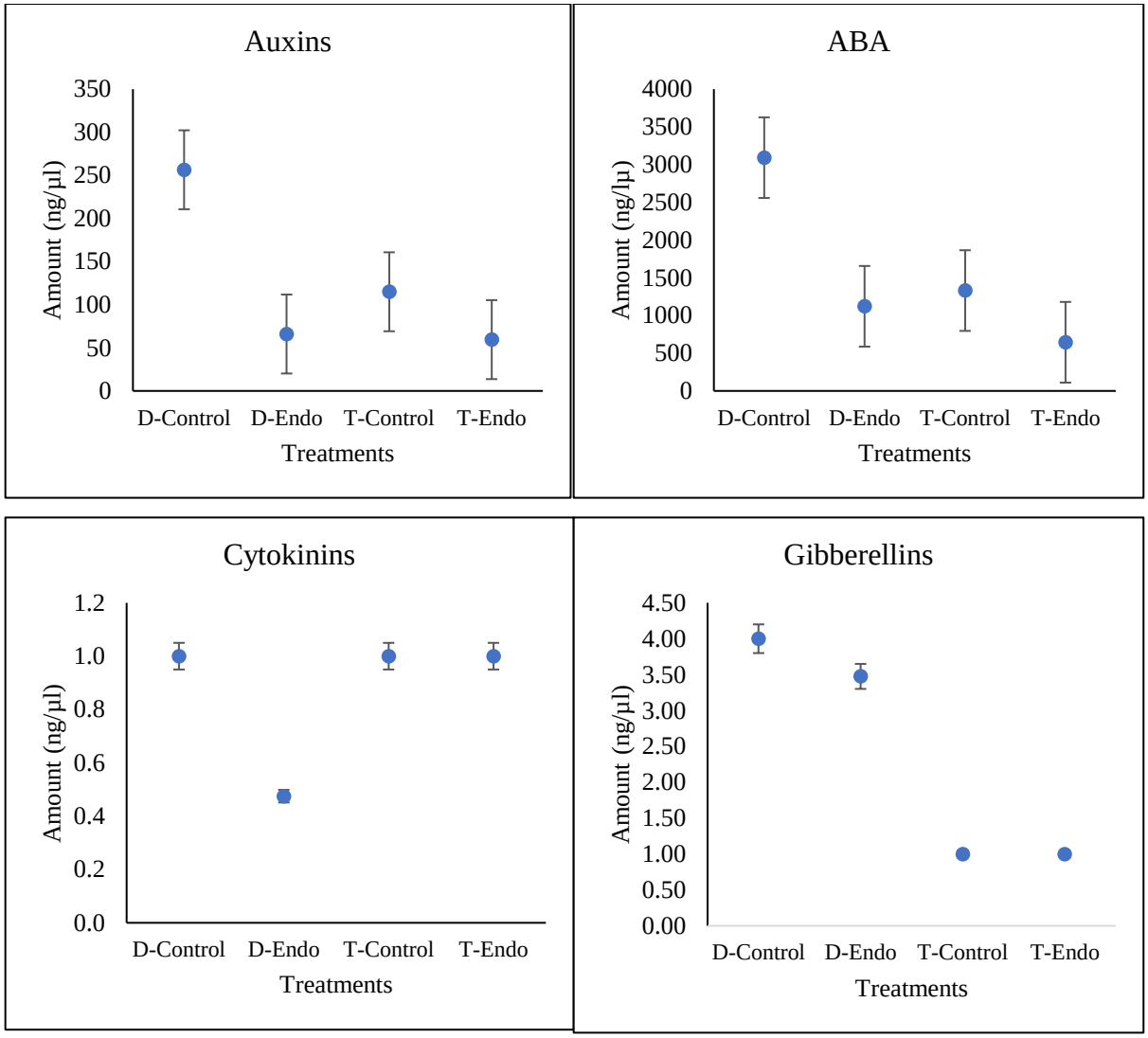


Figure 6.3: Different hormones were quantified (ng/µl) in diploid and triploid Suver haze plants.

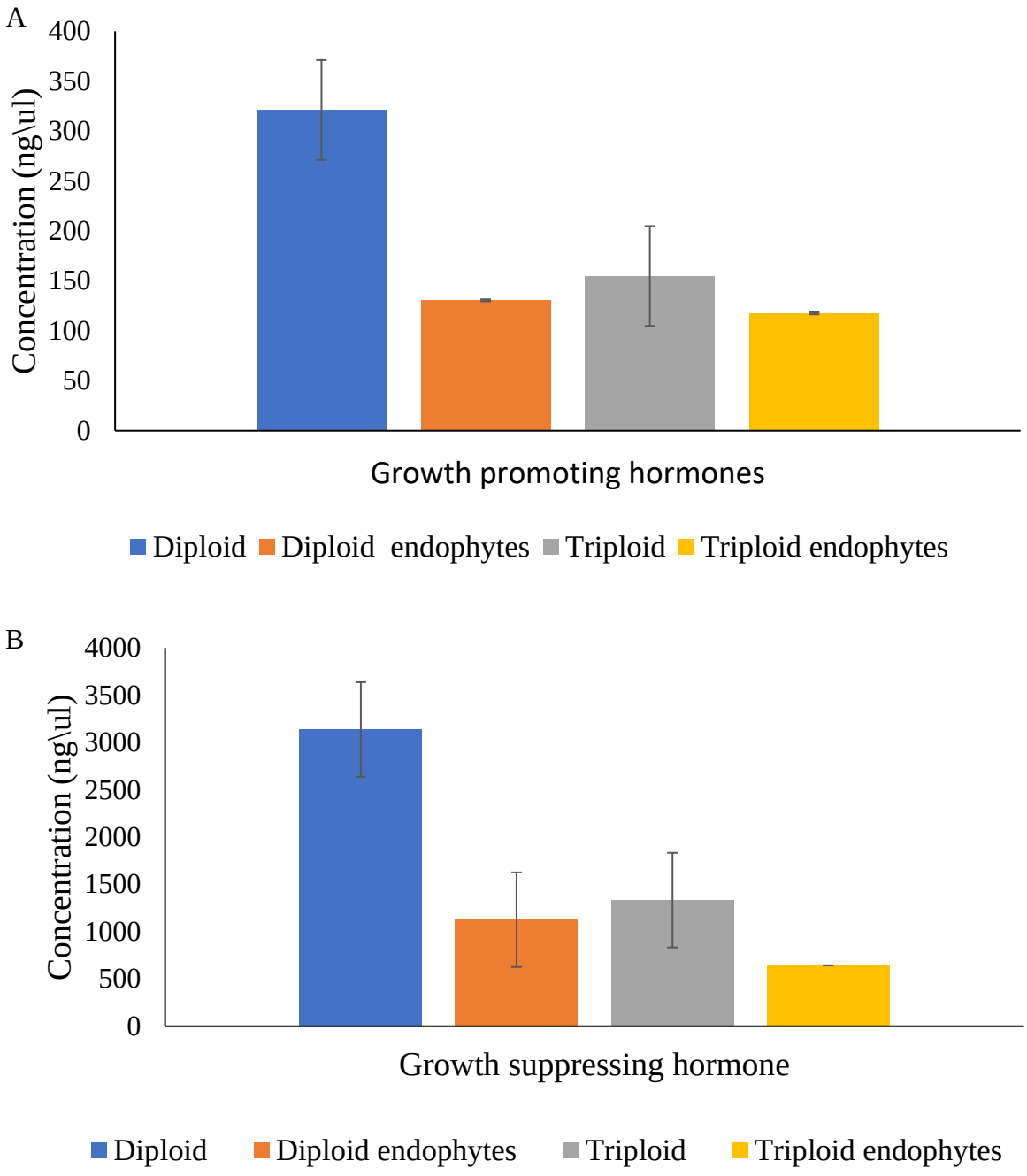
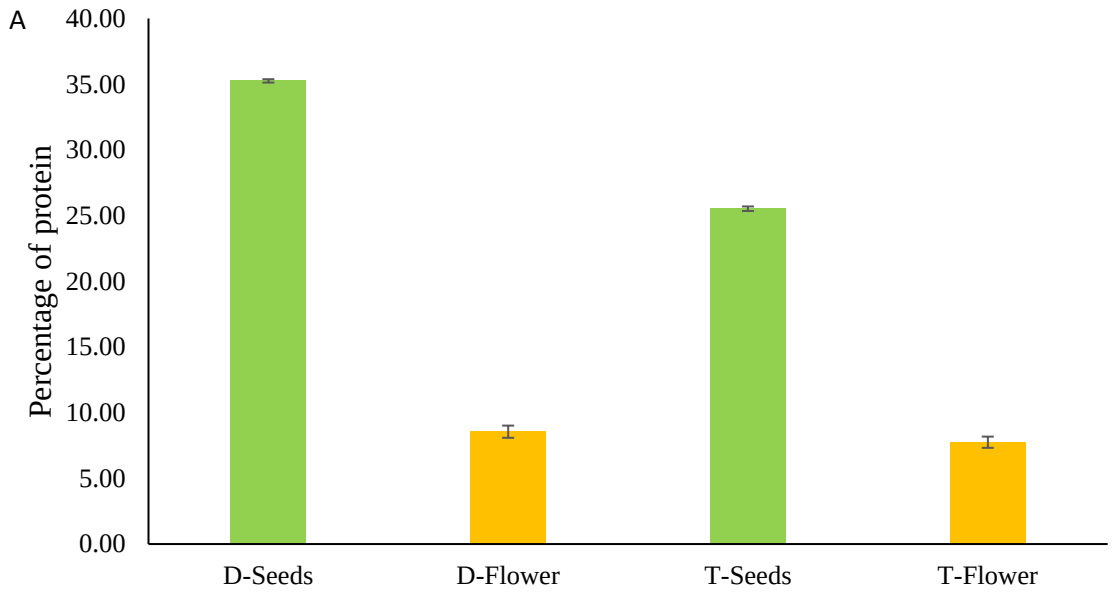


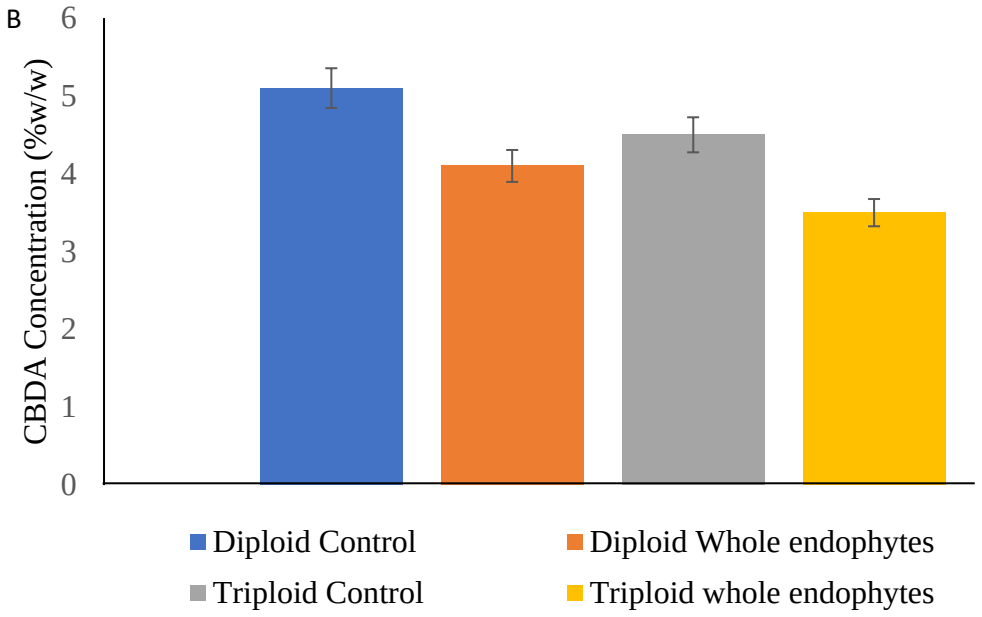
Figure 6.46: Growth-promoting and growth-suppressing hormone concentration variables in different cultivars affected by treatment with seed endophytes. Data are means and standard errors of three replicates (One-way ANOVA, $p < 0.01$).

6.5.3 Protein Concentration

The increased percentage of protein was registered in hemp seeds compared to flowers. It was 1.5 times higher in diploid than triploid seeds (A). The percentage of protein is reduced seven times in the flowering stage when compared to seeds. Both the diploid and triploid varieties have the same concentration of proteins in the flower (approx. 7%).

Protein content in seeds is not influenced by phytocannabinoids (CBDA) because they are not synchronously produced or accumulated. However, at the flower stage (C), the protein concentration was inversely proportional to the CBDA content; The CBDA concentration was decreased in endophyte-treated diploid and triploid cultivars (B), which corresponds to an increase in the protein/CBDA ratio (C) in those cultivars.





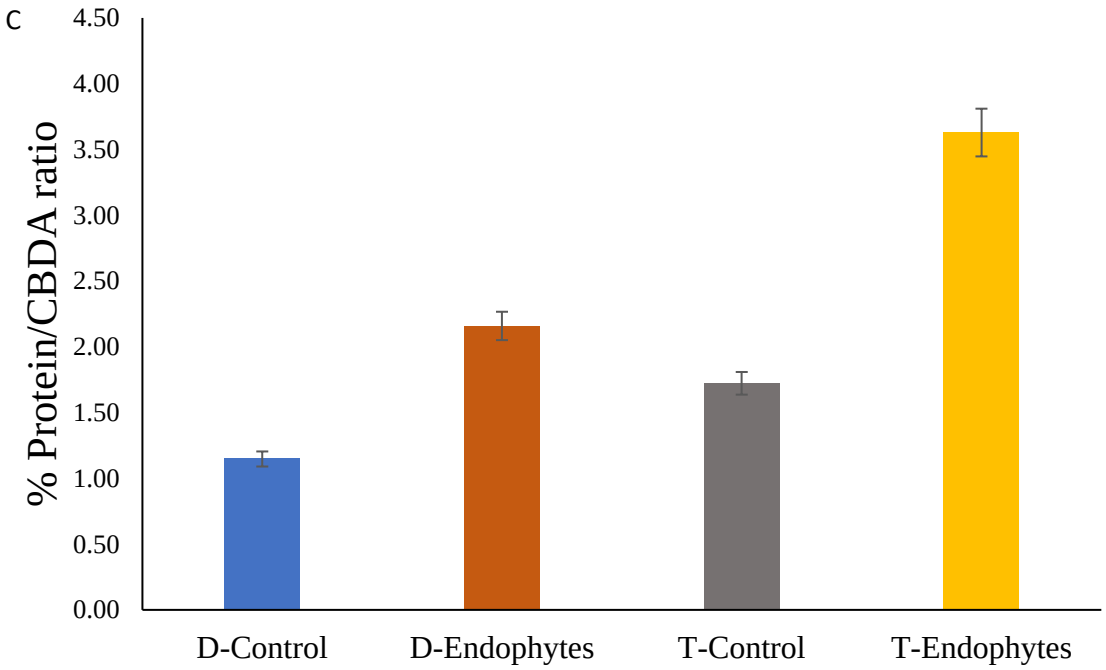
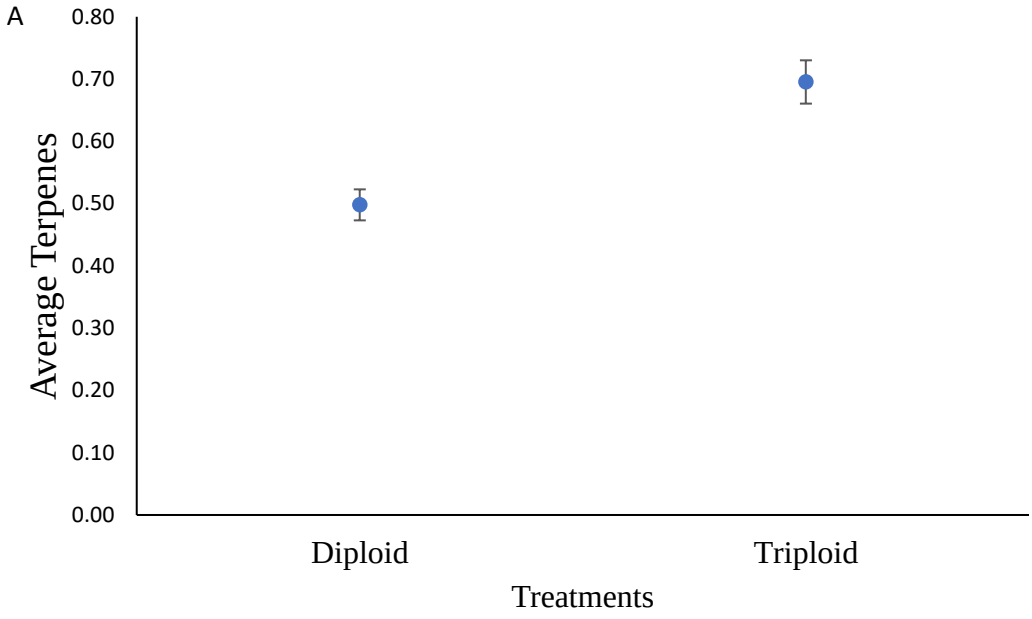
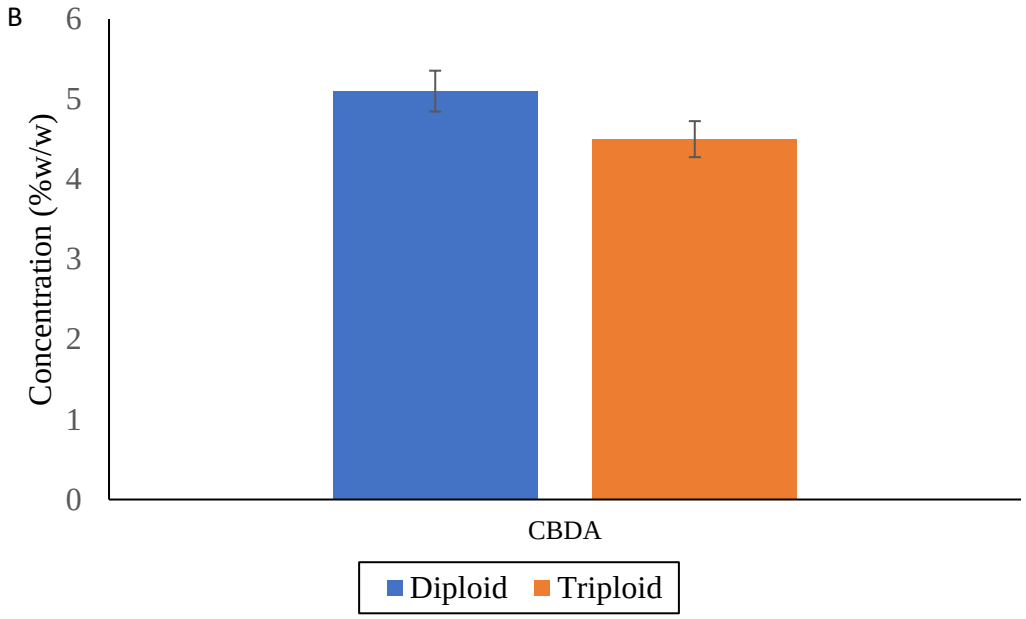


Figure 6.5: Percentage of protein in the seeds and flowers of untreated diploid and triploid plants (A) performed by the LECO test; CBDA concentration (%w/w) in untreated and seed endophyte-treated varieties of diploid and triploid cultivars (B); ratio of protein to CBDA in the flowers of different varieties of cultivar. Data are means and standard errors of three replicates (One-way ANOVA, $p < 0.05$).

6.5.4 Terpene Quantification

It was found that terpenes were found more in triploid cultivars as compared to diploid ones and they were inversely related to CBDA concentration same as like proteins. The higher the CBDA value, lower was the terpene value.





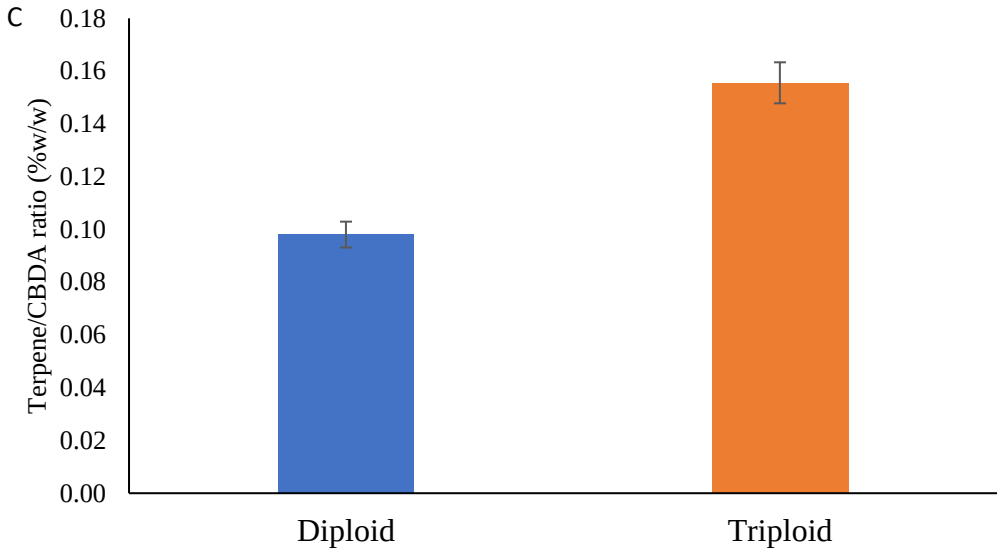


Figure 6.6: Total terpenes in diploid and triploid lines (A); total CBDA in diploid and triploid (B); and ratio of total terpenes/CBDA in untreated plants. Data are means and standard errors of three replicates (One-way ANOVA, $p < 0.05$).

6.5.5 Images of SEM

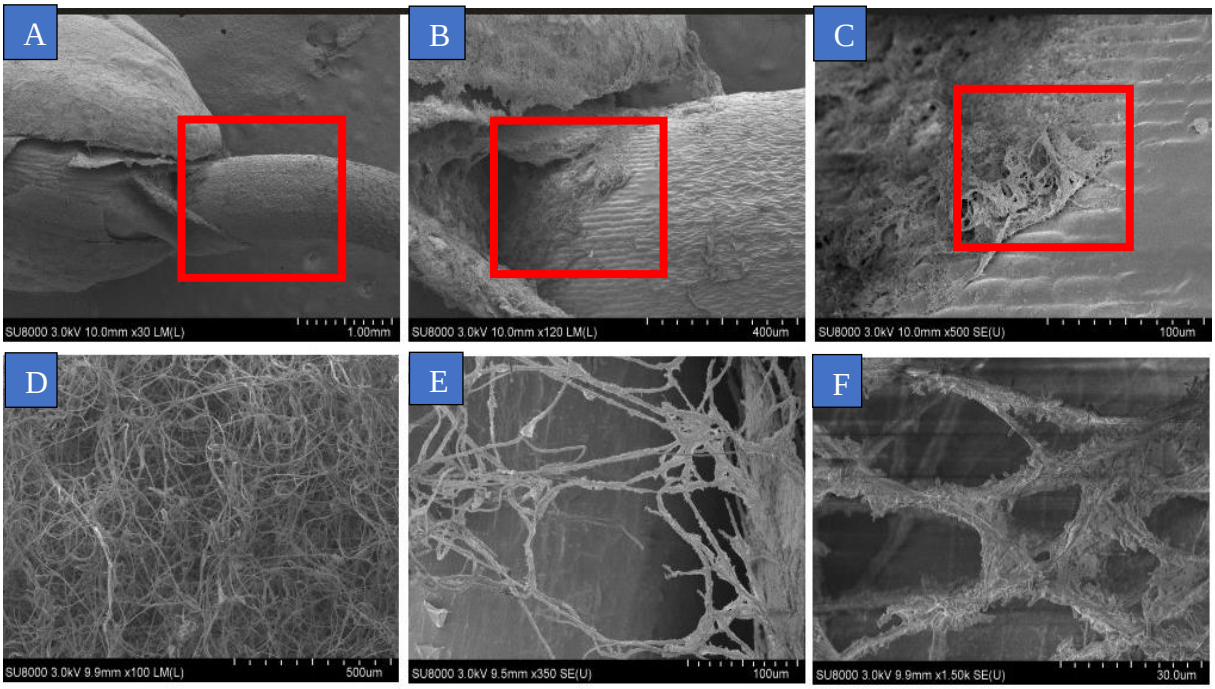


Figure 6.7: Scanning Electron Microscopy results showing interactive position of endophytes attached on the hemp seed surface. Cross sectional view of seed showing the region with red square where endophytes are situated, A-B-C. Endophytic fungi entangled in a mesh D-E, and F-endophytic bacteria forming a biofilm with colony/cells embedded in biopolymers.

6.6 Discussion

The increased phytocannabinoid ratio in endophyte-treated plants in diploid and triploid lines tested compared to untreated control underscores the role of microbiomes in influencing the concentration of THCA/CBDA ratios. This suggests that the microbiome of applied seed endophytes may directly or indirectly shape a diversity of host native microbiome and symbiotic host physiology, thus shifting the profile and accumulation of phytocannabinoid in plants treated. Second, the study registered variations in protein content in hemp seeds, with higher levels in diploid seeds—a noteworthy 1.5 times higher than in triploid seeds. However, during the flowering stage, protein content exhibited a significant reduction ($p < 0.05$), approximately sevenfold. This indicates that protein levels in seeds are not considerably

influenced by phytocannabinoid (Cattaneo et al., 2021; Pavlovic et al., 2019). However, during flower production, there is a reduced protein accumulation, which is inversely related to the increased CBDA content. It seems that the changes in these two compounds coincided with shifts in microbial diversity associated with the two plant stages, seed and flower, respectively. This study also found that the levels of growth-promoting and growth-suppressing hormones are noteworthy affected by seed endophyte treatments. The highest hormone levels were observed in the untreated diploid plants, with the second highest in untreated triploid cultivars. Surprisingly, the seed endophyte-treated plants in both host lines exhibited the lowest hormone levels, which implies that the microbiomes present in treated cultivars may play a role in promoting plant growth, thereby reducing the need for elevated hormone production by plants. Furthermore, the discussion highlights the relationship between CBDA content and terpene levels, indicating that higher CBDA values correspond to lower terpene levels, suggesting a potential trade-off between these two vital compounds. This phenomenon could be a result of resource allocation within the plant, where energy and resources may be diverted towards one pathway, such as CBDA synthesis, at the expense of terpene production.

The effect of phytocannabinoids and phytohormones on diploid and triploid hemp plants is a very complex topic. Cannabinoids and hormone concentrations are affected by the microbiomes present in a particular cultivar variety, which could be beneficial to plant health and its production in general. It is also well known that the presence and composition of terpenes can vary among plant varieties as they are regulated by various transcription factors present in the plant (Liu et al., 2021; Singh and Sharma, 2015). Therefore, further explorations of the genes regulating this plant terpenes would open a wide range of possibilities for understanding the development of crops with enhanced terpene profiles, benefiting industries such as agriculture, horticulture, and herbal medicine. Microbiomes hold the potential to reshape the phytocannabinoid profile, hormonal balance, protein composition, and secondary metabolite production in both diploid and triploid plants. Hence, understanding these complex interactions is essential not only for advancing our knowledge of plant-microbe relationships but also for unlocking the full potential of these plants in agriculture, medicine, and industry.

6.7 Conclusions

This study's findings showed that protocooperative endophytic microbiome-triploid hemp plants can eventually be an effective strategy to improve hemp host fitness. When assessing the microbiome diversity in diploid versus triploid plants, it became apparent that diploid plants naturally exhibited a higher degree of genetic variation (in plant) compared to their triploid counterparts. However, when both diploid and triploid lines received seed endophyte treatments, shifts in plant growth and reproductive performance appeared. Specifically, the triploid plants displayed more promising outcomes in several critical aspects, including growth potential, flowering potential, secondary metabolite production, and protein concentration. This intriguing observation suggests that despite the inherent genetic diversity gap between diploid and triploid plants, the introduction of external microbiomes, such as seed endophyte, effectively boosted the productivity of triploid plants. This, in turn, raises the compelling outcome that microbiomes play a pivotal role in fortifying the overall physiological health associated with improved phenotypical and molecular traits of hemp plants.

This research also highlights the remarkable effectiveness of seed endophytes in promoting the biologicals as an alternative to synthetic fertilizers and pesticides for the benefits of *Cannabis sativa* sustainable production. We discovered the remarkable potential of triploid plants, when coupled with external microbiome support, to outperform their diploid counterparts, despite their initially lower genetic diversity. This emphasizes the indispensable role of microbiomes in bolstering the overall vitality of plants, presenting exciting opportunities for the optimization of plant physiology and productivity.

7 General Discussion

Using seed endophyte treatment as a plant growth promoting and biological control agents presents a promising alternative for cultivating robust and high-yielding hemp plants, given their paramount significance in industries for their phytocannabinoids. This study intricately explores the well-established ecological triangle concept of interaction involving the environment, host (hemp plant), and microbiome, with a specific focus on hemp cultivation. The environment encompasses various factors such as soil composition, climate, and agricultural practices, all of which significantly influence hemp growth. The hosts, in this case, are diploid and triploid hemp plants as distinct host-genotype component in the interaction. The microbiome, a diverse community of microorganisms residing within these hemp plants, plays a pivotal role in its overall health and productivity. This intricate interplay involves symbiotic relationships where the microbiome contributes to nutrient uptake, disease resistance, and overall resilience of the hemp plant. Simultaneously, the hemp plant provides a niche environment for microbial communities, influencing their composition and functions.

Understanding the specific dynamics of the environment-host-microbiome triangle in the context of hemp cultivation has practical implications for optimizing hemp production. Harnessing beneficial microbial interactions can enhance soil health, nutrient cycling, and pest resistance, ultimately leading to healthier and more productive hemp crops. This holistic approach, considering the interconnectedness of these elements, is crucial for sustainable and efficient hemp cultivation practices in the ever-evolving landscape of agriculture and environmental stewardship.

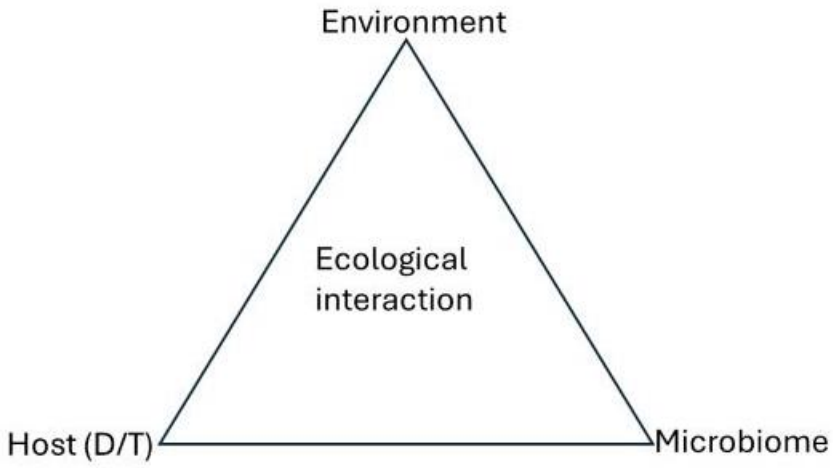


Figure 7.1: The protocooperative triangle model is comprised of environment, host (diploid/triploid) and microbiome

8 General Conclusion

In this study, following key findings were highlighted

Hemp traits and its health: Hemp is a versatile and sustainable plant that has a range of traits and potential health benefits. It's important to distinguish hemp from marijuana as both belong to the *Cannabis sativa* species but they have different traits and purposes. This study focused on making this plant healthy with the perspective of 5 key elements.

1. **Positive Impact of Endophytes**: The research demonstrated that both epiphyte and endophyte, along with whole seed, contributed significantly to the growth and overall health of hemp plants. Endophyte treatments showed a positive effect on plant health and were considered for future objectives, suggesting their potential in hemp cultivation.
2. **Microbiome Diversity**: The study revealed a diverse range of microbiomes in both diploid and triploid hemp cultivars. These findings underscore the importance of microbiomes in polyploidization - influencing plant health, growth, and characteristics.
3. **Core Microbiome Communities**: The identification of core microbiome communities opens avenues for further research to enhance hemp cultivation in different ploidy hemp plants. Understanding and manipulating these communities may lead to improved crop production.
4. **Complex Relationship Between Cannabinoids, Hormones, and Microbiomes**: This study hinted at the complex interplay between phytocannabinoids, phytohormones, and microbiomes in diploid and triploid hemp plants. The presence of specific microbiomes seemed to affect the concentrations of cannabinoids and hormones, which could have significant implications for plant health and productivity.
5. **Terpene Variation and Gene Regulation**: Terpene composition in hemp plants was found to vary among different plant varieties, influenced by various transcription factors (TFs). Exploring the genes that regulate terpenes could offer insights into developing crops with enhanced terpene profiles, benefiting various industries such as agriculture, horticulture, and herbal medicine. In the presence of presence and absence of endophyte.

9 References

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10 Appendix

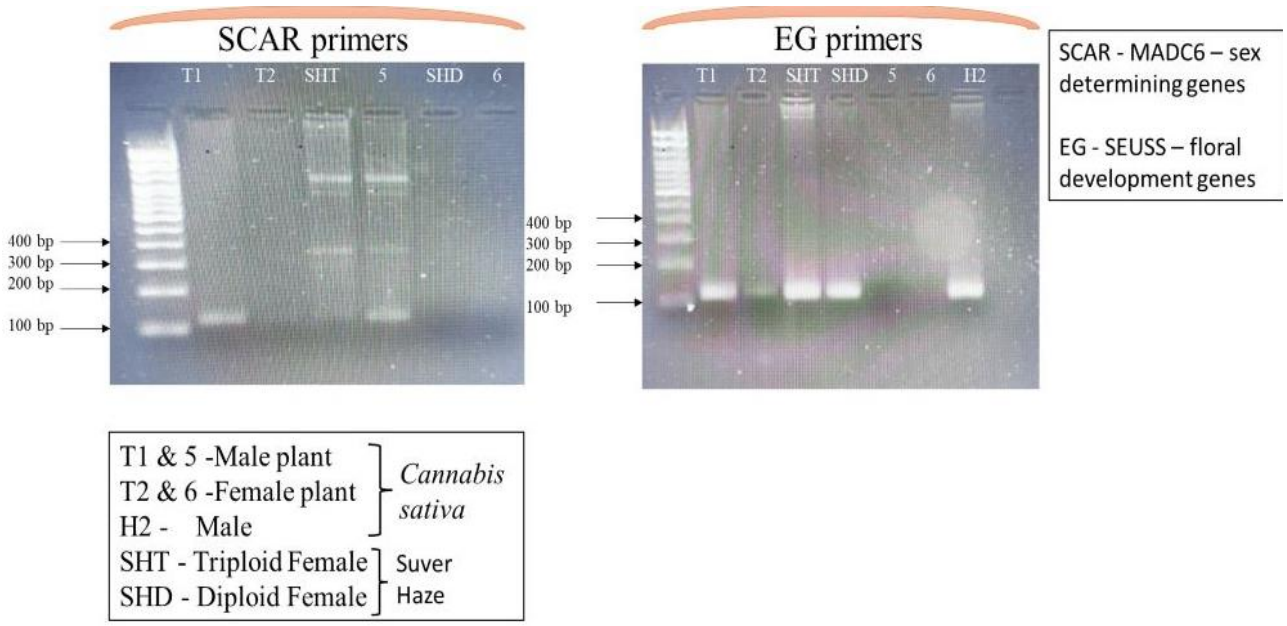

Figure 9.1: Polymerase chain reaction with SCAR and EG primers for *Cannabis sativa* leaves.