The effect of microbial inoculation and nutrient regimes on *Cannabis* stalked trichome development, growth, and cannabinoid profiles

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Abstract

With *Cannabis sativa* L. being a newly available agricultural crop for the rapidly growing medicinal and recreational-use sectors, the need to develop sustainable production approaches to be adopted by this novel industry is more important than ever. *Cannabis* is known for its cannabinoids, which are primarily produced within stalked glandular trichomes on inflorescences. The ability to optimize trichome formation while providing plants minimal soil/nutrient inputs is imperative to inform growers on how best to incorporate modern eco-friendly agricultural practices into their operations. Plant-growth promoting rhizobacteria (PGPR) are well-established for improving yields in a variety of crops. They have also been found to improve essential oil content and glandular trichome development of common garden plants, typically herbs; research in this area tailored for *Cannabis* cultivation is very limited. As PGPR are often used to mitigate stress effects, we postulated that PGPR-associated benefits by way of *Cannabis* stalked glandular trichomes and overall plant development could be maintained, if not improved, when plants are subjected to stress.

This thesis project aimed to evaluate how PGPR inoculation affects *Cannabis* stalked trichome development, and if an environmental stressor plays a role in stimulating PGPR response. Cultivar "CBD Kush" was cloned from mother plants by vegetative cuttings that were transplanted into soil, at which time strains of *Pseudomonas* sp. and *Bacillus* sp. were inoculated either separately or in combination. Plants were grown either under recommended or low nutrient regimes until week 6 of development, after which all plants were given recommended nutrient levels. Development variables were measured weekly. At time of harvest, one inflorescence per plant was dissected to individual calyces and bracts, and their epidermal surfaces were imaged. These surface areas were measured using ImageJ and a cell counter plugin was used to count individual stalked trichomes, determining density. In addition, developmental variables were

measured weekly from time of transplant to harvest to assess if nutrient stress impacts overall plant growth, and if PGPR inoculation can affect the plant response.

The results revealed that when plants were grown at recommended nutrient levels, PGPR treatments unexpectedly had a tendency to somewhat reduce the densities of stalked glandular trichomes on inflorescence organs. Consistent with this, the contents of 9 cannabinoids were slightly reduced. When plants were grown under the low nutrient regime there was a positive effect of PGPR on trichome densities. While this tendency was inconsistent for cannabinoid levels under the low nutrient regime, when comparing results between the two nutrient regimes, the prevalent cannabinoids were present in greater quantities under nutrient limitation. Additionally, *Bacillus* sp. inoculation coupled with the low nutrient regime was related to the greatest number of changes to the cannabinoid profile. There were no significant changes to development variables regardless of PGPR treatment for either nutrient regime. Leaf area was enhanced under limited nutrients compared to the recommended nutrient regime, by all PGPR treatments. In addition, average individual inflorescence mass was greater under the low nutrient regime, with the *Bacillus* sp. treatment resulting in the highest average. There were no visual indicators of poor plant health with low nutrient levels. By demonstrating a positive relationship between environmental stress and PGPR presence on cannabinoid yield, we have provided incentive for companies to both reduce soil nutrient inputs for *Cannabis* and incorporate the use of PGPR as biofertilizers to optimize cannabinoid yields. We recommend further studies be undertaken to identify ideal environmental stressors to further reduce chemical inputs, and to elucidate the interactions between rhizosphere microorganisms and trichome development.

Résumé

Le *Cannabis sativa* L. étant une culture agricole nouvellement disponible pour les secteurs en pleine expansion de la médecine et des loisirs, il est plus important que jamais de développer des méthodes de production durables qui seront adoptées par cette nouvelle industrie. Le cannabis est connu pour ses cannabinoïdes, qui sont principalement produits dans les trichomes glandulaires pédonculés des inflorescences. Il est impératif d'optimiser la formation des trichomes tout en fournissant aux plantes un apport minimal en sol et en nutriments afin d'informer les cultivateurs sur la meilleure façon d'intégrer les pratiques agricoles modernes et respectueuses de l'environnement dans leurs opérations. Les rhizobactéries favorisant la croissance des plantes (PGPR) sont reconnues pour leur capacité à améliorer les rendements de diverses cultures. On a également constaté qu'elles amélioraient la teneur en huile essentielle et le développement des trichomes glandulaires des plantes de jardin courantes, généralement des herbes aromatiques ; les recherches dans ce domaine adaptées à la culture du cannabis sont très limitées. Comme les PGPR sont souvent utilisées pour atténuer les effets du stress, nous avons supposé que les avantages associés aux PGPR par le biais des trichomes glandulaires des tiges de cannabis et du développement général de la plante pourraient être maintenus, voire améliorés, lorsque les plantes sont soumises à un stress.

Ce projet de thèse visait à évaluer comment l'inoculation de PGPR affecte le développement des trichomes de Cannabis, et si un facteur de stress environnemental joue un rôle dans la stimulation de la réponse des PGPR. Le cultivar "CBD Kush" a été cloné à partir de plantes mères par des boutures végétatives qui ont été transplantées dans le sol. Des souches de *Pseudomonas* sp. et de *Bacillus* sp. ont alors été inoculées soit séparément, soit en combinaison. Les plantes ont été cultivées selon les régimes nutritifs recommandés ou faibles jusqu'à la sixième semaine de développement, après quoi toutes les plantes ont reçu les niveaux nutritifs

recommandés. Les variables de développement ont été mesurées chaque semaine. Au moment de la récolte, une inflorescence par plante a été disséquée en calices et bractées individuels, et leurs surfaces épidermiques ont été imagées. Ces surfaces ont été mesurées à l'aide d'ImageJ et un plugin de compteur de cellules a été utilisé pour compter les trichomes pédonculés individuels, afin de déterminer la densité. En outre, les variables de développement ont été mesurées chaque semaine, de la transplantation à la récolte, afin d'évaluer si le stress nutritionnel a un impact sur la croissance globale de la plante et si l'inoculation de PGPR peut affecter la réponse de la plante.

Les résultats ont révélé que lorsque les plantes étaient cultivées aux niveaux de nutriments recommandés, les traitements PGPR avaient, de manière inattendue, tendance à réduire quelque peu les densités de trichomes glandulaires pédonculés sur les organes d'inflorescence. En conséquence, les teneurs en 9 cannabinoïdes ont été légèrement réduites. Lorsque les plantes ont été cultivées sous un régime pauvre en nutriments, la PGPR a eu un effet positif sur la densité des trichomes. Bien que cette tendance ne soit pas cohérente pour les niveaux de cannabinoïdes sous le régime de faibles nutriments, lorsque l'on compare les résultats entre les deux régimes de nutriments, les cannabinoïdes prédominants étaient présents en plus grandes quantités sous la limitation des nutriments. En outre, l'inoculation de *Bacillus* sp. associée à un régime de faibles nutriments a été liée au plus grand nombre de changements dans le profil des cannabinoïdes. Il n'y a pas eu de changements significatifs dans les variables de développement, quel que soit le traitement par PGPR et quel que soit le régime nutritif. La surface foliaire a été augmentée dans des conditions de nutriments limités par rapport au régime de nutriments recommandé, par tous les traitements PGPR. En outre, la masse moyenne des inflorescences individuelles était plus importante sous le régime de faibles nutriments, le traitement par *Bacillus* sp. donnant la moyenne la plus élevée. Il n'y a pas eu d'indicateurs visuels

de mauvaise santé des plantes avec de faibles niveaux de nutriments. En démontrant une relation positive entre le stress environnemental et la présence de PGPR sur le rendement en cannabinoïdes, nous avons incité les entreprises à réduire les apports de nutriments dans le sol pour le cannabis et à incorporer l'utilisation de PGPR comme biofertilisants pour optimiser les rendements en cannabinoïdes. Nous recommandons que d'autres études soient entreprises pour identifier les facteurs de stress environnementaux idéaux afin de réduire davantage les intrants chimiques, et pour élucider les interactions entre les micro-organismes de la rhizosphère et le développement des trichomes.

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Chapter 1 – General Introduction

1.1 Introduction

The *Cannabis* industry (hereafter, cannabis) is growing at a rapid rate within Canada, and the industry is expected to continue growing globally as more countries legalize it for recreational use. With these expanding markets, there will be further strain on the environment from both outdoor and greenhouse production facilities. In addition, the chance to provide sustainable production options for optimized yields at the start of a new agricultural industry is a particularly unique opportunity for the 21st century. While there are a variety of avenues to incorporate novel technologies for an industry, it is important to provide science-based information for producers to take them on.

The ability to reduce the need for soil inputs in production operations and implement the use of biofertilizers in the form of plant growth-promoting rhizobacteria (PGPR) would be of significant benefit not just to the ecosystem around outdoor grow operations and related wastewater facilities, but also to producers themselves. Applying high amounts of expensive synthetic fertilizers and pest control throughout plant development can lead to a range of environmental harms in the form of air and water pollution and increasing the rate of climate change. PGPR strains have been demonstrated to promote plant growth by improving root development and production of plant hormones, as well as stimulating natural defence pathways to mitigate infections (reviewed by Lyu et al. 2021a,b). For example, nitrogen application is of particular concern, with the agriculture sector being a major source of environmental degradation through overuse of the nutrient. As nitrogen is vital to plant yield, its use is directly linked to agricultural profits. Therefore, a reduction in its application must be compensated by an

improvement in nitrogen acquisition efficiency (Kanter et al., 2014); PGPR strains proven to increase nutrient acquisition for other crop species are seen as a realistic solution.

Reducing soil inputs for cannabis has so far been with regard solely to PGPR inoculations, with the consensus presently being that there is a benefit to their use as a replacement for traditional fertilizer applications (Pagnani et al., 2018; Lyu et al., 2019, Lyu et al. 2022). However, these have been with regards to whole-plant effects and cannabinoid concentrations, not the specific secretory structures, which are glandular trichomes on the inflorescences. Additionally, there has yet to be any studies on cannabis investigating the possibility of pushing the reduction of soil inputs by applying an environmental stressor, but there have been studies on common agricultural crops that indicate that this may be a boon to the industry when linked with PGPR inoculations (Eshaghi Gorgi et al. 2022); one study specifically linked this design with an increase in trichome counts (Mirzaie et al., 2020). This intriguing connection is worth exploring, as not only would it allow for a further environmentally friendly approach to cannabis production, but it would also be relatively simple to justify to producers to incorporate into their facilities by improving their financial returns.

In this study, the use of two PGPR strains previously established to have beneficial effects on plant development (Lyu et al., 2022) were applied to cannabis plants at time of transplant. In addition, further subsets of plants with same inoculation conditions were kept on a low nutrient regime until the start of the 6th week of development, which is when flowers primarily begin to develop. The goal was to determine if the density of stalked glandular trichomes will increase with the presence of these PGPR, and if the densities and the overall development of the plants are consistent under a low nutrient regime up until the week that flower development begins. Both these objectives are novel to the cannabis industry, and methodology for determining trichome

densities on cannabis floral tissue has not been previously described. The literature review regarding cannabis trichomes and questions surrounding their development has previously been published and was reformatted for this thesis.

1.2 Research Objectives and Hypotheses

Overall objective:

Determine if the presence of PGPR increases the density of stalked glandular trichomes on cannabis floral tissue.

Specific objectives:

1. Determine if incorporating a lower than conventionally recommended nutrient regime throughout vegetative development will enhance the effect of the PGPR on stalked glandular trichome densities.
2. Determine if a low nutrient regime for a partial period of development coupled with PGPR presence will have a significant effect on overall plant development and yield.

Hypothesis:

1. PGPR inoculation will increase the density of stalked glandular trichomes on cannabis floral organs, thereby increasing the cannabinoid concentration of inflorescences.
2. The stress response to a low nutrient regime will be mitigated by PGPR presence, preventing a reduction in trichome densities and maintaining normal plant development and yield.

Chapter 2 – Literature Review

Title: Cannabis Glandular Trichomes: A Cellular Metabolite Factory

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Abstract

Cannabis has been legalized for recreational use in several countries and medical use is authorized in an expanding list of countries; markets are growing internationally, causing an increase in demand for high quality products with well-defined properties. The key compounds of Cannabis plants are cannabinoids, which are produced by stalked glandular trichomes located on female flowers. These trichomes produce resin that contains cannabinoids, such as tetrahydrocannabinolic acid and cannabidiolic acid, and an array of other secondary metabolites of varying degrees of commercial interest. While growers tend to focus on improving whole flower yields, our understanding of the "goldmines" of the plant – the trichomes – is limited despite their being the true source of revenue for a multi-billion-dollar industry. This review aims to provide an overview of our current understanding of cannabis glandular trichomes and their metabolite products in order to identify current gaps in knowledge and to outline future research directions.

2.1 Introduction

Trichomes are formed on the plant surface across a range of taxonomically disparate species, providing a variety of functions and benefits to the plant. These can include simple tasks, such as affecting leaf temperature and photosynthesis, or more complicated functions, such as pest-deterrence *via* their physical structures or production of compounds (Wagner, 1991; Hare et al., 2003). Glandular trichomes are of particular commercial interest as they are one of the key plant structures that produce essential oils – an industry valued at 18.62 billion USD in 2020 (Grand View Research, 2020). Other oil-producing plant structures are internal glands and other trichome types, some of which are capable of producing resinous secretions. Trichome morphology is highly variable both among plant species and within the plant itself (Sangwan et al., 2001). In *Cannabis sativa* L. (hereafter, cannabis), stalked glandular trichomes are the trichome morph that produces substances of economic value (Fairbairn, 1972; Sirikantaramas et al., 2005). These trichomes develop a secretory cavity between secretory disk cells and the cuticle where secondary metabolites, including cannabinoids and terpenes, are deposited and stored (Kim and Mahlberg, 1991, 1997; Sirikantaramas et al., 2005; Marks et al., 2009). Though there are a variety of other trichome morphs found across the cannabis plant, they are beyond the scope of this review.

While male plants produce small amounts of cannabinoids, in cannabis cultivation, the primary products are the female flowers clustered in inflorescences (Ohlsson et al., 1971). Stalked glandular trichomes are primarily concentrated on the calyces and bracts (Figure 2.1A; Spitzer-Rimon et al., 2019; Leme et al., 2020) with populations extending to the inflorescence “sugar

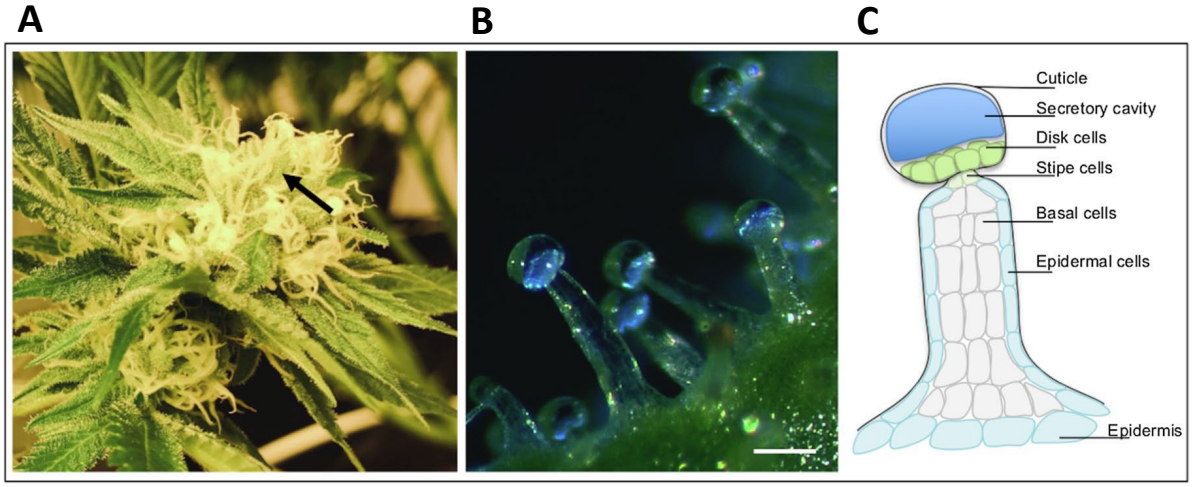

Figure 2.1: Cannabis (*Cannabis sativa L.*) inflorescence and trichomes. (A) An individual inflorescence, with majority of the organs covered in stalked glandular trichomes. Arrow indicates cluster of calyces and bracts covered with trichomes. (B) Dark field micrograph of stalked glandular trichomes protruding from calyx epidermis. Biosynthesis of secondary metabolites occurs in the secretory disk cells lining the base of the globular trichome head. The metabolites are stored in the clear subcuticular cavity above the secretory disk cells; this cavity will turn milky white to dark brown over the course of flower maturity. (C) Graphic illustration of stalked glandular trichome structure.

leaves"; these are the sites of accumulation for secreted metabolic products. These valuable secretions include tetrahydrocannabinolic acid (THCA), cannabidiolic acid (CBDA), terpenes, and flavonoids (ElSohly and Slade, 2005; Flores-Sanchez and Verpoorte, 2008). Cannabis plant morphology and cannabinoid profiles are influenced by genetics and the cultivation environment, highlighting the importance of controlled conditions for cannabis cultivation (Magagnini et al., 2018; Danziger and Bernstein, 2021a,b). With the gradual global increase in social and legal acceptance of cannabis, there has been considerable interest in producing consistent high-quality yields. In addition, as medicinal uses for cannabinoids are supported by peer-reviewed research and clinical trials, the global demand for medicinal cannabis products will continue to increase. This will create further pressure on growers to improve control over the concentration of specific cannabis metabolites and the associated cannabis genotypes. However, the genotypes and environmental conditions needed to obtain this level of precision remain poorly characterized.

Ultimately, these elusive methods need to be centered around trichomes as the "factories" of the plant. Current efforts have focused on the effects of breeding and cultivar selection, industrial growing conditions, and fertilization methods on flower yield and cannabinoid profiles (Vanhove et al., 2011; Campiglia et al., 2017; Tang et al., 2017; Hawley et al., 2018; Janatová et al., 2018; Burgel et al., 2020; Saloner and Bernstein, 2021). However, as undefined cannabis plant material in pioneering research papers formed the backbone for future cannabis/cannabinoid research, comparing data with uniform standards is impossible. Thus, the need for systematically validating results of these papers and cannabis production "folklore" is paramount yet challenging due to the impact of genotype and growing environment. Regardless of these challenges, since trichomes are ultimately responsible for yield and quality control, it is necessary to advance our understanding of how they, specifically, are affected by these efforts, as well as to investigate new approaches to broaden the scope of possible cost-effective applications for improving yield.

2.2 Trichome Profiles

2.2.1 Trichomes Across the Plant Kingdom

Trichomes are found across the plant kingdom, displaying a stunning variety of shapes and properties. Glandular trichomes, which arise from the epidermis on vegetative and reproductive organs, can be generally divided into secretory and non-secretory types with the former being able to secrete substances (Tian et al., 2017). Both the morphologies and metabolic secretions of trichomes are consistent within a plant species, and some species have different trichome morphs on the same plant organ (Muravnik, 2020). Secreted compounds, including THCA (Sirikantaramas et al., 2005), can be toxic to plant cells; therefore, metabolite storage in the cavity of the glandular head affords protection to the plant (Sirikantaramas et al., 2008). While different glandular

trichome morphs invoke different storage strategies, the architecture of the morph and cavity position in relation to the secretory cells determine secretion direction (Tissier et al., 2017). The molecular details surrounding the development of glandular trichomes and their secretions are beyond the scope of this article, and we refer to in-depth reviews by Muravnik (2020) and Tian et al. (2017).

Genomic studies are imperative to investigate the factors that influence trichome development in cannabis, both within and between cultivars. Trichome differentiation mechanisms have been investigated in *Arabidopsis thaliana*, with transcription factor (TFs) groups playing key roles in the transcriptional networks for trichome production and patterns (Tian et al., 2017). While genomic studies are available for other economic plants, including *Humulus lupulus* which belongs to the Cannabaceae family (Matoušek et al., 2016; Mishra et al., 2020), similar studies for cannabis are lacking despite their potentially important impacts for precise cannabis trichome control. Taking advantage of the genetic libraries available for related species with similar resin secretions will help guide these much-need studies (Braich et al., 2019; Zager et al., 2019; Liu et al., 2021).

2.2.2 Key Cannabis Metabolites

The decades-long stigma surrounding cannabis has led to a variety of misconceptions surrounding the plant and its products regarding cannabinoid biosynthesis. While THCA and CBDA are the major cannabinoids produced by the plant, their degradation products, THC and CBD, are of great interest for their psychoactive and therapeutic effects. Additional cannabinoids are gradually gaining interest as their effects on the human body are beginning to be understood (ElSohly and Slade, 2005; Aizpurua-Olaizola et al., 2016; Andre et al., 2016). Fresh cannabis flower tissue contains relatively low levels of THC and CBD and higher levels of THCA and

CBDA as the acid forms are converted to neutral forms *via* decarboxylation in post-harvest processing and storage; the rate of conversion is primarily dependent on temperature and light (Yamauchi et al., 1967). Metabolites, including cannabinoids, terpenes, and flavonoids, are formed within secretory disk cells that line the base of the glandular trichome head and stored in the subcuticular cavity (Figure 2.1 B,C; Kim and Mahlberg, 1991, 1997). Within the cells, cannabinoid biosynthesis starts in the cytosol, moves to the plastid, and finishes with oxidocyclization in the apoplastic space; transport between these areas is not yet resolved (Gülck and Møller, 2020). A plethora of cannabinoids have been identified in recent years, bringing the total known number to just over 110, which can be divided into 11 subclasses (ElSohly and Gul, 2014; Andre et al., 2016; Hanuš _et al., 2016; Berman et al., 2018). The biosynthesis pathways of the key cannabinoids, in particular THC and CBD, are described in detail in previous reviews (Gülck and Møller, 2020; Desaulniers Brousseau et al., 2021).

To date, over 120 terpenes have been identified in cannabis, which are broadly classified as monoterpenes and sesquiterpenes based on differences in their carbon skeletons (ElSohly and Slade, 2005; Degenhardt et al., 2009). Terpenes have a biosynthesis pathway similar to cannabinoids, and this process has been extensively reviewed (Booth et al., 2017; Desaulniers Brousseau et al., 2021). Terpenes impart floral aroma and flavor, making them important components for plant product applications, like essential oils, from many plant species. Terpene profiles vary among cannabis cultivars (Booth et al., 2017) and hemp oils containing more monoterpenes score better on olfactory evaluations than oils containing more sesquiterpenes while an oil containing a mix of both scored highest on scent tests (Mediavilla and Steinemann, 1997). Thus, the terpene composition of cannabis flowers at maturity can directly affect the olfactory quality of flower-based products and extracts, including essential oil-based goods.

Flavonoids are an additional major cannabis phytochemical group; however, this group of compounds has received less research focus compared to cannabinoids and terpenes. Similar to terpenes, flavonoids are found across a wide range of plant genera with a broad range of roles and benefits for the plant (Panche et al., 2016). There are over 20 identified flavonoids for cannabis, with three relatively unique compounds known as cannflavin A, B, and C (Bautista et al., 2021). The potential pharmaceutical uses of flavonoids, spanning from anti-inflammatories to anti-cancer therapies, are boosting interest in these compounds particularly as the entourage effects afforded by cannabis metabolite profiles become better understood (Tomko et al., 2020; Bautista et al., 2021). As flavonoids are produced primarily in cannabis leaves, not the inflorescences (Jin et al., 2020), the present article will focus on cannabinoids and terpenes.

2.3 Cannabis Glandular Trichomes

Previously, three types of glandular trichomes on cannabis flowers were described – referred to as capitate-sessile, capitate-stalked, and bulbous – based on structural assessments by scanning electron microscopy (Hammond and Mahlberg, 1973). The trichomes were differentiated based on their morphology, where bulbous trichomes were small and low, sessile trichomes were comprised of a globular head on a very short stalk, and stalked trichomes had a larger globular head on a long stalk; of the three trichome types, stalked trichomes produce the greatest amount of cannabinoids (Hammond and Mahlberg, 1973; Mahlberg and Kim, 2004; Livingston et al., 2020). Unfortunately, this non-specific differentiation between trichome types led to misidentification of trichomes due to the similar appearance of sessile and stalked morphs (Dayanandan and Kaufman, 1976; Livingston et al., 2020). However, a recent study on trichome anatomy revealed that sessile trichomes on vegetative leaves consistently have exactly eight

secretory disk cells while stalked glandular trichomes on mature flowers have 12–16; these numbers were consistent across hemp and drug-type varieties (Livingston et al., 2020). As sessile-presenting trichomes on immature cannabis flowers can contain more than eight disk cells and emit fluorescence at intermediate wavelengths, which true sessile trichomes cannot, sessile-presenting trichomes are now thought to be a precursor developmental stage of immature stalked trichomes (Livingston et al., 2020). These discoveries allow for improved accuracy of trichome classification during plant development, may provide more precise estimates of plant maturity and allow for identification of optimal points of metabolite production. This understanding further allows for greater accuracy when assessing the density of stalked glandular trichomes and the ability to predict mature flower trichome densities.

The causes of variable metabolite profiles found among varieties/genotypes and plant organs are genetic and environmental. For example, flowers sampled from the upper region of the plant produce significantly greater quantities of cannabinoids and terpenes than lower positions; light source and plant maturity are believed to be important factors influencing the concentration and/or amounts (Namdar et al., 2018; Eichhorn Bilodeau et al., 2019). Abiotic factors that influence cannabis growth are the same as those affecting other plant species, such as temperature, fertilization, photoperiod, and light intensity (Taschwer and Schmid, 2015; Conant et al., 2017; Pagnani et al., 2018; Bernstein et al., 2019; Eichhorn Bilodeau et al., 2019; Taghinasab and Jabaji, 2020). However, knowledge regarding how these factors influence growth and trichome formation is limited, with much work needed to produce scientific evidence to support links between metabolite production and environmental factors (Taghinasab and Jabaji, 2020). Research on cannabis is in the early stages, and future work is necessary to investigate signaling pathways that mediate the effect of external factors on metabolite production. Attention toward developing this

area of cannabis research is increasing (Mudge et al., 2019; Aliferis and Bernard-Perron, 2020; Conneely et al., 2021).

2.3.1 Potential Benefits of Cannabis Trichomes to the Plant

The exact benefit of cannabinoids and terpenes for the plant has yet to be discovered but several findings point to defense-related functions. This is consistent with a common role of trichomes in many plant species (Levin, 1973). Early studies have also hypothesized that THC protects against ultraviolet (UV) radiation, as cannabis plants produce significantly elevated levels of THC when exposed to higher levels of UVB radiation, possibly resulting in the development of geographical chemotypes (Pate, 1983). A recent study found that CBD could be a potential sunscreen additive as its application to human keratinocyte and melanocyte cells led to improved cell viability after exposure to UVB radiation, suggesting that cannabinoids protect cells against this type of potentially DNA-damaging radiation and supporting the geographical chemotype hypothesis (Gohad et al., 2020). These findings indicate that cannabinoids may be secreted and concentrated around flowers to protect the reproductive organs – and thereby the next generation – from the effects of sun damage; genotypes that originate from closer to the equator will produce higher levels of cannabinoids due to the higher incidence of UVB radiation in that region.

Terpenes may act as deterrents against herbivory, as the monoterpenes a-pinene and limonene repel insects are present in higher concentrations in flowers while sesquiterpenes, which are bitter to mammals, have greater concentrations in the lower leaves (Potter, 2009; Nerio et al., 2010; Russo, 2011). This apparent range of terpene profiles, dependent on organ and position, is in line with probable causes of damage, as insects would be more likely to damage the flowers and herbivorous mammals are likely to focus on the larger fan leaves. In addition, cannabinoids and

terpenes can complement each other to provide plants with a complex defense mechanism against insects. The ratio of monoterpenes to sesquiterpenes determines cannabis resin viscosity while CBGA and THCA are toxic to insects. Altering the ratio of terpene types to increase viscosity can trap insects while CBGA and THCA induce apoptosis as shown on cultured insect cell lines, thus protecting the plant and critical tissues like flowers as they develop (Sirikantaramas et al., 2005; Russo, 2011). Terpenes and cannabinoids also interact after ingestion by animals as terpenes were shown to contribute to the affinity of THC to cannabinoid receptor 1 receptors in humans, among other effects (Russo and McPartland, 2001; Andre et al., 2016). The interactions between terpenes and cannabinoids are thus subject to ongoing investigations, not only to gain insight into the role of terpenes for plants, but also due to the potential therapeutic benefits which the medicinal cannabis sector could leverage.

The role of cannabinoids in biotic stress tolerance is consistent with their elevated concentration in flowers where trichome densities are highest. In addition to reducing the risk of pest-related damage, cannabinoids also have antimicrobial properties. Five key compounds [THC, CBD, cannabichromene (CBC), cannabigerol (CBG), and cannabinol (CBN)] and their acid precursor forms have significant antibacterial activity against several methicillin-resistant *Staphylococcus aureus* strains through bacterial membrane targeting (van Klingeren and ten Ham, 1976; Appendino et al., 2008; Farha et al., 2020). This suggests that cannabinoids, including those that are typically secreted in low concentrations, have a broad range of benefits, acting both within and outside the plant, particularly with regards to cannabinoid production in flowers when compared to the rest of the plant (Farha et al., 2020). However, while there is an increasing understanding of the defensive properties of the major metabolic products produced by cannabis, the lesser-known compounds must also be given attention. As there have been over 200 identified

cannabinoid and terpene compounds combined, the costs for producing this vast number of secondary metabolites must be investigated to elucidate their individual benefits and roles in plant function. Transcriptomic studies into these lesser-known compounds and their expression in response to common stressors could provide an important start into answering these questions.

Overall, the range of potential benefits of these secondary metabolites strongly suggests that they play a key role in the general health and survival of cannabis plants and their progeny through a combination of factors. To corroborate this, genomics, transcriptomics, and metabolomics studies must be conducted to confirm hypothesized characteristics associated with various trichome morphs, their development patterns across different tissues, and their non-uniform metabolite secretions. Evidence is required to prove that these compounds are not simply by-products of other biological processes but truly have a primary role in defense mechanisms. To be meaningful, these studies should not only include cannabis cultivars that are the result of centuries of breeding, but also naturally occurring types that are not products of human selection activity, though these are rarely available. One hundred ten whole genomes of cannabis cultivars, from wild plants and historical varieties to modern hybrids, with a focus on Asian sources to account for the likely domestication origin, were recently sequenced and analyzed to provide an invaluable genetic framework for the history of the plant; the resulting information can be applied to secondary metabolite investigations (Ren et al., 2021). With time, the validity of these hypotheses is sure to be determined thanks to this new genomic information, along with valuable insight into the impressive complexity seen within them.

2.4 Conclusion and Future Prospects

Cannabis was left behind in the agricultural research boom of the last century because of its illegal status in most jurisdictions. While many of the advancements in plant science for a wide range of other species are applicable to cannabis, multiple species-specific traits require dedicated research both to gain fundamental insights and to provide evidence-based data to the growing industry. Since industrial agriculture practices became globally established and genomic studies became possible in the 20th century, researchers have been able to elucidate novel agricultural applications derived from molecular-scale understanding, while cannabis applications remain centered on breeding and environmental conditions; cultivation protocols were largely based on anecdotal rather than scientific evidence. For example, the soybean genome has been unraveled to identify genetic markers related to nematode resistance and this has been exploited to support precise breeding strategies (Kim et al., 2016); meanwhile, the simple taxonomy of cannabis remains controversial (Koren et al., 2020). The cannabis research field is slowly catching up to the level of investigation that is observed for other valuable crop species, with one example being a recent study demonstrating a high-throughput assay using genetic markers to identify sex and chemotype of cannabis germplasm (Toth et al., 2020). However, this study was primarily focused on THC:CBD ratios to determine chemotype and when modeling "total potential cannabinoids" only THC, CBD, CBG, and CBC were included, highlighting the limits of current genetic studies (Toth et al., 2020). Regardless of their limitations, these studies signal the beginning of cannabis truly entering 21st century agricultural research.

Trichomes and essential oils in other plant species have been well characterized in recent decades, and it is important that our understanding of cannabis trichomes reach similar levels of comprehension. The increasingly widespread legalization and public acceptance of cannabis suddenly brings a once-shunned plant into a position of intense interest and high demand in a time

of exceptional experimental standards, raising expectations that questions surrounding it be answered much more quickly than for previous crops. Simple breeding and agricultural production techniques for influencing metabolite profiles are not precise nor always consistent, leading to a host of potential complications for both producer and consumer. An example of this complication is the growing medicinal and recreational consumer demand for products with greater THC levels, causing a trend referred to as "lab shopping" that is observed where producers will test their products at several laboratories until they receive the desired cannabinoid concentration analysis for their products (Swider, 2021; Zoorob, 2021). The resulting lack of reliability in the identification might potentially lead to health complications and distrust by those who use cannabis for pain mitigation and as an appetite stimulant/anti-emetic. These issues highlight the need for not just a more reliable and ethical approach to cannabis product quality, but also for methods to reliably tailor metabolite production at the trichome source. New approaches, such as phytomicrobiome manipulation and exploitation, present interesting possibilities, as root inoculums have demonstrated similar effects on THC and CBD contents to nitrogen application (Pagnani et al., 2018; Lyu et al., 2019). If methods can be developed to consistently replicate specific metabolite concentrations and combinations within small ranges across cannabis plants at the trichome level, and if these methods were to become standard across the industry, the benefits for both producers, medical practitioners, and consumers would be great.

From a scientific perspective, multiple interesting questions are associated with the glandular trichomes. Primarily, these questions center around differences related to genotype and growing conditions. How changes to soil composition, light, nutrients, water levels, and other environmental factors affect trichome densities remain largely unknown for cannabis. Our knowledge on how the metabolite profiles themselves differ among varieties is limited and

primarily based on poor reporting from growers that are incomplete beyond the major cannabinoids and terpenes, leaving 100 of metabolites unknown. Our lack of knowledge in these areas of cannabis metabolism and composition make it difficult to directly hypothesize exactly where and how differences occur, stressing the need for rigorous uniform standards to allow unbiased and scientifically sound data comparisons. The more we understand about trichomes, the more applicable our knowledge of this plant will be to those along the chain of production and consumption.

2.5 References

Aizpurua-Olaizola, O., Soydaner, U., Öztürk, E., Schibano, D., Simsir, Y., Navarro, P., et al. (2016). Evolution of the cannabinoid and terpene content during the growth of *Cannabis sativa* plants from different chemotypes. *J. Nat. Prod.* 79, 324–331. doi:10.1021/acs.jnatprod.5b00949.

Aliferis, K. A., and Bernard-Perron, D. (2020). Cannabinomics: Application of metabolomics in cannabis (*Cannabis sativa L.*) research and development. *Front. Plant Sci.* 11: 554. doi:10.3389/fpls.2020.00554.

Andre, C. M., Hausman, J. F., and Guerriero, G. (2016). *Cannabis sativa*: The plant of the thousand and one molecules. *Front. Plant Sci.* 7: 19. doi:10.3389/fpls.2016.00019.

Appendino, G., Gibbons, S., Giana, A., Pagani, A., Grassi, G., Stavri, M., et al. (2008). Antibacterial cannabinoids from *Cannabis sativa*: A structure-activity study. *J. Nat. Prod.* 71, 1427–130. doi:10.1021/np8002673.

Bautista, J. L., Yu, S., and Tian, L. (2021). Flavonoids in *Cannabis sativa*: Biosynthesis, bioactivities, and biotechnology. *ACS Omega* 6, 5119–5123. doi:10.1021/acsomega.1c00318.

Berman, P., Futoran, K., Lewitus, G. M., Mukha, D., Benami, M., Shlomi, T., et al. (2018). A new ESI-LC/MS approach for comprehensive metabolic profiling of phytocannabinoids in Cannabis. *Sci. Rep.* 8: 14280. doi:10.1038/s41598-018-32651-4.

Bernstein, N., Gorelick, J., Zerahia, R., and Koch, S. (2019). Impact of N, P, K, and humic acid supplementation on the chemical profile of medical cannabis (*Cannabis sativa* L). *Front. Plant Sci.* 10: 736. doi: 10.3389/fpls.2019.00736

Booth, J. K., Page, J. E., and Bohlmann, J. (2017). Terpene synthases from *Cannabis sativa*. *PLoS One* 12: e0173911. doi:10.1371/journal.pone.0173911.

Braich, S., Baillie, R. C., Jewell, L. S., Spangenberg, G. C., and Cogan, N. O. I. (2019). Generation of a comprehensive transcriptome atlas and transcriptome dynamics in medicinal cannabis. *Sci. Rep.* 9: 16583. doi: 10.1038/ s41598-019-53023-6

Burgel, L., Hartung, J., Pflugfelder, A., and Graeff-Hönninger, S. (2020). Impact of growth stage and biomass fractions on cannabinoid content and yield of different hemp (*Cannabis sativa L.*) genotypes. *Agronomy* 10: 371. doi:10.3390/agronomy10030372.

Campiglia, E., Radicetti, E., and Mancinelli, R. (2017). Plant density and nitrogen fertilization affect agronomic performance of industrial hemp (*Cannabis sativa* L.) in Mediterranean environment. *Ind. Crop. Prod.* 100, 246–254. doi: 10.1016/j.indcrop.2017.02.022

Conant, R. T., Walsh, R. P., Walsh, M., Bell, C. W., and Wallenstein, M. D. (2017). Effects of a microbial biostimulant, mammoth PTM, on *Cannabis sativa* bud yield. *J. Hortic.* 4: 191. doi: 10.4172/2376-0354.1000191

Conneely, L. J., Mauleon, R., Mieog, J., Barkla, B. J., and Kretzschmar, T. (2021). Characterization of the *Cannabis sativa* glandular trichome proteome. *PLoS One* 16: e0242633. doi:10.1371/journal.pone.0242633.

Danziger, N., and Bernstein, N. (2021a). Light matters: effect of light spectra on cannabinoid profile and plant development of medical cannabis (*Cannabis sativa* L.). *Ind. Crop. Prod.* 164: 113351. doi: 10.1016/j. indcrop.2021.113351

Danziger, N., and Bernstein, N. (2021b). Plant architecture manipulation increases cannabinoid standardization in 'drug-type' medical cannabis. *Ind. Crop. Prod.* 167: 113528. doi: 10.1016/j.indcrop.2021.113528

Dayanandan, P., and Kaufman, P. B. (1976). Trichomes of *Cannabis sativa L*. (Cannabaceae). *Am. J. Bot.* 63, 578–591. doi:10.2307/2441821.

Degenhardt, J., Köllner, T. G., and Gershenzon, J. (2009). Monoterpene and sesquiterpene synthases and the origin of terpene skeletal diversity in plants. *Phytochemistry* 70, 1621–1637. doi:10.1016/j.phytochem.2009.07.030.

Desaulniers Brousseau, V., Wu, B. Sen, MacPherson, S., Morello, V., and Lefsrud, M. (2021). Cannabinoids and terpenes: How production of photo-protectants can be manipulated to

enhance *Cannabis sativa L.* phytochemistry. *Front. Plant Sci.* 12: 620021. doi:10.3389/fpls.2021.620021.

Eichhorn Bilodeau, S., Wu, B. Sen, Rufyikiri, A. S., MacPherson, S., and Lefsrud, M. (2019). An update on plant photobiology and implications for cannabis production. *Front. Plant Sci.* 10: 296. doi:10.3389/fpls.2019.00296.

ElSohly, M. A., and Gul, W. (2014). "Constituents of *cannabis sativa*," in *Handbook of Cannabis*. ed. R. Pertwee (Oxford: Oxford University Press), 3–22. doi: 10.1093/acprof:oso/9780199662685.003.0001

ElSohly, M. A., and Slade, D. (2005). Chemical constituents of marijuana: The complex mixture of natural cannabinoids. *Life Sciences* 78, 539–548. doi:10.1016/j.lfs.2005.09.011.

Fairbairn, J. W. (1972). The trichomes and glands of *Cannabis sativa L*. *BULL.STUPEF.* 24, 29–33.

Farha, M. A., El-Halfawy, O. M., Gale, R. T., Macnair, C. R., Carfrae, L. A., Zhang, X., et al. (2020). Uncovering the hidden antibiotic potential of cannabis. *ACS Infect. Dis.* 6, 338–346. doi:10.1021/acsinfecdis.9b00419.

Flores-Sanchez, I. J., and Verpoorte, R. (2008). Secondary metabolism in cannabis. in *Phytochemistry Reviews* 7, 615–639. doi:10.1007/s11101-008-9094-4.

Gohad, P., McCoy, J., Wambier, C., Kovacevic, M., Situm, M., Stanimirovic, A., et al. (2020). Novel cannabidiol sunscreen protects keratinocytes and melanocytes against ultraviolet B radiation. *J. Cosmet. Dermatol.* 20, 1350–1352. doi:10.1111/jocd.13693.

Grand View Research (2020). Essential Oils Market Size, Share & Trends Analysis Report by Application (Food & Beverages, Spa & Relaxation), By Product (Orange, Peppermint), By Sales Channel, And Segment Forecasts, 2020–2027. *San Francisco, CA.* Available at: https://www.grandviewresearch. com/industry-analysis/essential-oils-market#:~:text=The%20global%20essential%20oils%20market%20size%20was%20estimated%20at%20USD,USD%2018.6%20billion20in202020.&text=The%20global%20essential%20oils%20market%20is%20expected%20to%20grow%20at,USD%2033.3%20billion20by202027 (Accessed 05 June, 2021)

Gülck, T., and Møller, B. L. (2020). Phytocannabinoids: Origins and biosynthesis. *Trends Plant Sci.* 25, 985–1004. doi:10.1016/j.tplants.2020.05.005.

Hammond, C. T., and Mahlberg, P. G. (1973). Morphology of glandular hairs of *Cannabis sativa* from scanning electron microscopy. *Am. J. Bot.* 60, 524–528. doi: 10.1002/j.1537-2197.1973.tb05953.x

Hanuš, L. O., Meyer, S. M., Muñoz, E., Taglialatela-Scafati, O., and Appendino, G. (2016). Phytocannabinoids: A unified critical inventory. *Nat. Prod. Rep.* 33, 1357–1392. doi:10.1039/c6np00074f.

Hare, J. D., Elle, E., and Van Dam, N. M. (2003). Costs of glandular trichomes in *Datura wrightii*: A three-year study. *Evolution (N. Y).* 57, 793–805. doi:10.1111/j.0014-3820.2003.tb00291.x.

Hawley, D., Graham, T., Stasiak, M., and Dixon, M. (2018). Improving cannabis bud quality and yield with subcanopy lighting. *HortScience* 53, 1593–1599. doi: 10.21273/HORTSCI13173-18

Janatová, A., Fraňková, A., Tlustoš, P., Hamouz, K., Božik, M., and Klouček, P. (2018). Yield and cannabinoids contents in different cannabis (*Cannabis sativa L.*) genotypes for medical use. *Ind. Crops Prod.* 112, 363–367. doi:10.1016/j.indcrop.2017.12.006.

Jin, D., Dai, K., Xie, Z., and Chen, J. (2020). Secondary metabolites profiled in cannabis inflorescences, leaves, stem barks, and roots for medicinal purposes. *Sci. Rep.* 10: 3309. doi: 10.1038/s41598-020-60172-6

Kim, E. S., and Mahlberg, P. G. (1997). Immunochemical localization of tetrahydrocannabinol (THC) in cryofixed glandular trichomes of cannabis (*Cannabaceae*). *Am. J. Bot.* 84, 336–342. doi:10.2307/2446007.

Kim, E.-S., and Mahlberg, P. G. (1991). Secretory cavity development in glandular trichomes of *Cannabis sativa L*. (*Cannabaceae*). *Am. J. Bot.* 78, 220–229. doi:10.2307/2445245.

Kim, K. S., Vuong, T. D., Qiu, D., Robbins, R. T., Grover Shannon, J., Li, Z., et al. (2016). Advancements in breeding, genetics, and genomics for resistance to three nematode species in soybean. *Theor. Appl. Genet.* 129, 2295–2311. doi:10.1007/s00122-016-2816-x.

Koren, A., Sikora, V., Kiprovski, B., Brdar-Jokanović, M., Aćimović, M., Konstantinović, B., et al. (2020). Controversial taxonomy of hemp. *Genetika* 52, 1–13. doi:10.2298/GENSR2001001K.

Leme, F. M., Schönenberger, J., Staedler, Y. M., and Teixeira, S. P. (2020). Comparative floral development reveals novel aspects of structure and diversity of flowers in *Cannabaceae*. *Bot. J. Linn. Soc.* 193, 64–83. doi:10.1093/botlinnean/boaa004.

Levin, D. A. (1973). The role of trichomes in plant defense. *Q. Rev. Biol.* 48, 3–15. doi:10.1086/407484.

Liu, Y., Zhu, P., Cai, S., Haughn, G., and Page, J. E. (2021). Three novel transcription factors involved in cannabinoid biosynthesis in *Cannabis sativa* L. *Plant Mol. Biol.* 106, 49–65. doi: 10.1007/s11103-021-01129-9

Livingston, S. J., Quilichini, T. D., Booth, J. K., Wong, D. C. J., Rensing, K. H., Laflamme-Yonkman, J., et al. (2020). Cannabis glandular trichomes alter morphology and metabolite content during flower maturation. *Plant J.* 101, 37–56. doi:10.1111/tpj.14516.

Lyu, D., Backer, R., Robinson, W. G., and Smith, D. L. (2019). Plant growth-promoting Rhizobacteria for cannabis production: yield, cannabinoid profile and disease resistance. *Front. Microbiol.* 10: 1761. doi: 10.3389/ fmicb.2019.01761

Magagnini, G., Grassi, G., and Kotiranta, S. (2018). The effect of light Spectrum on the morphology and cannabinoid content of *Cannabis sativa* L. *Med. Cannabis Cannabinoids* 1, 19–27. doi: 10.1159/000489030

Mahlberg, P. G., and Eun, S. K. (2004). Accumulation of cannabinoids in glandular trichomes of cannabis (*Cannabaceae*). *J. Ind. Hemp* 9, 15–36. doi:10.1300/J237v09n01_04.

Marks, M. D., Tian, L., Wenger, J. P., Omburo, S. N., Soto-Fuentes, W., He, J., et al. (2009). Identification of candidate genes affecting Δ^9- tetrahydrocannabinol biosynthesis in *Cannabis sativa*. *J. Exp. Bot.* 60, 3715–3726. doi:10.1093/jxb/erp210.

Matoušek, J., Kocábek, T., Patzak, J., Bříza, J., Siglová, K., Mishra, A. K., et al. (2016). The "putative" role of transcription factors from HlWRKY family in the regulation of the final steps of prenylflavonid and bitter acids biosynthesis in hop (*Humulus lupulus* L.). *Plant Mol. Biologicals* 92, 263–277. doi: 10.1007/s11103-016-0510-7

Mediavilla, V., and Steinemann, S. (1997). Essential oil of *Cannabis sativa* L. strains. *J. Ind. Hemp* 4, 80-82.

Mishra, A. K., Kocábek, T., Nath, V. S., Awasthi, P., Shrestha, A., Killi, U. K., et al. (2020). Dissection of dynamic transcriptome landscape of leaf, bract, and lupulin gland in hop (*Humulus lupulus* L.). *Int. J. Mol. Sci.* 21: 233. doi: 10.3390/ijms21010233

Mudge, E. M., Brown, P. N., and Murch, S. J. (2019). The terroir of cannabis: terpene metabolomics as a tool to understand *Cannabis sativa* selections. *Planta Med.* 85, 781–796. doi:10.1055/a-0915-2550.

Muravnik, L. E. (2020). "The structural peculiarities of the leaf glandular trichomes: A review," in *Plant Cell and Tissue Differentiation and Secondary Metabolites. Reference Series in Phytochemistry*. eds. K. Ramawat, H. Ekiert, and S. Goyal (Cham: Springer), 63–97.

Namdar, D., Mazuz, M., Ion, A., and Koltai, H. (2018). Variation in the compositions of cannabinoid and terpenoids in *Cannabis sativa* derived from inflorescence position along

the stem and extraction methods. *Ind. Crops Prod.* 113, 376–382. doi:10.1016/j.indcrop.2018.01.060.

Navarro, G., Varani, K., Lillo, A., Vincenzi, F., Rivas-Santisteban, R., Raïch, I., et al. (2020). Pharmacological data of cannabidiol- and cannabigerol-type phytocannabinoids acting on cannabinoid CB1, CB2 and CB1/CB2 heteromer receptors. *Pharmacol. Res.* 159: 104940. doi:10.1016/j.phrs.2020.104940.

Nerio, L. S., Olivero-Verbel, J., and Stashenko, E. (2010). Repellent activity of essential oils: A review. *Bioresour. Technol.* 101, 372–378. doi:10.1016/j.biortech.2009.07.048.

Ohlsson, A., Abou-Chaar, C. I., Agurell, S., Nilsson, I. M., Olofsson, K., and Sandberg, F. (1971). Cannabinoid constituents of male and female *Cannabis sativa*. *Bull Narc.* 23, 29–32.

Pagnani, G., Pellegrini, M., Galieni, A., D'Egidio, S., Matteucci, F., Ricci, A., et al. (2018). Plant growth-promoting rhizobacteria (PGPR) in *Cannabis sativa* 'Finola' cultivation: An alternative fertilization strategy to improve plant growth and quality characteristics. *Ind. Crops Prod.* 123, 75–83. doi:10.1016/j.indcrop.2018.06.033.

Panche, A. N., Diwan, A. D., and Chandra, S. R. (2016). Flavonoids: An overview. *J. Nutr. Sci.* 5: e47. doi:10.1017/jns.2016.41.

Pate, D. W. (1983). Possible role of ultraviolet radiation in evolution of cannabis chemotypes. *Econ. Bot.* 37, 396–405. doi:10.1007/BF02904200.

Potter, D. J. (2009). The propagation, characterisation and optimisation of *Cannabis sativa* L. as a phytopharmaceutical. Doctoral thesis. London, England: King's College London.

Ren, G., Zhang, X., Li, Y., Ridout, K., Serrano-Serrano, M. L., Yang, Y., et al. (2021). Large-scale whole-genome resequencing unravels the domestication history of *Cannabis sativa*. *Sci. Adv.* 7: eabg2286. doi:10.1126/sciadv.abg2286.

Russo, E. B. (2011). Taming THC: Potential cannabis synergy and phytocannabinoid-terpenoid entourage effects. *Br. J. Pharmacol.*, 13-44–1364. doi:10.1111/j.1476-5381.2011.01238.x.

Russo, E. B., and McPartland, J. M. (2001). Cannabis and cannabis extracts: greater than the sum of their parts? *J. Cannabis Ther.* 1, 103–132. doi:10.1300/J175v01n03_08

Saloner, A., and Bernstein, N. (2021). Nitrogen supply affects cannabinoid and terpenoid profile in medical cannabis (*Cannabis sativa* L.). *Ind. Crops Prod.* 167: 113516. doi:10.1016/j.indcrop.2021.113516.

Sangwan, N. S., Farooqi, A. H. A., Shabih, F., and Sangwan, R. S. (2001). Regulation of essential oil production in plants. *Plant Growth Regul.* 34, 3–21. doi:10.1023/A:1013386921596.

Sirikantaramas, S., Taura, F., Tanaka, Y., Ishikawa, Y., Morimoto, S., and Shoyama, Y. (2005). Tetrahydrocannabinolic acid synthase, the enzyme controlling marijuana psychoactivity, is secreted into the storage cavity of the glandular trichomes. *Plant Cell Physiol.* 46, 1578–1582. doi:10.1093/pcp/pci166.

Spitzer-Rimon, B., Duchin, S., Bernstein, N., and Kamenetsky, R. (2019). Architecture and florogenesis in female *Cannabis sativa* plants. *Front. Plant Sci.* 10: 350. doi: 10.3389/fpls.2019.00350

Swider, J.. (2021). Lab Shopping: Highlighting the Need for Checks and Balances in Cannabis. Available at: https://cannabisindustryjournal.com/column/lab-shopping-highlighting-the-need-for-checks-and-balances-in-cannabis/ (Accessed 6 May, 2021).

Taghinasab, M., and Jabaji, S. (2020). Cannabis microbiome and the role of endophytes in modulating the production of secondary metabolites: An overview. *Microorganisms* 8: 335. doi:10.3390/microorganisms8030355.

Tang, K., Struik, P. C., Yin, X., Calzolari, D., Musio, S., Thouminot, C., et al. (2017). A comprehensive study of planting density and nitrogen fertilization effect on dual-purpose hemp (*Cannabis sativa* L.) cultivation. *Ind. Crop. Prod.* 107, 427–438. doi: 10.1016/j.indcrop.2017.06.033

Taschwer, M., and Schmid, M. G. (2015). Determination of the relative percentage distribution of THCA and δ9-THC in herbal cannabis seized in Austria - Impact of different storage temperatures on stability. *Forensic Sci. Int.* 254, 167–171. doi:10.1016/j.forsciint.2015.07.019.

Tian, N., Liu, F., Wang, P., Zhang, X., Li, X., and Wu, G. (2017). The molecular basis of glandular trichome development and secondary metabolism in plants. *Plant Gene* 12, 1–12. doi: 10.1016/j.plgene.2017.05.010

Tissier, A., Morgan, J. A., and Dudareva, N. (2017). Plant volatiles: going 'in' but not 'out' of trichome cavities. *Trends Plant Sci.* 22, 930–938. doi: 10.1016/j.tplants.2017.09.001

Tomko, A. M., Whynot, E. G., Ellis, L. D., and Dupré, D. J. (2020). Anti-cancer potential of cannabinoids, terpenes, and flavonoids present in cannabis. *Cancers (Basel).* 12: 1985. doi:10.3390/cancers12071985.

Toth, J. A., Stack, G. M., Cala, A. R., Carlson, C. H., Wilk, R. L., Crawford, J. L., et al. (2020). Development and validation of genetic markers for sex and cannabinoid chemotype in *Cannabis sativa L. GCB Bioenergy* 12, 213-222. doi:10.1111/gcbb.12667.

van Klingeren, B., and ten Ham, M. (1976). Antibacterial activity of Δ^9-tetrahydrocannabinol and cannabidiol. *Antonie Van Leeuwenhoek* 42, 9–12. doi:10.1007/BF00399444.

Vanhove, W., Van Damme, P., and Meert, N. (2011). Factors determining yield and quality of illicit indoor cannabis (Cannabis spp.) production. *Forensic Sci. Int.* 212, 158–163. doi:10.1016/j.forsciint.2011.06.006.

Wagner, G. J. (1991). Secreting glandular trichomes: More than just hairs. *Plant Physiol.* 96, 675–679. doi:10.1104/pp.96.3.675.

Yamauchi, T., Shoyama, Y., Aramaki, H., Azuma, T., and Nishioka, I. (1967). Tetrahydrocannabinolic acid, a genuine substance of tetrahydrocannabinol. *Chem. Pharm. Bull.* 15, 1076–1079. doi:10.1248/cpb.15.1075.

Zager, J. J., Lange, I., Srividya, N., Smith, A., and Markus Lange, B. (2019). Gene networks underlying cannabinoid and terpenoid accumulation in cannabis. *Plant Physiol.* 180, 1877–1897. doi: 10.1104/pp.18.01506

Zoorob, M. J. (2021). The frequency distribution of reported THC concentrations of legal cannabis flower products increases discontinuously around the 20% THC threshold in Nevada and Washington state. *J. Cannabis Res.* 3, 1–6. doi:10.1186/s42238-021-00064-2.

Connecting Text between Chapter 2 and Chapter 3

As Chapter 2 highlighted the extensive lack of knowledge surrounding cannabis stalked glandular trichomes, the first objective, addressed in Chapter 3, evaluates the potential of PGPR to improve their densities. In this objective, I have maintained all plant trials used in the analysis and created the experimental design. I have evaluated the stalked trichome densities of dissected cannabis inflorescences by imaging calyces and the abaxial and adaxial sides of bracts, followed by analysis using ImageJ software. I have conducted all statistical analysis related to the trichome densities reported here and drafting of the manuscript.

With the minimal insight into trichomes available, I have helped to close this gap identified in the literature review by determining how the application of PGPR may alter the densities of trichomes on cannabis tissue. This was evaluated by the creation of a novel technique for determining trichome densities for cannabis. The few studies to have described trichome counts for other plant species, mentioned in the previous chapter, involved trichomes that are low to the epidermal surface and typically uniform in appearance. The technique developed in the following chapter circumvents the inconsistent stalked glandular trichomes found on cannabis, and its simplicity allows it to be easily replicated across plant species with common laboratory equipment.

I have gone a step further in bridging the knowledge gap discussed in Chapter 2 by incorporating the evaluation of an alternative, more sustainable nutrient regime with the PGPR inoculation treatments, painting a deeper picture of the extent to which these environmentally-friendly applications affect cannabis. This is further compounded by the inclusion of investigating the response of 9 common cannabinoid concentrations to provide a thorough understanding of how potential changes in trichome density may – or may not – impact their relative contents.

Chapter 3 – Sub-optimal nutrient regime coupled with *Bacillus* and *Pseudomonas* sp. inoculation influences trichome density and cannabinoid profiles in drug-type *Cannabis sativa*

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Title: Sub-optimal nutrient regime coupled with plant growth-promoting rhizobacteria inoculation enhances *Cannabis* trichome development and influences cannabinoid profiles

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Abstract

Cannabis sativa remains under heavy legal restriction around the globe that prevent extensive investigations into agricultural applications for improving its development. This work aims to investigate the potential of specific plant growth-promoting rhizobacteria (PGPR) to improve *Cannabis* cannabinoid yield through increased trichome densities on floral organs, and to determine if sub-optimal environmental conditions would affect the outcomes of PGPR presence by altering plant development and cannabinoid profiles. Here, *Pseudomonas* sp. or *Bacillus* sp. were applied to the root system either separately or in a consortium to determine the effect of this bacterial treatment on the density of stalked glandular trichomes. Further, a low nutrient regime

was applied for the first half of plant development to determine if an environmental stressor interacts with the effects of the microbial treatments on stalked trichome densities. Following 8 weeks of flower development, trichome density on calyces and bracts of inflorescences were determined microscopically. Our findings unexpectedly indicate that recommended nutrient levels were linked to a decreasing shift in trichome densities with PGPR inoculations, but a low nutrient regime coupled with PGPR treatment increases them. Cannabinoid content is partially consistent with these results, in that a low nutrient regime increased the abundance of key cannabinoids compared to recommended regimes, with *Bacillus* sp. inoculation leading to the greatest number of significant changes between the two nutrient regimes. Overall, this work provides insight into how PGPR presence affects *Cannabis* stalked trichome development and cannabinoid profiles, and how environmental stressors can affect, and even enhance, trichome densities and influence major cannabinoid production, thereby pointing towards avenues for reducing the reliance on synthetic fertilizers during plant production without compromising yield.

3.1 Introduction

With Canada having set the precedent for nation-wide recreational *Cannabis* (hereafter, cannabis) legalization in North America, and Malta now the first European Union country to legalize it for personal use in 2021 (Authority on the Responsible Use of Cannabis Act, 2021), global demand for cannabis products is expected to rise sharply as more countries follow suit. Research on cannabis has slowly begun to catch up to the progress made in the agricultural science boom of the 20th century, but research remains primarily focused on medicinal and analytical aspects. This is largely due to accessibility, as despite increasing legalization, the regulations governing cannabis cultivation for agricultural research remain challenging. While a highly

profitable industry, with heavy legislation limiting product types and consumer availability, research focused on agricultural applications remains scarce. Projected to reach 102.2 billion USD by 2030 in the global legal market (Grand View Research, 2022), it is now time that validations of modern agricultural methods be carried out for cannabis to ensure this expanding industry benefit from research-backed practices.

Female cannabis inflorescences are the primary source of cannabinoids and terpenes (Ohlsson et al., 1971), with stalked glandular trichomes on the surface of floral organs and bracts being the key secretory structures (Mahlberg and Kim, 2004; Livingston et al., 2020). Recent work has used a deep learning pipeline to identify stages of trichome development based on their age-based transition through clear-milky-brown phenotypes, providing a sophisticated tool for cannabis product investigation (Sutton et al., 2023). As these trichomes are the source of the bulk of cannabinoids in cannabis products, it is imperative that research aimed at improving cannabinoid yield be directed towards these structures.

Efforts at manipulating cannabis yields are largely focused on abiotic environmental conditions for plant culture (reviewed by Jin et al., 2019; Desaulniers Brousseau et al., 2021; Backer et al., 2019) or on post-harvest processing (reviewed by Addo et al., 2021). Variables characterizing indoor growing conditions are being investigated for their potential to affect metabolite profiles and yields in cultivar-specific manner. Efforts are typically directed toward lighting systems, in the context of light spectrum and intensities (Hawley et al., 2018; Magagnini et al., 2018; Danziger and Bernstein, 2021; Westmoreland et al., 2021; Wei et al., 2021; Rodriguez-Morrison et al., 2021) and fertilizer nutrient applications (Caplan et al., 2017; Bernstein et al., 2019; Yep and Zheng, 2020, Bevan et al., 2021). The concept of optimizing nutrient applications for cannabis cultivation by precise manipulation of individual compounds is beginning to attract

research interest. Bevan et al. (2021) demonstrated how nitrogen, phosphorus, and potassium contents can impact inflorescence yield in drug-type cannabis. They pointedly identified how potassium had no bearing on inflorescence yield, suggesting that the administration of this nutrient is likely provided in excess and may be a resource drain. While this conclusion was tested only in soilless (deep water culture) environments, it is a step towards optimizing grow operations and opens avenues toward tailoring production protocols. Poorly understood hitherto is the potential of the use of microbial supplements to improve cannabis yield, and research to this end is warranted.

The potential of manipulating the phytomicrobiome to optimize cannabis yield is in its infancy. Plant growth-promoting rhizobacteria (PGPR) are well-established fertilizer supplements for other crop species. They are known to support plant development through improving water and nutrient acquisition and establishing synergistic relationships with their plant hosts through the production of phytohormones (Berendsen et al., 2012; Sivasakthi et al., 2014; Kundan et al., 2015) or signal compounds (Backer et al., 2019; Lyu et al., 2022a). Recent work has demonstrated that the administration of PGPR has potential applications for improving cannabis development and metabolic yields, but details are lacking. Lyu et al. (2022a) demonstrated an improvement of inflorescence fresh weight with three separate rhizobacterial species, of which two increased the number of inflorescences per plant. Using hemp cultivars, Pagnani et al. (2018) revealed that PGPR inocula also affected the metabolite profiles of their cultivars. With regard to pathogen control, Balthazar et al. (2022a) has recently demonstrated the efficacy of twelve strains of *Bacillus* and *Pseudomonas* against culturable cannabis fungal pathogens and found 5 strains had a significant biocontrol impact by reducing gray mold development *in planta*. They also confirmed that there were no recognized virulence or toxin factor genes in the genome of the favourable

potential strains (Balthazar et al. 2022a). These findings further support the use of beneficial microbes for sustainable cannabis yield improvement, but through pathogen control; a concept further explored in Balthazar et al. (2022b) with a focus on *Pseudomonas* sp. applications.

In addition to whole-plant effects, it has been demonstrated in other plant species that PGPR can help increase the content of essential oils, which in turn have been linked to increases in trichome counts (Copetta et al., 2006; Banchio et al., 2008). More specifically, an environmental stressor has been found to increase essential oil yields in *Melissa officinalis* (Eshaghi Gorgi, 2022), and in *Cymbopogon citratus* this has further been linked to increases in trichome counts (Mirzaie et al., 2020). With the link between PGPR, essential oil production, and trichome development established for other crops, we wanted to assess whether similar relationships could be detected in cannabis. To determine if PGPR can influence cannabis stalked trichome densities, and if an environmental stressor can amplify these results, we inoculated cannabis plants with two PGPR strains, separately and in a consortium. In addition, a low nutrient regime for the first 6 weeks of development was applied to determine if an environmental stressor enhances any PGPR effects with regards to trichome development.

3.2 Materials and Methods

3.2.1 Plant propagation and maintenance

Female Cannabis sativa L. plants of the cannabis variety “CBD Kush” were grown from cuttings sourced from in-house mother plants at Macdonald Campus, Saint-Anne-de-Bellevue, Quebec, in a Canada Revenue Agency and Health Canada approved research laboratory (license no. LIC-5AZZW7S4GM-2019). Mother plants were inspected for any signs of nutrient deficiency, pathogens, or pest damage. Medium-thick branches (~2 mm diameter) were cut and placed in water

to prevent wilting. All leaves, with the exception of the top three fully-formed leaves, were removed from the stem and the outermost halves of the remaining leaves were clipped off. The ends of cuttings were trimmed to a 45º angle and dipped in Stim Root No. 2 powder (Master Plant-Prod Inc., Brampton, ON, Canada), after which they were placed in 3 cm pre-soaked rockwool cubes (Grodan, Roermond, Netherlands) on mesh trays (53 × 27 × 6 cm, Bootstrap Farmer, Downington, PA, USA) inside propagation trays (54 × 28 × 6 cm, Mondi, Vancouver, BC, Canada). Two L of VeloKelp nutrient solution (pH 5.6, Remo Nutrients; Remo Brands Inc., Maple Ridge, BC, Canada) at Transplant concentration (Tables 3.1, 3.2) were poured into the trays and replaced once per week. Prepared trays were covered with a vented mini greenhouse (54 × 28 × 19 cm; Mondi, Vancouver, BC, Canada) and placed on a propagation rack for three to four weeks, until sufficient roots were observed (conditions – light at approximately 150 µmol m-2 s-1, 24 h photoperiod, 75-95% humidity, 24-25 ºC).

Table 3.1. Nutrient applications for inoculated cannabis cv. CBD Kush.

	Week											
	Transplant	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Average Daily Water Quantity (mL)	250	150	150	150	150	250	250	250	250	250	250	250	250
Low Nutrients	1.3 mL L^{-} VeloKelp	1.84 mL L^{-} of VeloKelp						2.2 mL L^{-} each of VeloKelp, Micro, MagNifiCal, Bloom, Astroflower					Water
Recommended Nutrients	1.3 mL L^{-} VeloKelp	1.84 mL L^{-} each of VeloKelp, Micro, Grow, MagNifiCal				2.2 mL L^{-} each of VeloKelp, Micro, MagNifiCal, Bloom, Astroflower							Water

Under each nutrient regime, three series of healthy cuttings were transplanted into 15 cm pots (Teris, Laval, QC, Canada) containing pre-soaked Agromix G6 soil (300 mL of water per 400

g; Farfad Inc., Saint-Bonaventure, QC, Canada) and grown under vegetative conditions (approximately 150 µmol m-2 s-1, 18 h photoperiod, 20-22 ºC, 65% relative humidity) for four weeks. Vegetative plants were given the recommended nutrient regime of 150 mL water and nutrient solution application according to week of vegetative growth (Table 3.1) as per manufacturer guidelines and Lyu et al. (2022a) (Nutrients: MagNifiCal, Micro, VeloKelp, Grow at pH 6.3, Remo Nutrients). Following this period, plants were transferred to flowering conditions (approximately 150 µmol m-2 s-1, 12 h photoperiod, 20-22 ºC, 65 % relative humidity) and given a regime of 250 mL water and nutrient solution application according to the week of flowering growth (Table 3.1) as per manufacturer guidelines and Lyu et al. (2022a) (Nutrients: MagNifiCal, Micro, VeloKelp, Astroflower, Bloom at pH 6.3, Remo Nutrients); only water was given in the final week of development, as per guidelines. Plants were grown under flowering conditions for a total of 8 weeks. Plants undergoing the low nutrient regime were given the same volume of nutrient-containing solution under the same growing conditions as the recommended nutrient regime. However, from Week 1 through 6, three of the four nutrient mixes were omitted and only the VeloKelp nutrient was provided at the same concentration as when combined with other nutrients as part of the recommended regime (pH 6.3, Remo Nutrients), creating a nutrient deficiency (Table 3.1). Following Week 6, complete nutrients, as described above, were given for

a total of 5 weeks until Week 12, during which only water was provided (Table 3.1). Table 3.2 provides the NPK content of each individual nutrient.

Table 3.2. NPK content of the nutrient solutions applied to cannabis cv. CBD Kush.

	VeloKelp	Micro	Grow	MagNifiCal	Bloom	Astroflower
Nitrogen	1%	1%	2%	3%	1%	1%
Phosphorous	1%	0%	3%	0%	4%	6%
Potassium	1%	1%	5%	0%	7%	11%

3.2.2 Bacterial inoculum preparation and delivery

Pseudomonas sp. (*Pseudomonas koreensis,* AF468452) and Bacillus sp. (*Bacillus mobilis*, KJ812449), originally isolated and identified by Fan et al. (2020) and previously studied with cannabis applications in Lyu et al. (2022a), were stored at -80 ºC in glycerol and were revived by streaking onto petri plates containing sterile (30 min, 121 °C) King's Medium B (KB; 20.0 g L-1 protease peptone, 1.5 g L-1 K2HPO4, 10.0 g L-1 glycerol, 0.25 g L-1 MgSO4•7 H2O) and incubating at 28 ºC overnight. Bacterial suspensions were prepared by scraping colonies from the plate surface into a beaker containing approximately 75 mL of sterile liquid KB medium and grown overnight at 28 ºC, rotating at 150 rev min^{-1}. The following day, 30 mL of the inoculated media was distributed into 50 mL falcon tubes. Tubes were centrifuged at 6000g for 10 min (Sorvall Biofuge Pico, Kendro Laboratory Products, Asheville, NC, USA). The supernatant was discarded, and pellets were washed in 10 mL of 10 mM MgSO4. Following the wash, resuspended pellets were diluted to 0.1 OD at 600 nm (Ultraspec 4050 Pro UV/Visible spectrophotometer), using 10 mM MgSO4 as the blank.

Prepared inoculations were dispensed onto the soil immediately surrounding the base of the transplanted cuttings, which remained in the rockwool cubes when moved to soil, using a serological pipette on the day of transplantation. Ten mL of each inoculum was dispensed onto each cutting, and for the consortium treatment five mL of each inoculum component was dispensed. Control treatments received 10 mL of 10 mM MgSO4. Four plants per inoculation treatment group were prepared per nutrient regime experimental series and organized in a randomized complete block design. Four blocks per experimental series were created, with 3 experimental series per nutrient regime grown, providing a total of 12 true replicates.

3.2.3 Quantification of trichome density

To maintain consistency when sampling and to account for differences in plant height, one inflorescence per plant was removed at 3-5 nodes down from the apex inflorescence. Inflorescences were chosen based on size to ensure enough organ tissue would be available from a single inflorescence for dissection. Inflorescences were dissected down to individual calyces and bracts using razor blades, forceps, tweezers, and dissecting scissors. A minimum of six calyces and eight bracts were isolated, of which four calyces, four bract abaxial epidermis, and four bract adaxial epidermis surfaces were imaged (Figure 3.1), resulting in a total of twelve images per inflorescence, per plant in each inoculation treatment group for all experimental series. Tissues were imaged on a clear petri dish lid under darkfield conditions (0.63x, 2.5 optivar; Zeiss SteREO Discovery V8, Carl Zeiss Canada Ltd, Toronto, ON, Canada).

ImageJ (https://imagej.nih.gov/ij/download.html) was used to determine organ surface area through manual selection of visible surface and calculated using the ROI manager. Stalked trichomes were manually counted using the Cell Counter plugin, allowing calculation of stalked trichomes per mm^2.

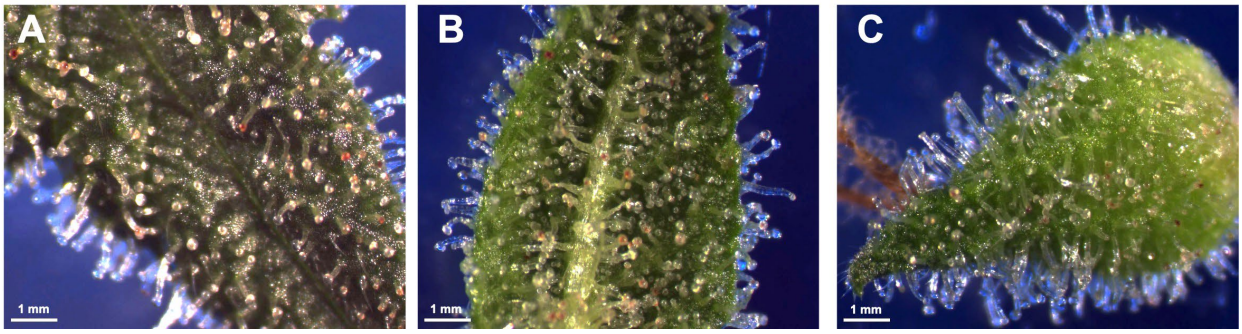

Figure 3.1: Examples of stereomicrographs used for determination of trichome density. Bract adaxial epidermis (A), bract abaxial epidermis (B), and calyx epidermis (C). Visual tissue area was selected and stalked trichomes were manually counted.

3.2.4 Quantification of cannabinoid concentrations

A random sampling of dried inflorescences collected post-harvest from three of the four plants per treatment for a single experimental series was ground, separately, to a fine homogenous powder following freeze drying using a lyophilizer (SNL216V freeze-dryer, Thermo Savant Co. Ltd. USA). For each replicate, 0.2 g of sample was mixed with 20 mL of 100% ethanol in a 50 mL centrifuge tube. Tubes were placed on their side and shaken on a rotator for 5 min. One mL of the extract was transferred to an Eppendorf tube and centrifuged at 12,000 rpm for 5 min. Solvent was transferred to a 2 mL vial, either at the original concentration or 20x diluted in 100% ethanol.

Nine commercially available standards (purity > 98%) for cannabigerolic acid (CBGA), tetrahydrocannabinolic acid (THCA), cannabidiolic acid (CBDA), Δ^9-tetrahydrocannabinol (Δ^9-THC), cannabidiol (CBD), cannabichromene (CBC), cannabigerol (CBG), cannabinol (CBN), and Δ^8-tetrahydrocannabinol (Δ^8-THC) were obtained from Cerilliant (Round Rock, Texas, USA).

Cannabinoid analysis was performed using the Agilent 1290 Infinity Ultra High-Performance Liquid Chromatography (UHPLC) system with an UV DAD detector (Agilent Technologies Inc., Santa Clara, CA, USA) set at 220 nm, for identification and quantification of the nine compounds, as per Lyu et al. (2022b). Due to sample overloading, CBDA and THCA required analysis at 20x dilution. All other cannabinoids were analyzed at their original concentrations.

3.2.5 Scanning electron microscopy

Individual calyces and bracts were submerged in two mL of 3.5% v/v formaldehyde in 0.025M PIPES buffer. Samples were rotated overnight, followed by three rinses with 0.025M PIPES buffer. Samples underwent ethanol ascensions of 30, 50, 70, 80, 95, and 100% for 30 min each, with three additional 100% ethanol rinses. Following this, samples were critical-point dried with solvent-substituted CO2 (Leica EM CPD300, Leica Microsystems, Concord, ON, Canada). Samples were mounted on aluminum stubs with carbon mounts and rotary coated with 4 nm gold layer (Leica EM ACE200, Leica Microsystems, Concord, Canada). Samples were imaged in a vacuumed chamber with a Hitachi TM-1000 scanning electron microscope operated at 15 kV (Hitachi Ltd., Chiyoda City, Japan).

3.2.6 Statistical analysis

Data analysis was performed using the statistical program SAS OnDemand for Academics, Enterprise Guide 8.3 for trichome data analysis (SAS Institute Inc. Cary, NC, USA). Version 9.4 of SAS OnDemand for Academics, Enterprise Guide was used for cannabinoid analysis (SAS Institute Inc. Cary, NC, USA). Differences in trichome densities between treatments and organs were evaluated using PROC GLM Tukey's studentized range with the Dunnett adjustment for multiple comparisons, using a nested model. The level of significance was set at $p < 0.05$. Analysis

of cannabinoid concentration was done using PROC GLIMMIX, using an interaction model with the same level of significance.

For the control and consortium treatment for the recommended nutrient regime, n was 44 whereas for Pseudomonas sp. and Bacillus sp. treatments n was 48. n was 48 for all four treatments under the low nutrient regime. This discrepancy is due to 1 plant dying in each of the control and consortium treatments under the recommended nutrient regime, preventing trichome data collection and reducing the n value for their respective treatment groups.

3.3 Results

3.3.1 Effect of PGPR inoculation on cannabis stalked trichome density

Across the three experimental series, generally, no increase in stalked trichome densities resulting from the bacterial treatments was observed when compared to the control (Figure 3.2). Only plants treated with *Pseudomonas* sp. displayed a statistically significant effect ($p < 0.05$) for the abaxial bract epidermis with a decrease in trichome density by 9.4%. Although not statistically significant, trichome densities tended to be slightly reduced in almost all other PGPR treated samples, except for the calyx of plants treated with the consortium inoculum, where a slight increase in trichome density of 3.5% was observed ($p = 0.0242$).

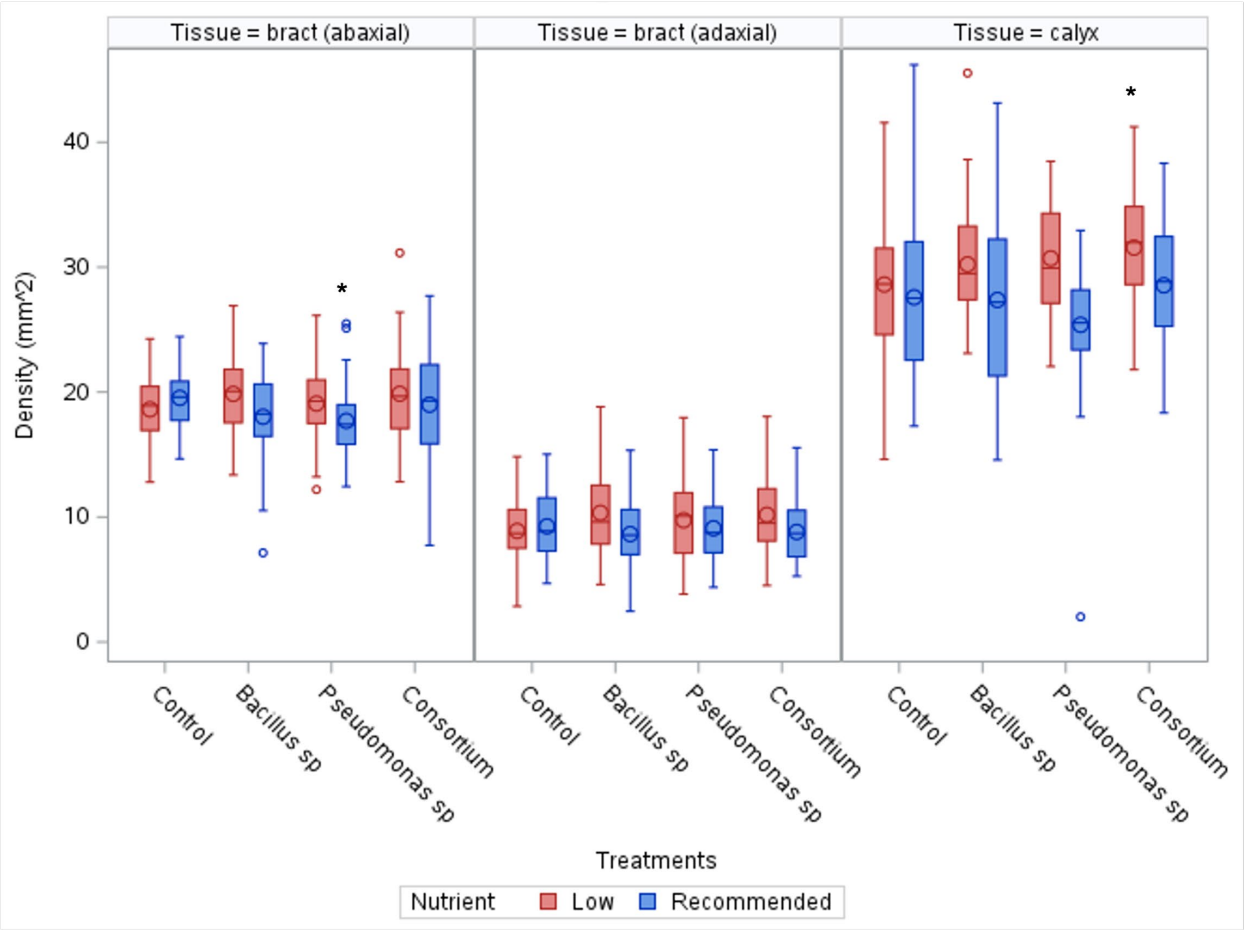

Figure 3.2: Comparisons of trichome densities by tissue and treatments under either recommended or low nutrients. No treatment had a statistically significant effect on improving trichome densities across cannabis inflorescence tissues under recommended nutrients, however the consortium under low nutrients was significant. Horizontal bar within box indicates median value, circle within boxes indicates the mean. 'o' indicates outlier data points, vertical bars indicate data range. Asterisk indicates significant results.

3.3.2 PGPR inoculation coupled with a low nutrient regime affects stalked trichome density

Under the low nutrient regime, the general tendency showed an increase in average stalked trichome densities on plants inoculated with PGPR compared to the recommended nutrient conditions (Figure 3.2). Within this tendency, only the increase observed on the calyces of plants inoculated with the consortium treatment were significant (p = 0.081) in comparison to non-

inoculated, low nutrient regime plants. The increase caused by the presence of PGPR in these inadequate nutrient-supply treated plants was 10.3% for consortium-treated calyx tissue compared to the control treatment for the low nutrient regime. The average densities of the other epidermal tissues under the PGPR treatments were not significant against the non-inoculated control.

When comparing the trichome densities between the two nutrient regimes coupled with PGPR inoculations, an absence of bacteria under the low nutrient regime was the only treatment to cause a decrease in trichome densities compared to the recommended condition counterpart; a decrease of 4.7% for bract abaxial tissue and 4.0% for bract adaxial tissue, though an increase of 3.7% for calyx tissue was observed (Figure 3.2). Nonetheless, the administration of PGPR showed a tendency to higher average trichome densities under low nutrient conditions than the level observed for the recommended nutrients under the same PGPR treatments (Figure 3.2). This is illustrated by Figure 3.3, where inflorescence epidermal tissue from the low nutrient regime remains covered by stalked trichomes. Comparing the changes in trichome densities between nutrient regimes, plants inoculated with the *Bacillus* sp. treatment showed the most consistent increase in stalked trichome densities on both organs under low nutrient conditions (Figure 3.2). Trichome densities on the bract abaxial, bract adaxial, and calyx epidermal tissues from low nutrient plants inoculated with *Bacillus* sp. increased by 10.1, 19.7, and 10.4%, respectively, compared to the recommended nutrient *Bacillus* sp. treatment group. For plants under *Pseudomonas* sp. inoculation, the low nutrient regime led to bract abaxial, bract adaxial, and calyx trichome densities increasing by 7.8, 7.0, and 21.0%, respectively, against their recommended nutrient counterpart. For plants treated with the consortium inoculum in the low nutrient regime, trichome densities on bract abaxial, bract adaxial, and calyx increased by 4.6, 15.5, and 10.6%, respectively, compared to consortium-inoculated plants grown under the recommended nutrients.

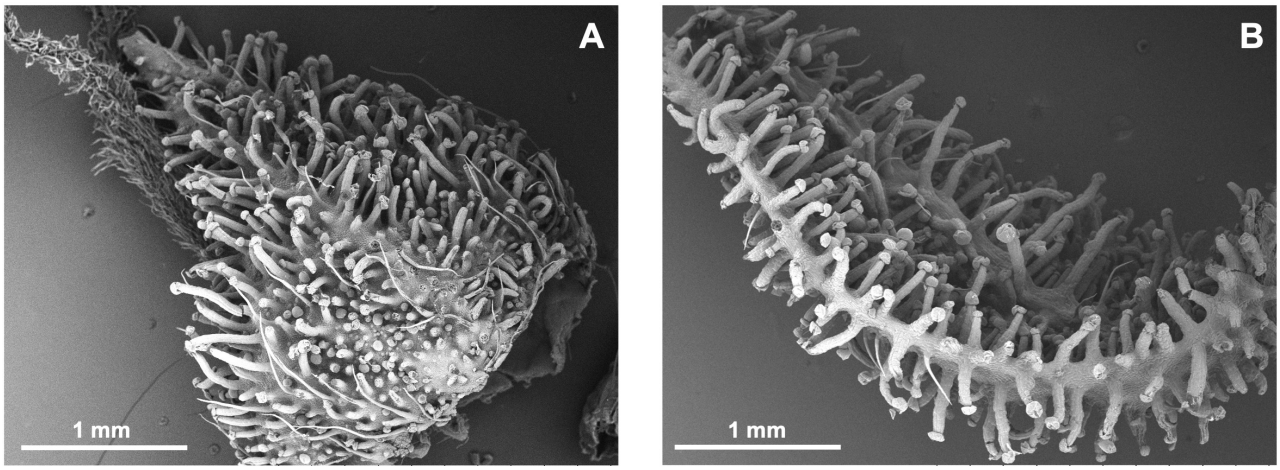

Figure 3.3: Scanning electron micrographs of cannabis inflorescence organs at Week 8 of flowering. Stalked trichomes completely cover the calyx (A) and bract (B) surfaces at time of harvest despite being under a low nutrient regime for the first half of development.

3.3.3 Cannabinoid content changes due to nutrient conditions and PGPR inoculations

In order to ascertain whether the minimal changes in trichome densities are linked to changes in cannabinoid production, quantifications of cannabinoid concentrations from ground inflorescences under PGPR treatments and both nutrient conditions were obtained. Under recommended nutrient conditions, none of the concentrations of the cannabinoids measured were found to be significantly different in plants inoculated with bacteria compared to plants without bacterial treatment (Figure 3.4); the test for the statistical interaction model (treatment*compound) under the optimal nutrient regime was not statistically significant ($p = 0.56$). This was consistent with the results of the trichome densities, as no PGPR treatment led to significantly greater trichome densities across all three inflorescence epidermal surfaces. When considering the two primary cannabinoids of interest, CBDA and THCA, PGPR-treated plants were found to have somewhat lower concentrations than those treated without PGPR, consistent with the trichome density patterns previously observed (Figure 3.5); none of the differences were statistically significant, however.

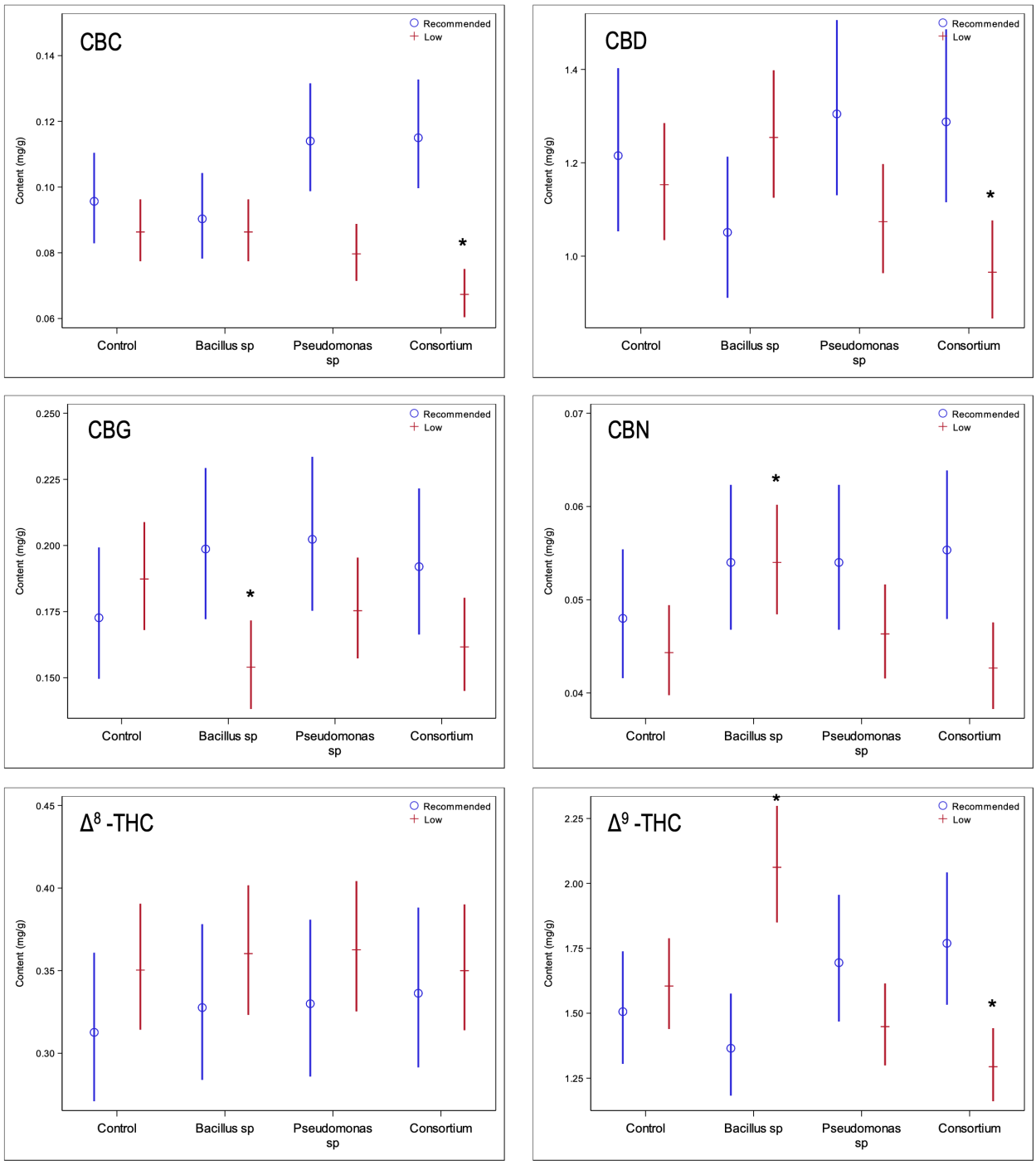

Figure 3.4: Comparisons of cannabinoid concentrations between low and recommended nutrient regimes across PGPR treatments. Under recommended nutrient conditions PGPR did not lead to any significant changes in cannabinoid concentration, whereas under low nutrient conditions, significant changes were observed for CBG and Δ^9-THC under Bacillus sp. treatment and CBC under consortium treatment. Circles indicate the average concentration, vertical bars indicate data range. Asterisk indicates significant results.

Under the low nutrient regime, plants treated with either the *Bacillus* sp. or the bacterial consortium treatments featured significant changes in the concentrations of three cannabinoids each (Figure 3.4). The effect of *Bacillus* sp. was significant for CBG, CBN, and Δ^9-THC, with CBG decreased by 21.6%, CBN increased by 17.9%, and Δ^9-THC increased by 22.2% compared to the non-inoculated control. When inoculated with the bacterial consortium the CBC, CBD, and Δ^9-THC cannabinoids were altered in significant quantities, with CBC decreased by 28.2%, CBD decreased by 19.4%, and Δ^9-THC decreased by 24.0% also when compared to the non-inoculated control.

The primary cannabinoid compounds of interest, CBDA, THCA, and CBGA, are also those that are the most abundant in cannabis inflorescence tissue. While the differences between their concentrations were not statistically significant compared to their uninoculated control, under the low nutrient regime, the concentrations of CBDA and THCA were greater than under recommended conditions but were reduced for CBGA across all treatment groups (Figure 3.5), as expected as CBGA is the precursor molecule for THCA and CBDA. In the absence of PGPR, the low nutrient group showed an increase by 6.1% for CBDA, increase by 21.9% for THCA, and a decrease by 13.4% for CBGA, when compared to the non-inoculated control under recommended nutrient levels. The PGPR treatments caused increases of 15.6, 14.1 and 8.3% for CBDA, 20.5, 18.5 and 13.9% for THCA, and reductions by 30.7, 3.6 and 8.02% for CBGA for the *Bacillus* sp., *Pseudomonas* sp. and the consortium treatments, respectively. Interestingly, despite the low nutrient non-inoculated plants being the only group to have lower average trichome densities than its recommended nutrient counterpart, it yielded some of the greatest differences for the three cannabinoids of interest (Figure 3.5).

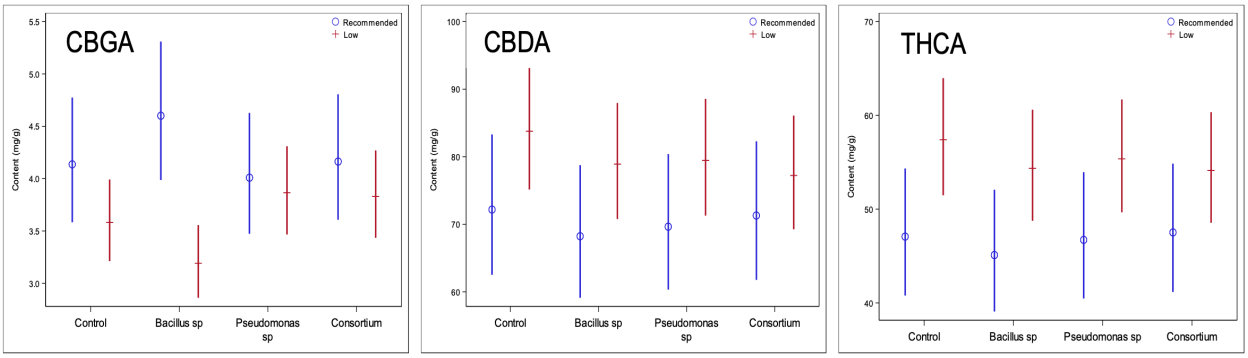

Figure 3.5: Comparisons of the three primary cannabinoids of interest between low and recommended nutrient regimes. Low nutrient regime consistently led to higher concentrations of CBDA and THCA than under recommended nutrients regardless of PGPR treatment. CBGA content on the other side was slightly higher under recommended nutrient conditions. Circles indicate the average concentration, vertical bars indicate data range.

When comparing the total tested cannabinoids between the two nutrient regimes under the same PGPR treatments, the differences appear to be related to the presence of *Bacillus* sp. Referring to Table 3.3, the differences between the two nutrient regimes for four of the nine cannabinoids tested were significant in plants treated with *Bacillus* sp., of which the low nutrient regime was higher except for CBGA and CBG. Plants inoculated with the consortium treatment led to the same number of significant differences, with the levels of four cannabinoids being altered, however these were at lower concentrations than under the recommended regime. Plants with *Pseudomonas* sp. treatment only had changes in the concentrations of two cannabinoids ($p < 0.05$), CBC and CBD, both of which were reduced compared to the recommended regime. Lastly, in plants grown in the absence of PGPR, only THCA was affected ($p < 0.05$) by the nutrient regime, with its concentration being higher under low nutrients than recommended nutrient conditions.

Table 3.3: Statistically significant differences in cannabinoid concentrations between low and recommended nutrient regimes of inoculated cannabis cv. CBD Kush. Positive values indicate an increase when under low nutrient conditions, and a negative value indicates a decrease when under low nutrients, in comparison to the recommended nutrient regime.

Treatment	Cannabinoids with Statistically Significant Differences between Nutrient Regimes	% Change of Low Nutrient to Recommended Nutrient	Upper Limit	Lower Limit	P values
Control	Tetrahydrocannabinolic acid	21.9%	0.02	0.38	0.0308
Bacillus sp.	Cannabigerol	-22.5%	-0.43	-0.07	0.0058
	Cannabigerolic acid	-30.7%	-0.54	-0.19	<0.0001
	Tetrahydrocannabinolic acid	20.5%	0.007	0.37	0.0419
	δ9-Tetrahydrocannabinol	51.0%	0.23	0.59	<0.0001
Pseudomonas sp.	Cannabichromene	-30.1%	-0.54	-0.18	0.0001
	Cannabidiol	-17.7%	-0.37	-0.01	0.0340
Consortium	Cannabichromene	-41.4%	-0.71	-0.35	<0.0001
	Cannabidiol	-25.0%	-0.47	-0.12	0.0019
	Cannabinol	-22.9%	-0.44	-0.08	0.0049
	δ9-Tetrahydrocannabinol	-26.9%	-0.49	-0.13	0.0008

3.4 Discussion

This study has demonstrated that while PGPR inoculations do not have a significant effect on stalked trichome densities on cannabis inflorescence organs when plants were grown under

recommended nutrient conditions, the application of an environmental stress for the first half of plant development reveals a benefit to applying these microbes. Under recommended nutrient conditions, there was a surprising downward shift of trichome densities on all three evaluated epidermal tissues, but this tendency was reversed under the low nutrient regime. Though non-inoculated plants treated with the low nutrient regime manifested a decrease in trichome densities compared with the recommended nutrient regime across all epidermal types, PGPR rescued this effect on trichome numbers. This is consistent with the limited studies available that have provided links to trichome densities with PGPR presence in *Ocimum basilicum* (Copetta et al., 2006) and environmental stressors in *Cymbopogon citratus* (Mirzaie et al., 2020). However, not only were these studies conducted on different plant species, but the stress was related to the effect of drought only. This makes ours an early-stage investigation to determine both if PGPR presence affect trichome quantities on cannabis plants and the impact of nutrient stress on trichome densities, but also how these two factors influence trichome development when simultaneously administered.

The true benefit of manipulating cannabis growth conditions lies in the application of an environmental stress, revealed by the cannabinoid concentrations. Under the recommended nutrient conditions, PGPR did not cause meaningful differences in the abundance of minor cannabinoids. The abundance of two cannabinoids of commercial interest, CBDA and THCA, was reduced by the PGPR but under the low nutrient regime PGPR increased their concentrations. For example, *Bacillus* sp. inoculation under the low nutrient regime increased the Δ^9-THC concentration, a degradation product of THCA, by 51% against the amount detected in the recommended nutrient regime's *Bacillus* sp. treatment. This effect of low nutrient stress is consistent with Caplan et al. (2017), who investigated the link between substrate and liquid fertilizer application rates. Caplan et al. (2017) found that while a lower fertilizer rate led to

reduced yield, there were higher cannabinoid concentrations with a particular substrate. As we have shown, this link is marginally reflected in enhanced trichome densities, though statistically insignificant for all but the calyx tissue under consortium inoculation; it seems to be more evident of an increased level of cannabinoid biosynthesis per trichome. What was most surprising, however, was bacterial inoculation increasing the amount of THCA and CBDA compared to non-inoculated plants, in both nutrient regimes. It should be noted that the reduction in CBGA, under low nutrient conditions, compared to recommended conditions is likely due to it being the precursor molecule to THCA and CBDA, and as these were present in higher amounts under low nutrient regimes; it is consistent that CBGA content would be lower than that of the recommended nutrient regime treatments. The observations on cannabinoid concentrations differ somewhat from previous work from our laboratory (Lyu et al., 2022b), an effect that is likely due to the type of rooting medium used. Plants in the present study were grown in soil with coconut fibre, which is recommended by the manufacturer for cannabis and cutting propagation, whereas Lyu et al. used soil with compost which provides additional beneficial microorganism and nutrient sources; this could have led to the differences in cannabinoid profiles between treatments. Clearly much remains to be learned in this regard, however as both Bevan et al. (2021) and Caplan et al. (2017) demonstrated, cannabis can have unique responses to tailored growth conditions and environments; future studies may be based on comparing how the effects of microbial inoculations need to be altered for cannabis based on the rooting medium used. Notably, in cannabis production, an increased 'yield' is not necessarily equated with an increase in biomass, but 'yield' may instead represent an increase in cannabinoid concentration per volume biomass, possibly at the cost of reduced overall biomass. Only experiments conducted at a larger scale will reveal whether this can be translated into higher cannabinoid yield per production surface. Secondly, 'yield' may relate to

a particular cannabinoid rather than the overall combined amount and may pertain to a desired ratio between certain compounds rather than their absolute abundance. Growth conditions must therefore be tailored to the desired outcome which may require a carefully formulated combination of growth-enhancing and growth-stressing conditions. As the current Canadian cannabis market demands increasingly higher THC contents, it is imperative that producers have as much guidance available as possible in this regard in order to make informed decisions about their production strategies.

This study has provided evidence to justify the incorporation of eco-friendly growth conditions into indoor cannabis production. While the PGPR treatments had marginal effects on the trichome densities and did not necessarily enhance effects on cannabinoid contents, it was the reduction in the amount of applied nutrients for the first half of plant development that led to a noticeable improvement in the primary cannabinoids of interest, namely THC and CBD and their counterparts, particularly when inoculated with *Bacillus* sp. This leads us to potentially recommend the practice of restricting nutrient applications for cannabis plants, and while in general the presence of PGPR only yielded moderate changes in trichome count and cannabinoid profile, the addition of *Bacillus* sp. led to the greatest number of changes in cannabinoid profiles between recommended and low nutrient regimes. Therefore, if the goal is specifically to manipulate the cannabinoid profile, we tentatively suggest the use of *Bacillus* sp. along with a partial low nutrient regime to produce the greatest degree of change. In doing so, there is the potential for producers to not only save production costs, but they could also increase their profit margins by obtaining greater amounts of THCA and CBDA; future work should investigate the true economic potential of these results for producers. In addition, by both reducing the amount of nutrients manufactured and diminishing the concentration of contaminated wastewater leaving

facilities, the stress on the environment from facility practices will be lessened. Overall, this study not only demonstrated that PGPR inoculation has a limited impact on cannabis stalked glandular trichomes, but notably how the application of an environmental stressor can elicit improved effects of these inoculations, thereby motivating changes towards production methods that minimize chemical inputs.

3.5 References

Addo, P. W., Desaulniers Brousseau, V., Morello, V., MacPherson, S., Paris, M., and Lefsrud, M. (2021). Cannabis chemistry, post-harvest processing methods and secondary metabolite profiling: A review. *Ind. Crops Prod.* 170: 113743. doi:10.1016/j.indcrop.2021.113743.

Authority on the Responsible Use of Cannabis Act, LXVI L.S. § 628 (2021). https://legislation.mt/eli/cap/628/20211218/eng.

Backer, R., Schwinghamer, T., Rosenbaum, P., McCarty, V., Eichhorn Bilodeau, S., Lyu, D., et al. (2019). Closing the yield gap for cannabis: A meta-analysis of factors determining cannabis yield. *Front. Plant Sci.* 10: 495. doi:10.3389/fpls.2019.00495.

Balthazar, C., Novinscak, A., Cantin, G., Joly, D.L., Filion, M. 2022a. Biocontrol activity of *Bacillus* spp. and *Pseudomonas* spp. against *Botrytis cinerea* and other cannabis fungal pathogens. Phytopathology 112, 549–560. doi: 10.1094/PHYTO-03-21-0128-R.

Balthazar, C., Joly, D.L., and Filion, M. 2022b. Exploiting beneficial *Pseudomonas* spp. For cannabis production. Front. Microbiol. 112, 549–560. doi: /10.3389/fmicb.2021.833172.

Banchio, E., Bogino, P. C., Zygadlo, J., and Giordano, W. (2008). Plant growth promoting rhizobacteria improve growth and essential oil yield in *Origanum majorana L*. *Biochem. Syst. Ecol.* 36, 766–771. doi:10.1016/j.bse.2008.08.006.

Berendsen, R. L., Pieterse, C. M. J., and Bakker, P. A. H. M. (2012). The rhizosphere microbiome and plant health. *Trends Plant Sci.* 17, 478–486. doi:10.1016/j.tplants.2012.04.001.

Bernstein, N., Gorelick, J., Zerahia, R., and Koch, S. (2019). Impact of N, P, K, and humic acid supplementation on the chemical profile of medical cannabis (*Cannabis sativa L*). *Front. Plant Sci.* 10: 736. doi:10.3389/fpls.2019.00736.

Bevan, L., Jones, M., and Zheng, Y. (2021). Optimisation of nitrogen, phosphorus, and potassium for soilless production of *Cannabis sativa* in the flowering stage using response surface analysis. *Front. Plant Sci.* 12: 2587. doi:10.3389/fpls.2021.764103.

Caplan, D., Dixon, M., and Zheng, Y. (2017). Optimal rate of organic fertilizer during the flowering stage for cannabis grown in two coir-based substrates. *HortScience* 52, 1307-1312. doi:10.21273/HORTSCI12401-17.

Copetta, A., Lingua, G., and Berta, G. (2006). Effects of three AM fungi on growth, distribution of glandular hairs, and essential oil production in *Ocimum basilicum L.* var. *Genovese*. *Mycorrhiza* 16, 485–494. doi:10.1007/s00572-006-0065-6.

Danziger, N., and Bernstein, N. (2021). Plant architecture manipulation increases cannabinoid standardization in 'drug-type' medical cannabis. *Ind. Crops Prod.* 167: 113528. doi:10.1016/j.indcrop.2021.113528.

Desaulniers Brousseau, V., Wu, B. Sen, MacPherson, S., Morello, V., and Lefsrud, M. (2021). Cannabinoids and terpenes: How production of photo-protectants can be manipulated to enhance *Cannabis sativa* L. phytochemistry. *Front. Plant Sci.* 12: 620021. doi:10.3389/fpls.2021.620021.

Eshaghi Gorgi, O., Fallah, H., Niknejad, Y., and Barari Tari, D. (2022). Effect of Plant growth promoting rhizobacteria (PGPR) and mycorrhizal fungi inoculations on essential oil in *Melissa officinalis L*. under drought stress. *Biologia (Bratisl).* 77, 11–20. doi:10.1007/s11756-021-00919-2.

Grand View Research (2022) Legal Marijuana Market Size, Share & Trends Analysis Report By Product Type (Flower, Oil And Tinctures), By Application (Medical, Adult Use), By Region (North America, Europe, APAC, LATAM, Africa), And Segment Forecasts, 2022 – 2030. *San Francisco, CA.* Available at: https://www.grandviewresearch.com/industry-analysis/legal-marijuana-market (Accessed 06 September, 2022).

Hawley, D., Graham, T., Stasiak, M., and Dixon, M. (2018). Improving cannabis bud quality and yield with subcanopy lighting. *HortScience* 53, 1593-1599. doi:10.21273/HORTSCI13173-18.

Jin, D., Jin, S., and Chen, J. (2019). Cannabis indoor growing conditions, management practices, and post-harvest treatment: A Review. *Am. J. Plant Sci.* 10: 925. doi:10.4236/ajps.2019.106067.

Kundan, R., Pant, G., Jadon, N., and Agrawal, P. K. (2015). Plant growth promoting rhizobacteria: mechanism and current prospective. *J. Fertil. Pestic.* 6: 9. doi:10.4172/2471-2728.1000155.

Livingston, S. J., Quilichini, T. D., Booth, J. K., Wong, D. C. J., Rensing, K. H., Laflamme-Yonkman, J., et al. (2020). Cannabis glandular trichomes alter morphology and metabolite content during flower maturation. *Plant J.* 101, 37–56. doi:10.1111/tpj.14516.

Lyu, D., Backer, R., and Smith, D. L. (2022a). Three plant growth-promoting rhizobacteria alter morphological development, physiology, and flower yield of *Cannabis sativa L*. *Ind. Crops Prod.* 178:114583. doi:10.1016/j.indcrop.2022.114583.

Lyu, D., Backer, R., Ruan, E., and Smith, D. L. (2022b). Evaluation of PGPR inoculants on cannabinoid and accumualation in inflorescences of *Cannabis sativa* L. [Submitted for publication], *Department of Plant Science, McGill University.*.

Magagnini, G., Grassi, G., and Kotiranta, S. (2018). The effect of light spectrum on the morphology and cannabinoid content of *Cannabis sativa L*. *Med. Cannabis Cannabinoids* 1, 19–27. doi:10.1159/000489030.

Mahlberg, P. G., and Eun, S. K. (2004). Accumulation of cannabinoids in glandular trichomes of Cannabis (Cannabaceae). *J. Ind. Hemp* 9, 15–36. doi:10.1300/J237v09n01_04.

Mirzaie, M., Ladanmoghadam, A. R., Hakimi, L., and Danaee, E. (2020). Water stress modifies essential oil yield and composition, glandular trichomes and stomatal features of lemongrass (*Cymbopogon citratus l.)* inoculated with arbuscular mycorrhizal fungi. *J. Agric. Sci. Technol.* 22, 1575-1585.

Mitchell Westmoreland, F., Kusuma, P., and Bugbee, B. (2021). Cannabis lighting: Decreasing blue photon fraction increases yield but efficacy is more important for cost effective production of cannabinoids. *PLoS One* 16: e0248988. doi:10.1371/journal.pone.0248988.

Ohlsson, A., Abou-Chaar, C. I., Agurell, S., Nilsson, I. M., Olofsson, K., and Sandberg, F. (1971). Cannabinoid constituents of male and female *Cannabis sativa*. *Bull Narc.* 23, 29–32.

Pagnani, G., Pellegrini, M., Galieni, A., D'Egidio, S., Matteucci, F., Ricci, A., et al. (2018). Plant growth-promoting rhizobacteria (PGPR) in *Cannabis sativa* 'Finola' cultivation: An alternative fertilization strategy to improve plant growth and quality characteristics. *Ind. Crops Prod.* 123, 75–83. doi:10.1016/j.indcrop.2018.06.033.

Rodriguez-Morrison, V., Llewellyn, D., and Zheng, Y. (2021). Cannabis yield, potency, and leaf photosynthesis respond differently to increasing light levels in an indoor environment. *Front. Plant Sci.* 12: 646020. doi:10.3389/fpls.2021.646020.

Sivasakthi, S., Usharani, G., and Saranraj, P. (2014). Biocontrol potentiality of plant growth promoting bacteria (PGPR) - *Pseudomonas fluorescens* and *Bacillus subtilis*: A review. *African J. Agric. Res.* 9, 1265–1277. doi:10.5897/AJAR2013.7914.

Wei, X., Zhao, X., Long, S., Xiao, Q., Guo, Y., Qiu, C., et al. (2021). Wavelengths of LED light affect the growth and cannabidiol content in *Cannabis sativa L*. *Ind. Crops Prod.* 165: 113433. doi:10.1016/j.indcrop.2021.113433.

Yep, B., and Zheng, Y. (2021). Potassium and micronutrient fertilizer addition in a mock aquaponic system for drug-type *Cannabis sativa L*. Cultivation. *Can. J. Plant Sci.* 101, 341–352. doi:10.1139/cjps-2020-0107.

Connecting Text between Chapter 3 and Chapter 4

In Chapter 3, the focus was on determining if PGPR presence affects stalked glandular trichomes on cannabis inflorescences, both with and without an environmental stressor, evaluated by determining their density using stereomicroscopy. While this was found to not be the case in a significant manner under recommended conditions, there was a marginal effect (not statistically significant overall) observed under a low nutrient regime. These differences were reflected in the associated cannabinoid profiles, though the development of the overall plant under an environmental stress was not explored.

Despite the lack of statistical significance found in the trichome densities of Chapter 3, it is important to evaluate the whole-plant potential of these PGPR inoculations and alternative nutrient regime. Linking back to Chapter 2, the novelty of agricultural research for cannabis leaves many questions unanswered, and Chapter 4 continues from the work in Chapter 3 to address some of these in order to provide producers with informed expectations should they desire to incorporate elements described in this work. In this chapter, I have maintained all plants used in the trials and created the experimental design. I have collected all data presented and conducted the statistical analysis, followed by the drafting of the manuscript.

As Chapter 3 identified that cannabinoids underwent greater changes in their concentrations under a low nutrient regime, notably in the presence of *Bacillis mobilis*, it is necessary to relate these changes to any potential deviations in expected biomass yields. Having previously discussed how cannabis yield can refer to either cannabinoid contents or inflorescence (flower) mass, the following chapter delves into how common indicators of overall plant development may be impacted by the same inoculation and nutrient regime trials presented in Chapter 3. Thus, the investigation into stress (low nutrition) effects on overall plant health, development, and biomass yield continues in Chapter 4.

Chapter 4 – Minimization of nutrient input during early plant development does not adversely affect inflorescence formation in *Cannabis sativa*

This manuscript is currently submitted for publication consideration in Frontiers in Plant Science.

Title: Reduction of nutrient input during Cannabis production does not affect development

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Abstract

Cannabis is very under-researched in the context of agricultural production, primarily due to the severe legal restrictions placed on it. As a result of these limitations, it has been largely left behind with regards to the validation of modern technologies for optimizing yield in terms of inflorescence mass and overall plant development. This work aims to help close this gap by determining if inoculation with plant growth-promoting rhizobacteria (PGPR), coupled with a low nutrient regime, affects *Cannabis* development. *Cannabis* plants were inoculated with either *Pseudomonas* sp. or *Bacillus* sp., separately or in a consortium, and subjected to a low nutrient regime for the first half of development. After 6 weeks of development, plants were switched to a recommended nutrient regime for the remaining 6 weeks of growth. Development variables were measured throughout the growth period in addition to harvest data. Our findings determine that there was no statistically significant impact on plant development, using plant height and leaf area as metrics, or inflorescence yields under low nutrient regimes. Unexpectedly, a low nutrient regime led to greater leaf area across all treatments and reduced data variation when compared to recommended nutrient conditions. In addition, plants with *Bacillus* sp. inoculation under a low nutrient regime featured bigger inflorescences compared to inflorescences from the same PGPR

treatment under recommended nutrient conditions; all treatment groups had, on average, bigger inflorescences under low nutrient conditions than their recommended nutrient counterparts. Overall, this work provides novel insight into how *Cannabis* can be grown at reduced nutrient conditions without sacrificing developmental health, providing validation for environmentally friendly production methods that reduce production costs in greenhouse conditions.

4.1 Introduction

Cannabis (hereafter, cannabis) is in the unique position of being a novel agricultural crop in the 21st century while remaining highly regulated for both its production practices and supply to the public. While the cannabis sector is a highly profitable industry despite the heavy legislation limiting product types and availability to consumers, research focused on agricultural applications is lacking. With an industry predicted to surpass 100 billion USD by 2030 in the global legal market (Grand View Research, 2022), it is time that validation of modern agricultural methods be carried out for cannabis to ensure this growing industry is operating under applicable research-backed practices.

Cannabis producers primarily focus on female plants, as their inflorescences are the primary source of cannabinoids and terpenes which are produced in glandular trichomes (Ohlsson et al., 1971; Mahlberg and Kim, 2004; Livingston et al., 2020). Little is known about trichome development although it is well documented that growth conditions can influence their density on the surface of plant organs as well as the abundance and chemical composition of the metabolites that they produce. For example, the administration of plant growth-promoting rhizobacteria (PGPR) has been linked to an increase in the content of essential oils, and thus trichome quantities, in *Ocimum bascilicum* (Copetta et al., 2006) and *Organum majorana* (Banchio et al., 2008). These essential oils are similar to the cannabinoid-containing resin produced in cannabis trichomes. This

link between PGPR presence and trichome yields raises the possibility to employ the manipulation of the cannabis phytomicrobiome with the aim to optimize yield. For cannabis, yield is primarily assessed in terms of cannabinoid content at harvest. PGPR are well-established in their ability to support plant development in a range of plant species. This effect is mediated by improving water and nutrient acquisition, enhancing stress tolerance and by establishing synergistic relationships with the plant host that involve the production of phytohormones (Berendsen et al., 2012; Sivasakthi et al., 2014; Kundan et al., 2015) and signal compounds (Backer et al., 2018; Lyu et al., 2022). In cannabis, the relation between PGPR and yield is poorly understood. In 2017, Conant et al. evaluated the efficacy of a commercial microbial biostimulant on hemp plants, revealing an increase in both vegetative development, reflected in plant height and bud yield. Though the authors did not assess CBD yield, the increase in bud mass are consistent with later findings of Pagnani et al. (2018), who measured cannabinoids. Pagnani et al. determined cannabidiol (CBD), tetrahydrocannabinol (THC), and cannabinol (CBN) concentration in hemp cultivars grown under greenhouse condition and exposed to a variety of cultivation conditions involving PGPR inoculum concentrations (Pagnani et al., 2018). Among their findings, this group indicated that with regards to biomass, an inoculum of 106 cells mL^{-1} had comparable effects to traditional nitrogen fertilizer. The abundance of three cannabinoids measured was consistently increased by both bacterial inoculum concentrations (Pagnani et al., 2018). The idea of using PGPR to increase cannabinoid yields continues to gain interest (Ahmed and Hijri, 2021).

PGPR can help to mitigate the effects of environmental stressors and improve crop productivity (reviewed by Backer et al., 2018; Oleńska et al., 2020). This has been demonstrated in major agricultural species such as corn (Lin et al., 2019; Pereira et al., 2020; Mishra et al., 2020) and rice (Hafez et al., 2019; Joshi et al., 2020; Durairaj et al., 2021), leading to the promising

potential for applications in cannabis. However, the limited work on PGPR inoculations with cannabis has been performed in unstressed conditions (Pagnani et al., 2018; Lyu et al., 2022). The ability to minimize production costs by reducing artificial fertilizer inputs without sacrificing yield is a promising venture to consider for an industry that is so new, but it is important that cannabis health and development are not compromised by these conditions.

As previous work has shown that the two PGPR evaluated here have either neutral or positive impacts on developmental variables of cannabis plants (Lyu et al., 2022), we attempted to assess whether the same *Pseudomonas* sp. and *Bacillus* sp. are capable of mitigating an environmental stress. The potential for this outcome could lead to a method that is not only more sustainable by reduction of natural resource consumption and chemical output from production facilities but will also cut production costs in order to support their applications. By reducing the amount of nutrients provided through the elimination of all but 1 of the recommended nutrients, of what is recommended for the first half of development, our goal is to determine – by assessing plant height, leaf area, and inflorescence data – whether there is a recovery of overall cannabis health and yield.

4.2 Materials and Methods

4.2.1 Bacterial inoculum preparation and delivery

Referring to Tanney et al. (2023; Chapter 3), *Pseudomonas* sp. (*Pseudomonas koreensis*, AF468452) and *Bacillus* sp. (*Bacillus mobilis*, KJ812449) were stored at -80 ºC in glycerol and were revived by streaking onto petri plates containing sterile (30 min, 121 °C) King's Medium B (KB; 20.0 g L^{-1} protease peptone, 1.5 g L^{-1} K_2HPO_4, 10.0 g L^{-1} glycerol, 0.25 g L^{-1} $MgSO_4 \bullet 7\ H_2O$) and incubating at 28 ºC overnight. These strains were originally isolated by Fan et al. (2020), and have been previously studied for their potential application in cannabis production by Lyu et al. (2022). Bacterial suspensions were prepared and grown overnight at 28 ºC, rotating at 150 rev min^{-}

[1]. The following day, 30 mL of the inoculated media was distributed into 50 mL falcon tubes. Tubes were centrifuged at 6000 g for 10 min (Sorvall Biofuge Pico, Kendro Laboratory Products, Asheville, NC, USA). The supernatant was discarded, and pellets were washed in 10 mL of 10 mM $MgSO_4$. Following the wash, resuspended pellets were diluted to 0.1 OD at 600 nm (Ultraspec 4050 Pro UV/Visible spectrophotometer), using 10 mM $MgSO_4$ as the blank.

Prepared inoculations were dispensed onto the soil of cuttings at the base of the stem using a serological pipette on the day of transplantation. Ten mL of each inoculum was dispensed onto each cutting, and for the consortium treatment five mL of each inoculum component was dispensed. Control treatments received 10 mL of 10 mM $MgSO_4$.

4.2.2 Plant propagation and maintenance

Referring to Tanney et al. (2023; Chapter 3), female *Cannabis sativa* L. plants of the marijuana variety "CBD Kush" were grown from cuttings sourced from in-house mother plants at Macdonald Campus, Saint-Anne-de-Bellevue, Quebec, in a Canada Revenue Agency and Health Canada approved research laboratory (license no. LIC-5AZZW7S4GM-2019). Medium-thick branches (~2 mm diameter) were cut and all but the top three fully-formed leaves were removed from the stem and the outermost halves of the remaining leaves were clipped off. The ends of cuttings were trimmed to a 45º angle and dipped in Stim Root No. 2 powder (Master Plant-Prod Inc., Brampton, ON, Canada), after which they were placed in 3 cm pre-soaked rockwool cubes (Grodan, Roermond, Netherlands) on mesh trays (53 x 27 x 6 cm, Bootstrap Farmer, Downington, PA, USA) inside propagation trays (54 x 28 x 6 cm, Mondi, Vancouver, BC, Canada). Two L of VeloKelp nutrient solution (pH 5.6, Remo Nutrients; Remo Brands Inc., Maple Ridge, BC, Canada) were poured into the trays and replaced once per week. Prepared trays were covered with a vented mini greenhouse (54 x 28 x 19 cm; Mondi, Vancouver, BC, Canada) and placed on a

propagation rack for three to four weeks, until sufficient roots were observed (conditions – light at approximately 150 µmol m^{-2} s^{-1}, 24 h photoperiod, 75-95% humidity, 24-25 ºC). Tanney et al. (2023; Chapter 3), provides the NPK contents of the nutrients used in this study.

Three series of healthy cuttings were transplanted into 15 cm pots (Teris, Laval, QC, Canada) containing pre-soaked Agromix G6 soil (300 mL of water per 400 g; Farfad Inc., Saint-Bonaventure, QC, Canada) and grown under vegetative conditions (approximately 150 µmol m^{-2} s^{-1}, 18 h photoperiod, 20-22 ºC, 65 % relative humidity) for four weeks. Vegetative plants were given the recommended nutrient regime of 150 mL water and nutrient application according to week of vegetative growth as per manufacturer guidelines and Lyu et al. (2022) (Nutrients: Magnifical, Micro, Velokelp, Grow at pH 6.3, Remo Nutrients). Following this period, plants were transferred to flowering conditions (approximately 150 µmol m^{-2} s^{-1}, 12 h photoperiod, 20-22 ºC, 65 % relative humidity) and given a regime of 250 mL water and nutrient application according to the week of flowering growth as per manufacturer guidelines and Lyu et al. (2022) (Nutrients: Magnifical, Micro, Velokelp, Astroflower, Bloom at pH 6.3, Remo Nutrients); only water was given in the final week of development, as per guidelines. Plants were grown under flowering conditions for a total of 8 weeks. Plants undergoing the low nutrient regime were given the same volume of nutrient-containing solution under the same growing conditions as the recommended regime. However, from Week 1 to 6, three of the four nutrient mixes were omitted and only the Velokelp nutrient was provided at the same concentration as when combined with other nutrients as part of the recommended regime (pH 6.3, Remo Nutrients), creating a nutrient deficiency. Following Week 6, complete nutrients, as described above, were given for a total of 5 weeks until Week 12, during which only water was provided. Tanney et al., (2023; Chapter 3), provides a reference table for the provided nutrients over time.

4.2.3 Developmental and harvest measurements

Following transplantation, non-destructive measurements of plant height were taken on a weekly basis. Under flowering light, weekly inflorescence counts were taken, in addition to plant height; an inflorescence was counted when stigma elongation was observed. Twelve weeks post-transplant, plants were harvested and separated into inflorescence buds, leaves, and stems. Leaf area was measured using a leaf area meter (Li-3100 C, Lincoln, NE, USA). The inflorescences were weighed for fresh mass and counted, and the total leaf area was recorded. The dry inflorescence mass was recorded following freeze drying using a lyophilizer (SNL216V freeze-dryer, Thermo Savant Co. Ltd. USA).

4.2.4 Statistical analysis

Data analysis was performed using the statistical program SAS (SAS OnDemand for Academics, Enterprise Guide 8.3, SAS Institute Inc. Cary, NC, USA). Differences between treatments and tissues and the control were evaluated using PROC GLM; the level of significance was set at $p < 0.05$.

Four plants per inoculation treatment group were prepared per nutrient regime experimental series and organized in a randomized complete block design. A total of 12 replicates were created, through four blocks per experimental series with 3 experimental series per nutrient regime grown. For the control treatment and the consortium treatment for the recommended nutrient regime have n was 11 for all measurements, whereas for the *Pseudomonas* sp. and *Bacillus* sp. treatments n was 12. For the low nutrient regime leaf area measurements n was 8, whereas for the recommended regime measurements n was 12.

4.3 Results

4.3.1 Plant height is marginally affected by low nutrient regime

In order to assess if overall development is stunted by a restricted nutrient regime, plant height was measured across vegetative and flowering periods, from transplantation to harvest. Under a low nutrient regime, there were no significant differences in plant height during the full 12 weeks of development with the PGPR treatments, when compared to the uninoculated control treatment (Figure 4.1). Across the weeks, plants treated with the consortium inoculum consistently had the same height as the corresponding non-inoculated control, while the height of plants treated with *Bacillus* sp. and *Pseudomonas* sp. had the tendency to be higher. As expected, the greatest variation occurred during the vegetative period (weeks 1-4), with differences due to bacterial inoculation diminishing across all treatments after Week 6.

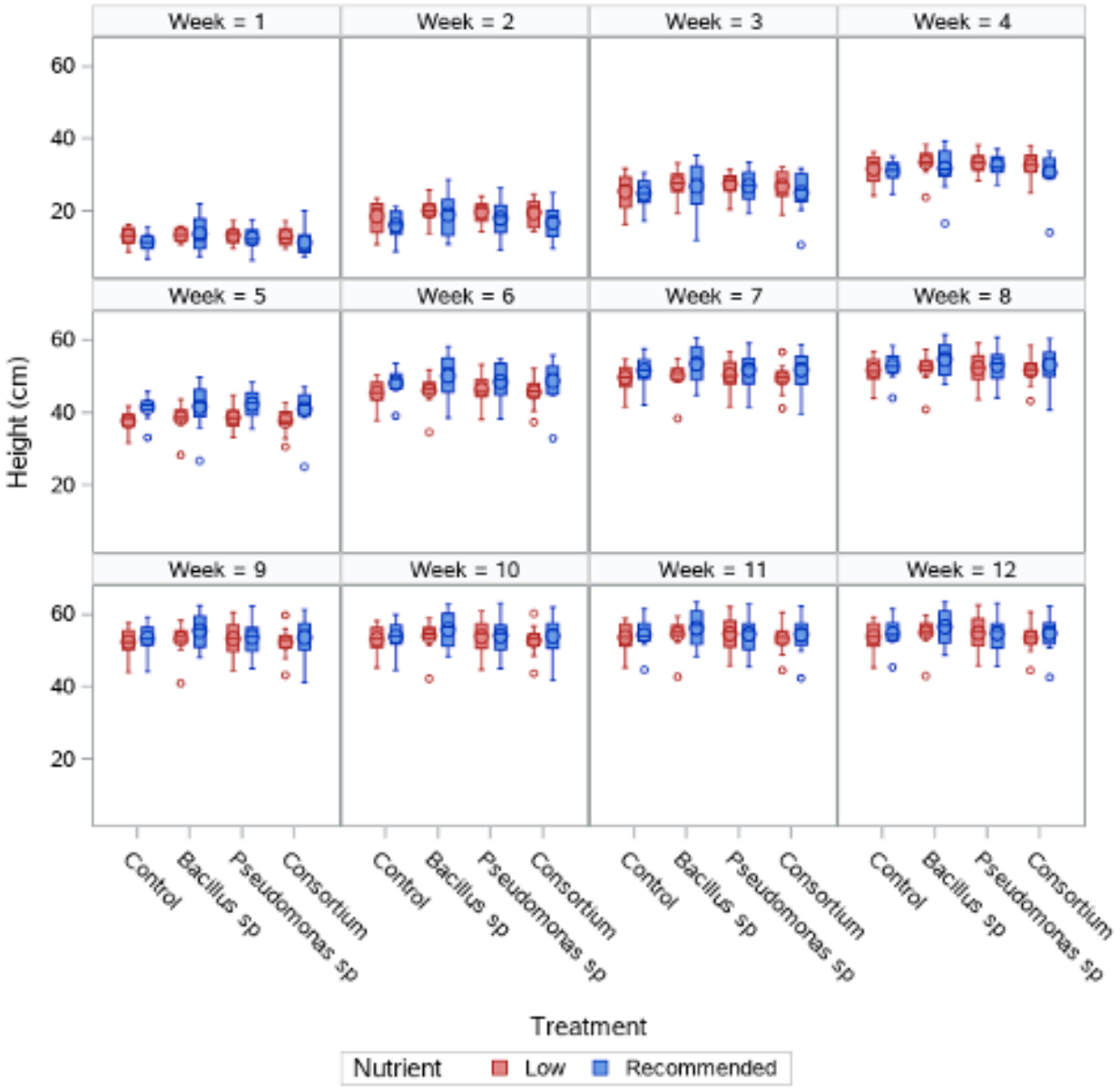

Figure 4.1: Comparison of average plant height of non-inoculated and inoculated cannabis cv CBD Kush under two nutrient regimes across development. Circle indicates mean height, bar indicates median height, vertical bars indicate data range.

Under the recommended nutrient regime there were no significant effects of bacterial inoculation on plant height either, but plant height was in general slightly greater than under low nutrient conditions (Figure 4.1). The tendency towards this slight increase were also similar between the PGPR groups across the two nutrient regimes, in that height of plants inoculated with

the consortium was consistently close to the corresponding uninoculated control, though with age plants treated with the *Pseudomonas* sp. inoculation had an increasingly smaller gap in height with the uninoculated control. By the time of harvest, little difference remained between the two groups. This left the *Bacillus* sp. treatment to be the only inoculation that resulted in a tendency to be greater in height compared to the non-inoculated samples, but not significantly so ($p > 0.05$). When compared against the low nutrient regime, following the key time point of Week 5, after which the low nutrient regime was switched to the recommended regime, there were noticeable differences in height between plants inoculated with bacteria and the uninoculated control. The difference between low and recommended nutrient regimes at Week 5 was a decrease of 9.40, 7.45, 8.75, and 7.82% for the control, *Bacillus* sp., *Pseudomonas* sp., and consortium treatments, respectively, indicating a marginal recovery of height under PGPR treatment at the time when the nutrient restriction on the low nutrient regime ended. However, at time of harvest the difference between the regimes was reduced to a reduction of just 1.46, 2.83, 2.37% for the control, *Bacillus* sp., and consortium groups ($p > 0.05$). Plants treated with the low nutrient regime and *Pseudomonas* sp. inoculation had a slightly higher average height at time of harvest, though the difference was only by 0.18%. Interestingly, under the recommended nutrient regime the variability of plant heights within the sample set was considerably higher than that under low nutrient conditions.

4.3.2 Leaf area is greater under low nutrients with PGPR inoculation

As leaves are a key indicator of overall plant health, fan leaf area was assessed at harvest to evaluate if plant development was negatively affected under low nutrient conditions. This was complemented by visually monitoring plant health over the growth period. At no point in development were there visual indications of poor plant health under this restrictive regime, with plants that had been subjected to the low nutrient regime being indistinguishable at time of harvest

from those having been consistently growing under the recommended nutrient regime (Figure 4.2). Plants subjected to low nutrient conditions, had no significant difference in leaf area at time of harvest between the non-inoculated control and plants inoculated with bacteria. Decreases by 0.71, 1.46, and 0.44% under *Bacillus* sp., *Pseudomonas* sp., and consortium treatments, respectively, were non-significant ($p > 0.05$) (Table 4.3). This demonstrates that under a low nutrient regime, PGPR are not effective in altering the effects of a partial nutrient stress in cannabis with regards to fan leaf development.

Figure 4.2: Comparison of plant development between nutrient regimes using randomly selected plants from each PGPR-treated batch. A-D show plants grown under the recommended nutrient regime and E-F show plants grown under low nutrient regime without bacterial inoculation (A,E), inoculated with *Bacillus* sp. (B,F), *Pseudomonas* sp. (C,G), and consortia inoculations (D,H). Images show plants at Week 12, just prior to harvest.

By comparison, under recommended nutrient regime the administration of PGPR resulted in differences with the non-inoculated control (Figure 3). In the presence of *Bacillus* sp. and the consortium inoculants, fan leaf area was decreased by 7.90 and 6.43%, respectively, but plants inoculated with *Pseudomonas* sp. featured an increase by 1.63%. Interestingly, across all bacterial

treatment groups, the average leaf area was greater with plants having been under low nutrient conditions than under recommended nutrient conditions. This effect was largest for the *Pseudomonas* sp. (11.6% increase) and consortium treatments (10.1% increase). Similar to plant height, leaf area was substantially more variable under recommended nutrient conditions compared to the low nutrient regime, indicating that a low nutrient stress leads to greater consistency in developmental variables.

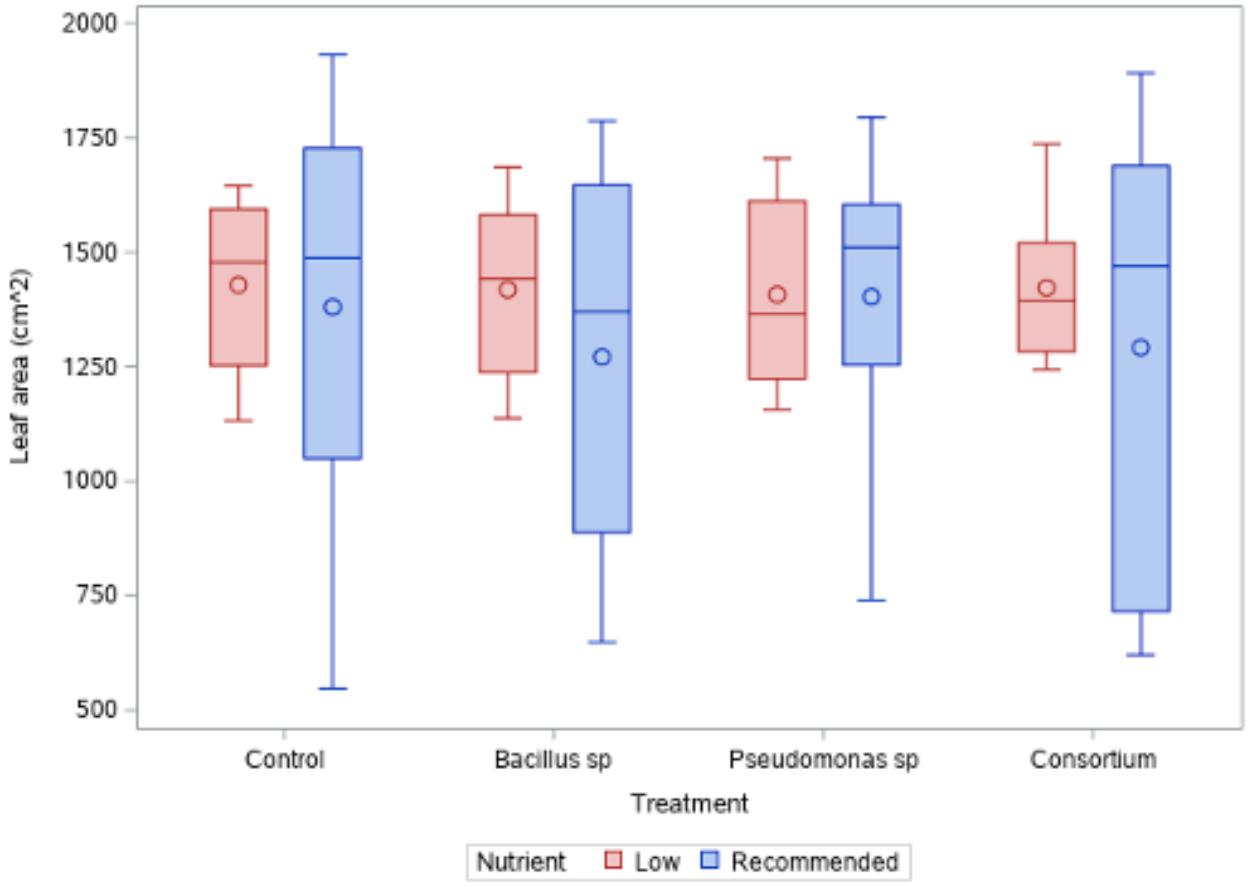

Figure 4.3: Comparison of average leaf area of non-inoculated and inoculated cannabis cv CBD Kush under two nutrient regimes. Circle indicates mean leaf area, bar indicates median leaf area, vertical bar indicates data range.

4.3.3 Recommended nutrient conditions are associated with more variation in inflorescence counts and mass than the low nutrient regime

Cannabis inflorescences are the primary yield factor in cannabis. To assess inflorescence development under both nutrient regimes, their numbers were assessed weekly. This was followed by standard harvest assessments of the final inflorescence number and the total fresh and dry masses. Notably, the number of inflorescences per plant was higher than under low nutrient conditions, though the variability between plants was greater (Figure 4.4). However, the number of inflorescences determined during weekly counts was not influenced by the application of PGPR under low or recommended nutrient regimes (Figure 4.4; week 5 not shown due to inflorescences not developing until Week 6, Week 12 not shown as final harvest counts occur at this time). Furthermore, for plants under the low nutrient regime the final inflorescence count did not increase much between Week 11 and harvest at Week 12, with the number of inflorescences at Week 11 being only 6.49, 4.24, 3.86, and 4.67% below the final inflorescence counts for control, *Bacillus* sp., *Pseudomonas* sp., and consortium treatments, respectively. Meanwhile for plants under recommended nutrient conditions, the same comparison resulted in reduction of 8.91, 9.7, 10.9, and 11.7% for the same treatments, respectively. The difference between Week 11 and final harvest counts is due to cannabis inflorescences developing in stacks, so that by the time of harvest the larger inflorescence "clumps" have often separated into smaller individual inflorescences only identified during harvest, leading to slightly higher final counts.

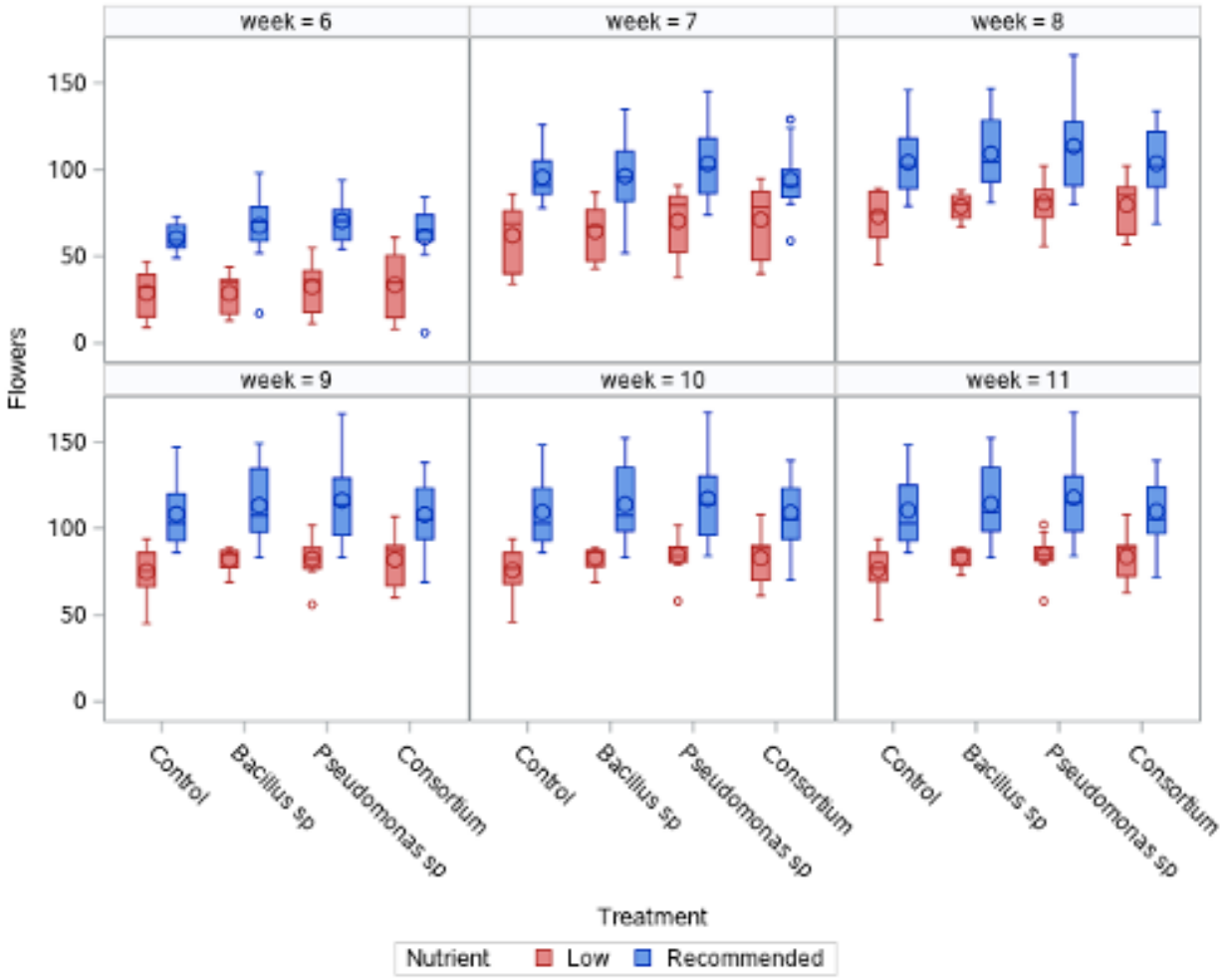

Figure 4.4: Comparison of average counts of inoculated cannabis cv CBD Kush under two nutrient regimes across flowering development. Circle indicates mean height, bar indicates median height, vertical bars indicate data range. Week 5 not shown. Final flower count is higher than Week 11 values due to the exposure of individual inflorescences when clusters are broken down.

For variables collected at harvest, namely the final inflorescence counts and their fresh and dry masses, the PGPR treatments did not lead to consistent improvements against the control. However, under low nutrient conditions, treatment with *Bacillus* sp. resulted in the greatest differences in average fresh and dry mass (g) per plant compared to the absence of PGPR, with increases by 1.86 and 17.46%, respectively. While *Bacillus* sp. treatment resulted in a slightly lower final inflorescence count at harvest than the other two PGPR treatments, the dry mass of

individual inflorescences was higher with 0.170 g inflorescence^{-1}, compared to 0.154 g inflorescence^{-1} for the control treatment (Table 1). While *Pseudomonas* sp. resulted in a higher inflorescence count than the non-inoculated control, the respective combined fresh and dry mass of these was not significantly altered, suggesting that individual inflorescences were smaller. The average dry mass of a single inflorescence was 0.142 g in plants treated with *Pseudomonas* sp. vs 0.154 g in the non-inoculated control. The consortium treatment led to higher inflorescence number per plant compared to the control, but the respective combined fresh and dry mass of all inflorescences per plant was decreased by 4.06 and 1.59%, respectively, with an average dry weight of 0.141 g inflorescence^{-1}.

Table 4.1: Average inflorescence count and combined mass (g) of non-inoculated and inoculated cannabis cv. CBD Kush under two nutrient regimes.

	Control	***Bacillus* sp.**	***Pseudomonas* sp.**	**Consortium**
Low Nutrients				
Final count	81.7 ± 13.1	87.3 ± 8.64	88.1 ± 11.3	87.7 ± 12.7
Fresh mass (g)	59.2 ± 8.14	60.3 ± 7.58	59.6 ± 6.73	56.8 ± 6.60
Dry mass (g)	12.6 ± 2.09	14.8 ± 4.54	12.5 ± 1.35	12.4 ± 1.77
Optimal Nutrients				
Final Count	121.2 ± 18.6	126.3 ± 24.4	132.2 ± 28.1	124.4 ± 24.8
Fresh mass (g)	70.5 ± 13.4	74.8 ± 17.8	73.0 ± 15.1	70.5 ± 13.1
Dry mass (g)	14.9 ± 2.90	15.8 ± 3.59	15.38 ± 3.26	15.0 ± 2.95

Under the recommended nutrient regime, all the final inflorescence counts per plant and their fresh and dry weights were higher for all inoculated and non-inoculated samples compared to the low nutrient equivalents. Dry mass of all inflorescences combined per plant was 15.4, 6.33, 18.8, and 17.3% higher under recommended nutrients compared to low nutrient regimes for control, *Bacillus* sp., *Pseudomonas* sp., and consortium treatments, respectively (Table 4.1). Interestingly however, due to the high inflorescence counts for the recommended nutrient groups, the recommended nutrient regime resulted in an average dry weight per individual inflorescence of just 0.123, 0.125, 0.116, 0.120 g for the control, *Bacillus* sp., *Pseudomonas* sp., and consortium treatments, respectively. This indicates that while there is a higher total inflorescence mass and

number under a recommended nutrient regime, the individual inflorescences were smaller on average. Thus, under low nutrient conditions, the plants appear to allocate resources to produce fewer but bigger inflorescences, with *Bacillus* sp. able to maintain the closest to recommended conditions with regards to overall dry mass. Alternatively, this could be due to reduced inflorescence splitting; more studies on the architecture of cannabis inflorescences and how they are determined during development are needed in order to deduce the true cause.

4.4 Discussion

With cannabis now being a viable plant for study of modern agricultural methods under research settings, we aimed to determine if the application of PGPR inoculants would allow cannabis to tolerate environmental stressors without sacrificing yield. The ultimate aim was to validate a production method that would require fewer soil nutrient inputs. This study has demonstrated that restricting nutrients until the start of Week 6 does not negatively impact drug-type cannabis plant development and leads to bigger inflorescences. In addition, there was generally reduced variance in developmental variables under this partial low nutrient regime, compared to the continuous application of recommended conditions, leading to more reproducible, consistent results. In a surprising twist, it was revealed that the non-inoculated control treatments of the low nutrient regime adapted better to the stressor than those with PGPR inoculants, suggesting that general plant development does not benefit from these bacterial additives. However, across the treatments, the presence of *Bacillus* sp. in the low nutrient regime led to inflorescence dry mass to be most similar to dry masses produced under recommended nutrient conditions and the biggest inflorescences. This, coupled with previous work (Tanney et al., unpublished) that demonstrated inoculation with *Bacillus* sp. under low nutrients is consistently related to the most significant differences in cannabinoid concentration compared to recommended

nutrients, such as increasing in the concentration of tetrahydrocannabinolic acid (THCA) by 20.5%, indicates that the loss of inflorescence dry mass could potentially be recovered in the cannabinoid concentrations. This is particularly useful information for producers aiming to create oil-based products, as they will be able to isolate higher amounts of cannabinoids of interest with less processing material, thus saving time and resources.

The results of this study add to the scarce science-based knowledge on cannabis development. Caplan et al. (2017) previously investigated the link between higher and lower substrate container capacity coupled with varying rates of liquid fertilizer application, indicating that a lower container capacity leads to higher inflorescence dry mass, THC yields, and growth index, and an increase in fertilizer rate led to higher development rates and yields to the detriment of THC, THCA, and CBGA concentrations. Similarly, in their high container capacity trials, lower rates of fertilizer applications increased their cannabinoid concentration at the expense of inflorescence yield (Caplan et al., 2017). Meanwhile, the findings in the present study complement Lyu et al. (2022) who investigated cannabis development on the same cultivar and PGPR species. While inflorescence yields are dissimilar between the two studies, likely due to the use of different substrates, Lyu et al. (2022) observed no effect of PGPR administration on height and leaf area measurements. As Lyu et al. (2022) used soil with compost as substrate while the present study used coco, the consistency in plant development but inconsistency in inflorescence yield likely indicate that the substrate used for potted cannabis has a more pronounced impact than expected on the effects of PGPR with regards to yield. Thus, while these studies lead to the recommendation that nutrient restriction can be used to obtain larger inflorescences, the choice to implement PGPR inoculations depends on the selected potting material with regards to inflorescence yield.

By determining that overall plant health is not negatively affected by a low nutrient regime and that it provides a benefit in terms of leaf area and inflorescence densities, we have established that the risk of plant loss due to nutrient deprivation is minimal. Even in absence of PGPR, the control treatment for the low nutrient regime maintained developmental robustness and there were no visual indicators of nutrient deficiency. Overall, this study provides validation for the application of an environmental stressor in the form of nutrient restriction at early stages of cannabis development, allowing for minimization of chemical inputs for cannabis production.

4.5 References

Ahmed, B., and Hijri, M. (2021). Potential impacts of soil microbiota manipulation on secondary metabolites production in cannabis. *J. Cannabis Res.* 3, 1–9. doi:10.1186/s42238-021-00082-0.

Backer, R., Rokem, J. S., Ilangumaran, G., Lamont, J., Praslickova, D., Ricci, E., et al. (2018). Plant growth-promoting rhizobacteria: Context, mechanisms of action, and roadmap to commercialization of biostimulants for sustainable agriculture. *Front. Plant Sci.* 9: 1473 doi:10.3389/fpls.2018.01473.

Banchio, E., Bogino, P. C., Zygadlo, J., and Giordano, W. (2008). Plant growth promoting rhizobacteria improve growth and essential oil yield in *Origanum majorana* L. *Biochem. Syst. Ecol.* 36, 766–771. doi:10.1016/j.bse.2008.08.006.

Berendsen, R. L., Pieterse, C. M. J., and Bakker, P. A. H. M. (2012). The rhizosphere microbiome and plant health. *Trends Plant Sci.*, 17, 478–486. doi:10.1016/j.tplants.2012.04.001.

Conant, R. T., Walsh, R. P., Walsh, M., Bell, C. W., and Wallenstein, M. D. (2017). Effects of a microbial biostimulant, Mammoth PTM, on *Cannabis sativa* bud yield. *J. Hortic.* 4: 2376-0354. doi:10.4172/2376-0354.1000191.

Copetta, A., Lingua, G., and Berta, G. (2006). Effects of three AM fungi on growth, distribution of glandular hairs, and essential oil production in *Ocimum basilicum* L. var. Genovese. *Mycorrhiza* 16, 485–494. doi:10.1007/s00572-006-0065-6.

Durairaj, J., Thankappan, B., Prabina, J., Binodh, A. K., and Rajammal, T. S. J. (2022). Rhizosphere engineering of rice with plant growth promoting rhizobacteria (PGPR) elicits crop growth and soil microcosm in Blue-R Dye contaminated soil. *Commun Soil Sci Plant Anal* 53, 2434–2446. doi: 10.1080/00103624.2022.2071929

Fan, D., Subramanian, S., and Smith, D. L. (2020). Plant endophytes promote growth and alleviate salt stress in *Arabidopsis thaliana*. *Sci. Rep.* 10: 12740. doi:10.1038/s41598-020-69713-5.

Grand View Research (2022). Legal Marijuana Market Size Worth $102.2 Billion by 2030. *San Francisco, CA.* Available at: https://www.grandviewresearch.com/press-release/global-legal-marijuana-market (Accessed 16 October 2022).

Hafez, E. M., Alsohim, A. S., Farig, M., Omara, A. E. D., Rashwan, E., and Kamara, M. M. (2019). Synergistic effect of biochar and plant growth promoting rhizobacteria on alleviation of water deficit in rice plants under salt-affected soil. *Agronomy* 9: 847. doi:10.3390/agronomy9120847.

Joshi, B., Chaudhary, A., Singh, H., and Kumar, P. A. (2020). Prospective evaluation of individual and consortia plant growth promoting rhizobacteria for drought stress amelioration in rice (*Oryza sativa* L.). *Plant Soil* 457, 225–240. doi:10.1007/s11104-020-04730-x.

Kundan, R., Pant, G., Jadon, N., and Agrawal, P. K. (2015). Plant growth promoting rhizobacteria: Mechanism and current prospective. *J. Fertil. Pestic.* 6: 9. doi:10.4172/2471-2728.1000155.

Lin, Y., Watts, D. B., Kloepper, J. W., Feng, Y., and Torbert, H. A. (2020). Influence of plant growth-promoting rhizobacteria on corn growth under drought stress. *Commun. Soil Sci. Plant Anal.* 51, 250–264. doi:10.1080/00103624.2019.1705329.

Livingston, S. J., Quilichini, T. D., Booth, J. K., Wong, D. C. J., Rensing, K. H., Laflamme-Yonkman, J., et al. (2020). Cannabis glandular trichomes alter morphology and metabolite content during flower maturation. *Plant J.* 101, 37–56. doi:10.1111/tpj.14516.

Lyu, D., Backer, R., and Smith, D. L. (2022). Three plant growth-promoting rhizobacteria alter morphological development, physiology, and flower yield of Cannabis sativa L. *Ind. Crops Prod.* 178: 114583. doi:10.1016/j.indcrop.2022.114583.

Mahlberg, P. G., and Eun, S. K. (2004). Accumulation of cannabinoids in glandular trichomes of Cannabis (Cannabaceae). *J. Ind. Hemp* 9, 15–36. doi:10.1300/J237v09n01_04.

Mishra, S. K., Khan, M. H., Misra, S., Dixit, V. K., Gupta, S., Tiwari, S., et al. (2020). Drought tolerant *Ochrobactrum* sp. inoculation performs multiple roles in maintaining the homeostasis in *Zea mays* L. subjected to deficit water stress. *Plant Physiol. Biochem.* 150, 1–14. doi:10.1016/j.plaphy.2020.02.025.

Ohlsson, A., Abou-Chaar, C. I., Agurell, S., Nilsson, I. M., Olofsson, K., and Sandberg, F. (1971). Cannabinoid constituents of male and female *Cannabis sativa*. *Bull Narc.* 23, 29–32.

Oleńska, E., Małek, W., Wójcik, M., Swiecicka, I., Thijs, S., and Vangronsveld, J. (2020). Beneficial features of plant growth-promoting rhizobacteria for improving plant growth and health in challenging conditions: A methodical review. *Sci. Total Environ.* 743: 140682. doi:10.1016/j.scitotenv.2020.140682.

Pagnani, G., Pellegrini, M., Galieni, A., D'Egidio, S., Matteucci, F., Ricci, A., et al. (2018). Plant growth-promoting rhizobacteria (PGPR) in *Cannabis sativa* 'Finola' cultivation: An alternative fertilization strategy to improve plant growth and quality characteristics. *Ind. Crops Prod.* 123, 75–83. doi:10.1016/j.indcrop.2018.06.033.

Pereira, S. I. A., Abreu, D., Moreira, H., Vega, A., and Castro, P. M. L. (2020). Plant growth-promoting rhizobacteria (PGPR) improve the growth and nutrient use efficiency in maize (Zea mays L.) under water deficit conditions. *Heliyon* 6: e05106. doi:10.1016/j.heliyon.2020.e05106.

Sivasakthi, S., Usharani, G., and Saranraj, P. (2014). Biocontrol potentiality of plant growth promoting bacteria (PGPR) - *Pseudomonas fluorescens* and *Bacillus subtilis*: A review. *African J. Agric. Res.* 9, 1265–1277. doi:10.5897/AJAR2013.7914.

Tanney, C. A. S., Lyu, D., Schwinghamer, T., Geitmann, A., Ruan, E., and Smith, D. L. S. (2023). Sub-optimal nutrient regime coupled with *Bacillus* and *Pseudomonas* sp. inoculation influences trichome density and cannabinoid profiles in drug-type *Cannabis sativa*. [Submitted for publication]. *Department of Plant Science, McGill University.*

Chapter 5 – General discussion and conclusions

The agricultural sector is vast in its array of interconnected components. This leads to a variety of points along the production chain, from germination to point of purchase, that are sinks for financial cost and sources of environmental degradation. However, these same points are areas of focus where efforts can be directed to improve efficiency and incorporate sustainability. Fertilizer application is one such area, as it is a crucial part of maintaining and improving crop yield but is also a key cause of environmental harm within agriculture. From sourcing natural resources to be formulated into final synthetic chemical plant fertilizer products, global transport costs to individual farmers, to increased water and air pollution in local ecosystems, there are many opportunities to optimize this system to reduce both financial and environmental costs.

"External costs" refers to the cost of damage and environmental impact that synthetic fertilizers impart, with investigations into these effects having been conducted over a number of decades. In 2007, von Blottnitz et al. estimated that in Europe, the 0.5 € kg_N^{-1} market price of synthetic nitrogen fertilizers results in a 0.3 € kg_N^{-1} damage cost (60% of market price) over its life cycle. This is primarily related to the climate change impacts from N_2O and CO_2 emissions during both their production and from agricultural fields; it is estimated that 5% of total climate change stems from N_2O emissions due to nitrate conversion under agricultural field conditions (von Blottnitz et al., 2007). While this study was focused on the damage costs within Europe, one can imagine the scale of costs from areas with vast amounts of land dedicated solely to agriculture, as found across North America. This was considered in a 2011 publication focused on the United States, where it was determined that the country acquires external costs between $5.7-16.9 billion USD per year, of which $4,969-16,150 million per year is in crop production alone (Tegtmeier and Duffy, 2011). Calculations for these estimates stemmed from damages to water, soil and air resources, wildlife and ecological biodiversity, and human health with regard to the control of

pathogens and pesticides (Tegtmeier and Duffy, 2011). Clearly, the cost of industrial agricultural practices extends past simple financial burdens.

PGPR applications are growing in agricultural settings, and their range of benefits continue to be explored. Coming to the forefront of novel agricultural techniques primarily in the 21st century, to sustain a growing global population, the phytomicrobiome and its constituents are being increasingly investigated for their potential to enhance crop yields in low-cost, sustainable ways. A core issue with organic and conservation farming is that they are unable to meet the yields demanded of our current population; conventional farming must identify new applications to reduce environmental impacts. As land degradation is estimated to affect 23% of terrestrial areas globally, a more widespread use of microbial inputs to retain and improve the use efficiency of water and nutrients would help slow down the rate of degradation (Stavi and Lal, 2015).

With research turning to the soil ecosystem, the holobiont concept has been formed to articulate the complex relationships between the plant-associated microbial community and the plant host. The holobiont consists of the plant host and its associated phytomicrobiome, encompassing both the commensal and parasitic microbes that work with each other and the plant itself to create a web of responses to environmental variations (Lyu et al., 2021). Within this ecosystem, the microbial diversity is largely unexplored, primarily due to the inability to culture the majority of the microbes with standard laboratory techniques; the percent estimate of microbes that can be cultured is both debateable and dependent on the locale of study (Epstein, 2013; Martiny, 2019; Steen et al., 2019). Regardless, the failure to culture and investigate so many species is likely due to their having important, interdependent roles within their community, possibly even helping shape the evolution of the holobiont itself (Zilber-Rosenberg & Rosenberg, 2008; Lyu et al., 2021). This relates to the ongoing problem of biostimulant greenhouse trials being

successful under controlled conditions but failing to obtain the same results in the field. The process of screening for growth-promoting traits under the artificial laboratory environment and testing with ideal model plants in greenhouses is unable to account for the complexity, stressors, and microbial establishment requirements of field conditions. Regrettably, the failure of these trials hinders microbial biostimulant application from becoming more widespread in agriculture (Sessitsch et al., 2019). Furthermore, introducing microbes to an environment where products for consumption are grown requires regulatory approval for human and ecological safety, which in some cases can take several years; the process is better than that for genetically modified plants or chemical inputs, but is nonetheless an additional burden (Sessitsch et al., 2019). This leads to a bottleneck on the road to widespread application of biostimulants, with major roadblocks involving farmer hesitancy, PGPR formulation and strain specificity, and improper handling at application – all this on top of the established concerns related to sometimes inconsistent performance in the field and obtaining approval for use (Tabassum et al., 2017).

Despite the complications of large-scale applications, it has nonetheless been demonstrated that soil with high organic matter content and dynamic microbial communities tend to have lower fertilizer needs than conventionally farmed soil (reviewed in Bender et al., 2016). A PGPR consortium of *Bacillus* sp. and arbuscular mycorrhiza fungi (AMF) were evaluated on tomatoes in conjunction with variable inorganic fertilizer rates (Adesemoye et al., 2009). The study observed that only 75% of the recommended fertilizer rate, coupled with the microbial inputs, was necessary to maintain development, yield, and nutrient uptake on par with the 100% recommended rate, albeit the work was conducted in greenhouse trials (Adesmoye et al., 2009). In trials across 5 different locations of mature tea fields with 3 separate soil series, inoculants of 2 PGPR species were applied along with either reduced or recommended fertilizer rates (Tennakoon et al., 2019).

Similar to the aforementioned tomato trial, the group found that they were able to produce yields comparable to those resulting from application of recommended fertilizer rates when the PGPR inoculants were applied with a third of the nitrogen and half of the phosphorus recommended rates (Tennakoon et al., 2019). This study, having been conducted across a range of fields and soil types provides confidence in continuing research into incorporating PGPR into sustainable agricultural practices. As drug-type cannabis must be grown indoors, in accordance with regulatory requirements, the application of PGPR inoculants is all the more appealing based on the success of controlled trials for other crop species, justifying swifter incorporation of sustainable production methods.

While the marginal, albeit statistically insignificant, effects of PGPR treatment on cannabis trichome formation and overall development were unexpected under recommended conditions, it nonetheless revealed that opportunities to improve cannabis development lie in alternative methodology. This work has further explored this concept, through the inclusion of a low nutrient regime for the first half of development. In this design, where the nutrients typically applied were restricted to a fifth of their recommended components, there were compelling results that demonstrated a slight tendency for increasing stalked trichome densities across PGPR treatments. Notably, the profiles of major cannabinoids were markedly increased under the low nutrient conditions, at no cost to overall plant health and development. This leads me to the conclusion that while PGPR presence is not a requirement to improve cannabis products, an environmental stressor is likely to lead to an increase in cannabinoid concentrations, particularly for the primary cannabinoids of interest: THCA and CBDA. It should be noted that this work is just the beginning; there needs to be additional investigations regarding effects of both restrictive conditions and PGPR applications on cannabis yield. This research was limited to just two strains of common

PGPR genera and only a single inoculation level was evaluated. The concentration used was determined in previous work (Lyu et al., 2022) and was found to be sufficient for root inoculations, however as there was a difference observed in the development outcomes from the two strains, manipulating their concentrations could potentially affect the variables assessed in this research. As well, there are vast numbers of unexplored potential PGPR species with their possible impacts on cannabis yield presently unknown. Much still remains to be discovered in this area for common agricultural crops, with even more remaining to be determined with cannabis. In regard to applying an environmental stressor, our focus remained on limiting all but the single nutrient provided at the initial cutting stage. However, the remaining nutrients, which are applied throughout the course of development, may indeed have their own effects on how cannabis grows and its final yields; these nutrients differ in both their components and concentrations, leaving the potential for stark contrasts between results. Additionally, while the focus of this project was on nutrient restriction, additional stressors such as drought, salt, and temperature are all extremely relevant to the current climate change impacts observed globally. This leaves the door open to further investigations looking to push the limits of cannabis yield across a range of scenarios, all in conjunction with PGPR applications, to reveal the most optimal procedures for grow operations in the ever-evolving realm of crop production.

Chapter 6 – Future directions

The ability to conduct studies on an entirely novel plant for the agricultural sector is an opportunity not often afforded in the 21st C. Through the work described in this thesis I have provided a simple methodology for determining cannabis trichome densities, and have also demonstrated how cannabinoid yields, with PGPR inoculation, are not necessarily linked to an increase in trichome development; they are instead likely linked to the environmental responses of PGPR. Given this, future research into this field could benefit from an investigation into a variety of environmental stressors to evaluate the metabolic responses they evoke at the core secretory site: the glandular trichomes. Given our current understanding of the situation, the following should be points of focus for further research:

1. Under the recommended nutrient regime demonstrated the control treatment unexpectedly performing better than the PGPR treatments. While the strains used are from two of the most common rhizobacterial genera, they are nonetheless an extremely small fraction of potentially beneficial microbial species. Therefore, I suggest further research into a wider variety of genera, and of species within the two genera used, to confirm the results found here. This would allow for a more reliable conclusion of the observations made with these experiments and would provide a deeper understanding of the limitations of the rhizosphere to affect cannabis development and metabolic profiles.
2. The more positive PGPR effects under low nutrient regime conditions suggest a variety of potential benefits. While the implementation of nutrient stress to crops is not a novel approach, the concept of environmental stressors being applied to cannabis to improve yields is. This leaves the other types of stressors – e.g., drought and salt stress – unknown factors that could also cause improvements on yield when in the presence of PGPR biofertilizers. Thus, I suggest future experiments should be undertaken to evaluate the

effects of a broader range of environmental stressors on PGPR inocula demonstrated to improve yields under recommended conditions, to determine if there is scope for further improvement.

7 Master References

Adesemoye, A. O., Torbert, H. A., and Kloepper, J. W. (2009). Plant growth-promoting rhizobacteria allow reduced application rates of chemical fertilizers. *Microb. Ecol.* 58, 1–12. doi:10.1007/s00248-009-9531-y.

Bender, S. F., Wagg, C., and van der Heijden, M. G. A. (2016). An underground revolution: biodiversity and soil ecological engineering for agricultural sustainability. *Trends Ecol. Evol.* 31, 440–452. doi:10.1016/j.tree.2016.02.016.

Epstein, S. S. (2013). The phenomenon of microbial uncultivability. *Curr. Opin. Microbiol.*, 636–642. doi:10.1016/j.mib.2013.08.003.

Eshaghi Gorgi, O., Fallah, H., Niknejad, Y., and Barari Tari, D. (2022). Effect of plant growth promoting rhizobacteria (PGPR) and mycorrhizal fungi inoculations on essential oil in *Melissa officinalis* L. under drought stress. *Biologia (Bratisl).* 77, 11–20. doi:10.1007/s11756-021-00919-2.

Kanter, D. R., Zhang, X., and Mauzerall, D. L. (2015). Reducing nitrogen pollution while decreasing farmers' costs and increasing fertilizer industry profits. *J. Environ. Qual.* 44, 325–335. doi:10.2134/jeq2014.04.0173.

Lyu, D., Backer, R., Robinson, W. G., and Smith, D. L. (2019). Plant growth-promoting rhizobacteria for cannabis production: Yield, cannabinoid profile and disease resistance. *Front. Microbiol.*, 1761. doi:10.3389/fmicb.2019.01761.

Lyu, D., Backer, R., and Smith, D. L. (2022). Three plant growth-promoting rhizobacteria alter morphological development, physiology, and flower yield of *Cannabis sativa* L. *Ind. Crops Prod.* 178 : 114583. doi:10.1016/j.indcrop.2022.114583.

Lyu, D., Msimbira, L. A., Nazari, M., Antar, M., Pagé, A., Shah, A., et al. (2021). The coevolution of plants and Microbes underpins sustainable agriculture. *Microorganisms* 9 : 1036. doi:10.3390/microorganisms9051036.

Lyu, D., Zajonc, J., Pagé, A., Tanney, C. A. S., Shah, A., Monjezi, N., et al. (2021). Plant Holobiont Theory: The Phytomicrobiome Plays a Central Role in Evolution and Success. *Microorganisms* 9: 675. doi:10.3390/microorganisms9040675.

Martiny, A. C. (2019). High proportions of bacteria are culturable across major biomes. *ISME J*, 13, 2125–2128. doi:10.1038/s41396-019-0410-3.

Mirzaie, M., Ladanmoghadam, A. R., Hakimi, L., and Danaee, E. (2020). Water stress modifies essential oil yield and composition, glandular trichomes and stomatal features of lemongrass (Cymbopogon citratus l.) inoculated with arbuscular mycorrhizal fungi. *J. Agric. Sci. Technol.* 22, 1575–1585.

Pagnani, G., Pellegrini, M., Galieni, A., D'Egidio, S., Matteucci, F., Ricci, A., et al. (2018). Plant growth-promoting rhizobacteria (PGPR) in Cannabis sativa 'Finola' cultivation: An alternative fertilization strategy to improve plant growth and quality characteristics. *Ind. Crops Prod.* 123, 75–83. doi:10.1016/j.indcrop.2018.06.033.

Sessitsch, A., Pfaffenbichler, N., and Mitter, B. (2019). Microbiome applications from lab to field: facing complexity. *Trends Plant Sci.* 24, 194–198. doi:10.1016/j.tplants.2018.12.004.

Stavi, I., and Lal, R. (2015). Achieving zero net land degradation: Challenges and opportunities. *J. Arid Environ.* 112, 44–51. doi:10.1016/j.jaridenv.2014.01.016.

Steen, A. D., Crits-Christoph, A., Carini, P., DeAngelis, K. M., Fierer, N., Lloyd, K. G., et al. (2019). High proportions of bacteria and archaea across most biomes remain uncultured. *ISME J*, 13, 3126–3130. doi:10.1038/s41396-019-0484-y.

Tabassum, B., Khan, A., Tariq, M., Ramzan, M., Iqbal Khan, M. S., Shahid, N., et al. (2017). Bottlenecks in commercialisation and future prospects of PGPR. *Appl. Soil Ecol.* 121, 102–117. doi:10.1016/j.apsoil.2017.09.030.

Tegtmeier, E. M., and Duffy, M. D. (2004). External costs of agricultural production in the United States. *Int. J. Agric. Sustain.* 2, 1–20. doi:10.1080/14735903.2004.9684563.

Tennakoon, P. L. K., Rajapaksha, R. M. C. P., and Hettiarachchi, L. S. K. (2019). Tea yield maintained in PGPR inoculated field plants despite significant reduction in fertilizer application. *Rhizosphere* 10: 100146. doi:10.1016/j.rhisph.2019.100146.

Von Blottnitz, H., Rabl, A., Boiadjiev, D., Taylor, T., and Arnold, S. (2006). Damage costs of nitrogen fertilizer in Europe and their internalization. *J. Environ. Plan. Manag.* 49, 413–433. doi:10.1080/09640560600601587.

Zilber-Rosenberg, I., and Rosenberg, E. (2008). Role of microorganisms in the evolution of animals and plants: The hologenome theory of evolution. *FEMS Microbiol. Rev.*, 723–735. doi:10.1111/j.1574-6976.2008.00123.x.