Tissue culture and genetic transformation of *Cannabis sativa* L.

by

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Abstract

Cannabis sativa L. is grown for medicinal and recreational uses in Canada. Factors affecting tissue culture response and genetic transformation using *Agrobacterium* were investigated. Variables including genotype, explant source, light intensity, explant preparation, *Agrobacterium* strain, and resistance marker selection influenced the ability to obtain transformed callus, which occurred at a maximum frequency of 65% via RT-PCR. Genetic transformation was confirmed by PCR and RT-PCR using primers to confirm the presence of the bialaphos (bar) selection marker as well as the *Arabidopsis thaliana* NPR1 gene (*AtNPR1*). *AtNPR1* was under the control of the constitutive CaMV 35S promoter. Natural infection by *Agrobacterium* to produce crown galls was observed on cannabis plants and confirmed by artificial inoculation and molecular analysis. *Cannabis* is naturally susceptible to *Agrobacterium* and in vitro transformation can lead to transgenic tissue development, providing opportunities for plant improvement through biotechnology. Further research is needed, however, to regenerate whole plants from transgenic callus.

Keywords: *Cannabis sativa*; Crown gall; *Agrobacterium*-mediated transformation; Tissue culture; Marijuana; Non-expressor of PR-1 (NPR1)

Dedication

To Jesus who loves everyone and to my parents, Brian and Karen Holmes, whose prayer, encouragement, and belief in me gave me the courage to face the many obstacles, failures, and successes of life so far.

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Table of Contents

List of Tables

List of Figures

List of Acronyms

AFK	Afghan Kush *Cannabis* strain
BLC	Black Cherry *Cannabis* strain
CBD	Pure CBD *Cannabis* strain
CPH	Copenhagen Kush *Cannabis* strain
DEB	Death Bubba *Cannabis* strain
DKW	Driver and Kuniyuki Walnut
HAP	Hash Plant *Cannabis* strain
ISH	Island Honey *Cannabis* strain
MBD	Moby Dick *Cannabis* strain
MS	Murashige and Skoog
O.D.	Optical Density
PD	Powdered Donuts *Cannabis* strain
PNK	Pink Kush *Cannabis* strain
PWE	Pennywise *Cannabis* strain
RT	Room Temperature
SWD	Sweet Durga *Cannabis* strain
WHR	White Rhino *Cannabis* strain

Chapter 1. Introduction

1.1. The biology and production of *Cannabis sativa* L.

Cannabis sativa L. is an annual flowering plant from the family *Cannabaceae* that originated in China but has been cultivated all over the world for hundreds of years (Chandra et al., 2017). *Cannabis* has been subdivided into drug-type strains (marijuana) and fiber-type strains (hemp) by governmental/law enforcement authorities (Monthony *et al*., 2021). In most countries around the world, cultivation of this plant is highly restricted, and possession of its products is often considered illegal. In 2018, the Canadian government legalized *Cannabis* for recreational use which has opened the doors for important research on this historically restricted plant to be performed as limited research was available previously. *C. sativa* is utilized for many purposes ranging from pharmaceuticals, including the cannabinoids THC and CBD (in marijuana), to strong, high quality fibers and protein from its seed (in hemp).

Cannabis is a diecious plant where male and female flowers are present on separate plants (Punja and Holmes, 2020). However, monecious varieties of *Cannabis* also exist (Razumova et al., 2016; Monthony et al., 2021). Although various cannabinoids are present in both sexes of plants, female plants are much more highly prized for their inflorescences due to their high concentrations of cannabinoids formed in trichomes. Tetrahydrocannabinol (THC) and Cannabidiol (CBD) are the most common cannabinoids found in *Cannabis*. THC is most known for its psychoactive capacity while CBD is valuable for its calming effects and has been shown to have useful properties which include anti-inflammatory, anti-nausea, and anti-anxiety capacity (Andre et al., 2016). Male plants are often discarded as reproduction between male and female plants can cause seeds to form in the female inflorescences which lower the market value of the product (Punja and Holmes, 2020).

Cannabis was legalized for recreational use across Canada in October of 2018, although it had been legalized for medicinal purposes prior to 2018. Since legalization for recreational use, the indoor area for licensed *Cannabis* production has increased from 452,896 m^2 to 1,756,642 m^2 and the amount of available dried *Cannabis* stock in licensed facilities has increased from 41,677 kg to 133,154 kg (Health Canada, 2021). In addition, the sale of *Cannabis* extracts for non-medical use increased approximately 8-

fold from 2019 to 2021 (Health Canada, 2021). With recreational *Cannabis* being legalized in Canada only recently and medical marijuana having already been legal, the demand for high quality *Cannabis* and subsequent products has increased. With the increase in demand for *Cannabis* comes the demand for a better understanding of the pathogens that affect it, reliable tissue culture protocols and the development of biotechnological methods that can improve important features in *Cannabis*.

1.2. Reported pathogens of C. sativa

There have been many previously reported pathogens of *Cannabis* (McPartland, 1996; 2003). McPartland (1996) reported around 88 species of fungal pathogens including, but not limited to, *Fusarium oxysporum* which cause damping off, *Sclerotinia sclerotiorum* which causes hemp canker, and grey mold caused by *Botrytis cinerea*. However, *B. cinerea*, *F. oxysporum*, and powdery mildew (*Golovinomyces ambrosiae*) are the most commonly encountered phytopathogens in licensed production facilities in British Columbia, Canada (Punja, 2021; Punja et al., 2019). *Botrytis cinerea* causes bud rot which results in mycelial growth on the buds and eventually the browning of female inflorescences (Punja et al., 2019). This disease is particularly devastating as it results in unsalvageable product (dried *Cannabis*) and a complete loss of revenue. *Fusarium oxysporum* is another devastating pathogen as it affects the root system and internal vascular tissue of *Cannabis* plants, causing root rot and damping off which can be particularly damaging to young plants and recently rooted cuttings undergoing propagation (Figure 1.1) (Punja et al., 2019).

Figure 1.1. Major phytopathogens of *Cannabis sativa* L. A.) Botrytis bud rot caused by *Botrytis cinerea*. Mycelium grows over the female inflorescences and causes browning and rotting of the bud compared to healthy female inflorescences. B.) Damping off of young *Cannabis* plantlets and browning of the vascular tissue in well established plants caused by *Fusarium oxysporum*. C.) Powdery mildew caused by *Golovinomyces ambrosiae* covering the inflorescence and leaves of *Cannabis*.

Managing these diseases in *Cannabis* can be challenging due to various factors. Firstly, Health Canada that oversees *Cannabis* regulation nationally has set strict chemical residue and microbial count thresholds which licensed producers must not

exceed in order for their product to be able to be taken to market. Secondly, producers are limited in the products which are available to control for various pathogens on *Cannabis* as each product must undergo rigorous regulation testing and evaluation before it is approved for use on *Cannabis* which can be a costly and time consuming endeavour (Pest Management Regulatory Agency, 2022). Lastly, little is still known about disease resistance within *Cannabis* from both a genomic and genotype point of view. There is some research, however, that has recently identified genotypes with resistance to powdery mildew and the genes involved in resistance in *C. sativa* (Mihalyov and Garfinkel, 2021; Stack et al., 2021). More research needs to be done to evaluate the extent of resistance to other pathogens, their underlying genetics, and the future of biotechnology applications for disease resistance for *Cannabis*.

1.3. Crown gall

Crown gall caused by the bacterial pathogen *Agrobacterium tumefaciens* was reported to infect *Cannabis* (McPartland 1996). Crown gall is a plant disease characterized by the presence of fleshy, tumor-like outgrowths, or “galls”, on the crown or roots of the plant. It results from infection by the plant pathogen, *Agrobacterium tumefaciens*, syn. *Agrobacterium radiobacter* (Parte et al., 2020). *Agrobacterium* species belonging to the family *Rhizobiaceae* live in the rhizoplane, the soil which is closely associated with the roots of a plant and infects the host via wound sites. It is a rod shaped, gram negative, soil-borne bacterium. Its growth is strictly aerobic, and it thrives in well aerated soils like sandy loams (Kado, 2002). These bacteria are also motile due to the presence of flagella that are arranged subpolarly and span the circumference of the cell which is known as circumthecal flagellation (Kado, 2002).

1.3.1. *Agrobacterium* taxonomy and role as plant pathogens

Since the beginning of the genomic era, the taxonomic group previously known as “*Agrobacterium tumefaciens*” has undergone significant changes based on new genomic and phylogenetic information (Mafakheri et al., 2019). Previously, *Agrobacterium* species were named based on their pathogenicity and were separated into biovars based on host infection, however, this naming system did not take into account the fact that tumor inducing plasmids, which confer pathogenicity or virulence of

a strain, can be transferred to non-pathogenic strains of *Agrobacterium* (Puławska, 2016). In addition, there exists a close genetic relationship between the genus *Agrobacterium* and the genus *Rhizobium* which had garnered some debate as to the correct taxonomic structure. Young et al. (2001) proposed that all species in the genus *Agrobacterium* be moved to the genus *Rhizobium*. In response to this proposal, Farrand et al. (2003), suggested that although *Agrobacterium rhizogenes* (biovar 2) clusters more closely to species in *Rhizobium*, there was enough evidence to support the retention of the genus name *Agrobacterium* for biovars 1 and 3, which garnered a response from Young et al. (2003) critiquing Farrand et al.'s (2003) suggestion and providing continued support for their proposal. Currently, sufficient evidence has been found to support the reclassification of *A. rhizogenes* and *A. vitis* to *Rhizobium rhizogenes* and *Allorhizobium vitis*, respectively (Mousavi et al., 2014; 2015). In addition, several species that once belonged to *Rhizobium* have now been placed in the genus *Agrobacterium* (Mousavi et al., 2015).

Just as the taxonomy of *Agrobacterium* remains unclear, the taxonomy of *Agrobacterium tumefaciens* (biovar 1) also remains to be resolved. *A. tumefaciens* is no longer considered to be a taxonomic group but is instead considered to be a complex of various different genomospecies which, until each species has been formally identified, will continue to be referred to as the *A. tumefaciens* species complex (Lindstrom and Young, 2011; Kuzmanovic et al., 2015; Puławska, 2016; Mafakheri et al., 2019). Species which have been identified from the *A. tumefaciens* species complex are as follows: *A. arsenijevicii* (Kuzmanovic et al. 2015), *A. bohemicum* (Zahradnik et al., 2018), *A. deltaense* (Yan et al., 2017a), *A. larrymoorei*, *A. nepotum*, *A. radiobacter*, *A. rosae* (Kuzmanovic et al., 2018a), *A. rubi*, *A. salinitolerans* (Yan et al., 2017b), *A. skierniewicense*, *Allorhizobium vitis* (synonym *A. vitis*), *Rhizobium rhizogenes* (Mousavi et al., 2015; Ormeno-Orrillo et al., 2015), and *Rhizobium tumorigenes* (Kuzmanovic et al., 2018b). Currently, 11 genomospecies have been identified. G2 has been named *A. pusense* (Panday et al., 2011), G4 is *A. radiobacter* (Costechareyre et al. 2010; Lindstrom and Young, 2011), G7 is *A. deltaense* (Yan et al., 2017a), G8 is *A. fabrum* (Lassalle et al., 2011), G9 is *A. salinitolerans* (Yan et al., 2017b), and G14 is *A. nepotum* (Puławska et al., 2012).

Agrobacterium vitis (renamed *Allorhizobium vitis*) and *Agrobacterium rubi* are strains of gall-forming bacteria from the family *Rhizobiaceae* which infect mostly

grapevine and specifically members of the *Rubus* genus (blackberry and raspberry), respectively. Instead of causing crown gall, *A. rhizogenes* (renamed *Rhizobium rhizogenes*) causes hairy root formation on plants due to carrying a root inducing (RI) plasmid. Some *Agrobacterium* spp. can combat other pathogenic species of *Agrobacterium* by producing an antibiotic called Agrocin-84 (Kado, 2002). Agrocin-84 mimics a certain opine which other *Agrobacterium* utilize as a food source and therefore gets taken into the cell via the same uptake pathways as opines. Once inside the cell Agrocin-84 inhibits DNA replication (Kado, 2002). *Agrobacteria* commonly infect dicotyledonous plants (dicots) whereas infection of monocotyledonous plants (monocots) is much less common (De Cleene and De Ley, 1976). *Agrobacterium* recognizes phenolic compounds, like acetosyringone, and sugars that are released into the environment when plants are wounded which help direct the bacteria cells to the wound site. These sugars and phenolics are detected by tumorigenic *Agrobacteria* like A. *radiobacter* and in turn induce virulence genes in the bacteria (Ream, 1989; Gelvin, 2000). The bacteria then travel towards the wounded plant and infect. *Agrobacteria* infect woody and herbaceous plants. Stone and pome fruit, like cherries and apples, and nut trees, including walnut and almond, are susceptible to *Agrobacterium* infection and such infection has an economic impact on these crops (Kado, 2002, 2010, 2014). Sunflower in particular is highly susceptible to infection and is one of the plant species used to determine the virulence of various *Agrobacterium* strains (Kado, 2002; Braun, 1941). Some ornamental crops like roses, Marguerite daisies, Chrysanthemum spp., and morning glory can be systemically infected by *Agrobacterium*. Therefore, galls are not limited to the crown and roots among systemically infected crops. Grapevine can also be systemically infected. Monocotyledonous plants (monocots), unlike dicots, do not produce some of the phenolic compounds that are needed to induce virulence. This lack of specific phenolics effectively protects wounded monocots from being detected by *Agrobacterium* in nature. In the lab, however, researchers have been able to create *Agrobacterium* strains which can infect monocots (Cheng, 2004). Kado (2002) reports that crown gall has also been observed on field crops such as tomatoes, beans, alfalfa, and cotton but, unlike fruit and nut trees, the incidence of crown gall on field crops has not been of economic importance. Dr. Siva Sabaratnam from the Ministry of Agriculture Canada located in Abbotsford, BC, Canada had observed an increase in the frequency of crown gall affecting blackberry and raspberry plants along the Fraser Valley in British Columbia over the last 2 years (Sabaratnam, 2019).

1.3.2. *Agrobacterium radiobacter*

The disease cycle of crown gall begins with soil infested with *Agrobacterium radiobacter* (syn. *Agrobacterium tumefaciens*). When plants that are planted in the contaminated soil become wounded via insect damage, herbivory, or farming practices, *A. radiobacter* infects at the wounding site. The *Agrobacterium* transfers a section of DNA, called the T-DNA, into the plant genome. This T-DNA codes for proteins that promote unregulated plant cell growth and opine production. Once infected, a tumor-like outgrowth of cells begins to form at the primary infection site. These galls are easily detached (Kado, 2002; Hwang et al., 2017)), and once detached they can re-infect the soil as the galls house *A. radiobacter* and serve as an inoculum source for future years.

The discovery of *Agrobacterium* spp. and the mechanisms by which they can induce tumorigenesis have been of paramount importance to scientists studying plants and pathology. Due to its genetic editing/altering abilities, *Agrobacterium* has been used to alter plant genomes for the purposes of studying the function of previously unknown genes or to improve the natural qualities of the existing plant via gene insertion and editing. *Agrobacterium radiobacter* has been used to create gene knockout lines within *Arabidopsis* (Qu and Qin, 2014). When *A. radiobacter* inserts its T-DNA into the plant host genome it does so randomly (Gelvin, 2003). Sometimes this means that the T-DNA fragment will be inserted in the middle of a gene sequence and therefore knock out the protein function of that gene. This method has been used in studies aimed at identifying the function of genes within host genomes (Qu and Qin, 2014).

Crown gall has been recorded in many countries of the world such as Canada, USA, Australia, Italy, Iran, etc. (Pulawska, 2010; Kado, 2014; Mafakheri et al., 2019). Fruit and nut trees are especially affected in nurseries where conditions are the most favorable for tumor induction (Schroth, 1971; Pulawska, 2010). Once infected, rootstocks and plants need to be uprooted and destroyed, as they cannot be cured after infection, which can result in large financial losses for nurseries and growers who are trying to grow susceptible plant species. In *Cannabis*, crown gall had been listed as a pathogen by McPartland (1996; 2003) but has not been reported since. In this research, both large and small galls were found near the crown of 2 week old cuttings and established *C. sativa* plants from 2019 to 2022 in a licensed production facility. This was investigated further and is described in more detail in Chapter 2.

1.4. Tissue culture of C. sativa

Cannabis growers desire uniform identical propagation material and plants that are disease free. The *Cannabis* industry, like many economic crop industries, needs reliable tissue culture techniques which growers can use to store and maintain their strains and create disease free plant sources. Reliable tissue culture methods are also needed to support the development of *C. sativa*-specific biotechnology. Lata et al. (2009a, 2009b, 2010, 2016) have contributed much to the development of early *C. sativa* tissue culture protocols. Lata et al. (2009a) has shown that using 5.0 µM thidiazuron instead of benzyladenine or kinetin resulted in more and higher quality shoots from nodes containing axillary buds. Lata et al. (2009b) has also regenerated *Cannabis* plants using synthetic seed technology methods. Instead of using somatic embryos which are commonly used, Lata et al. (2009b) used axillary buds encapsulated in calcium alginate beads. These studies were conducted on specific strains of Cannabis, such as strain MX-1, and MX.

Various media for callus induction in *Cannabis* have been developed using different combinations of phytohormones and explant sources. Feeney and Punja (2003) showed callus formation after 4 weeks from stem and leaf explants on Murashige and Skoog (MS) medium with Gamborg B5 vitamins supplemented with 5 mM 2,4-dichlorophenoxyacetic acid (2,4-D) and 1 mM kinetin, 3% sucrose, and 8 g/L agar. Movahedi et al. (2015) obtained optimal callus growth from cotyledon explants on MS medium supplemented with 3 mg^{-1} Tdz + 0.5 mg^{-1} IBA. Lata et al. (2010) also developed an efficient plant regeneration protocol from callus and found that optimal callus growth was observed on MS media containing 0.5 µM NAA and 1 µM Tdz. Callus development on MS media supplemented with 0.5 µM and 1 µM Tdz was also observed after 4 weeks by Holmes et al. (2021) using leaf and petiole explants taken from greenhouse conditions. However, Page et al. (2021) shows that callus growth on tissue culture media using DKW basal salts supplemented with various concentrations of 2,4-D resulted in callus with greater surface area than those grown on an equivalent media supplemented with MS basal salts using explants which were previously in tissue culture conditions.

Various endophytes and microbial contaminants of *C. sativa* have been previously reported (McPartland, 1994; Kusari et al., 2013; 2014; Scott et al., 2018; Punja et al. 2019; Collyer, 2021; Holmes et al., 2021). The presence of endophytes in

tissue explants can negatively impact the development and performance of tissue culture methods. Some specific protocols and methods for control of endophytes in *C. sativa* have been evaluated such as the addition of various antibiotics or fungicides to tissue culture media and alternative sterilization methods (Collyer, 2021; Holmes et al., 2021). Out of the many reported fungal and bacterial endophytes the most common endophytes present in the *C. sativa* strains used in this research were *Penicillium*, *Fusarium*, *Pseudomonas*, and *Bacillus*. This is described in more detail in Chapter 3 of this thesis.

1.5. The potential for biotechnology to improve *C. sativa*

1.5.1. Transformation methods

In one of the earliest published reports of *C. sativa* transformation, Feeney and Punja (2003) were able to transform callus suspension cells of the hemp strain, Anka, using *Agrobacterium* strain EHA101 carrying the binary vector pNOV3635 with a gene encoding phosphomannose isomerase (PMI). They found an average transformation frequency of 31.23% for all of the transformation experiments, however, regeneration of stably transformed plants was not achieved. Recently, a method of transient transformation involving Agroinfiltration of *C. sativa* tissues has shown to be promising. Deguchi et al. (2020) used vacuum infiltration and the *Agrobacterium* strain GV3101, containing a plasmid with either the GUS or GFP reporter genes, to transform leaf, male and female flowers, stem, and root explants in the hemp strain CRS-1. GUS staining was observed in leaf discs, mature leaves, pollen sacs, anthers and sepals, pollen sac clusters, filaments, pollen grains, non-glandular trichomes, female flowers, and pistils. GFP fluorescence was observed in the hemp leaf discs, pollen sac clusters, anthers and sepals, filaments, capitate-stalked trichomes, and roots in the hemp seedlings although there was no mention of transformation frequency for tissues transformed with GUS or GFP. Deguchi et al. (2020) also showed their transformation method can be used for RNAi silencing studies in *C. sativa*. They designed a vector which included both sense and antisense strands of the CsPDS gene that encodes phytoene desaturase resulting in a hairpin RNA structure. After 4 days, they reported an albino phenotype in mature leaves, male flowers and female flowers along with a decrease in CsPDS gene expression to less than 20% compared to the control. Another transformation method

resulting in transient transformation was developed utilizing DNA bound to nano-particles (Ahmed et al., 2021). Two different soybean genes tagged with GFP were bound to nano-particles then a syringe of the nan-particle suspension was used to infiltrate leaves of 1 month old *C. sativa* plants of the low-THC strain, Tygra. The genes chosen were GmMYB29A2 or GmNAC42-1 which are transcriptions factors that regulate biosynthesis of glyceollin. Researchers showed expression of the soybean genes through RT-PCR and qRT-PCR and observed GFP fluorescence in the nuclei of all *Cannabis* leaf cell types in addition to the trichomes (Ahmed et al., 2021). Stable *Agrobacterium*-mediated transformation has previously been achieved using hypocotyl tissue from hemp strain DMG278 (Zhang et al., 2021). Hypocotyl explants were co-cultivated with *Agrobacterium* strain AGL1 containing a plasmid with 1 of 5 different cDNA sequences of *C. sativa* genes involved in regulating development. Enhanced regeneration is reported along with the presence of chimeric transgenic tissue. Zhang et al. (2021) also developed a CRISPR/Cas9 system for *C. sativa* using protoplasts as explants. Galan-Avila et al. (2021) also used hypocotyl, cotyledon, and developed meristem explants of various varieties of hemp (low-THC strains) to achieve transgenic *C. sativa* plants. Researchers used *Agrobacterium* strain LBA4404 containing the plasmid pBIN19 which contained the GUS, *nptII*, and kanamycin resistance genes for transformation. They obtained transgenic plants from each of the explant types where 120 hypocotyl explants resulted in 6 transgenic plants, 2 cotyledon explants resulted in 2 transgenic explants, and 130 developed meristems resulted in 1 transgenic plant. Both chimeric and uniformly transformed tissue was observed via GUS staining (Galan-Avila et al., 2021). Although there has been promising progress in *Cannabis* biotechnology among hemp strains, the development of biotechnological tools utilizing drug-type (high-THC) strains of *C. sativa* has yet to be reported.

1.5.2. *Arabidopsis thaliana* NPR1

Systemic Acquired Resistance (SAR) is the process by which a plant increases its defense in uninfected areas based on the defense signaling pathway initiated by an infected part of the plant. When a pathogen infiltrates the host and is detected by the plant's internal defense receptors, the plant mounts a hypersensitive response (HR) which results in localized cell death and the buildup of salicylic acid (SA). The increase in defense hormones like SA signals the rest of the plant to boost defenses (Malamy et

al., 1990; Vlot et al., 2009). Non-expressor of pathogenesis-related gene 1 (NPR1) is an important protein involved in the SA-dependent pathway of SAR. NPR1 functions as the transcription activation domain of pathogenesis related (PR) genes in SAR. Upon pathogen infection, a systemic signal from the infected tissue induces NPR1 transcription in the rest of the plant. NPR1 remains in the cytosol, however, upon receipt of SA, NPR1 crosses the nuclear membrane. In the nucleus, NPR1 interacts with TGA proteins through ankyrin repeat domains (Zhang et al., 1999). The resulting protein complex initiates transcription of various PR genes involved in plant defense by binding to a region within the PR genes promoter sequence called the as-1-like region (Izawa et al., 1993). Previous research has shown that overexpression of *NPR1* and its homolog in *Arabidopsis* can confer disease resistance to different pathogens in various plants such as apple (Malnoy et al., 2007), cotton (Kumar et al., 2013), *Brassica juncea* (Ali et al., 2017), *Arabidopsis thaliana* (Cao et al., 1998), rice (Chern et al., 2001), and soybean (Matthews et al., 2014). In this research, we used *AtNPR1* for use in our transformation studies on *C. sativa* and provide the first report of development of transgenic callus from marijuana strains of *Cannabis* expressing a disease resistance gene.

1.6. Research objectives

A first objective of this research was to assess if *Agrobacterium tumefaciens* can induce galls on *C. sativa*. Two week old cuttings of drug-type *Cannabis* plants from a licensed production facility were found with small galls near the crown of the plant above the wounding site. In addition, several flowering plants were found with large galls near the crown and roots. A second objective of this research was to identify endophytes of *Cannabis* present in tissue culture and evaluate alternative tissue culture media and sterilization methods to control for contamination in tissue culture as well as evaluate differing responses to tissue culture conditions among the *Cannabis* strains used in this research. Previous studies of *Cannabis* have reported various pathogens, microbes, and endophytes which are present in and on the plant (McPartland, 1994; 1996; 2003; Kusari et al., 2013; 2014; Punja et al., 2019); however, there has been little report of how these endophytes negatively impact tissue culture (Collyer, 2021). The third objective of this research was to evaluate the potential of transformation methods for the development of transgenic *Cannabis.* Therefore, variables that influence transformation frequency were assessed. Explant type, light intensity, explant preparation, cytokinin source, selection

pressure, and *Agrobacterium* strain were assessed for their effect on transformation frequency using plasmids containing a reporter gene (bialaphos resistance) and the *NPR1* disease resistance gene from *Arabidopsis thaliana*.

Chapter 2. Crown gall development on *Cannabis sativa* L. (marijuana)

2.1. Introduction

Crown gall, caused by *Agrobacterium radiobacter* (previously *Agrobacterium tumefaciens* biovar 1, syn. *Rhizobium radiobacter*) (CABI 2021; The Uniprot Consortium 2021) induces tumor-like outgrowths or galls on the crowns, roots and stems of plant species in over 93 families (Kado, 2002). Symptoms on affected plants include stunted growth, chlorotic foliage and reduced vigor (Kado, 2002). *Agrobacterium radiobacter* and other *Agrobacterium* spp. identified from the "*Agrobacterium tumefaciens* species complex"*,* as well as related species such as *Rhizobium rhizogenes, A. vitis*, and *A. rubi*, infect mostly dicotyledonous plant species although several monocotyledonous species are also hosts (Matthysse, 2006). *Agrobacterium* spp. are Gram-negative, aerobic bacteria that thrive in well aerated soils and in the rhizosphere of plants and infect via wound sites (Kado, 2002). During infection, the wounded cells release phenolics and sugar compounds that induce transcription of *Agrobacterium* virulence proteins, followed by transfer of the T(transfer)-DNA region on the Ti (tumour-inducing) -plasmid into plant cells (Gelvin, 2000, 2003). The T-DNA region contains phytohormone genes and opine synthase genes within its left and right borders (LB and RB) (Gelvin, 2000, 2003). The phytohormone genes encode auxins and cytokinins which induce unregulated plant cell division that results in visible gall formation, while the opine synthase genes produce opines that are used as a nutrient source by the bacterium (Gelvin, 2000, 2003; Kado, 2002, 2014). Under laboratory conditions, *Agrobacterium* can be differentiated from other microbes using differential growth media, carbon utilization tests, pathogenicity tests, and other biochemical tests (Moore, et al. 2001; Suzaki, et al. 2004; Islam, et al. 2010). In addition, PCR of various housekeeping genes, such as *Elongation factor G* (*fusA*), *Recombinase A* (*recA*), *RNA polymerase sigma factor* (*rpoD*), as well as virulence genes and regions of T-DNA, are often used to identify *Agrobacterium* at the species level (Bini et al., 2008; Pulawska et al., 2016).

Nursery and greenhouse crops are particularly susceptible to infection by *A. tumefaciens* as the ambient environmental conditions are ideal for pathogen establishment (Schroth et al., 1971; Pulawska, 2010). A range of fruit and nut trees,

such as cherry, apple, walnut and almond, are affected by crown gall and disease development has a large economic impact on these hosts (Kado, 2002, 2010, 2014). Sunflower is highly susceptible to "*A. tumefaciens"* and has been used to determine the virulence of various *Agrobacterium* strains (Braun and White, 1941; Kado, 2002). Other ornamental hosts, such as roses, Marguerite daisies, chrysanthemum spp., and morning glory can be systemically infected by *Agrobacterium* (Kado, 2002). Crown gall development also occurs on field crops such as tomatoes, beans, alfalfa, and cotton but, unlike fruit and nut trees, the incidence of disease has not been of economic importance.

On *Cannabis sativa* L, which includes both hemp and drug-type *Cannabis* (marijuana), crown gall is a relatively uncommon disease reported previously to occur on hemp (McPartland, 1994; 2003). The disease has not been reported on drug-type *Cannabis* (marijuana). With the expanding cultivation of *Cannabis* in greenhouses across Canada, a range of emerging diseases have been reported to occur (Punja, 2021), of which bacterial pathogens are still relatively uncommon. In this study, we report the occurrence of symptoms resembling crown gall on the crown, roots, and stems of *Cannabis* plants originating from two licensed production facilities in British Columbia. The objectives of this research were to: (i) assess the presence of *Agrobacterium* in diseased tissues using culture-based and molecular approaches; (ii) evaluate the susceptibility of different tissues (roots, crowns, stems, petioles) of *Cannabis* to infection; and (iii) compare the susceptibility of five different genotypes (strains) of *Cannabis* to artificial inoculation. Symptoms of the disease on *Cannabis* plants are described for the first time.

2.2. Materials and methods

2.2.1. Crown gall symptoms and diseased tissue analysis

Four diseased plant samples were obtained in 2019 from two commercial *Cannabis* licenced production facilities in British Columbia (BC). One sample consisted of galls that had developed on roots of a *Cannabis* plant of genotype 'Sour Kush' grown in an indoor licenced production facility (sample A) (Figure 1 A, B). The remaining samples displayed large galls (up to 5 cm in diameter) on the crown of affected plants under greenhouse conditions at a second facility (samples B, C, D) (Figure 1 D-F). The galls in samples B and C developed on *Cannabis* genotype 'White Rhino' (Figure 1D, E)

during the flowering phase on 6 week old plants. Sample D was obtained from a plant of 'Pink Kush' at harvest time (10 weeks of age) and was not included in the analysis (Figure 1 F). In 2021, additional samples of stem cuttings of 'Pink Kush' with galls measuring 2-3 mm in diameter were observed in the propagation room of the second facility (samples E1-6) (Figure 2A). In 2022, 16 galls were found on stem cuttings of strain "Powdered Donut (PD)" and 40 galls were found on stem cuttings of strain "Black Cherry (BLC)" with sizes ranging from 3 mm to 1 cm. None of the plants showed any other symptoms of disease. Sample A (roots), sample E (stem cuttings), PD galls, and BLC galls were used for the isolation of putative *Agrobacterium* species. For sterilization, sample A was immersed in 70% EtOH for 1 min, followed by 10% bleach with 0.1% (v/v) Tween 20 for 20 min, whereas sample E, PD galls, and BLC galls, being smaller is size, were immersed in 70% EtOH for 30 s, followed by 10% bleach with 0.1% (v/v) Tween 20 for 1 min. All galls were rinsed in sterile distilled water for 1 min and then blotted dry with sterile blotting paper. For sample A, the gall was cut in half longitudinally with a sterile scalpel and tissue pieces (0.5 $cm^{2)}$ were taken from the region between the center and the outer surface of the gall and minced into small pieces using a sterile scalpel. The tissue was suspended in 3-5 mL of sterile water and serially diluted up to 10^{-5}. For each dilution, 300 µL was plated onto Petri dishes containing MacConkey agar, a medium selective for Gram-negative bacteria (Islam et al. 2010). For sample E, PD galls, and BLC galls, the entire gall was minced and added to 1 mL of sterile distilled water or 1 mL of YEB. The 16 PD galls were grouped into 2 groups then minced and the 40 BLC galls were cut in half and half of each gall was grouped into 8 groups then minced. The other halves were saved for PCR using *Agrobacterium* specific primers. Serial dilutions were not performed for samples A and E and 300 µL was plated onto MacConkey agar medium but serial dilutions to 10^{-5} were made for the PD and BLC galls. For PD galls, 50, 100, and 200 µL of the 10^{-4} and 10^{-5} dilutions were first plated onto King's B medium then replica plated onto MacConkey Agar and finally replica plated onto D1 medium (Kado and Heskett, 1970), an *Agrobacterium* selection media, along with *Pseudomonas* and *Agrobacterium* (*Rhizobium radiobacter* ATCC 15955) controls. For BLC galls, 50, 100, and 200 µL of the 10^{-5} dilution were plated onto D1 medium along with a *Pseudomonas* and *Agrobacterium* (*Rhizobium radiobacter* ATCC 15955) control. The plates were incubated at 28℃ overnight – 72 hrs depending on the growth of the single colonies. Single bacterial colonies that displayed varying shades of pink on MacConkey Agar and dark olive green on D1 medium (like the "WT" *Agrobacterium* control) were

subcultured in 1-3 mL of Luria Broth or YEB medium and shaken overnight at 200 rpm and 26℃ after which they were transferred to fresh MacConkey or Nutrient agar plates. Samples were then sent to the University of Guelph, Agriculture and Food Laboratory, for microbial 16S rDNA sequencing. For comparison, a confirmed isolate of *A. tumefaciens* containing plasmid GV3101 (obtained from Dr. Yu Xiang, Agriculture and Agri-Food Canada, Summerland, BC) was included for identification purposes.

2.2.2. Molecular analyses

Crown gall samples A, C, E (1-6), the growing substrate (cocofibre) originating from the root zone of sample C, and the 8 groups of BLC galls were used for total DNA extraction using Qiagen's DNeasy Plant Mini Kit (cat. 69104). To determine the appropriate primers to be used for PCR identification of *Agrobacterium,* a number were selected based on previous studies that used primers specific to the nuclear genome, T-DNA region and virulence genes on the Ti-plasmid of various *Agrobacterium* strains. Plasmid DNA from *Agrobacterium* strains 15955 (*Rhizobium radiobacter* ATCC 15955) and GV3101 (provided by Dr. Yu Xiang) was isolated from overnight liquid cultures of single colonies according to the manufacturer's protocol of the Monarch Plasmid Miniprep Kit (NEB cat. T1010S). The Qiagen Taq PCR Core Kit (cat. 201223) was used according to the manufacturer's directions and PCR reactions were conducted in an Applied Biosystems 2720 Thermocycler (Thermofisher). The results from these PCR reactions were analyzed by gel electrophoresis on a 1% agarose gel. The controls included DNA from wild-type *R. radiobacter* (ATCC 15955), *Agrobacterium* strain GV3101 and healthy *Cannabis* genotype 'White Rhino (WHR)'. The negative control was DNase/RNase free water. The results of the PCR were analyzed on a 1% agarose gel.

Following these initial primer analyses, two primer sets were selected: iaaH-F2/iaaH-R1 and iaaH-F10/iaaH-R10. Both are designed to amplify conserved regions in the *indole-acetic acid H* (*iaaH*) gene of *A. tumefaciens* and *A. vitis* T-DNA (Bini et al. 2008). When both primer sets were tested, primer set iaaH-F2 (ACATGCATGAGTTATCGTTTGGAAT) and iaaH-R1 (GCATCAAGGTCATCGTAAAAGTAGGT) provided more consistent results (Figure 3A). Primers iaaH-F10 and iaaH-R10 amplified bands from the *Cannabis* DNA controls (Figure 3C), yielding results that were difficult to explain. The PCR conditions for the iaaH-F2/iaaH-R1 primers were as follows: 1 cycle of 94° C for 3 min, then 40 cycles of

94° C for 30 s, 50° C for 45 s, 72° C for 1 min and a final extension of 72° C for 7 min. The positive *A. tumefaciens* (*Rhizobium radiobacter* ATCC 15955)control and DNA from healthy *Cannabis* plants were included. The PCR product was visualized on a 1% agarose gel. Bands of interest were extracted and purified using NEB's Monarch DNA Gel Extraction Kit (cat. T1020S) according to the manufacturer's instructions. The bands were sent to Eurofins Genomics for sequencing. Sequence identity was compared using NCBI's BLAST (Zhang et al. 2000; Morgulis et al. 2008).

2.2.3. Artificial inoculation studies

To assess the ability of *A. tumefaciens* to induce gall formation on *Cannabis* plants, the wild-type strain (*Rhizobium radiobacter*) ATCC 15955 was used. Unrooted cuttings of genotype 'Pink Kush' (PNK) and rooted cuttings of *Cannabis* genotypes 'Copenhagen Kush' (CPH), 'Hash Plant' (HAP), 'Moby Dick' (MBD), and 'Sweet Durga' (SWD) obtained as described by Punja et al. (2021) were used. Unrooted cuttings were inserted into rockwool or peat plugs while rooted cuttings were transferred to potting medium comprised of cocofibre and perlite (3:1, v/v) and grown under two Sunblaster brand 54-watt 6400k T5HO lights with a 24 h photoperiod. Plants were watered as needed with a solution of 1 ml/L Sensi Grow Coco pH Perfect A+B and 1 ml/L General Hydroponics Calimagic adjusted to a pH of 5.8-6.2 using Advanced Nutrients pH-Down (Scott and Punja 2020). After 2 weeks, plants were inoculated with an overnight culture of *R. radiobacter* grown in nutrient broth with an estimated $O.D._{600}$ reading of ~1.1. Four to 9 plants of CPH, HAP, MBD and SWD were inoculated at three wound sites made along the main stem (at the bottom near the crown, in the centre, and towards the top) of each plant as well as on two petioles/plant (for SWD and MBD genotypes). Unrooted cuttings of PNK were only inoculated at the base of the cuttings. The inoculation sites were first wiped with 70% ethanol and a sterile syringe was inserted into the stem. A 10 uL droplet of the inoculum was placed on the wound and a piece of Parafilm was wrapped around the inoculation site. Control plants/cuttings received 10 uL of nutrient broth on the inoculation site. The plants were grown at ambient temperature (21-25℃) under a 24 hr photoperiod for three weeks. To determine whether *Agrobacterium* could infect the roots of *Cannabis* plants, roots of SWD near the surface of the potting medium were wiped with 70% ethanol and wound-inoculated as described above. A 10 uL drop of *Agrobacterium* suspension was applied to the wound and covered with soil. Photos of

galls were taken 3 weeks post-inoculation and the circular tool in ImageJ (Schneider et al. 2012) was used to measure the area of each gall. The gall sizes at each inoculation site on the four strains were analyzed for differences using ANOVA and means separation achieved post hoc using Tukey's HSD test (P=0.05) (Table 1). Gall area was also compared between inoculation sites (bottom, middle, top) of all strains using ANOVA and means separation achieved post hoc using Tukey's HSD test (P=0.05).

Table 2.1. Comparison of crown gall development on four genotypes of *Cannabis* (*Cannabis sativa* L.) plants at 3 weeks post-inoculation at three inoculation sites.

Cannabis Genotype	Average Gall Area (mm)			
	Base of stem	Middle of stem	Top of stem	Petioles
CPH	30.06	35.55	47.86	nd
HAP	11.34	74.63	76.75	nd
MBD	12.03	84.05	85.56	52.18
SWD	24.57	63.79	71.11	47.32
ANOVA	p = 0.0651	p = 0.0733	p = 0.261	N/A

Note: Comparisons were made between each genotype for each inoculation site.

For re-isolation of *Agrobacterium* to fulfill Koch's postulates, two galls each on CPH plants inoculated at the top of the stem after 4 weeks and at 10 weeks were assayed. The galls were surface sterilized in 70% EtOH for 1 min, followed by 10% bleach (0.625% NaOCl) with 0.1% (v/v) Tween 20 for 15 min, rinsed twice in sterile double distilled water for 1 min and dried on sterile blotting paper. The outer layer of tissues surrounding the gall was removed using a sterile scalpel and the inner tissues were minced into small pieces, which were suspended in 10 mL of sterile distilled water for 15 min and then 100-300 µL was plated onto Petri dishes containing MacConkey agar. Dishes were incubated for 48-72 h at ambient room temperature (21-23℃). Single colonies that were light pink in color were transferred to Luria Broth medium and grown overnight at 26℃ with shaking at 225 rpm. A sterile inoculation loop was used to streak out the cell culture onto fresh MacConkey agar plates. Cultures were sent for microbial identification to the Agriculture and Food Laboratory, University of Guelph.

To identify other microbes aside from *Agrobacterium* that had the potential to form galls, 17 microbes previously isolated from *C. sativa* galls were grown overnight in 3 mL of nutrient broth at 28℃ with ~250 rpm shaking. Cuttings of Pink Kush (PNK) were

rooted in rockwool cubes and 6 days later were inoculated with 10 µL of one of the 17 microbes via a wound site created by a sterile needle on the stem of the cutting near the crown. Parafilm was wrapped around the inoculated wound and plantlets were incubated under a 16hr photoperiod for 3 weeks at ambient room temperature (21-23℃) under a humidity dome. Microbes that showed gall formation on the rooted cuttings were grown on nutrient agar plates and sent for microbial identification via 16S rDNA sequencing to the Agriculture and Food Laboratory, University of Guelph. This experiment was repeated a second time with the gall forming microbes that had been identified in the previous experiment (2 microbes) and 3 other isolates of the same species of microbes that had not yet been used to inoculate *C. sativa*. Each of the 5 microbes were used to inoculate two 6-day-old cuttings for a total of 10 inoculated cuttings and 1 negative control cutting inoculated with sterile nutrient broth.

2.3. Results

2.3.1. Crown gall symptoms and diseased tissue analysis

The symptoms observed on *Cannabis* plants that displayed crown and root galls are shown in Figure 2.1. On genotype ‘Sour Kush’, galls measuring 1-1.5 cm in diameter were observed on roots below the soil surface (Figure 2.1A), and when removed from the soil, the root gall (sample A) measured up to 2 cm in diameter (Figure 2.1B). Following artificial inoculations made on roots and the crown tissue at the soil line of genotype SWD using A. *tumefaciens* strain 15955 (ATCC), similar-appearing galls were formed within 4 weeks that were yellowish-white in color and 1-1.5 cm in diameter. On the crowns of plants of genotype ‘White Rhino’ (WHR), galls measuring 4-5 cm in diameter were observed (Figure 2.1 D, E) (representing samples B, C); on genotype ‘Pink Kush’ (PNK), similar-sized galls were observed (Figure 2.1 F) (sample D). On naturally infected stem cuttings of PNK, galls were creamy white in appearance and measured 2-3 mm in diameter (Figure 2.2 A, B). They all formed close to surface of the rockwool and developed at nodal sites where a leaf had been removed (Figure 2.2B).

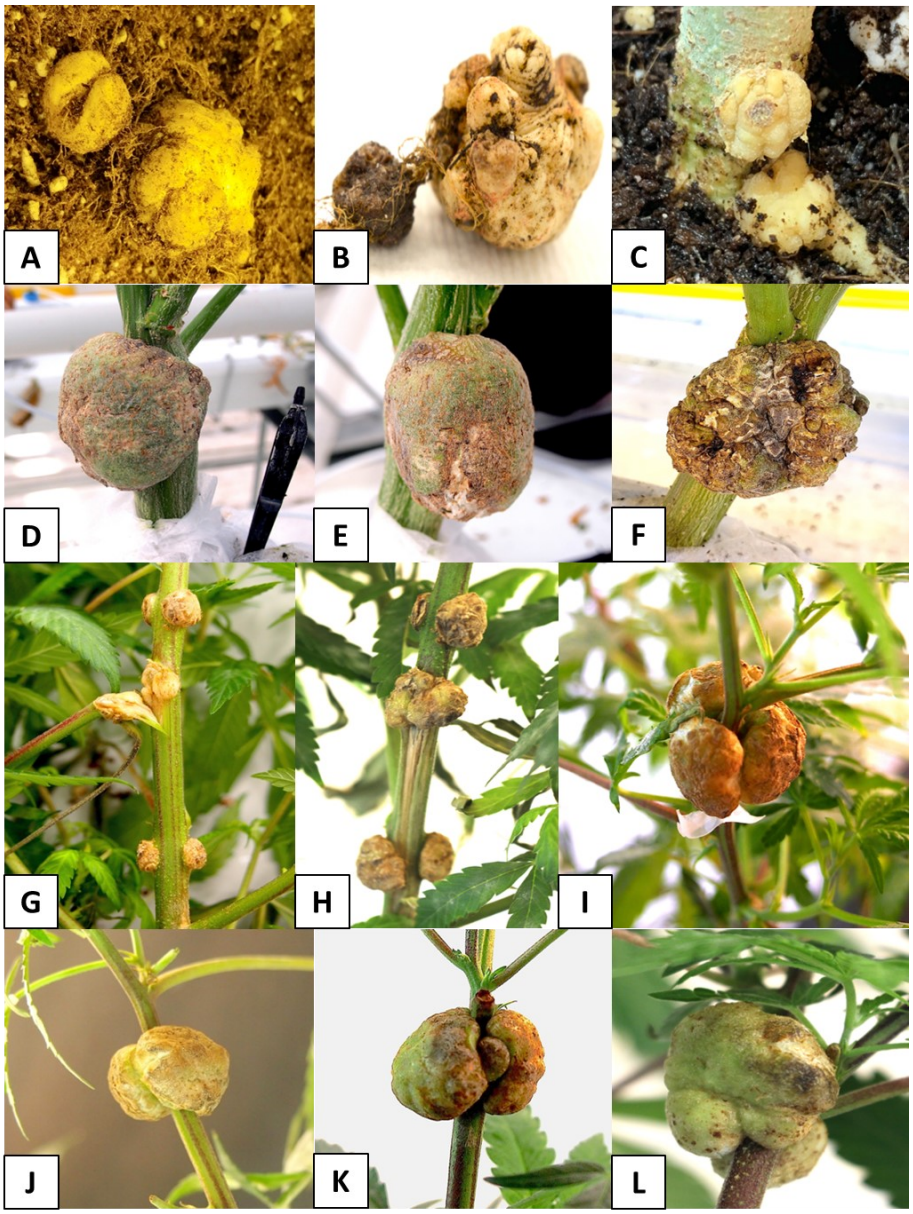


Figure 2.1. **Symptoms of crown gall caused by *Agrobacterium tumefaciens* on naturally infected and artificially inoculated *Cannabis* plants. A) Gall development on roots of genotype 'Sour Kush'. B) Close-up of gall shown in (A). C) Development of galls at the crown and roots of genotype SWD four weeks after artificial inoculation. D, E) Galls at the crown of plants of genotype WHR resulting from natural infection. F) Gall at the crown of a plant of genotype 'Pink Kush'. G) Galls on the middle stem of plants of genotype CPH following artificial inoculation. H) Same as (G) but on genotype MBD. I) Galls on the top of the stems of genotype SWD following artificial inoculation. J) Same as (I) but on genotype HAP. K) Same as (I) but on genotype CPH. Photos shown in I-L were taken 3 weeks after inoculation with A. *tumefaciens*.**

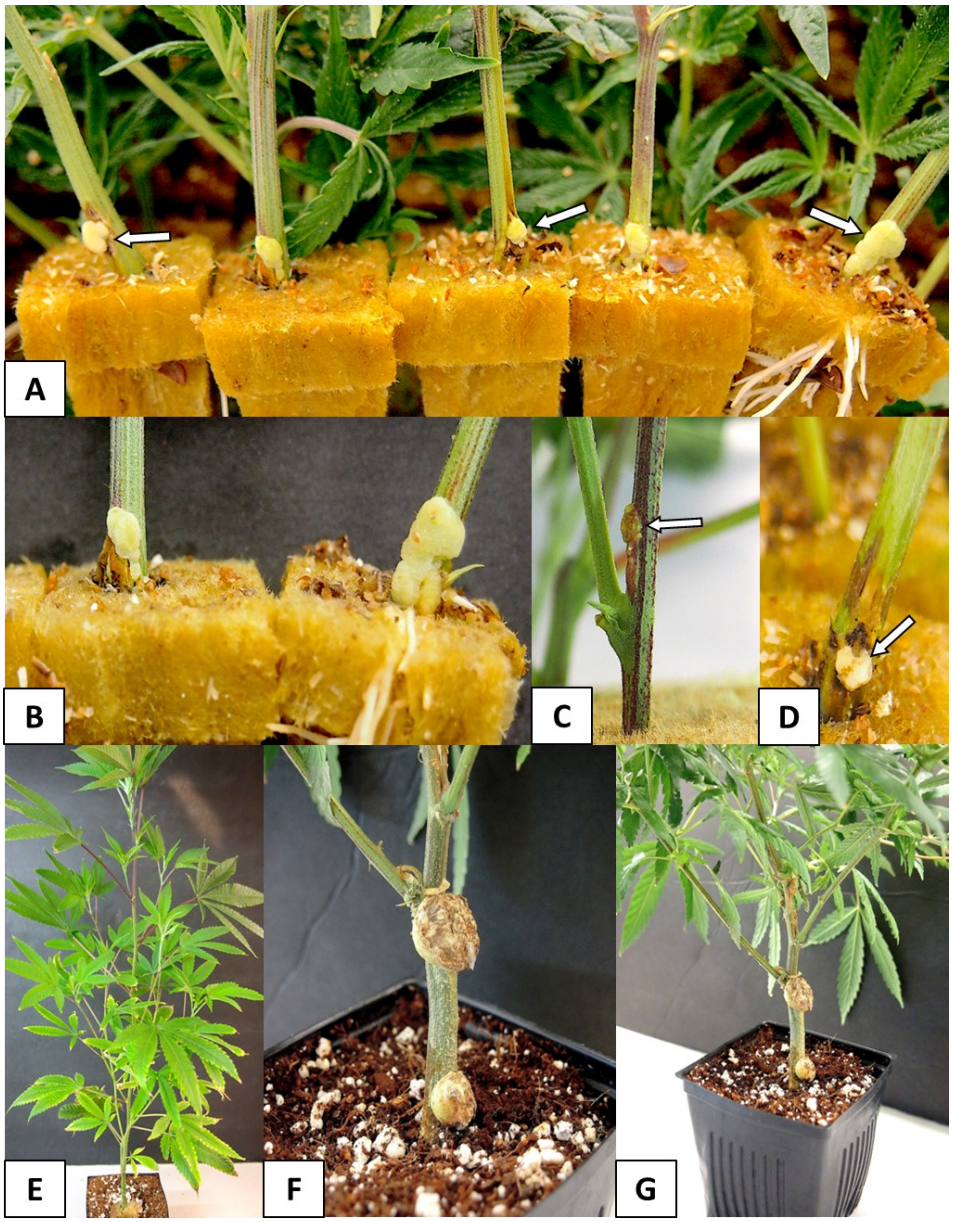


Figure 2.2. **Symptoms of crown gall caused by *Agrobacterium tumefaciens* on naturally infected and artificially inoculated *Cannabis* stem cuttings. A) Gall development at the base of five cuttings in a propagation room. B) Close-up of gall development. C, D) Galls developing following artificial inoculation with A. *tumefaciens* after 7 days (C) and 14 days (D). E) Healthy *Cannabis* plant with no obvious symptoms grown from a cutting that had developed a gall at the base of the cutting. F) Comparison of gall sizes on a *Cannabis* plant genotype CPH following artificial inoculation with A. *tumefaciens*. The gall on the middle of the stem section is larger than on the crown. G) An inoculated *Cannabis* plant shows no foliar symptoms as a result of gall development at two sites. Photo was taken 4 weeks after inoculation.**

Root gall sample A and stem cutting gall sample C were surface-sterilized and tissues were minced using a sterile scalpel and plated onto MacConkey agar to isolate *Agrobacterium*. Several putative pink colored colonies were identified following 16S rDNA sequencing as follows: *Raoultella terrigena* and either *Pseudomonas canadensis*, *P. fluorescens*, *P. simiae* or *P. poae* from sample A and *Kluyvera cryocrescens*, *Enterobacter hormaechei* or *E. cloacae*, and *Pseudomonas* or *Stenotrophomonas* from sample C. *Agrobacterium* was not recovered from either of these gall samples.

In addition, the dark olive green colonies isolated on *Agrobacterium* selection medium D1 (Kado and Heskett, 1970) from PD galls did not result in the isolation of *Agrobacterium*. The isolated microbes were identified using 16S rDNA sequencing as *Pseudomonas migulae*, *Enterobacter mori* or *E. ludwigii* or *E. roggenkampii* or *E. asburiae*, *Pseudomonas tensinigenes* or *P. umsongensis*, *Pseudomonas veronii* or *P. fildesensis* or *P. extremaustralis*, and *Pseudomonas lutea*. Microbes isolated from BLC galls were not sent for sequencing due to the PCR using iaaH-F2/iaaH-R1 primers did not return any potential *Agrobacterium* bands.

2.3.2. Molecular analyses

DNA was extracted from gall sample A (on roots), sample C (on crown), samples E (1-6) (on stem cuttings), and the 8 groups of Black Cherry (BLC) galls and PCR was conducted with primers iaaH-F2 and iaaH-R1. A band positioned between 250 bp and 500 bp was amplified from the following samples: gall sample C (Figure 3A, lane 2), *A. tumefaciens* ATCC 15955 (Figure 3A, lane 3), and galls of genotypes CPH and SWD artificially inoculated with *A. tumefaciens* 15955 (Figure 3A, lanes 5 and 7). The band in gall sample C was faint (Figure 3A, lane 2) compared to the bright bands seen in the positive *Agrobacterium* controls (Figure 3A, lanes 3, 5, and 7). No band of the expected size was obtained from root sample A (Figure 3A, lane 1) or BLC galls groups 1-8. Positive bands were obtained from tissue sample E (1-6) (Figure 3B lanes 3-14) as well as from *A. tumefaciens* ATCC 15955 (Figure 3B, lane 1), and galls of genotype CPH artificially inoculated with *A. tumefaciens* 15955 (Figure 3B, lane 2). The bands from samples C and E were extracted, purified and sequenced and aligned to produce a consensus sequence of 390 bp in size, with 99.74% similarity (and 100% query coverage) to *A. tumefaciens* accession CP033030.1 (strain A6 plasmid pTiA6), accession CP032920.1 (strain 15955 plasmid pTi15955), accession CP026926.1 (strain

1D1609 plasmid pTiD1609), accession KY000061.1 (strain CFBP2413 plasmid pTi_CFBP2413), and *A. fabacearum* accession KY000030.1 (strain CFBP5767 plasmid pTi_CFBP5767).

The sequences were analyzed using NCBI's Conserved Domain Database and domains identified as indoleacetamide hydrolase (PRK07488 super family, Accession# cl30060) were found for the bands isolated from gall sample C (Figure 3A, lane 2), stem cutting sample E (Figure 3B, lane 4) and the positive *Agrobacterium* controls (Figure 3A, lanes 3, 5, and 7). For the negative controls of *Cannabis* DNA, namely CPH and SWD, the iaaH-F2 and iaaH-R1 primers produced faint bands between 100 bp and 250 bp in size (Figure 3A, lanes 4 and 6). These bands were extracted and sequenced and the sizes of the query sequences were 205 bp (lane 4) and 251 bp (lane 6). NCBI BLAST did not show any significant sequence similarity to these bands. However, when the bands were aligned using Geneious Prime software (2021.2.2) to a draft *Cannabis* genome from strain 'Purple Kush' (canSat3, available on The *Cannabis* Genome Browser at http://genome.ccbr.utoronto.ca), they were shown to be present in the Purple Kush Genome, but their identity is unknown. No domains were identified in gall sample A (Figure 3A, lane 1) or in the *Cannabis* DNA controls (Figure 3A, lanes 4 and 6) using NCBI's Conserved Domain Database.

PCR using primers iaaH-F10 and iaaH-R10 resulted in strong amplification of two bands of sizes ~750 bp and >500 bp in *Cannabis* DNA samples (Figure 3C). These bands were also amplified in the *Agrobacterium* controls and infected gall tissues (Figure 3C, lanes 4, 5, 8, 9, 12, 13); however, the ~750 bp band was very faint compared to that in the *Cannabis* DNA samples (Figure 3C, lanes 1, 2, 6, 7, 10, 11, 14, 15). Using NCBI BLAST, the ~750 bp band in *Cannabis* DNA samples showed ~98-99% similarity to *Cannabis sativa* UBP1-associated protein 2A (Accession XM_030648587.1, XM_030648586.1, and XM_030648585.1). The >500 bp band showed ~98-99% similarity to *Cannabis sativa* early endosome antigen 1 (Accession XR_004008807.1, XM_030629599.1, XM_030629594.1, and XM_030629590.1). No domains were identified in these bands using NCBI's Conserved Domain Database. The >500 bp band in the WHR *Cannabis* DNA sample (Figure 3, lanes 6 and 7) showed chimeric identity, where 365 bp (88%) of the query sequence showed 98% identity to *Cannabis sativa* early endosome antigen 1 while 34 bp (8%) towards the 3' end of the query sequence showed 100% identity to various *Agrobacterium tumefaciens* Ti plasmids. The ~750 bp

band in *Agrobacterium* positive controls (Figure 3C, lanes 4, 5, 8, 9, 12, 13) was too faint to isolate; however, the >500 bp band showed ~98-99% identity with various *Agrobacterium tumefaciens* Ti plasmids (Accession CP033030.1, CP032920.1, and CP026926.1). The sequences were analyzed using NCBI's Conserved Domain Database and indoleacetamide hydrolase (PRK07488 super family, Accession# cl30060) was identified in the >500 bp band of the *Agrobacterium* controls only (Figure 3C, lanes 4, 5, 8, 9, 12, 13). Domains were not identified in the other *Cannabis* DNA sequences.

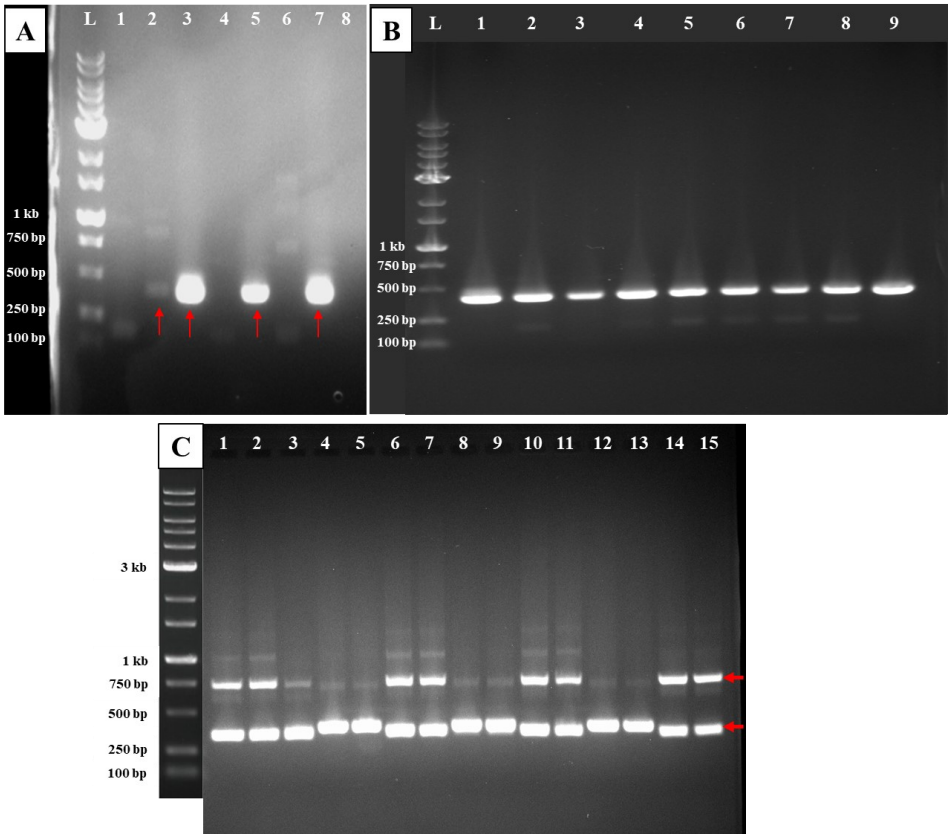


Figure 2.3. **Figure 3. PCR analysis for presence of *Agrobacterium* in *Cannabis* tissue samples using primers iaaH-F2 and iaaH-R1. A) Lane L= 1 kb Plus DNA ladder; lane 1 = root gall sample A; lane 2 = crown gall sample C; lane 3 = *R. radiobacter* (ATCC 15955) positive control; lane 4 = *Cannabis* genotype WHR negative control; lane 5 = artificially inoculated gall using *R. radiobacter* (ATCC 15955) on genotype CPH; lane 6 = *Cannabis* genotype CPH negative control; lane 7 = artificially inoculated gall using *R. radiobacter* (ATCC 15955) on genotype SWD; lane 8 = *Cannabis* genotype SWD negative control. Red arrows indicate sequences that were identified as *Agrobacterium* using NCBI BLAST. B) Lane L = 1 kb Plus DNA ladder; lane 1 = *R. radiobacter* (ATCC 15955) positive control; lane 2 = artificially inoculated gall using *R. radiobacter* (ATCC 15955) on genotype CPH; lanes 3-8 = galls on stems from 6 different cuttings of genotype PNK; lane 9 = artificially inoculated gall using *R. radiobacter* (ATCC 15955) on genotype SWD. C) PCR analysis using primers iaaH-F10 and iaaH-R10. Lanes 1 and 2 = root gall sample A; lane 3 = crown gall sample C of genotype WHR; lanes 4 and 5 = *R. radiobacter* (ATCC 15955) positive control; lanes 6 and 7 = *Cannabis* genotype WHR negative control; lanes 8 and 9 = artificially inoculated gall using *R. radiobacter* (ATCC 15955) on genotype CPH; lanes 10 and 11 = *Cannabis* genotype CPH negative control; lanes 12 and 13 = artificially inoculated gall using *R. radiobacter* (ATCC 15955) on genotype SWD; and lanes 14 and 15 = *Cannabis* genotype CPH negative control. The red arrows indicate the size of highly amplified bands in all samples.**

2.3.3. Artificial inoculation studies

The symptoms induced in *Cannabis* genotypes that were wound-inoculated with *A. tumefaciens* strain 15955 are shown in Figures 2.1 and 2.2. The galls that formed on stems of genotypes CPH and MBD at the middle inoculation site are shown in Figure 2.1 G, H. The galls that formed at the top of the stems were larger compared to those further down and can be seen for genotypes MBD (Figure 2.1 I), SWD (Figure 2.1 J), HAP (Figure 2.1 K) and CPH (Figure 2.1 L). The galls that formed on stem cuttings of genotype PNK are shown in Figure 2.2 C, D. To determine if there were any differences in crown gall development among *Cannabis* genotypes, the gall area sizes were compared 3 weeks after inoculation at each of the inoculation sites (Table 2.1). The ANOVA analysis was not significant ($p>0.05$), indicating that gall area was not affected by genotype. To assess gall development by inoculation site, gall area was compared between inoculation sites using ANOVA and post hoc Tukey's HSD test. Gall area was significantly affected by inoculation site (ANOVA, p=6.71e-06) (Figure 2.4**),** with galls at the middle and top inoculation sites being similar but significantly different from the crown (bottom) inoculation site (p=2.512e-04 and p=1.36e-05, respectively). A size comparison of the galls that developed at the crown and middle of the stem on genotype CPH can be seen in Figure 2.1 G, H. These galls appeared to have no effect on the growth of the *Cannabis* plant 8 weeks after inoculation. However, when galls developed on stems at the top of the plant, some caused girdling and death of the affected stem (unpublished observations). To confirm that these galls were the result of *Agrobacterium* infection and not wounding per se, galls were sampled from genotype CPH at 4 and 10 weeks after inoculation with *A. tumefaciens* 15955 and plated onto MacConkey medium. Colonies sent for sequencing were positively identified as *A. tumefaciens* (99% identity).

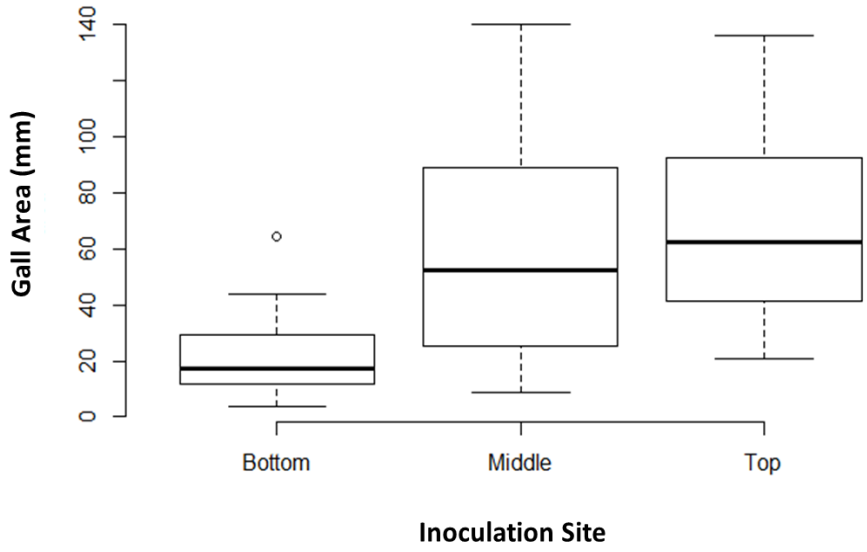


Figure 2.4. **Comparison of gall area caused by *A. tumefaciens* at various inoculation sites. Gall area was compared between each of the 3 inoculation sites (n=22) using the data from all genotypes. The box of each dataset represents the interquartile range (IQR) which contains the 3rd quartile (Q3 - top side of the box), the median value of all data (the middle line), and the first quartile (Q1 - bottom side of the box). The bars represent the maximum (Q3+1.5*IQR) and minimum (Q1-1.5*IQR) of the data. Outliers are represented by data points below or above the minimum and maximum, respectively. An ANOVA resulted in a p>0.05 and a Tukey's HSD test showed significant differences between top/bottom gall areas and middle/bottom gall areas. No significant difference was found between top/middle gall areas.**

Out of the 17 microbes that were used to inoculate *C. sativa* plants, 2 of them showed small galls forming on the stem (unpublished data). The 2 microbes were identified as *Kluyvera cryocrescens* and either *Enterobacter mori, E.cloacae, E. roggenkampii,* or *E. asburiae*. Other microbes, isolated from *C. sativa* galls, that were identified as the same species as *Kluyvera cryocrescens* (2 isolates) and either *Enterobacter mori, E.cloacae, E. roggenkampii, E. ludwigii,* or *E. asburiae* (3 isolates) were also used to inoculate 2 *C. sativa* 6 day-old rooted cuttings for each isolate. Out of the isolates only 1 of the 2 replicates of microbe PD#27 (*Enterobacter mori, E.cloacae, E. roggenkampii, E. ludwigii,* or *E. asburiae*) showed any gall formation, however, the negative control plant also displayed gall formation. Based on these results, it is likely

that these small galls are simply a callus response rather than caused by any of the isolated microbes.

2.4. Discussion

Crown gall development on plants infected by *Agrobacterium tumefaciens* (syn. *Rhizobium radiobacter*) is the result of insertion of the transfer DNA (T-DNA) on the Ti-plasmid into plant cells, where it integrates into the host genome (Chilton et al. 1977; Stachel et al. 1986; Gelvin 2000; Kado 2002; Gelvin 2003). The expression of genes encoding auxins, cytokinins and opines from the T-DNA causes plant cells to continue to proliferate without the presence of *Agrobacterium* and on tissue culture media without phytohormones (Braun and White 1941). The successful recovery of *Agrobacterium* from crown gall tissues, however, can be affected by several factors (Cubero et al. 1999; Kado 2002). Frequently, these infections harbour small numbers of bacteria inside tumours, depending on the plant species. Conventional disease diagnosis based on the isolation of *Agrobacterium* on non-selective or selective media, followed by inoculation into herbaceous hosts, such as tomato or tobacco, is generally used but can be time-consuming (Cubero et al. 1999). Therefore, PCR methods utilizing primers that amplify regions within the T-DNA are more efficient in confirming presence of *Agrobacterium* in plant galls compared to bacterial isolation on selective media or PCR targeting virulence genes on the Ti-plasmid or in the nuclear genome (Cubero et al. 1999). Results from our initial primer screening confirmed that primers targeting the T-DNA regions were better able to confirm *Agrobacterium* presence in *Cannabis* gall tissues than other primer sets tested (data not shown). From the two T-DNA primer sets that were used to test for *Agrobacterium* presence in this study, the iaaH-F2 and iaaH-R1 primers were the most reliable. Primers iaaH-F10 and iaaH-R10 produced bands in both *Agrobacterium* and *Cannabis* DNA controls. When sequenced, similarity to early endosome antigen 1 was found in *Cannabis* while in *Agrobacterium*, sequences showed indoleacetamide hydrolase conserved domains. This indicates that this primer set is not specific to *Agrobacterium* DNA as nontarget *Cannabis* DNA was amplified as well.

Agrobacterium was not recovered from the root galls (sample A) plated on MacConkey agar perhaps due to the age of the gall or the presence of competing micro-organisms in soil. Gall age and tissue condition can influence the success of recovery of *Agrobacterium* from presumed crown gall tissues (Cubero et al. 1999; Kado 2002). In

addition, the PCR result were negative for root galls and the BLC galls using the iaaH primers for *Agrobacterium* in this study. Due to their small size, these galls may be the result of callus growth from a wound site on the roots and stem (Moore and Putnam 2020). In contrast, the gall from sample C showed a weak positive band for *A. tumefaciens* T-DNA. Additionally, we confirmed presence of the *Agrobacterium* iaaH gene in galls produced on 5 young stem cuttings of PNK which displayed strong PCR bands. These bands were sequenced and showed high sequence similarity to *Agrobacterium tumefaciens* Ti plasmids in the NCBI Genbank database. *Agrobacterium* was not recovered from the PD galls or the BLC galls, however, inoculation studies using microbes isolated from the PD galls did show the ability of some of the microbes to result in gall formation. Due to the small size of the galls originally found on the PD and BLC galls it is difficult to determine whether these galls were caused by the microbe itself or simply plant callusing in response to the presence of the microbes. Successful recovery of the pathogen was demonstrated from artificially inoculated galls on MacConkey agar medium, indicating the isolation medium used initially was appropriate and could support the growth of *Agrobacterium*. We could not isolate *Agrobacterium* from sample C that was shown to be positive for the iaaH gene as that sample was utilized for molecular analysis and isolations made from sample E (stem cuttings) were unsuccessful. In the absence of a naturally occurring strain of *A. tumefaciens* from *Cannabis* plants, we selected a wild-type strain ATCC 15955 to demonstrate pathogenicity to *Cannabis* plants. We demonstrated that *Cannabis* plants are highly susceptible to *Agrobacterium* infection regardless of genotype. Gall size was larger higher up the stem of inoculated plants compared to the crown, perhaps because of greater translocation of nutrients and increased growth rate of these plant tissues. The occurrence of crown gall on *Cannabis* plants is considered to be of minimal economic importance during production given the low frequency of affected plants observed (0.01%) and the fact that affected plants did not show any foliar symptoms. However, the presence of galls has been a concern for growers as they show their product to investors (personal communication). The source of initial inoculum is unknown but was present early during propagation of stem cuttings. The crop preceding *Cannabis* in this particular licenced facility was tomato, which is known to be susceptible to *Agrobacterium* (Kado, 2002); whether this served as a potential source of the pathogen remains to be determined.

The high susceptibility of four genotypes of *Cannabis* to infection by *A. tumefaciens,* as demonstrated in this study, highlights the opportunity to conduct genetic transformation studies to introduce agronomically important genes using *Agrobacterium* as a vector, eg. to enhance disease resistance (Holmes and Punja 2021). In the first report of successful tissue transformation, Feeney and Punja (2003) transformed suspension culture cells of hemp with *A. tumefaciens* strain EHA101 containing a phosphomannose isomerase (PMI) gene. Transgenic calli displayed a pink colour when induced with the appropriate substrate. Recently, Deguchi et al. (2020) developed a method for Agroinfiltration to transiently express β-glucuronidase in flowers, leaves, stem, and roots of hemp cultivars. This approach was also used in gene silencing where the phytoene desaturase gene was silenced via transient hairpin RNA expression, resulting in an albino phenotype (Deguchi et al. 2020). Ahmed et al. (2021) developed a nanoparticle-based transient gene transformation approach for *C. sativa* in which gold nanoparticles coated with various plasmid constructs were introduced through syringe infiltration. Zhang et al. (2021) used *Agrobacterium*-mediated transformation to overexpress homologous developmental genes to promote callogenesis and shoot production in *C. sativa*. These recent studies highlight the ongoing interest in conducting transformation experiments with *Cannabis* and hemp. However, at the present time, there are no reports of the successful recovery of transgenic plants of either *Cannabis* or hemp, due in part to the difficulty of regenerating plantlets from transformed cells (Feeney and Punja 2003). Recent advances in tissue culture methods should accelerate progress towards the recovery of such transgenic plants in the future (Andre et al. 2016; Holmes et al. 2021; Page et al. 2021). In conclusion, the results from the present study demonstrate the occurrence of *A. tumefaciens* in *Cannabis* production facilities, causing gall development on crown and stem tissues but with no other apparent visible symptoms of reduced growth or vigour of affected plants.

Chapter 3. Tissue culture approaches for *Cannabis sativa* L.

This chapter has been published in Frontiers in Plant Science volume 12 under the title “Variables affecting shoot growth and plantlet recovery from tissue cultures of drug-type *Cannabis sativa* L.”

3.1. Introduction

Cannabis sativa L., a member of the *Cannabaceae* family, is a dioecious, annual flowering plant that has been cultivated for thousands of years for its fiber (as hemp) and medicinal and psychotropic properties (as *Cannabis* or marijuana). Vegetative cuttings are the conventional method for commercial propagation of *Cannabis* to ensure rapid propagation of desired genotypes without the introduction of genetic variability resulting from sexual reproduction as *C. sativa* is allogamous (Punja and Holmes, 2020). However, vegetative cuttings can lose vigor, since donor (mother or stock) plants can be affected by fungal pathogens and viruses that can reduce their growth and quality (Punja et al., 2019; Punja, 2021). In addition, maintaining donor plants to be used as a source of vegetative cuttings can be time-consuming and space intensive. Hence, there is interest in using tissue culture methods to propagate *Cannabis*, as it is recognized as a means to potentially increase plant numbers of desired genotypes (micropropagation) and maintain them in a controlled and stable environment (preservation) (Monthony et al., 2021). In addition, callus production (callogenesis) from explants in tissue culture can potentially be used to increase plant numbers through organogenesis (Page et al., 2021). However, there are still many challenges remaining in establishing a micropropagation and callusing system for *Cannabis* plants (Monthony et al., 2021). The quality of source (donor) plants, genotype or strain used, surface sterilization methods, and tissue culture medium and growth regulators can all influence the success rate of recovery of plantlets in stage 1 of tissue culture (the introduction of explant material for establishment of cultures). Rooting and acclimatization of plantlets are challenging aspects to tissue culture of hemp and *Cannabis* as well. Lata et al. (2009) reported successful propagation of strain MX-1 of *Cannabis* in tissue culture, but it is not known whether this method can be applied to other genotypes of *Cannabis*. Monthony et al. (2021) described a procedure for the micropropagation of six *Cannabis* genotypes but

rooting and plantlet recovery were not addressed. The success of a tissue culture method for *Cannabis* is contingent upon obtaining a high frequency of plantlets growing independently in growth media.

In this research, we investigated various factors that can influence the recovery of plantlets of *Cannabis* in tissue culture. The objectives of this study were to (1) assess the efficacy of sterilization methods to reduce the frequency of contaminants originating from donor plants; (2) recover and identify the various microbes present as contaminants in tissue cultures; (3) evaluate the effect of different basal salts on shoot growth from nodal explants; (4) evaluate the effect of different cytokinins on callus development; and (5) evaluate the response of 8 different genotypes to callus development to develop research methods for drug-type strains of *C. sativa* L.

3.2. Methods

3.2.1. Genotypes and Growing Conditions

The genotypes used were Copenhagen Kush (CPH), Pennywise (PWE), Death Bubba (DEB), Afghan Kush (AFK), Island Honey (ISH), Pink Kush (PNK), Pure CBD (CBD), and White Rhino (WHR). Various combinations of genotypes were used in experiments, depending on their availability. Donor plants of the various genotypes were grown by a licensed producer according to Health Canada requirements in a controlled-environment room or under commercial greenhouse conditions The growing medium for the donor plants was either pure coco fibers or a coco fiber:vermiculite mix (3:1). The plants in the controlled-environment room were placed under two Sunblaster brand 54-watt 6400 k T5HO lights with a 24-h photoperiod. The plants were watered as needed with a solution of 1 ml/L Sensi Grow Coco pH Perfect A+B (Advanced Nutrients, Los Angeles, CA, Untied States) and 1 ml/L CALiMAGIc (General Hydroponics, Santa Rosa, CA, United States) adjusted to a pH of 5.8–6.2 using pH-Down (Advanced Nutrients, Los Angeles, CA, Untied States) (Scott and Punja, 2020). The plants in the commercial greenhouse were grown according to the standards for the industry and kept under a 16h photoperiod to maintain vegetative growth. Explants were taken as needed for the tissue culture experiments described.

3.2.2. Explants

The explants tested were nodal segments and leaf tissues. Nodal segments with axillary buds were removed from lateral stems of the plants and trimmed to ~1 cm in length. Most of the external primordial leaves were removed from the axillary buds, however, a few were left intact to prevent over sterilization of the meristem. Mature leaves were trimmed from the plants and used as leaf explants in callus induction experiments. They were cut into pieces measuring 0.5 to 1 cm^2.

3.2.3. Sterilization

The standard sterilization protocol involved placing explants in a stainless steel tea strainer and immersing them in 70% EtOH in a glass beaker for 1min while stirring with a magnetic stir bar. The explants were transferred to a 10% bleach solution (0.625% NaOCl) with 0.1% Tween 20 for 10 – 20 min, followed by three rinses in sterile distilled water, 1 min each. In some of the experiments, the bleach concentration, length of sterilization, and length of rinsing were adjusted according to explant type and source. The explants were blotted dry on sterile blotting paper placed in a laminar flow hood and used immediately for tissue culture experiments.

3.2.4. Media

The medium containing Murashige and Skoog basal salts, as described by Lata et al. (2009), was used in initial tissue culture experiments. The medium was supplemented with myo-inositol (0.1 g/L) and activated charcoal (1 g/L). The growth regulators added were thidiazuron (TDZ, 1μM) and naphthaleneacetic acid (NAA, 0.5μM). This combination of ingredients constitutes a multiplication medium, referred to as MM (Table 3.1) and was altered from Lata et al. (2009) multiplication media (Holmes et el., 2021). All the chemical reagents were from Sigma-Aldrich (St. Louis, MO). All media was adjusted to pH 6.6 ± 0.01 with KOH before autoclaving for 15-20 min at 121℃ as following autoclaving, the pH dropped to ~5.8. The medium was dispensed into 220 ml culture jars C1770 with Magenta B caps (Phytotechnology Laboratories R , Lenexa, KS, United States), and each jar received ~20ml of the medium. A single meristem or nodal explant was placed inside each jar. For callus induction, MM without

activated charcoal (MM-AC) was used (Table 3.1). Each 90-mm Petri dish received ~20ml of the medium onto which 5 to 7 leaf sections were placed.

Table 3.1. Composition of various tissue culture media.

Medium Name	Medium Composition (Per 1 L)
MM	4.34 g MS Basal Salts, 20 g sucrose, 0.1 g Myo-inositol, 1 mL Gamborg B5 Vitamins (1000X), 1µM TDZ, 0.5µM NAA, 1.0 g Activated Charcoal, and 3.0 g Phytagel
MMC	4.34 g MS Basal Salts, 20 g sucrose, 0.1 g Myo-inositol, 1 mL Gamborg B5 Vitamins (1000X), and 3.0 g Phytagel
MM-AC	4.34 g MS Basal Salts, 20 g sucrose, 0.1g Myo-inositol, 1 mL Gamborg B5 Vitamins (1000X), 1µM TDZ, 0.5µM NAA, and 3.0 g Phytagel
DKW	5.22 g DKW Basal Salts, 20 g sucrose, 0.1 g Myo-inositol, 1.0 mL Gamborg B5 Vitamins (1000X), 1µM TDZ, 0.5 µM NAA, 1.0 g Activated Charcoal, and 3.0 g Phytagel
MMA1	4.34 g MS Basal Salts, 20 g sucrose, 0.1 g Myo-inositol, 1 mL Gamborg B5 Vitamins (1000X), 1µM TDZ, 0.5µM NAA, and 7g Agar powder
MMA1-BA	4.34 g MS Basal Salts, 20 g sucrose, 0.1 g Myo-inositol, 1 mL Gamborg B5 Vitamins (1000X), 1µM BA, 0.5µM NAA, and 7g Agar powder
MMA1-Kin	4.34 g MS Basal Salts, 20 g sucrose, 0.1 g Myo-inositol, 1 mL Gamborg B5 Vitamins (1000X), 1µM Kin, 0.5µM NAA, and 7g Agar powder

3.2.5. Alternative sterilization methods

Tissue culture contamination by fungi, bacteria, and yeasts was frequently observed from nodal and leaf explants derived from greenhouse plants, often ranging from 50% to 100% (100% for WHR and PNK taken from a licensed facility), which caused many of the explants to die. To assess the effect of systemic fungicides on controlling endophytic contamination, the fungicide Luna (fluopyram) was applied to donor plants of genotypes CPH and PWE at 5 ml/L as a foliar spray until run-off. Plants were grown for 3 weeks before nodal stem segments were collected. Nodal stem segments from treated and control plants were dissected and sterilized with ethanol:bleach as described previously and rinsed with sterile distilled water three times for 1 min each. The nodal stem segments were transferred to MM and grown under a 16-h photoperiod for 8 weeks, after which the proportion of jars with microbial contamination was evaluated. For the CPH strain, 44 nodes were in the Luna treatment group and 34 were in the control group. For the PWE strain, 24 nodal explants were

included for each treatment group. The data obtained from the CPH genotype met the requirements for a chi-square test, but the data from PWE did not, so Fisher's Exact test was performed. The experiment was conducted once.

Lengthy incubations in bleach which are required to control many of the endophytes in leaf and nodal explants can also cause damage to the tissue. To avoid over bleaching, Plant Preservative Mixture (PPM), a common biocide added to tissue culture media, was evaluated for its efficacy in surface sterilization of nodes. Fifteen CPH nodes were sterilized in a 5% PPM solution supplemented with 3x MS basal salts for 4.5 hrs at RT (~21-23℃) with 150rpm shaking (according to manufacturer's direction). The nodes were taken from the PPM mixture and placed directly on MM media in tissue culture jars and grown under 16hrs light and 8 hrs dark at RT (~21-23℃) for 8 weeks. Nineteen CPH nodes were sterilized according to the standard sterilization protocol (mentioned in a previous section) but were rinsed 3 times with sterile double distilled water for 1 min. The nodes were then placed on MM media in tissue culture jars and grown under 16hrs light and 8 hrs dark at RT (~21-23℃) for 8 weeks. A Fisher's Exact Test of Two Proportions was used to evaluate the two categorical variables of sterilization method (PPM or EtOH/bleach) and contamination (contaminated or uncontaminated) as the data did not meet the requirements of the Chi-squared test. PPM had previously been added to the tissue culture media, however, contamination was still present almost as frequently as before, so PPM was not utilized as an additive to the tissue culture media. Instead, it was recommended by the manufacturer of PPM to try immersing nodes in the above solution of 5% PPM for 4-12 hrs to reduce contamination.

In an effort to control internal contamination in leaf explants, the fungicide Luna (fluopyram) was applied to donor plants of genotype PNK at 5 ml/L as a foliar spray until run-off. Plants were grown for 3 weeks before leaves were collected. Leaf sections from treated and control plants were cut into 0.5-1 cm2 sections and sterilized with ethanol:bleach as described previously then rinsed with sterile distilled water three times for 1 min each. The leaf sections were transferred to MMA1 (Table 3.1) and incubated at room temperature (20-25℃) in the dark for 4 weeks, after which the proportion of leaf sections with microbial contamination was evaluated.

3.2.6. Identification of Tissue Culture Contaminants

Bacterial/yeast-like contaminants were streaked on nutrient agar and sent to the Microbial Identification Services Laboratory at the University of Guelph, Ontario for 16S rDNA sequencing. Fungal colonies representing the most common contaminants seen in tissue culture were transferred to potato dextrose agar + streptomycin (150 mg/L) for 2-4 weeks. The mycelium was harvested, and DNA was extracted using the DNeasy Plant Mini Kit (cat. No. 69104; QIAGEN, Hilden, Germany). For PCR, the internal transcribed spacer regions (ITS1 and ITS2) as well as the 5.8S gene of ribosomal rDNA were amplified using the universal eukaryotic primers UN-UP18S42 (5′-CGTAACAAGGTTTCCGTAGGTGAAC-3′) and UN-LO28S576B (5′GTTTCTTTTCCTCCGCTTATTAATATG-3′) to produce a DNA template for sequencing. PCR conditions were as follows: initial denaturation at 94C for 3min, followed by 40 cycles of denaturation at 94℃ for 30 s, annealing at 55℃ for 45 s, and extension at 72℃ for 2min, and a final extension at 72℃ for 7min, followed by 4℃ hold. PCR products were purified from the Agarose gel and sent to Eurofins Genomics (Eurofins MWG Operon LLC 2016, Louisville, KY, United States) for sequencing. The resulting sequences were compared with the corresponding ITS1-5.8SITS2 sequences from the National Center for Biotechnology Information (NCBI) GenBank database to confirm species identity using only sequence identity values above 99%.

3.2.7. Comparison of DKW and MS Salts

Two basal salts media, Driver and Kuniyuki Walnut (cat.#D190; Phytotechnology Laboratories R , Lenexa, KS, United States) and Murashige and Skoog (cat.#M5524; Sigma Aldrich, Saint Louis, MO, United States), were compared for nodal explant growth using genotypes CPH and PWE. The composition of DKW medium, as used in this study, is as follows: 5.22 g/L DKW basal salts, 20 g/L sucrose, 0.1 g/L myo-inositol, 1 ml/L Gamborg B5 vitamins, 0.5 uM NAA, 1 uM TDZ, 1 g/L activated charcoal, and 3 g/L Phytagel (Table 3.1). Explants were sterilized as described above but rinsed for 1min instead of 3min. They were transferred to either DKW or MM. Twenty jars, each containing a nodal explant, were placed under the lighting conditions described above. The initial height of the nodal explants was measured 4 days after placement on each medium to account for differences between initial explant size. The explants were transferred to the respective fresh media after 2 weeks, and 2 weeks later, the heights of

the shoot from the base where it attached to the original explant to the top were measured using ImageJ. The height differences from the start to the end of the experiment represented the shoot height for each explant. The number of leaves on nodal explants on each medium was also assessed. The experiment was conducted twice. Only data from nodal stem segments that grew were included in the statistical analysis. The shoot height data for CPH on both media were compared by an unpaired two-sample t-test. For the data on leaf numbers for CPH and the shoot length data and number of leaves data for PWE, Wilcoxon Rank Sum non-parametric tests were performed as the data did not meet the “normality” assumptions of the unpaired two-sample t-test.

3.2.8. Inducing root and shoot formation from callus

To determine which cytokinin would produce the best callus from leaf explants, 1uM of Thidiazuron (Tdz), 6-Benzylaminopurine (BA), and Kinetin (Kin) were compared in the MM-AC callus media described by Holmes et al. (2021) (Table 3.1). PNK leaf tissue was obtained from a licensed producer and kept at 4℃, wrapped in damp paper towel, for ~24 hrs then cut into ~0.5-1cm^2 sections and sterilized as indicated above. Leaf sections were then placed on callus media supplemented with either 1uM of Tdz (n=19), BA (n=20), or Kin (n=19) and 0.5uM NAA and incubated at room temperature (20-25℃) for 8 weeks. After 8 weeks, photos were taken of each plate and ImageJ was used to measure callus area and the length of the longest root for each leaf section. Data did not conform to the assumptions of the ANOVA test so a Kruskal-Wallis non-parametric test was performed with a post-hoc Dunn’s test.

An attempt to induce shoot formation from CPH callus was made. Leaf tissue from CPH (n=20) was cut into 1 cm^2 pieces and sterilized according to the protocol above. Leaf tissue was placed on MM-AC media for 8 weeks and incubated at room temperature (20-23℃) in the dark. The resulting calli was then transferred to MM-AC supplemented with 2μM Tdz and incubated in the dark at room temperature for 3 weeks. After 3 weeks plates were evaluated for shooting from callus.

It was previously observed that when leaf tissue was placed on MMC media without phytohormones (Holmes et al., 2021) slight root formation would occur due the level of endogenous phytohormones present in the leaf tissue. In order to induce more

robust and reliable root formation, CPH leaf tissue (n=30) was cut into ~1cm^2 sections, sterilized as above, and placed on MM-AC supplemented with 5μM NAA for 3 weeks at room temperature in the dark. After 3 weeks, plates were evaluated for root formation.

3.2.9. Callus growth

Genotypes AFK, CPH, DEB, ISH, PWE, PNK, CBD, and WHR were compared for their efficiency at callus development. Leaf segments from greenhouse-grown plants were sterilized in the bleach solution (0.625% NaOCl) for 15 min and rinsed 3 times for 1 min in sterile double distilled water then placed on MM-AC (Table 3.1). The dishes were wrapped in Al foil and incubated at 21–23℃ for a maximum of 8 weeks. The callus area was measured using the “freehand” tool in ImageJ from photos taken of the Petri dishes that included a ruler for scale. If the data did not meet the assumptions of parametric tests (i.e., ANOVA, unpaired two-sample t-test, etc.), then non-parametric tests were used (i.e., Welch’s ANOVA, and Wilcoxon Rank Sum test). If the data met the assumptions of the ANOVA test, then they were used followed post-hoc by Tukey’s HSD test.

3.3. Results

3.3.1. Alternative Sterilization Methods

Due to the high frequency of microbial contaminants from nodal stem explants and leaf sections when grown on tissue culture medium, the application of systemic fungicides to control internal contamination was assessed. For CPH donor plants treated with Luna fungicide, a chi-square test was significant at $p < 0.05$, which suggests that fungicide treatment of donor plants could reduce the endophytic contamination observed in tissue culture. The CPH control group showed 88.2% contamination, and the Luna treatment group showed 31.8% contamination. However, the PWE plants treated with Luna did not differ significantly from the untreated control as overall contamination rates were much lower (12.5 vs. 16.7%, Fisher’s exact test $p > 0.05$). These results indicate that genotypes may differ in terms of the extent of microbial contamination. Nodal stem explants surface sterilized with 5% PPM + 3x basal salt solution or 70% EtOH and 10% bleach +0.1% Tween 20 showed no differences in microbial contamination in genotype CPH (60.5 vs. 60%, chi-square test $p > 0.05$).

After 4 weeks on MMA1 medium, there was no contamination observed on PNK leaf sections in the control group or the Luna treatment group. There was no difference between Luna treated or control PNK leaf sections. This may indicate that explant tissue from licensed producers may be more or less internally contaminated at specific times.

3.3.2. Identification of Tissue Culture Contaminants

In general, an average of 50% of nodal stem explants (a range of 10 to 80%) and up to 100% of leaf explants (in WHR and PNK taken from a licensed producer) were found to be contaminated by microbes and therefore discarded, despite the rigorous sterilization methods used. Various species of bacterial and fungal microbes were observed from both nodal and leaf explant sources (Table 3.2).

Table 3.2. Internal contamination present in tissue culture media.

Genus of contaminating microbe	Type of microbe
Pseudomonas	Bacterial
Bacillus	Bacterial
Serratia marcescens	Bacterial
Penicillium	Fungal
Fusarium	Fungal
Moesziomyces	Fungal
Aspergillus	Fungal
Mortierella elongata	Fungal

3.3.3. Comparison of DKW and MS Salts

Shoot length and leaf numbers from nodal stem segments were evaluated for genotypes CPH and PWE after 4 weeks of growth on DKW or MM salts (Figure 3.1). For CPH, 78.4% of explants on DKW and 89.2% on MM basal salts grew and were included in a statistical comparison by an unpaired two sample t-test. For PWE, 70% of explants on DKW and 77.5% on MM grew and were included in a statistical comparison by the Wilcoxon Rank Sum test. The data showed that there was a significant difference between DKW and MS basal salts in shoot length for CPH ($p = 0.02947$; Figure 3.1A).

The average CPH shoot length on DKW and MM was 6.4 and 8.6mm, respectively. In comparing shoot length for PWE on DKW and MM, there was no significant difference (p = 0.08354). The average PWE shoot length on DKW and MS was 7.7 and 9.2mm, respectively (Figure 3.1B). For CPH leaf number, there was no difference between DKW and MM (p = 0.6882; Figure 3.1C). The average number of leaves on DKW and MM was 3.31 and 3.12, respectively. For PWE leaf number, there was a significant difference between DKW and MM (p = 0.04468; Figure 3.1D). The average number of leaves on DKW and MM was 4.1 and 5.6, respectively.

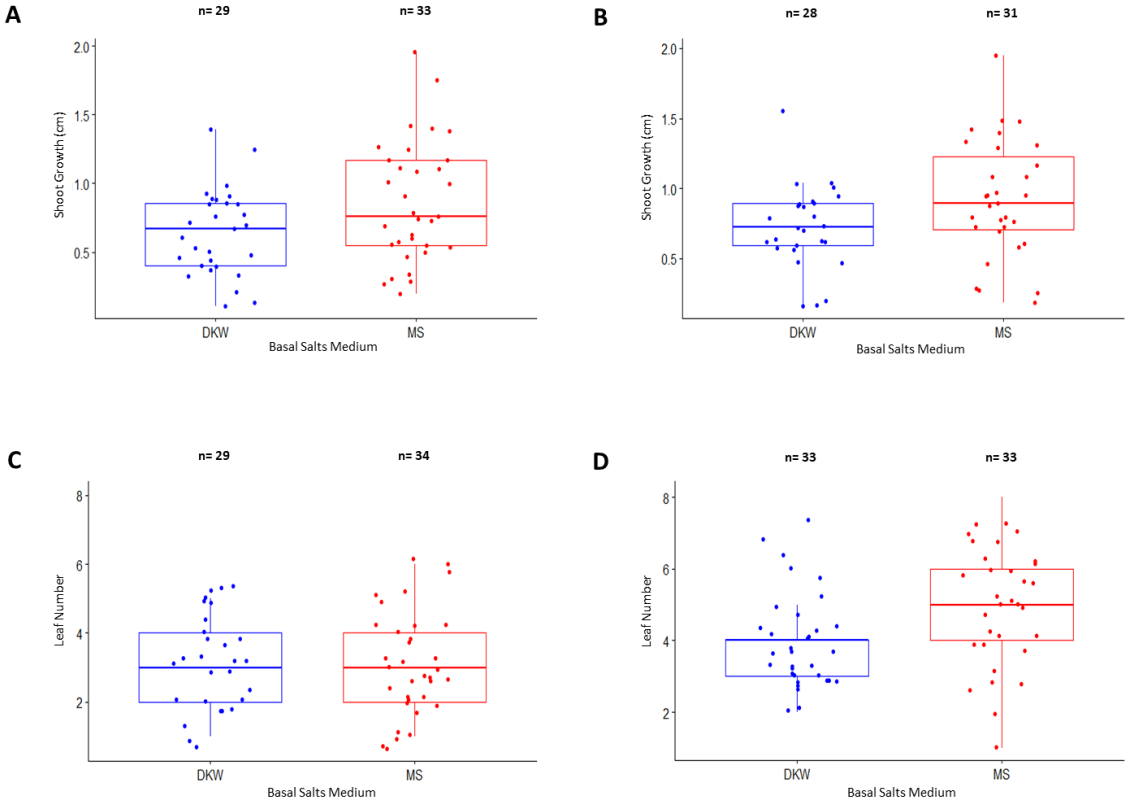
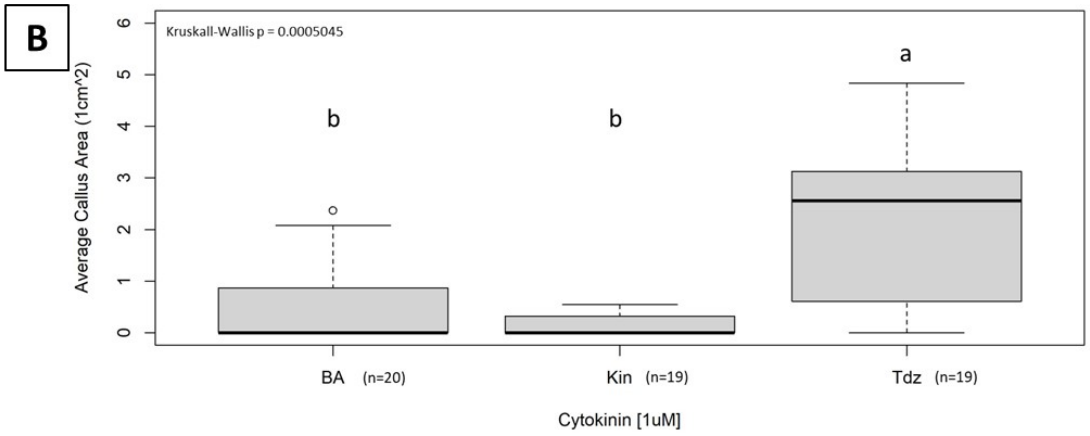


Figure 3.1. Comparison of shoot growth and leaf number from nodal stem explants of two *Cannabis* strains placed on basal salts medium containing either DKW or MS salts. (A) Shoot length of strain CPH. (B) Shoot length of strain PWE. (C) Leaf number of strain CPH. (D) Leaf numbers of strain PWE. The box of each dataset represents the interquartile range (IQR) which contains the 3rd quartile (Q3 - top side of the box), the median value of all of the data (the middle line), and the first quartile (Q1 - bottom side of the box). The bars represent the maximum (Q3+1.5*IQR) and minimum (Q1-1.5*IQR) of the data. The numbers (n) above the bars depict explant numbers used.

3.3.4. Inducing root and shoot formation from callus

After 8 weeks on tissue culture medium supplemented with different cytokinins at 1μM + 0.5μM NAA, callus formation was evaluated. Callus was expected from media with Tdz added as the cytokinin, however, the formation of roots was observed on the same medium only supplemented with BA or Kin as the source of cytokinin (Figure 3.2A). Leaf sections on media supplemented with Tdz showed predominantly callus with one leaf section developing a very small “arial” root which did not touch the media. Leaf sections on media supplemented with BA showed a similar amount of callusing and root formation whereas leaf sections on media supplemented with Kin showed predominantly root formation and little callus (Figure 3.2A). When callus area was compared between the 3 cytokinin treatments, the callus area from leaf sections on media with Tdz was significantly larger than callus produced on either media with BA or Kin (Figure 3.2B). Root length, however, was significantly larger from leaf sections on media supplemented with Kin than either media with BA or Tdz (Figure 3.2C). CPH calli cultivated on MMC supplemented with 2μM Tdz did not result in any observed shooting from 8 week old calli after 3 weeks. In addition, no rooting was observed from the CPH leaf tissue on MM-AC supplemented with 5μM NAA after 3 weeks.


A


B
Kruskall-Wallis p = 0.0005045
Average Callus Area (1cm^2)
0
1
2
3
4
5
6
b
b
a
BA (n=20)
Kin (n=19)
Tdz (n=19)
Cytokinin [1uM]

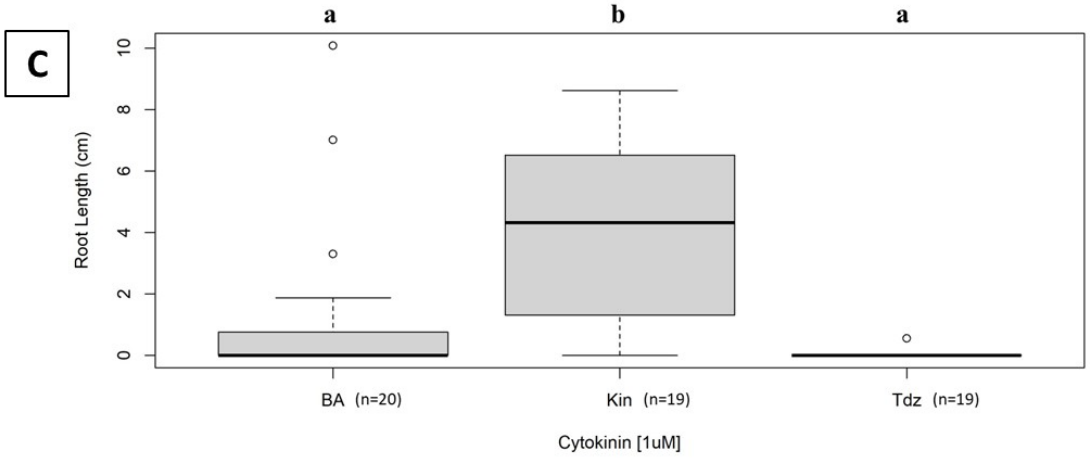

C
a
b
a
Root Length (cm)
0
2
4
6
8
10
BA (n=20)
Kin (n=19)
Tdz (n=19)
Cytokinin [1uM]

Figure 3.2. The effect of different cytokinins on callus formation. The effect of different cytokinins on callus area from leaf sections of *C. sativa*. Three different cytokinins were added to tissue culture media + 0.5µM NAA at a final concentration of 1uM. The box of each dataset represents the interquartile range (IQR) which contains the 3rd quartile (Q3 - top side of the box), the median value of all of the data (the middle line), and the first quartile (Q1 - bottom side of the box). The bars represent the maximum (Q3+1.5*IQR) and minimum (Q1-1.5*IQR) of the data. A) A side-by-side comparison of PNK leaf sections on tissue culture media containing different cytokinins after ~8 weeks. B) Callus area (cm^2) was measured using Image J software. The average area of calli from PNK leaf tissue on media containing Tdz was significantly larger than calli formed on tissue culture media containing BA or Kinetin. Whereas there was no significant difference between the average callus area of PNK leaf tissue on media containing BA and Kinetin. C) Cytokinin effect on root length. Data represents the length of the longest root (cm) for each leaf section. The freehand tool in ImageJ was used to measure root length. A Kruskal-Wallis non-parametric test and a post hoc Dunn's test were performed. A significant difference in root length was found for the comparison of Kin to BA and also for Kin to Tdz. The comparison of BA and Tdz was not significant.

3.3.5. Callus growth

Callus development from leaves, measured as callus area, was genotype-dependent (Figure 3.3). The mean callus area for the eight genotypes was compared by Welch's one-way ANOVA ($p < 0.05$). A Games–Howell non-parametric post-hoc test was then performed and identified that the following genotype comparisons were significantly different from one another: AFK vs. PNK, CPH vs. PNK, DEB vs. PNK, DEB vs. PWE, PNK vs. PWE, and PNK vs. WHR (Figure 3.3). Within 4 to 8 weeks in culture, extensive callus growth could be observed from leaf segments. None of the calli showed evidence of differentiation toward shoot development or somatic embryo development. Further transfers of calli to new media eventually resulted in browning and death.

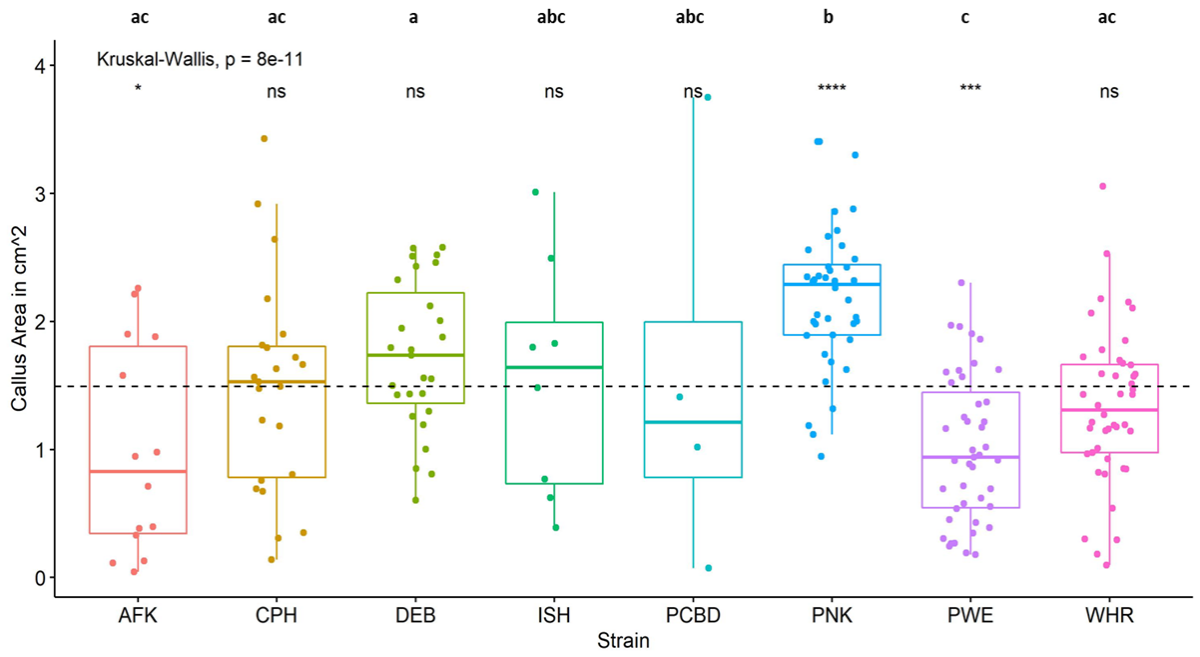


Figure 3.3. Comparison of callus area between strains. Callus area from leaf explants of 8 *Cannabis* strains compared to the mean callus area across all strains (represented by the dotted line). The '*' represents significance level and 'ns' represents not significant relationships between each strain and the across all strain mean (dotted line). A Kruskal-Wallis test resulting in a p-value of 8.0×10^{-11} and a Games-Howell post hoc test were performed. Significant differences identified in the Games-Howell post hoc test are shown using letters above the boxplots of each strain. The box of each dataset represents the interquartile range (IQR) which contains the 3rd quartile (Q3 - top side of the box), the median value of all of the data (the middle line), and the first quartile (Q1 - bottom side of the box). The bars represent the maximum (Q3+1.5*IQR) and minimum (Q1-1.5*IQR) of the data.

3.4. Discussion

The results from this study conducted on drug-type *Cannabis* (marijuana) show that the response to tissue culture conditions is influenced by the genotype of the plants tested. In addition, the results support the use of alternative surface and internal sterilization methods for tissue culture. Knowledge of these variables can enhance the successful recovery of plantlets from tissue culture and aid in the development of methods for *C. sativa*. Research on controlling contamination and variables affecting callus formation are valuable to establish the range of tissue culture responses of

Cannabis strains and to provide microbial reduction for tissue culture. Previous reports of tissue culture of *Cannabis sativa* L. have utilized hemp varieties because of legal restrictions placed on the cultivation of drug-type *Cannabis* in most regions of the world (Adhikary et al., 2021; Monthony et al., 2021). However, Lata et al. (2009a; 2009b; 2010; 2016) and Page et al. (2021) researched some of the variables that can influence the response of drug-type *C. sativa* to tissue culture conditions. In these studies, nodal segments with axillary buds were used for direct regeneration of shoots in stage 1 micropropagation (Lata et al., 2009b, 2016; Page et al., 2021). The differential responses of various genotypes to tissue culture conditions, was also noted by Monthony et al. (2021). Prior research has shown that the response to tissue culture conditions in other plant species is affected by genotype (Islam et al., 2005; Martínez et al., 2017). If the development of research methods for *Cannabis* is to be successful, an assessment of the response of genotypes of interest to tissue culture conditions would be valuable.

Since *C. sativa* L. is reported to harbor fungi and bacteria internally as endophytes (Scott et al., 2018; Punja et al., 2019), consequently, explants taken from mother plants that naturally carry endophytes or pathogens have a higher risk of becoming contaminated after transfer to tissue culture media, as observed in this study. Monthony et al. (2021) circumvented this problem by first establishing in vitro plants in stage 1 that were subsequently used to provide an explant material for studies on micropropagation and callogenesis in stage 2. While this approach is advantageous to provide clean explants and maintain desired genotypes in vitro, the explants used in the present study were derived from donor plants grown under commercial greenhouse conditions, as they provided nearly unlimited quantities of tissues, and the plants could be evaluated for commercially desired traits prior to introduction into the tissue culture environment. Callus can be easily induced from both greenhouse and in vitro explants which provides readily available plant cell culture for metabolite production and research as well as other genetic studies in *C sativa*. To avoid higher contamination rates from these tissues, a range of decontamination methods were evaluated. Nodes that had been surface-sterilized in 5% PPM for 4 hr showed no difference in contamination levels compared with nodes sterilized with 10% bleach +0.1% Tween 20. Interestingly, the application of a systemic fungicide (Luna) to the indoor-grown donor plants, followed 3 weeks later by the removal of nodal explants, showed reduced contamination levels in

tissue cultures of one strain by almost 3-fold. Contaminating microbes in *Cannabis* explants have been reported to reside within the central pith tissues (Punja et al., 2019). This could explain the difficulty in eradicating them with surface-sterilization methods.

Previous studies have demonstrated that an extensive array of fungal and bacterial endophytes can colonize hemp and *Cannabis* plants (Kusari et al., 2013; Scott et al., 2018; Punja et al., 2019). Kusari et al. (2013) found 30 different species of fungal endophytes, of which *Penicillium copticola* was the most prevalent. Scott et al. (2018) found 134 bacterial and 54 fungal strains in three hemp cultivars. The most abundant fungal genera were *Aureobasidium*, *Alternaria*, and *Cochliobolus*, and the most abundant bacterial genera were *Pseudomonas*, *Pantoea*, and *Bacillus*. Punja et al. (2019) showed that endophytic and pathogenic fungi, such as species of *Chaetomium*, *Trametes*, *Trichoderma*, *Penicillium*, and *Fusarium*, could colonize *Cannabis* plants internally. In this work, fungal contamination from *Moesziomyces* a genus in the family *Ustilaginaceae* , *Mortierella elongate*, and bacterial contamination from *Serratia marcescens* were observed from leaf tissue explants in tissue culture which have not been previously reported as endophytes in *Cannabis*. *Moesziomyces* spp. (previously referred to as *Pseudozyma*) have been known to be a biocontrol agent for various phytopathogens, including powdery mildew, and is commonly isolated from the plant surface (Liu et al., 2019). *Mortierella elongata* is known to be a beneficial plant-growth-promoting fungi found in the soil (Liao, 2021) and *Serratia marcescens* is also a known bacterial biocontrol agent (Ordentlich et al., 1987; Purkayastha et al., 2018). However, *Serratia marcescens* is a diverse group and some strains have been reported to cause diseases in plants such as Cucurbit Yellow Vine Disease (CYPD) (Besler and Little, 2017) and a leaf spot disease on *Cannabis* (Schappe et al., 2020). It is unclear as to whether *Moesziomyces* spp. survived through the rigorous surface sterilization process or whether this fungal contaminant was present as an endophyte, however, *Mortierella elongata* has been known to be an endophyte of roots (Liao, 2021; Purkayastha et al., 2018)

Previous tissue culture studies on *Cannabis* have not described problems with tissue culture contaminants. It is likely that the coco fiber used as a substrate for growing plants in this study harbored microbes that eventually colonized the internal tissues of the stems and became established (Punja et al., 2019). Other growing media, such as rockwool, may contain lower levels of contaminating microbes. Recent microbiome

studies have demonstrated that the bulk soil and rhizosphere of *Cannabis* and hemp plants are the most influential in determining the subsequent composition of internal microbes in other regions of the plant (Barnett et al., 2020; Comeau et al., 2021). Therefore, attention should be given to the condition of donor plants with regard to the substrate they are grown in and their duration in the growing environment. Young plants grown in relatively sterile growth substrates should be selected for tissue culture studies.

Axillary buds have been used as starting explant sources for shoot induction in tissue culture experiments of various plant species. For example, axillary buds are commonly used for the propagation of fruit and nut trees (Hussain et al., 2012). Growth of axillary buds in tissue culture has been studied in mint (*Mentha* species) (Rech and Pires, 1986), Cancer tree (*Camptotheca acuminate*) (Liu and Li, 2001), hops (*Humulus lupus*) (Roy et al., 2001), Andean blueberry (*Vaccinium floribundum*) (Cobo et al., 2018), and other woody plants (Sahoo and Chand, 1998). The multiplication media reported in Holmes et al. (2021) containing Murashige and Skoog (MS) basal salts supplemented with myo-inositol and activated charcoal was utilized in this study for growing nodal explants (Table 3.1). The growth regulators added were thidiazuron (TDZ, 1.0μM) and naphthaleneacetic acid (NAA, 0.5μM). On this medium, nodes responded favorably, and shoot growth was observed in the initiation phase. However, continuous subcultures over extended time periods on MS salts medium tended to produce shoots that displayed hyperhydricity and developed signs of nutrient deficiency with low multiplication rates (Holmes et al., 2021, unpublished observations). These symptoms were also described by Page et al. (2021) on MS basal salts medium. Page et al. (2021) reported that a tissue culture medium based on DWK basal salts supported better canopy growth than MS basal salts from nodal explant segments that were intended for stage 2 micropropagation. They compared five *Cannabis* genotypes and used two-node explants originating from micro-propagated plantlets grown on a DWK salts medium. Their results showed that explants grown on DKW produced a larger canopy area and had a higher multiplication rate than explants grown on MS. In this study, comparisons between the two basal salts media using two *Cannabis* genotypes did not show consistent differences in shoot growth that could be attributed to the effect of medium composition during stage 1 micropropagation. Wang et al. (2009) used an MS basal salts medium with 30 g/L sucrose, 6.8 g/L phytagel, and 1μM of TDZ to produce axillary buds from shoot tips of hemp during micropropagation. Lubell-Brand et al. (2021) used

an MS salts medium described by Lata et al. (2016) in which TDZ was replaced with the growth regulator meta-topolin (mT). They reported that hyperhydricity was reduced by modifying the agar content of the medium, coupled with improved vessel ventilation and enhanced nitrogen levels. Therefore, both DKW and MS salts media can support short-term growth in tissue culture media during the initiation of cultures. However, continuous subcultures and multiplication on a DKW-based medium appear to yield healthier and more vigorous plants (Page et al., 2021) or on an MS medium supplemented with enhanced levels of nitrogen (Lubell-Brand et al., 2021).

Rooting is often the most challenging step in micropropagation, especially in woody plant species (Ranaweera et al., 2013). In callus cultures, KIN and 2,4-D induced rooting (Feeney and Punja, 2003). When added to tissue culture media without phytohormones (MMC), neither KIN nor 2,4-D alone or in combination induced rooting in plantlets to the extent reported from callus (Holmes et al., 2021). To promote root induction, the concentration of the auxin NAA in the MM-AC callus media reported in Holmes et al. (2021) was increased from 1μM to 5μM, however, no rooting was observed, although some callusing was observed. Slusarkiewicz-Jarzina et al. (2005) studied the effect of plant growth regulators on the development of callus and subsequent regeneration in five hemp strains. The results showed that strain was an important factor for callus growth and regeneration. The hemp cultivar Fibrimon-24 produced the most calli (83%) of the strains compared. In previous studies, callus formation has been induced from both hemp and *Cannabis* explant tissues using combinations of the auxins 2,4-dichlorophenoxyacetic acid (2,4- D), naphthaleneacetic acid (NAA), and indole-3-butyric acid (IBA), and the cytokinins kinetin (Kin) and thidiazuron (TDZ) (Braemer and Paris, 1987; Feeney and Punja, 2003; Lata et al., 2009a; Wahby et al., 2013; Movahedi et al., 2015). Holmes et al. (2021) previously described robust callus formation on media supplemented with 1 μM Tdz + 0.5μM. In the current work, it was observed that a combination of 1μM BA + 0.5μM NAA produced both calli and roots from the same leaf section whereas a combination of 1μM Kin + 0.5μM NAA produced robust rooting from leaf sections of PNK.

Efforts have been made toward indirect regeneration of shoots from callus cultures of hemp and *Cannabis*. These have not been successful because of the recalcitrant nature of this species (Monthony et al., 2021). This is further supported by this current work where no shooting was observed from 8 week old calli on MMC

supplemented with 2 µM Tdz after 3 weeks. Differences in callus area from leaves were attributed to different genetic backgrounds of the plants tested. Results from a comparison of 8 different *C. sativa* strains showed that callus area was significantly different between AFK vs. PNK, CPH vs. PNK, DEB vs. PNK, DEB vs. PWE, PNK vs. PWE, and PNK vs. WHR (Figure 3.3). It also showed that AFK and PWE callus area was significantly smaller than the across all strains mean for callus area (dotted line in Figure 3.3), whereas PNK callus was significantly larger than the across all strains mean for callus area. The across all strains mean is affected by sample size and therefore a larger sample size of each strain would be beneficial to confirm these results.

Various explants have been studied for callus induction in *Cannabis*, such as cotyledons and epicotyls (Wielgus et al., 2008; Movahedi et al., 2015), leaves (Mandolino and Ranalli, 1999; Page et al., 2021), and petioles (Slusarkiewicz-Jarzina et al., 2005). In this study and in that of Page et al. (2021), the genotype was shown to influence the extent of callus formation. Page et al. (2021) found that the addition of 2,4-dichlorophenoxyacetic acid to media was required for callus production, and that media containing DWK salts yielded healthier and faster-growing calluses than the MS medium. Callus growth on the DWK salts medium was not tested in this research. The interest in deriving calli from *Cannabis* explants followed by regeneration of shoots is to allow genetic transformation studies to succeed (Feeney and Punja, 2003; Galan-Avila et al., 2021). In addition, there are numerous applications of tissue culture methods for *Cannabis* and hemp improvement, and these have been described elsewhere (Adhikary et al., 2021). To date, however, there are few reports describing the successful utility of tissue culture approaches for *C. sativa* on a large and cost-effective scale. The interest in micropropagation through tissue culture is to produce genetically identical, pathogen-free plants year-round in a limited amount of space (Yancheva and Kondakova, 2018; Lubell-Brand et al., 2021; Monthony et al., 2021).

The results of this study, and those of previous investigators (Lata et al., 2009a; 2010; Wang et al., 2009; Page et al., 2021; Holmes et al., 2021) show that the process of developing research methods for *Cannabis* requires a detailed assessment of the genotypic response, evaluation of the effect of basal salts medium, establishment of conditions promoting shoot growth and rooting frequency, and effective control of tissue culture contaminants. In addition, distinguishing between the requirements of stage 1 micropropagation (initiation of tissue cultures) verses stage 2 micropropagation

(multiplication of shoots), as pointed out by Page et al. (2021), may result in differing media compositions being developed. In contrast to the vegetative clonal propagation method that is widely used in the *Cannabis* industry, tissue culture approaches will still play a minor role in commercial production until research efforts have resolved many of the remaining challenges and the cost-effectiveness of this approach is proven. This study has attempted to assess genotypic responses to callus formation, microbial contamination, and axillary bud growth in tissue culture that can affect success in the development of methods for *Cannabis* research and plantlet recovery from stage 1 micropropagation using nodal stem explants in order to provide future directions in this area.

Chapter 4.

Variables Affecting Agrobacterium-mediated Transformation

4.1. Introduction

Agrobacterium-mediated transformation is a commonly used method for genome editing in plant research (De Bondt et al., 1996; Feeney and Punja, 2003; Komari et al., 2004; Dutt et al., 2007; Bakhsh, 2020; Deguchi et al., 2020). In nature, *Agrobacterium radiobacter* (syn. *Agrobacterium tumefaciens*, Parte et al., 2020) transforms plant cells upon detection of wounding compounds by using T-DNA from their Ti plasmid. The T-DNA is then transferred into the plant cell through a type IV (pillus) secretion system where the DNA is incorporated into the plant host genome (Gelvin, 2000; 2003). In lab settings, the strains of *Agrobacterium* used have had the T-DNA region removed from the Ti plasmid and placed onto a separate plasmid containing desired genes and features. In addition, the genes within the T-DNA that would cause gall development are replaced with selectable markers and target genes for use in experiments involving genetic transformation.

In *Cannabis*, there have been a few reports of successful transformation of hemp strains using *Agrobacterium* (Feeney and Punja, 2003; Deguchi et al., 2020; Galan-Avila et al., 2021; Zhang et al., 2021). *Cannabis* transformation frequencies, in hemp strains, ranging from 0.8% to 31.23% have been reported in the literature (Feeney and Punja, 2003; Galan-Avila et al., 2021). However, to date, there have been no reports of *Agrobacterium*-mediated transformation in drug-type strains of *Cannabis*. This research aims to understand the effect that different variables have on transformation frequency in callus tissue of drug-type *Cannabis* using a plasmid containing the reporter gene β-glucuronidase (GUS) and bialaphos resistance gene, *BlpR* (bar gene), selectable marker. In addition, the first report of transformed drug-type *Cannabis* callus from leaf tissue with the disease resistance gene, AtNPR1, is presented.

4.2. Methods

4.2.1. AtNPR1 and pEGgus Expression Plasmid Designs

The *Arabidopsis thaliana* NPR1 gene sequence (A.t.NPR1) was amplified from a plasmid that was provided by Dr. Yuelin Zhang at the University of British Columbia (UBC). A.t NPR1 was amplified using New England Biolabs (NEB) Q5® High-Fidelity DNA Polymerase (cat. M0491) and primers attB1/NPR1 and attB2/NPR1 which introduced the gateway cloning attB1 and attB2 sites onto either end of the AtNPR1 gene (Table 4.1). PCR conditions began with an initial denaturation step at 98℃ for 30 sec followed by 5 cycles of 98℃ for 10 sec, 50℃ for 30 sec, and 72℃ for 50 sec then 30 cycles of 98℃ for 10 sec, 70℃ for 30 sec, and 72℃ for 50 sec then ended with a cycle at 72℃ for 2 mins and a hold at 4℃ indefinitely. This created a single PCR product with gateway attB sites introduced at both ends of the fragment. The attB1-A.t. NPR1-attB2 sequence was purified from the PCR reaction using NEB's Monarch PCR and DNA cleanup kit (cat. T1030S) and cloned into the pEarleyGate100 plasmid (TAIR stock #CD3-724) (Earley et al., 2006), between the left and right T-DNA borders, via Thermo Fisher Scientific's Gateway Cloning protocols (cat. 12535037 & 11791020). The final expression plasmid containing the A.t NPR1 sequence was named pEGNPR1 (Figure 4.1). The pEarleyGate100 also contains a bialaphos resistance gene (BlpR) for use as a selectable marker within the left and right borders of the T-DNA. *Agrobacterium* GV3101 was transformed with the pEGNPR1 expression plasmid and used for transformation experiments. A positive control plasmid was also created following the same steps and materials as above however the β-glucuronidase (GUS) gene replaced the *At*NPR1 gene in the same backbone plasmid (Figure 4.1). The GUS gene came from the pENTR-gus plasmid that came with the gateway cloning kit. This control plasmid was named pEGgus and was not sent for sequencing.

Table 4.1. Primer sequences for Gateway cloning and validation.

Primer Name	Sequence (5' →3')	Purpose
attB1/NPR1	GGGGACAAGTTTGTACAAAAAAGCAGGCTCGC AAAAAATGGACACCACCATTGAT	For gateway cloning *AtNPR1* into pDONR/Zeo vector
attB2/NPR1	GGGGACCACTTTGTACAAGAAAGCTGGGTTCTA GTCGACGGCCCATGA	For gateway cloning *AtNPR1* into pDONR/Zeo vector
AtNPR1-F	AGCAGCACTAGTTTCGTCGC	Amplifies a 500bp band within the 1st exon of *AtNPR1* cDNA sequence from TAIR
AtNPR1-R	TCAGGGATCTTGAAGATGAAAGCC	Amplifies a 500bp band within the 1st exon of *AtNPR1* cDNA sequence from TAIR
BlpR-F	GTCTGCACCATCGTCAACC	Amplifies a 440bp fragment from the BlpR resistance gene in the pEarleyGate100 vector backbone
BlpR-R	GTCCAGCTGCCAGAAACC	Amplifies a 440bp fragment from the BlpR resistance gene in the pEarleyGate100 vector backbone

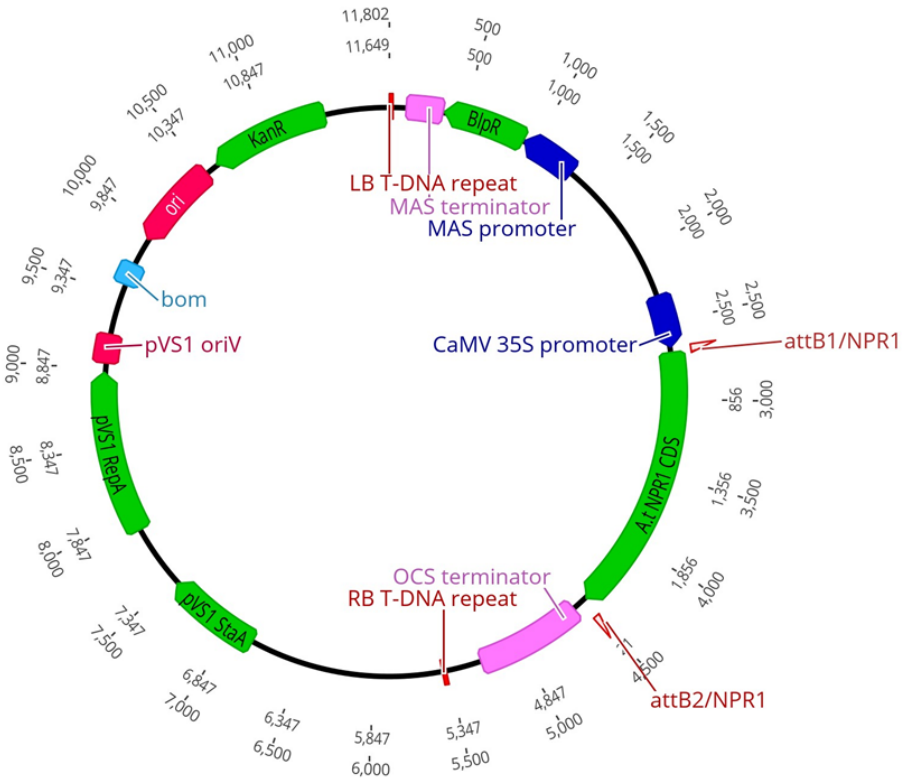


Figure 4.1. Plasmid map of pEGNPR1. The final expression plasmid from gateway cloning A.t. NPR1 into the pEarleyGate100 plasmid. pEGNPR1 contains herbicide resistance via the Bialaphos resistance gene and the A.t. NPR1 CDS sequence within the T-DNA region and a Kanamycin resistance gene for selection within the bacterial host.

4.2.2. Preparation of *Agrobacterium* suspensions for transformation

The first part of a protocol developed for Agroinfiltration from Dr. Yu Xiang's lab at AAFC Summerland, Canada was followed. *Agrobacterium* strains GV3101 (provided by Dr. Yu Xiang) or EHA105 (provided by Sherry Sun) transformed with either pEGNPR1 or pEGgus from a glycerol stock was used to inoculate a 3 mL overnight (O/N) culture of YEB + 50 µg/mL kanamycin (for expression plasmid selection) + 40 µg/mL rifampicin (for genomic selection) + 20 µg/mL gentamycin (for plasmid GV3101 selection). The O/N culture was incubated at 28℃ with shaking at 200-250rpm. The next day 0.5–1 mL of O/N culture was used to inoculate 50mL YEB + 50 µg/mL kanamycin (Millipore Sigma, cat.60615) + 40 µg/mL rifampicin (Millipore Sigma, cat.R3501) + 20

μg/mL gentamycin (GoldBio, cat.G-400-1) + 20 μM acetosyringone + 10 mM MES. The culture was incubated O/N at 28℃ with shaking at ~200 rpm. The *Agrobacterium* culture was then transferred into a sterile 50 mL centrifuge tube (FroggaBio, cat. TB50-500) and cells were centrifuged at 4℃ and 4000xg for 8 mins. In a laminar flow hood, the supernatant was poured off and the cell pellet was resuspended in sterile infiltration buffer (10 mM MES, 10 mM MgCl2, and 200 μM acetosyringone in distilled water, all components were sterile and combined in the laminar flow hood right before use) to an O.D.600 of 0.4-0.6. The *Agrobacterium* suspension was incubated at RT (~21-23℃) for at least 3 hours before it was used.

4.2.3. Preparation of tissue for transformation

Leaf tissue obtained from *Cannabis* plants grown either in an indoor room or under greenhouse conditions were cut into 0.5-1 cm^2 sections while petiole tissue was cut into 1 cm long sections. Axillary meristems were removed of most of their primordial leaves; however, some were left on to prevent over sterilization of the meristem. The explants were taken from cuttings which were stored at 4℃ for ~48 hr. Tissue was sterilized in 70% EtOH for 1 min, 10% bleach (0.625% NaOCl) for 10 min, then rinsed 3 times in sterile distilled water for 1 min. Excess water was removed on sterile paper towel and fresh edges of leaf and petiole sections were cut. Tissue was then immersed in the *Agrobacterium* suspension above for 15-30 min and excess liquid was removed on sterile paper towel. Tissue was then placed on sterile filter paper on co-cultivation medium (MMA1, Table 3.1) and incubated at RT (20-23℃) for 4 days. After co-cultivation, tissue was transferred to selection medium (MMA1 + 0.7 mg/L glufosinate (Cayman Chemical, cat.16675) + 500 mg/L timentin (GoldBio, cat.T-104-2)). Unless otherwise stated, this was the transformation protocol followed. After 8-9 weeks on selection media, putatively transformed calli were identified by their visible growth on selection medium and then cut in half for both DNA and RNA extractions. For each experiment, 50 tissue samples were prepared for each treatment group, however, the final number of tissue samples evaluated excluded any samples which showed endophytic microbial contamination. Therefore, final sample sizes were often less than 50 tissue samples and uneven across groups.

4.2.4. Effect of glufosinate on *C. sativa* L. callus growth

Glufosinate concentrations of 0, 0.25, 0.5, 1, and 3 mg/L were added to MMA1 medium (Table 3.1) to determine the concentration at which glufosinate prevents callus formation. Leaf sections from the *Cannabis* strain Moby Dick (MBD) were prepared as indicated above and placed on the media with varying glufosinate concentrations. After 6 weeks, photos were taken, and the callus area was measured using the "polygon" feature in Image J software. Since the data did not meet the requirements for an ANOVA test, a Kruskal-Wallis test was performed with a post-hoc Dunn's test.

4.2.5. *Cannabis* transformation with pEGNPR1

Leaf tissue was taken from CPH plants grown indoors under two Sunblaster brand (Langley, British Columbia, Canada) 54 watt 6400 k T5HO lights with a 24-h photoperiod. Plants were watered as needed with a solution of 1 mL/L Sensi Grow Coco pH Perfect A + B (Advanced Nutrients, West Hollywood, California, USA) and 1 mL/L General Hydroponics Calimagic (General Hydroponics, Santa Rosa, California, USA) adjusted to a pH of 5.8–6.2 using Advanced Nutrients pH-Down (Advanced Nutrients, West Hollywood, California, USA) (Scott and Punja, 2020). Leaves were then cut into approximately 1 cm^2 pieces and sterilized in 70% EtOH for 1 min, then 10% bleach (0.625% NaOCl) with 0.1% Tween20 for 15-20 mins and washed with sterile water 3 times for 1 min. Excess water was blotted off using sterile paper and fresh edges were cut on each leaf section. After fresh edges were cut the leaf section was immersed in an *Agrobacterium* GV3101/pEGNPR1 suspension with an O.D.600 of 0.433 for 15-30 mins. Excess *Agrobacterium* suspension was blotted off on autoclaved paper towel and leaf tissue was plated on MM-callus media with sterile filter paper on the media surface and incubated in aluminum foil at RT (21-23℃) for 52 hrs. Leaf sections were transferred to MM+9uM Tdz+1.4um NAA media containing 1mg/L of glufosinate and 500mg/L of timentin and incubated inside aluminum foil at RT until putatively transformed callus was seen (approx. 6-7 weeks). Previous experiments showed that callus development was inhibited between 0.5mg/L and 1mg/L glufosinate (Figure 4.1). Therefore, 1mg/L glufosinate was used for transformant selection. This selection was decreased to 0.7mg/L Glufosinate for all other experiments as it was observed that 1mg/L may have been too toxic for transformed cells.

To confirm the expression of *A.t.NPR1* in putatively transformed callus, RNA was extracted from callus samples which grew well on selection media containing 1mg/L glufosinate at 7-9 weeks. NEB's Monarch® Total RNA Miniprep Kit (T2010S) was used for RNA extraction. The RNA was used as the template in an RT-PCR reaction using the primers AtNPR1-F (AGCAGCACTAGTTTCGTCGC) and AtNPR1-R (TCAGGGATCTTGAAGATGAAAGCC) (Table 1). The primers were designed to amplify a 500bp region towards the 3' end of the first exon in the A.t.NPR1 mRNA sequence taken from the TAIR database. NEB's OneTaq® One-Step RT-PCR Kit (E5315S) was used for RT-PCR. The RT-PCR kit's recommended reaction conditions were followed with the annealing temperature for the primers set at 50°C. A 6ul sample of the RT-PCR reactions were visualized on a 1% agarose gel and the remaining cDNA of positive samples was purified with NEB's Monarch PCR and DNA Cleanup Kit (cat. T1030S) then sent to Eurofins Genomics for sequencing. NCBI's BLAST was used to determine sequence identity.

4.2.6. Effect of explant type on *Cannabis* transformation

Leaf, petiole, and meristem explants of cuttings from the *Cannabis* strain Pennywise (PWE) that had been in the fridge for ~48hr, were cut into 1cm or ~1cm^2 sections (leaves and petioles only) and sterilized in 70% EtOH for 1 min then in 20% bleach + 0.1% Tween20 for 10 min. Tissue was rinsed 3 times with sterile double distilled water for 2 mins and excess water was removed. Fresh edges of the tissues were cut, and they were immersed for 15-30 min in an *Agrobacterium* GV3101/pEGgus suspension of O.D.600 0.56-0.67. Excess suspension was removed using sterile paper towel and tissues were co-cultivated on callus induction media (MM-AC) overlaid with sterile filter paper for ~3 days at RT (~21-23℃) on a lab bench. After 3 days, tissue was transferred to selection media (MM-AC) supplemented with 0.7 mg/L glufosinate and 500 mg/L timentin and incubated in the dark at RT for 8 weeks. Previous work showed glufosinate prevented plant growth at 1mg/L. This experiment was repeated with PNK explants; however, the general tissue preparation method was followed. After 8 weeks, DNA was extracted from the putatively transformed calli and PCR was conducted using BlpR primers (BlpR-F/BlpR-R, Table 4.1) designed to amplify a 440bp fragment to confirm transformation. The Qiagen DNeasy Plant Mini Kit was used for DNA extraction and the Qiagen Taq Core Kit was used for PCR. The manufacturer's instructions for both

kits were followed and PCR conditions were 1 cycle at 94℃ for 3 min; 40 cycles of 94℃ for 30 sec, 58℃ for 45 sec, and 72℃ for 1 min; and 1 cycle at 72℃ for 4 min. Total RNA was also extracted using NEB's Monarch RNA Extraction Kit (#T2010) and RT-PCR, using the One*Taq* One-Step RT-PCR Kit (E5315S) from NEB, was carried out using both the BlpR-F/BlpR-R primers. Presence of a positive band in the RT-PCR results was recorded and a Fisher's Exact Test was performed to compare transformation frequency between whole leaves and sectioned leaves.

4.2.7. Effect of leaf preparation on *Cannabis* transformation

Leaves of *Cannabis* strain PNK were either cut into leaf sections (n=21) and sterilized as reported above or the leaflets (n=20) of the *Cannabis* leaves were separated, sterilized as above then a sterile scalpel was used to make light cuts along the length of the midrib vein of the leaflet. Both leaflets and leaf sections were then immersed in the *Agrobacterium* GV3101/pEGNPR1 suspension (as detailed above) and then co-cultivated on sterile filter paper on MMA1 media for 4 days. After 4 days, leaf tissue was transferred to selection media (MMA1 supplemented with 0.7 mg/L glufosinate + 500 mg/L timentin.) and incubated at room temperature (~20-25℃) in the dark. After 8 weeks, DNA was extracted from the putatively transformed calli and PCR was conducted using AtNPR1 primers (AtNPR1-F/AtNPR1-R, Table 4.1) designed to amplify a 500bp fragment to confirm transformation. The Qiagen DNeasy Plant Mini Kit was used for DNA extraction and the Qiagen Taq Core Kit was used for PCR. The manufacturer's instructions for both kits were followed PCR conditions were as follows: 1 cycle at 94℃ for 3 min; 40 cycles of 94℃ for 30 sec, 50℃ for 45 sec, and 72℃ for 1 min; and 1 cycle at 72℃ for 4 min. Total RNA was also extracted using NEB's Monarch RNA Extraction Kit (#T2010) and RT-PCR, using the One*Taq* RT-PCR Kit (E5310S) from NEB, was carried out using both the AtNPR1-F/AtNPR1-R primers. Presence or absence of a positive band in the RT-PCR results was recorded and a Fisher's Exact Test was performed to compare transformation frequency between whole leaves and sectioned leaves.

4.2.8. Effect of light on *Cannabis* transformation

Leaves of *Cannabis* strain PNK were cut into leaf sections and sterilized as above then leaf sections were immersed in the *Agrobacterium* GV3101/pEGgus

suspension (as detailed above) and co-cultivated on sterile filter paper on MMA1 media for 4 days. After 4 days, leaf tissue was transferred to selection media (MMA1 supplemented with 0.7 mg/L glufosinate + 500 mg/L timentin.) and incubated at room temperature (~20-25℃) in high light, low light, or in the dark for 8 weeks. For low light conditions, sheets of white printer paper were placed over the tissue culture plates and T5 Sunblaster lights were positioned 20-30cm above the plates. The light under the paper was measured to be ~20 µmol/m^2/sec. For high light conditions, the printer paper was not applied, and light was measured to be ~55 µmol /m^2/sec. Dark conditions were created by wrapping the plates in aluminum foil. After 8 weeks, DNA was extracted from the putatively transformed calli and PCR was conducted using BlpR primers (BlpR-F/BlpR-R, Table 4.1) designed to amplify a 500bp fragment to confirm transformation. The Qiagen DNeasy Plant Mini Kit was used for DNA extraction and the Qiagen Taq Core Kit was used for PCR. The manufacturer's instructions for both kits were followed PCR conditions were as follows: 1 cycle at 94℃ for 3 min; 40 cycles of 94℃ for 30 sec, 58℃ for 45 sec, and 72℃ for 1 min; and 1 cycle at 72℃ for 4 min. Total RNA was also extracted using NEB's Monarch RNA Extraction Kit (#T2010) and RT-PCR, using the Qiagen OneStep RT-PCR Kit (cat.210212), was carried out using BlpR-F/BlpR-R primers. The RT-PCR conditions were as follows: 1 cycle of 50℃ for 30 min, 1 cycle of 95℃ for 15 min, 40 cycles of 94℃ for 1 min, 58℃ for 30℃ sec, and 72℃ for 1 min then a final cycle of 72℃ for 10 min. Presence of a positive band in the RT-PCR results was recorded and a Fisher's Exact Test was performed to compare transformation frequency between whole leaves and sectioned leaves.

4.2.9. Effect of glufosinate concentration on transformation

Leaves of *Cannabis* strain PNK were cut into leaf sections and sterilized as above then leaf sections were immersed in the *Agrobacterium* GV3101/pEGgus suspension (as detailed above) and co-cultivated on sterile filter paper on MMA1 media for 4 days. After 4 days, leaf tissue was transferred to selection media, MMA1 supplemented with either 1 mg/L or 0.7 mg/L glufosinate + 500 mg/L timentin and incubated at room temperature (~20-25℃) in the dark for 8 weeks. After 8 weeks, DNA was extracted from the putatively transformed calli and PCR was conducted using BlpR primers (BlpR-F/BlpR-R, Table 4.1) designed to amplify a 500bp fragment to confirm transformation. The Qiagen DNeasy Plant Mini Kit was used for DNA extraction and the

Qiagen Taq Core Kit was used for PCR. The manufacturer's instructions for both kits were followed PCR conditions were as follows: 1 cycle at 94℃ for 3 min; 40 cycles of 94℃ for 30 sec, 58℃ for 45 sec, and 72℃ for 1 min; and 1 cycle at 72℃ for 4 min. Total RNA was also extracted using NEB's Monarch RNA Extraction Kit (#T2010) and RT-PCR, using the One*Taq* One-step RT-PCR Kit (E5315S) from NEB, was carried out using both the BlpR primers. Presence of a positive band in the RT-PCR results was recorded and a Fisher's Exact test was performed to compare transformation frequency between calli on 1mg/L glufosinate and 0.7mg/L glufosinate.

4.2.10. Effect of *Agrobacterium* strain on transformation

Agrobacterium strains GV3101 and EHA105 transformed with pEGNPR1 were evaluated for their effect on transformation frequency of *C. sativa* L. Leaves of *Cannabis* strain PNK were cut into leaf sections and sterilized as above then leaf sections were immersed in either an *Agrobacterium* GV3101/pEGNPR1 or *Agrobacterium* EHA105/pEGNPR1 suspension (as detailed above) and co-cultivated on sterile filter paper on MMA1 media for 4 days. After 4 days, leaf tissue was transferred to selection media (MMA1 supplemented with 0.7 mg/L glufosinate + 500 mg/L timentin.) and incubated at room temperature (~20-25℃) in the dark for 8 weeks. After 8 weeks, DNA was extracted from the putatively transformed calli and PCR was conducted using AtNPR1 primers (AtNPR1-F/AtNPR1-R, Table 4.1) designed to amplify a 500bp fragment to confirm transformation. The Qiagen DNeasy Plant Mini Kit was used for DNA extraction and the Qiagen Taq Core Kit was used for PCR. The manufacturer's instructions for both kits were followed PCR conditions were as follows: 1 cycle at 94℃ for 3 min; 40 cycles of 94℃ for 30 sec, 50℃ for 45 sec, and 72℃ for 1 min; and 1 cycle at 72℃ for 4 min. Total RNA was also extracted using NEB's Monarch RNA Extraction Kit (#T2010) and RT-PCR, using the One*Taq* One-step RT-PCR Kit (E5315S) and Onet*Taq* RT-PCR kit (E5310S) from NEB, was carried out using both the AtNPR1-F/AtNPR1-R primers. Presence or absence of a positive band in the RT-PCR results was recorded and a Fisher's Exact Test was performed to compare transformation frequency between GV3101 and EHA105.

4.3. Results

4.3.1. Effect of glufosinate on *C. sativa* L. callus growth

After 6 weeks on MMA1 with varying concentrations of glufosinate callus development from MBD leaf sections was evaluated. There were notable visual differences between each of the glufosinate concentrations (Figure 4.1A), and a significant difference was observed between callus area from leaf sections cultivated on MMA1+0.25mg/L glufosinate and MMA1+0.5mg/L glufosinate (Figure 4.1B). Based on these results, [glufosinate] selection for transformation experiments was set at 1mg/L due to the notable presence of callus on MMA1+0.5mg/L glufosinate.

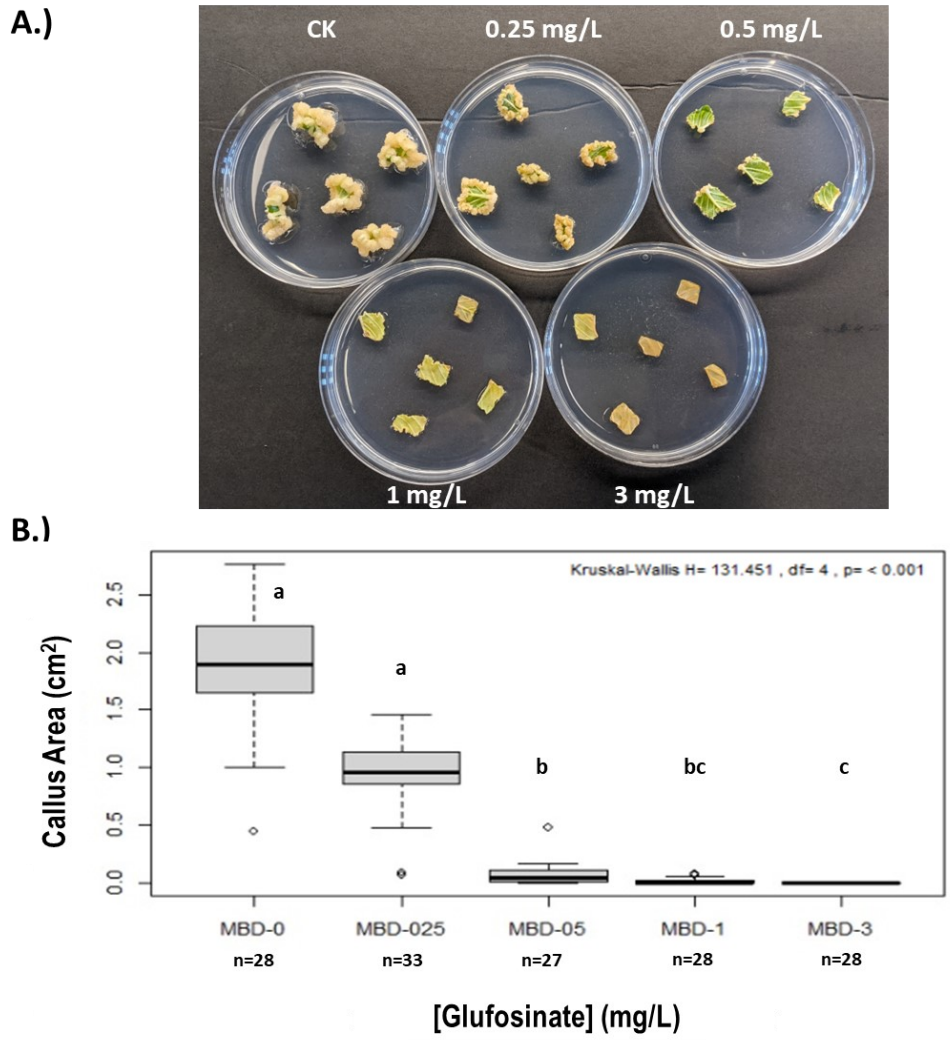


Figure 4.2. **Glufosinate concentration for transformed calli selection. A.) MBD leaf sections were plated on MMA1 callus media with varying [glufosinate] from 0 to 3mg/L glufosinate. There is a notable visual difference between the CK, 0.25, and 0.5mg/L glufosinate plates. Whereas, 1 and 3mg/L plates show little visual difference. B.) Average callus area comparisons were performed using a Dunn's post-hoc test. All comparisons except between 0-0.25, 0.5-1, and 1-3mg/L glufosinate were significantly different. Boxplots of data show the spread of data points. The bottom edge of the boxplot indicates the 1st quartile where 25% of the data is represented under the bottom of the box. The dark black line represents the mean of the data. The top edge of the box indicated the 3rd quartile below which 75% of the data is represented. The error bars represent the standard deviation.**

4.3.2. *Cannabis* transformation with pEGNPR1

After 5-6 weeks on MS+9uM Tdz+1.4um NAA medium supplemented with 1mg/L glufosinate and 500µg/mL timentin, RNA from CPH callus was extracted and used as the template in RT-PCR reactions with the primers AtNPR1-F and AtNPR1-R. No shoots were observed on this medium at 6 weeks. Out of the 6 putatively transformed CPH callus samples, 4 showed the expected 500bp banding pattern (Figure 4.3). NCBI BLAST results showed that the positive 500bp band had high sequence similarity (99.78-100%) to *Arabidopsis thaliana* NPR1 mRNA and genomic sequences. However, over the weeks of culturing following RT-PCR, 3 of the 4 transformed calli turned brown and died. The remaining one was propagated in cell suspension for 2 months then plated back onto solid callus induction media without glufosinate selection, however, this callus eventually died out as well. It was observed that 1mg/L glufosinate may have been too high of a selection pressure for transformed callus, as transformation can be a stressful event for tissue, so the glufosinate selection was reduced to 0.7mg/L glufosinate for all subsequent transformation experiments.

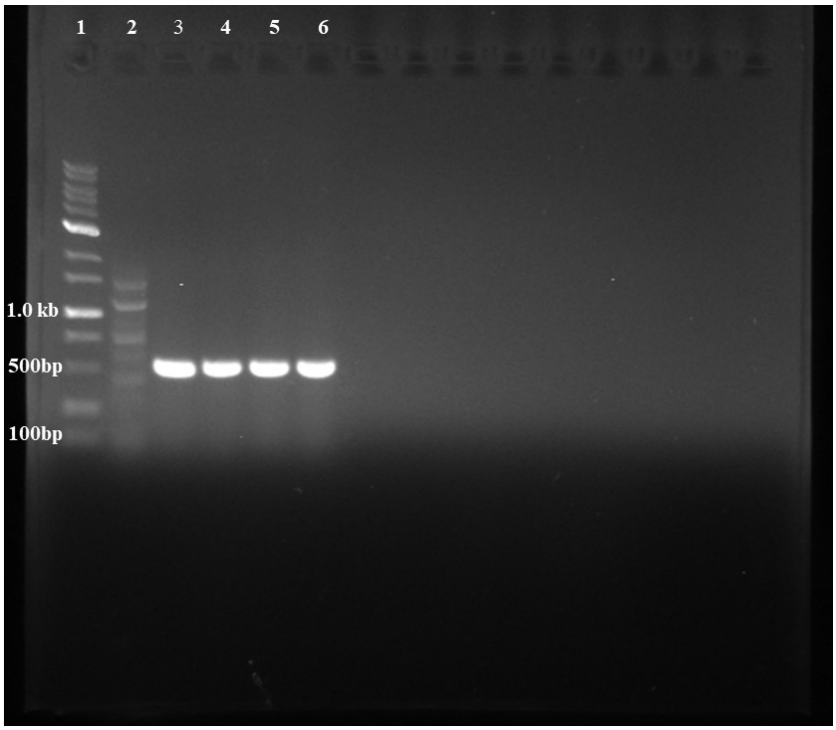


Figure 4.3. **RT-PCR of transformed CPH callus. RNA was extracted from 5 CPH callus samples growing on glufosinate selection media and reverse transcribed into cDNA. Primers AtNPR1-F and AtNPR1-R were used to amplify towards the 3' end of the first exon in the A.t. NPR1 mRNA sequence from TAIR. The expected size fragment that these primers amplify is 500bp.**

4.3.3. Effect of explant type on *Cannabis* transformation

After 8-9 weeks on selection media, a transformation frequency of 43.8% was observed for PNK calli that developed from leaf sections and 65% for calli that developed from petiole sections as confirmed through RT-PCR by the detection of *BlpR* (the bialaphos resistance gene) in callus tissue (Figure 4.4). For leaf explants, 15 out of 16 leaf sections produced putatively transformed calli, 7 of which were confirmed *BlpR* transformed by RT-PCR. For petiole sections, 18 out of 20 were identified as putatively transformed and 13 were confirmed *BlpR* transformed via RT-PCR. False positive PCR calli developed likely due to slight warping of the leaf sections after being plated on the tissue culture media so that parts of the leaf sections were not in direct contact with the selection media. A Chi-square test comparing transformation frequency (based on the total number of samples for each group) between leaf and petiole sections resulted in a

p-value of 0.3485. This experiment had previously been performed using PWE leaf tissue, however, no putative calli were observed.

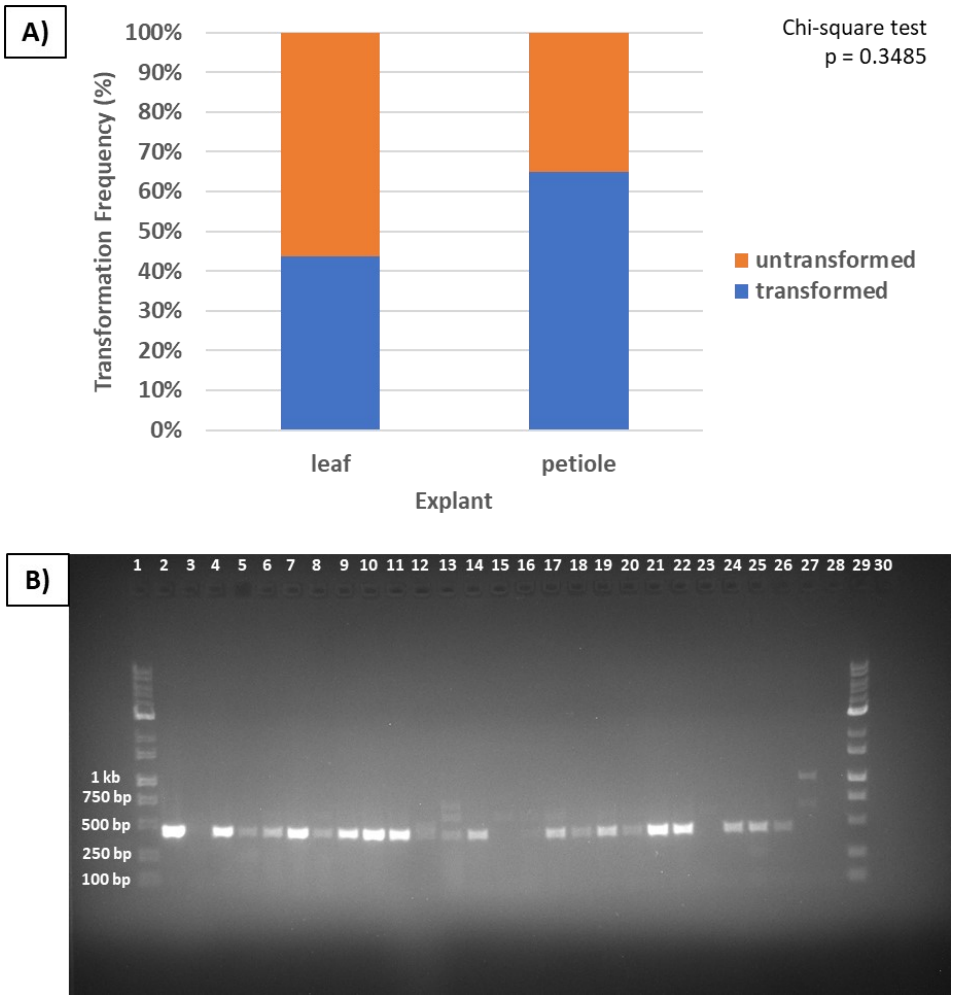
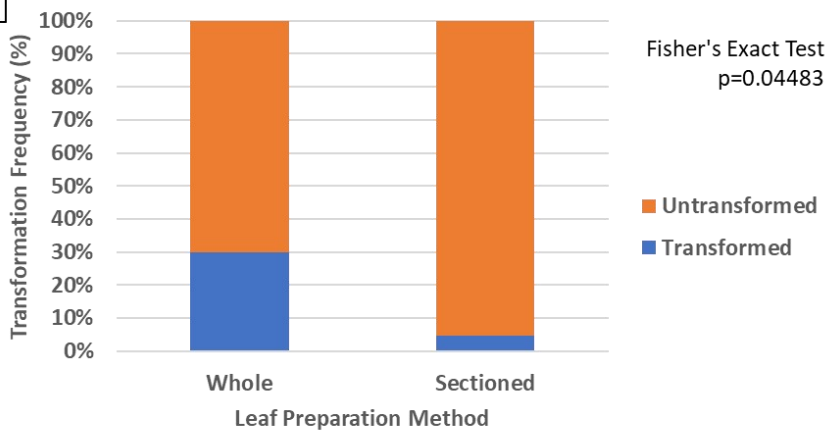


Figure 4.4. **Comparison of transformation frequency between leaf and petiole explants. A.) Leaf explants showed a 43.8% transformation frequency and petiole explants resulted in a 65% transformation frequency. The *Cannabis* strain PNK was used, however, the difference in transformation frequency was not significant using a Chi-square test (p=0.3485). B.) RT-PCR with BlpR primers of putatively transformed leaf, petiole, and meristem calli which were previously positive in a PCR test using the same primers. Lane 1 and 29 show the FroggaBio 1kb plus DNA ladder. Lane 2 is the pEGgus plasmid (positive control). Lanes 3-10 show the RT-PCR results of putatively transformed calli from leaf explants. Lane 11 shows the RT-PCR result of a putatively transformed callus from nodal meristem. Lanes 12-26 show the RT-PCR results of calli derived from petiole callus. Lane 27 shows the RT-PCR results of a negative calli DNA control. Lane 28 shows the RT-PCR results of a negative water control. Lane 30 is empty. The expected band size of the BlpR primers is 440bp and only samples with the 440bp band were considered transformed.**

4.3.4. Effect of leaf preparation on *Cannabis* transformation

After 8-9 weeks on selection media, a transformation frequency of 30% was observed for PNK calli that developed from whole leaflets and 4.8% for calli that developed from leaf sections as confirmed through RT-PCR by the detection of *AtNPR1* (the *Arabidopsis thaliana* resistance gene *NPR1*) in callus tissue (Figure 4.5). For whole leaves wounded along the midrib, 9 out of 20 whole leaves produced putatively transformed calli, 6 of which were confirmed *AtNPR1* transformed by RT-PCR. For leaf sections, 2 out of 21 leaf sections were identified as putatively transformed and 1 was confirmed *AtNPR1* transformed via RT-PCR. A Fisher's exact test of two proportions comparing transformation frequency (based on the total number of samples for each group) between whole leaflets and leaf sections resulted in a p-value of 0.04483. This experiment had previously been performed using PWE leaf tissue, however, no putative calli were observed.

A)


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Transformation Frequency (%)
Whole
Sectioned
Leaf Preparation Method
Fisher's Exact Test
p=0.04483
Untransformed
Transformed

B)

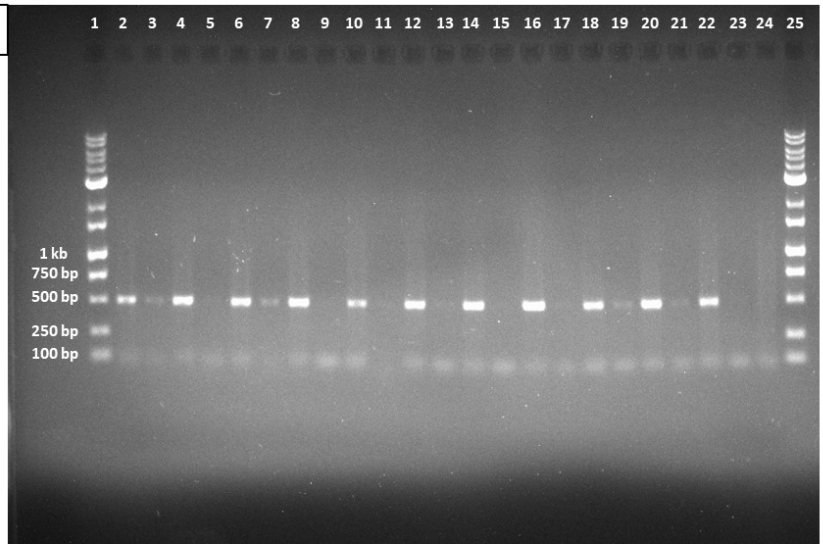

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
1 kb
750 bp
500 bp
250 bp
100 bp

Figure 4.5. Comparison of transformation frequency between whole leaflets and leaf sections. Whole leaflets showed a 30% transformation frequency and leaf sections resulted in a 4.8% transformation frequency. The *Cannabis* strain PNK was used and the difference in transformation frequency was significant based on a Fishers Exact test (p=0.04483). B.) RT-PCR with AtNPR1 primers of putatively transformed calli from wholeleaflets or leaf sections which were previously positive in a PCR test using the same primers. Lane 1 and 25 show the FroggaBio 1kb plus DNA ladder. Lanes 2 & 4 show the RT-PCR results of putatively transformed calli from leaf sections. Lanes 3 & 5 are the control reactions for lanes 2 & 4 without reverse transcriptase to estimate the quality of the RNA extraction and visualize the amount of residual DNA. Lanes 6, 8, 10, 12, 14, 16, 18, 20, & 22 show the RT-PCR results of putatively transformed calli from whole leaflets. Lanes 7, 9, 11, 13, 15, 17, 19, 21, and 23 are the control reactions for the wholeleaflet RT-PCR reactions without reverse transcriptase to estimate the quality of the RNA extraction and visualize the amount of residual DNA. Lane 24 shows the RT-PCR results of a negative calli cDNA control. The expected band size of the AtNPR1 primers is 500bp and only samples with the 500bp band were considered transformed.

4.3.5. Effect of Light on *Cannabis* transformation

After 8-9 weeks on selection media, transformation frequencies of 11.8% was observed for PNK calli that developed under high light conditions, 20% was observed for calli that developed under low light conditions, and 28.6% for calli that developed under no light conditions via RT-PCR by the detection of *BlpR* in callus tissue (Figure 4.6). For high light conditions, 8 calli out of 34 leaf sections were identified as putatively transformed and 4 were confirmed *BlpR* transformed via RT-PCR. For low light conditions, 9 out of 35 leaf sections produced putatively transformed calli, 7 of which were confirmed *BlpR* transformed by RT-PCR. For no light conditions, 8 calli out of 28 leaf sections were identified as putatively transformed and 8 were confirmed *BlpR* transformed via RT-PCR. A Chi-square test comparing transformation frequency (based on the total number of samples for each group) between calli grown under high light, low light, and no light conditions resulted in a p-value of 0.2516. The cDNA tissue culture negative control (Figure 4.6, lane 26) in this experiment was contaminated and so the 440bp band was sent for sequencing along with the 440bp band from 8 putatively transformed calli. All 440bp bands aligned with 99-100% identity to the bar (bialaphos resistance) gene. This experiment had previously been performed using PWE leaf tissue, however, no putative calli were observed.

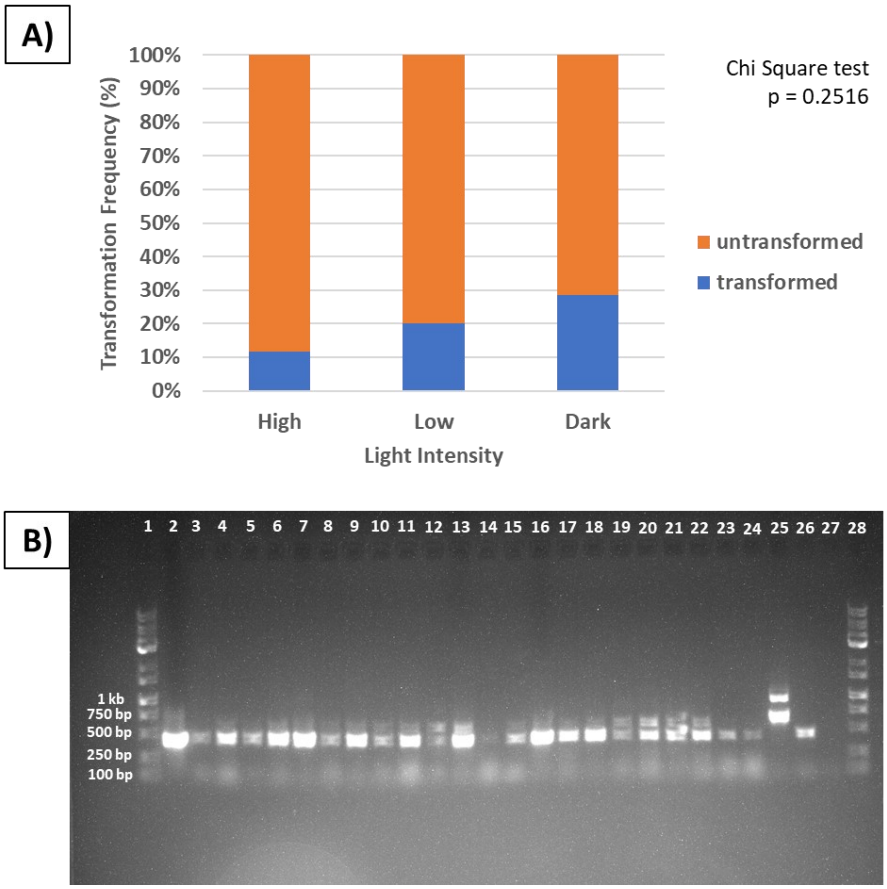


Figure 4.6. **Comparison of transformation frequency between calli grown under high, low, and no light conditions. A.) Leaf calli produced under high light conditions showed an 11.8% transformation frequency, calli under low light conditions showed a transformation frequency of 20%, and calli under no light conditions resulted in a 28.6% transformation frequency. The *Cannabis* strain PNK was used, however, the difference in transformation frequency was not significant using a Chi-square test (p=0.3485). B.) RT-PCR with BlpR primers of putatively transformed calli developed under high, low, and no light conditions which were previously positive in a PCR test using the same primers. Lane 1 and 28 show the FroggaBio 1kb plus DNA ladder. Lane 2 shows the RT-PCR results of the pEGgus plasmid control. Lanes 3-6 show the RT-PCR results from 4 putatively transformed calli produced under high light conditions. Lanes 7-14 show the RT-PCR results of 8 putatively transformed calli produced under low light conditions. Lanes 15-22 show the RT-PCR results of 8 putatively transformed calli produced under no light conditions. Lanes 23-24 show the RT-PCR results of two samples from a different transformation experiment. Lane 25 shows the RT-PCR results from the DNA of a negative tissue culture control. Lane 26 shows the RT-PCR results of a negative calli cDNA control. Lane 27 shows the RT-PCR results of negative water control. The expected band size of the BlpR primers is 440bp and only samples with the 440bp band were deemed transformed.**

4.3.6. Effect of glufosinate concentration on transformation

After 8-9 weeks on selection media, a transformation frequency of 8.6% was observed for PNK calli that developed on 1mg/L glufosinate and 15% for calli that developed on 0.7mg/L glufosinate via RT-PCR by the detection of *BlpR* in callus tissue (Figure 4.7). However, this difference was not significant. For 1mg/L glufosinate, 12 out of 35 leaf sections were identified as putatively transformed and 3 was confirmed *BlpR* transformed via RT-PCR. For 0.7mg/L glufosinate, 15 out of 40 whole leaves produced putatively transformed calli, 6 of which were confirmed *BlpR* transformed by RT-PCR. A Fisher's exact test of two proportions comparing transformation frequency (based on the total number of samples for each group) between calli produced on 1 mg/L and 0.7 mg/L glufosinate resulted in a p-value of 0.4895.

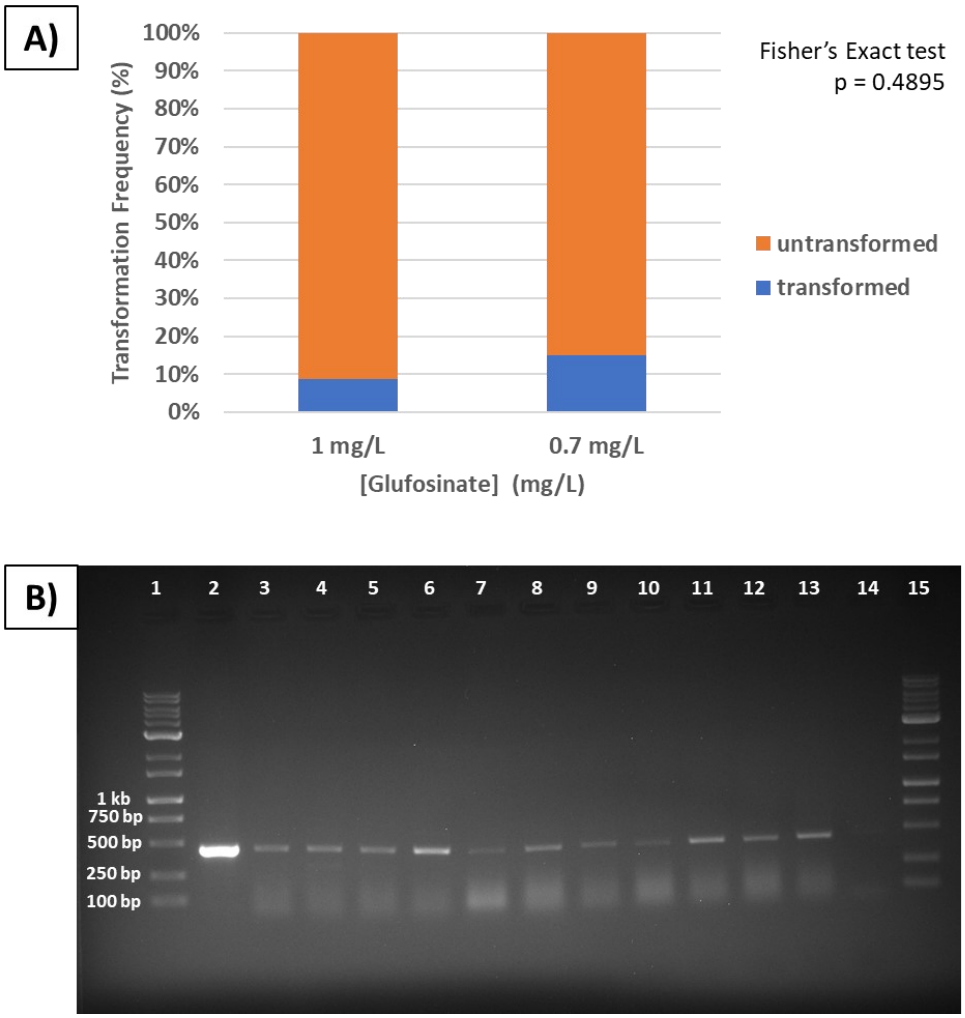
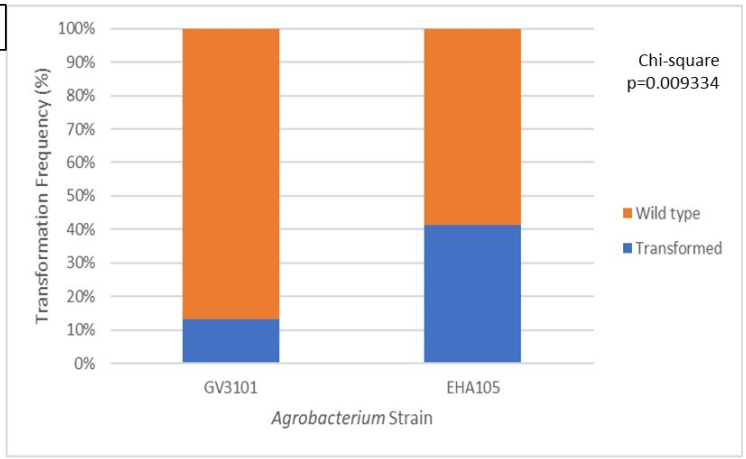

A)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Transformation Frequency (%)
1 mg/L
0.7 mg/L
[Glufosinate] (mg/L)
Fisher's Exact test
p = 0.4895
untransformed
transformed
B)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
1 kb
750 bp
500 bp
250 bp
100 bp

Figure 4.7. Comparison of transformation frequency of calli from leaf sections between 1 mg/L and 0.7 mg/L. Leaf calli that developed on 1 mg/L glufosinate showed a 8.6% transformation frequency and leaf calli that developed on 0.7 mg/L glufosinate resulted in a 15% transformation frequency. The *Cannabis* strain PNK was used and the difference in transformation frequency was significant based on a Fishers Exact test (p=0.04895). B.) RT-PCR with BlpR primers of putatively transformed calli developed on media with 1mg/L or 0.7mg/L glufosinate which were previously positive in a PCR test using the same primers. Lanes 1 and 15 show the FroggaBio 1kb plus DNA ladder. Lane 2 shows the RT-PCR results of positive control plasmid pEGgus (DNA). Lanes 3-5 show the RT-PCR results of 3 calli samples grown on 1 mg/L glufosinate that showed positive BlpR bands in previous PCR reactions. Lane 6 shows the RT-PCR results of a callus sample grown on 1mg/L glufosinate that was negative (no BlpR band) in a previous PCR reation. Lanes 7-12 show the RT-PCR results of 6 calli samples grown on 0.7mg/L glufosinate that showed positive BlpR bands in previous PCR reactions. Lane 13 shows the RT-PCR results of a callus sample grown on 0.7mg/L glufosinate that was negative (no BlpR band) in a previous PCR reation. Lane 14 shows the RT-PCR results of a water control. The expected band size of the BlpR primers is 440bp and only samples with the 440bp band were considered transformed.

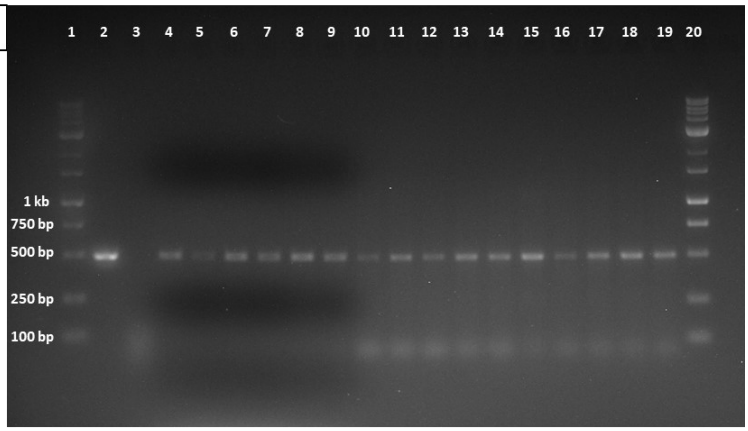
4.3.7. Effect of *Agrobacterium* strain on Transformation

After 8-9 weeks on selection media, a transformation frequency of 13.2% was observed for PNK calli that developed from leaf sections immersed in GV3101 and 41.3% for calli that developed from leaf sections immersed in EHA105 as confirmed through RT-PCR by the detection of *AtNPR1* in callus tissue (Figure 4.8). For GV3101 treated leaf sections, 5 out of 38 produced putatively transformed calli, all of which were confirmed *AtNPR1* transformed by RT-PCR. For EHA105 treated leaf sections, 19 out of 46 leaf sections were identified as putatively transformed and all 19 were confirmed *AtNPR1* transformed via RT-PCR. A Chi-square test comparing transformation frequency (based on the total number of samples for each group) between GV3101 and EHA105 groups resulted in a p-value of 0.009334.

A)


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Transformation Frequency (%)
GV3101
EHA105
Agrobacterium Strain
Chi-square
p=0.009334
Wild type
Transformed

B)


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
1 kb
750 bp
500 bp
250 bp
100 bp

C)

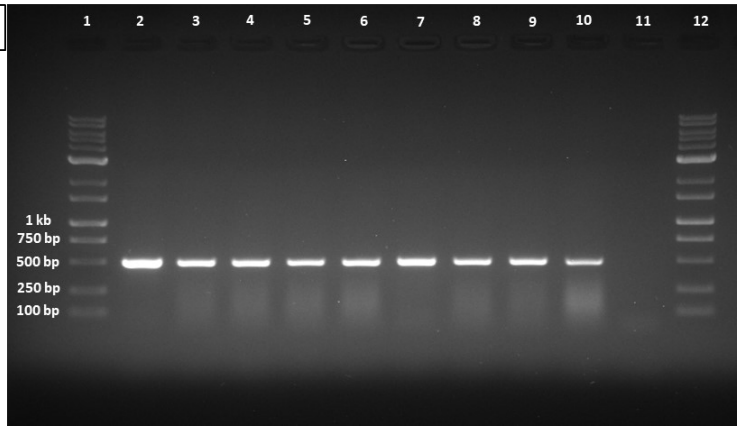

1 2 3 4 5 6 7 8 9 10 11 12
1 kb
750 bp
500 bp
250 bp
100 bp

Figure 4.8. Comparison of transformation frequency between leaf sections treated with GV3101 and EHA105. Leaf sections treated with *Agrobacterium* strain GV3101 showed a 13.2% transformation frequency and leaf sections treated with *Agrobacterium* strain EHA105 resulted in a 41.3% transformation frequency. The *Cannabis* strain PNK was used and the difference in transformation frequency was significant based on a Chi-square test (p=0.009334). B.) RT-PCR results from putatively transformed PNK calli exposed to *Agrobacterium* GV3101 or EHA105. Lanes 1 & 20 are the FroggaBio 1kb plus ladder. Lane 2 is the pEGNPR1 plasmid control. Lane 3 is a sample from a different expriment. Lanes 4-8 show the RT-PCR results from 5 previously PCR positive calli treated with *Agrobacterium* GV3101. Lanes 9-19 show the RT-PCR results from 11 previously PCR positive calli treated with *Agrobacterium* EHA105. C.) Lanes 1 & 12 are FroggaBio 1kb plus ladder. Lane 2 shows the pEGNPR1 plasmid control. Lanes 3-10 show the RT-PCR results of an additional 8 previously PCR positive calli treated with *Agrobacterium* EHA105 . Lane 11 shows the RT-PCR results of a negative water control.

4.4. Discussion

Using Gateway cloning and *Agrobacterium* GV3101, the *Arabidopsis thaliana* NPR1 gene was successfully transformed into the *Cannabis* strain Copenhagen Kush. The sequenced 500bp RT-PCR bands from CPH callus samples growing on 1mg/L glufosinate selection indicated that *A.t NPR1* had been successfully integrated into the *Cannabis* genome and was transcribed into RNA. A glufosinate selection level of over 0.5 mg/L and under 3 mg/L of glufosinate was found to be most effective for selection in *Cannabis* which is consistent with the bialaphos selection levels that others have used (Spencer, 1990). Of the variables that were evaluated for their effect on transformation, leaf preparation method and *Agrobacterium* strain showed a significant difference in transformation frequency between treatment groups. There were no significant differences observed between leaf or petiole as explants, high, low, or dark light conditions, and 1mg/L or 0.7 mg/L glufosinate selection. It was observed that leaving leaflets whole and cutting the mid rib produced a higher transformation frequency than leaf sections, 30% and 4.8%, respectively. This could be due to whole leaves being able to produce more calli as more endogenous phytohormones are present in whole leaflets than in leaf sections. This experiment had been performed using PWE leaves, however, no putative calli were observed using this strain. This suggests that Cannabis genotype likely has an effect on transformation frequency. We did not assess the effect of

Cannabis strain on transformation frequency although all strains tested (CPH, PNK, PWE) produced transformed calli at different frequencies.

Evaluation of the effect of *Agrobacterium* strain on transformation frequency resulted in a significant difference between leaf sections treated with *Agrobacterium* GV3101 and *Agrobacterium* EHA105 where the transformation frequencies were 13.2% and 41.3%, respectively. Results suggest that *Agrobacterium* EHA105 is a more effective strain for transformation in *Cannabis sativa*. *Agrobacterium* strain EHA105 has previously been used by Sorokin et al. (2020) to develop a rapid transient expression method for medical *Cannabis* seedlings utilizing the pCAMBIA1301 construct which contains the *uidA* gene. Contamination by endophytic microbes was also responsible for decreasing the total number of viable tissue samples able to produce callus and may have had an effect on transformation frequency in all experiments. In addition, tissue samples which were identified in previous PCR experiments as negative could have been chimeric and therefore, the reported transformation frequencies could be underestimations. However, similar transformation frequencies have been reported using hemp callus suspension culture transformed with *Agrobacterium* EHA101 (Feeney and Punja, 2003) and medical *Cannabis* cotyledons and young true leaves transformed with *Agrobacterium* EHA105 (Sorokin et al., 2020).To be confident in these results, the experiments should be repeated with the same *Cannabis* strain PNK and larger sample sizes to confirm transformation frequencies as contamination and sterilization could have affected the total number of viable tissue samples able to produce calli. These experiments should also be performed using different strains to evaluate any strain specific responses to transformation.

Chapter 5. Final Discussion

Crown gall can affect numerous plant hosts and has been noted as a disease that can affect *Cannabis sativa* L. by McPartland (1996; 2003). In this research, *Agrobacterium* T-DNA had been sequenced and identified in the DNA from 6 galls found on the *Cannabis* strain Pink Kush (PNK), however, a strain of *Agrobacterium* was not isolated from these galls due to their small size and was not recovered from the other galls evaluated in this research. Small galls can often be difficult to diagnose as crown gall caused by *Agrobacterium* can also be the result of a callus response to wounding of the plant (Ikeuchi et al., 2013) or other gall producing bacteria such as *Pantoea agglomerans pv. gypsophila* or *Erwinia*, or *Enterobacter* (Chalupowicz et al., 2006; Davis et al., 2016). In addition, a commercially available strain of *Agrobacterium* was used to show gall formation in *Cannabis*. The size of galls differed with inoculation site location. The commercial strain of *Agrobacterium* that was used to create the galls was re-isolated on MacConkey Agar. This research shows that *Cannabis* is susceptible to *Agrobacterium* and therefore can be transformed for biotechnological purposes. Future directions of this work should explore the potential of other gall forming bacteria as the causal agent behind the *Cannabis* galls. In addition, further attempts should be made to isolate and identify *Agrobacterium* from future galls.

Developing reliable tissue culture methods for *Cannabis sativa* L. is crucial for many genetic studies in *Cannabis* as well as for future biotech applications. Although tissue culture protocols for *Cannabis* have been reported, few reports mention the negative impact that endophytes, microbes living in the plant tissue without causing disease, can have on tissue culture in *Cannabis* (Collyer, 2021; Holmes et al., 2021). Reliable tissue culture protocols can be severely impacted by endophyte contamination as this work reports up to 100% of leaf explants showing contamination in some cases even after significant surface sterilization. This research shows that application of a systemic fungicide, Luna, to mother plants of *Cannabis* can reduce contamination in tissue culture of nodal explants. Stable transformation of callus, cotyledons, hypocotyl, and meristems had previously been achieved in various hemp strains of *Cannabis* (Feeney and Punja, 2003; Galan–Avila et al., 2021; Zhang et al., 2021). This research provides the first report of stable transformation of leaf calli of a drug-type strain of *Cannabis sativa* L. using *AtNPR1*, a disease resistance gene. *Cannabis* strain is an

important factor to consider when choosing tissue for transformation as this work shows that various *Cannabis* strains can have differences in their response to callus induction media. In addition, of the five variables evaluated in this research, leaf preparation method and *Agrobacterium* strain showed significant differences in transformation frequency. Pink Kush (PNK) whole leaves with shallow slices down the midrib and leaf sections immersed in *Agrobacterium* EHA105 resulted in more *AtNPR1* transformed calli than calli produced from leaves cut into sections and leaf sections immersed in *Agrobacterium* GV3101, respectively.

For the results of this research to be fully utilized, the development of a reliable shooting method from callus for *C. sativa* is essential to create biotechnology protocols for *Cannabis sativa*. However, the development of a reliable callus transformation protocol can aid in future research which utilize callus cell culture for various genetic studies such as metabolite production and biosynthesis pathway studies. Future research directions should include the development of better tissue culture sterilization methods, a reliable callus shooting protocol, the evaluation of strain differences in shooting protocols from callus and larger studies of variables that affect transformation. Recently, methods which report shooting from callus in hemp strains of *Cannabis* have been published which utilize meristem, hypocotyl, and cotyledon explants for callus transformation (Hesami and Jones, 2021; Gal´an-Avila et al, 2021). Further experiments should also evaluate these methods using the strains of *Cannabis* present in our lab.

References

Adhikary, D., Kulkarni, M., El-Mezawy, A., Mobini, S., Elhiti, M., Gjuric, R., et al. (2021). Medical *Cannabis* and industrial hemp tissue culture: present status and future potential. Frontiers in Plant Science 12:275. doi: 10.3389/fpls.2021.627240.

Ahmed, S., Gao, X., Jahan, A., Adams, M., Wu, N., and Kovinich, N. (2021). Nanoparticle-based genetic transformation of *Cannabis sativa*. Journal of Biotechnology 326:48–51. doi: 10.1016/j.jbiotec.2020.12.014.

Ali, S., Mir Zahoor, A., Tyagi, A., Mehari, H., Meena, R.P., Bhat, J.A., Yadav, P., Papalou, P., Rawat, S., and Grover, A. (2017). Overexpression of *NPR1* in *Brassica juncea* confers broad spectrum resistance to fungal pathogens. Frontiers in Plant Science 8:1693. doi: 10.3389/fpls.2017.01693.

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

Bakhsh, A. (2020). Development of efficient, reproducible and stable *Agrobacterium*-mediated genetic transformation of five potato cultivars. Food Technology Biotechnology 58(1):57-63. doi: 10.17113/ftb.58.01.20.6187.

Barnett, S.E., Cala, A.R., Hansen, J. L., Crawford, J., Viands, D.R., Smart, L.B., et al. (2020). Evaluating the microbiome of hemp. Phytobiomes Journal 4:351–363. doi: 10.1094/PBIOMES-06-20-0046-R.

Besler, K.R., and Little, E.L. (2017). Diversity of *Serratia marcescens* strains associated with cucurbit yellow vine disease. Georgia Plant Disease 101:129-136. doi: 10.1094/PDIS-05-16-0618-RE.

Bini, F., Kuczmog, A., Putnoky, P., Otten, L., Bazzi, C., Burr, T.J., and Szegedi, E. (2008). Novel pathogen-specific primers for the detection of *Agrobacterium* vitis and *Agrobacterium tumefaciens*. Vitis 47(3):181-189. doi: 10.5073/vitis.2008.47.181-189.

Braemer, R., and Paris, M. (1987). Biotransformation of cannabinoids by a cell suspension culture of *Cannabis sativa* L. Plant Cell Reports 6:150–152. doi: 10.1007/BF00276675.

Braun, A.C., and White, P.R. (1941). Crown gall production by bacteria-free tumor tissues. Science 94:239-241.

CABI. (2021). *Rhizobium radiobacter* (crown gall). In: Invasive species compendium. Wallingford, UK: CAB International. www.cabi.org/isc.

Cao, H., Xin, L., and Xinnian, D. (1998). Generation of broad-spectrum disease resistance by overexpression of an essential regulatory gene in systemic acquired resistance. Proceedings of the National Academy of Science 95:6531–6536.

Chalupowicz, L., Barash, I., Schwartz, M., Aloni, R., and Manulis, S. (2006). Comparative anatomy of gall development on *Gypsophila paniculata* induced by bacteria with different mechanisms of pathogenicity. Planta 224(2):429–437. doi: 10.1007/s00425-006-0229-9.

Chandra, S., Lata, H., Khan, I.A., and ElSohly, M.A. (2017). *Cannabis sativa* L.: botany and horticulture. In: *Cannabis sativa* L. botany and biotechnology, eds S. Chandra, H. Lata, M. ElSohly M (Cham: Springer), pg. 79-100. doi: 10.1007/978-3-319-54564-6.

Chern, M.S., Fitzgerald, H.A., Yadav, R.C., Canlas, P.E., Dong, X., and Ronald, P.C. (2001). Evidence for a disease-resistance pathway in rice similar to the NPR1-mediated signaling pathway in *Arabidopsis*. The Plant Journal 27(2):101-113.

Chilton, M.D., Drummond, M.H., Merlo, D.J., Sclaky, D., Montoya, A.L., Gordon, M.P., and Nester, E.W. (1977). Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. Cell 11:263-271.

Cobo, M., Gutiérrez, B., and Torres, M. (2018). Regeneration of Mortiño (*Vaccinium floribundum Kunth*) plants through axillary bud culture. In Vitro Cellular and Developmental Biology - Plant 54:112–116. doi: 10.1007/s11627-018-9884-3.

Collyer, D. (2021). A method for tissue culture of *Cannabis sativa* L. [Masters thesis, Simon Fraser University]. Summit.

Comeau, D., Novinscak, A., Joly, D.L., and Filion, M. (2021). Spatio-temporal and cultivar-dependent variations in the *Cannabis* microbiome. Frontiers in Microbiology 11:491. doi: 10.3389/fmicb.2020.00491.

Costechareyre, D., Rhouma, A., Lavire, C., Portier, P., Chapulliot, D., Bertolla, F., Boubaker, A., Dessaux, Y., and Nesme, X. (2010). Rapid and efficient identification of *Agrobacterium* species by *recA* allele analysis: *Agrobacterium recA* diversity. Microbial Ecology 60:862-872. doi: 10.1007/s00248-010-9685-7.

Cubero, J., Martínez, M.C., Llop, P., and López, M.M. (1999). A simple and efficient PCR method for the detection of *Agrobacterium tumefaciens* in plant tumours. Journal of Applied Microbiology 86(4):591-602. doi:10.1046/j.1365-2672.1999.00700.x.

Davis Ii, E.W., Weisberg, A.J., Tabima, J.F., Grunwald, N.J., and Chang, J.H. (2016). Gall-ID: tools for genotyping gall-causing phytopathogenic bacteria. PeerJ 4:e2222. doi: 10.7717/peerj.2222.

De Bondt, A., Eggermont, K., Penninckx, I., Goderis, I., and Broekaert, W.F. (1996). *Agrobacterium*-mediated transformation of apple (*Malus x domestica* Borkh.): an assessment of factors affecting regeneration of transgenic plants. Plant Cell Reports 7:549-554. doi: 10.1007/BF00232992.

De Cleene, M., and De Ley, J. (1976). The host range of crown gall. The Botanical Review 42:389-466. doi: 10.1007/BF02860827.

Deguchi, M., Bogush, D., Weeden, H., Spuhler, Z., Potlakayala, S., Kondo, T., et al. (2020). Establishment and optimization of a hemp (*Cannabis sativa* L.) agroinfiltration system for gene expression and silencing studies. Scientific Reports 10:3504. doi:10.1038/s41598-020-60323-9.

Dutt, M., Li, Z.T., Dhekney, S.A., and Gray, D.J. (2007). Transgenic plants from shoot apical meristems of *Vitis vinifera* L. ‘‘Thompson Seedless’’ via Agrobacterium-mediated transformation. Plant Cell Reports 26:2101-2110. doi: 10.1007/s00299-007-0424-6.

Earley, K., Haag, J.R., Pontes, O., Opper, K., Juehne, T., Song, K., and Pikaard, C.S. (2006). Gateway-compatible vectors for plant functional genomics and proteomics. The Plant Journal 45:616-629.

Farrand, S.K., van Berkum, P.B., and Oger, P. (2003). *Agrobacterium* is a definable genus of the family *Rhizobiaceae*. International Journal of Systematic and Evolutionary Microbiology 53(5):1681-1687. doi: 10.1099/ijs.0.02445-0.

Feeney, M., and Punja, Z.K. (2003). Tissue culture and *Agrobacterium*-mediated transformation of hemp (*Cannabis sativa* L.). In Vitro Cellular Developmental Biology - Plant 39:578–585. doi: 10.1079/IVP2003454.

Gal´an-Avila, A., Gramazio, P., Ron, M., Prohens, J., Herraiz, F.J. (2021). A novel and rapid method for *Agrobacterium*-mediated production of stably transformed *Cannabis sativa* L. plants. Industrial Crops and Products 170:113691. doi: 10.1016/j.indcrop.2021.113691.

Gelvin, S.B. (2000). *Agrobacterium* and plant genes involved in T-DNA transfer and integration. Annual Review of Plant Physiology and Plant Molecular Biology 51:223–256. doi:10.1146/annurev.arplant.51.1.223.

Gelvin, S.B. (2003). *Agrobacterium*-mediated plant transformation: the biology behind the “gene-jockeying” tool. Microbiology and Molecular Biology Review 67(1):16-37. doi: 10.1128/MMBR.67.1.16–37.2003.

Health Canada. (2021). *Cannabis* market data. https://www.canada.ca/en/health-canada/services/drugs-medication/*Cannabis*/research-data/market.html.

Hesami, M., Jones, A.M.P. (2021). Modeling and optimizing callus growth and development in *Cannabis sativa* using random forest and support vector machine in combination with a genetic algorithm. Applied Microbiology and Biotechnology 105:5201-5212. doi: 10.1007/s00253-021-11375-y.

Holmes, J.E., Lung, S., Collyer, D., and Punja, Z.K. (2021). Variables affecting shoot growth and plantlet recovery from tissue cultures of drug-type *Cannabis sativa* L. Frontiers in Plant Science 12. doi:10.3389/fpls.2021.732344.

Hussain, A., Qashi, I., Nazir, H., and Ullah, I. (2012). Plant tissue culture: current status and opportunities. In Recent advances in plant in vitro culture, eds A. Leva and L. Rinaldi, pg. 2–28. doi: 10.5772/50568.

Hwang, H.H., Yu, M., and Lai, E.M. (2017). *Agrobacterium*-mediated plant transformation: biology and applications. The Arabidopsis Book 15. doi: 10.1199/tab.0186.

Ikeuchi, M., Sugimoto, K., and Iwase, A. (2013). Plant callus: mechanisms of induction and repression. The Plant Cell. 25(9):3159-73. doi: 10.1105/tpc.113.116053.

Islam, M.M., Ahmed, M., and Mahaldar, D. (2005). In vitro callus induction and plant regeneration in seed explants of rice (*Oryza sativa* L.). Research Journal of Agriculture and Biological Sciences 1:72–75.

Islam, M.S., Akter, M., Rahman, M.A., Rahman, M.M., Akhtar, M.M., and Alam, M.F. (2010). Isolation of *Agrobacterium tumefaciens* strains from crown gall samples of dicots plants in Bangladesh. Current Research in Bacteriology 3(1):27-36. doi: 10.3923/crb.2010.27.36.

Izawa, T., Foster, R., and Chua, N. (1993). Plant bZIP protein DNA binding specificity. Journal of Molecular Biology 230(4):1131-1144.

Kado, C.I. (2002). Crown gall. The Plant Health Instructor. doi:10.1094/PHI-I-2002-1118-01.

Kado, C.I. (2010). Plant bacteriology. St. Paul, MN: APS Press.

Kado, C.I. (2014). Historical account on gaining insights on the mechanism of crown gall tumorigenesis induced by *Agrobacterium tumefaciens*. Frontiers in Microbiology 5(340):1-15. doi:10.3389/fmicb.2014.00340.

Kado, C.L., Heskett, M.G.(1970). Selective media for isolation of *Agrobacterium*, *Corynebacterium*, *Erwinia*, *Pseudomonas*, *Xanthomonas*. Phytopathology 60:969-976.

Komari, T., Ishida, Y., and Hiei, Y. (2004). Plant transformation technology: *Agrobacterium*-mediated transformation. In: Handbook of plant biotechnology (eds P. Christou and H. Klee). doi: 10.1002/0470869143.kc014.

Kumar, V., Joshi, S.G., Bell, A.A., and Rathore, K.S. (2013). Enhanced resistance against *Thielaviopsis basicola* in transgenic cotton plants expressing *Arabidopsis* NPR1 gene. Transgenic Research 22:359–368. doi: 10.1007/s11248-012-9652-9.

Kusari, P., Kusari, S., Spiteller, M., and Kayser, O. (2013). Endophytic fungi harbored in *Cannabis sativa* L.: diversity and potential as biocontrol agents against host plant-specific phytopathogens. Fungal Diversity 60:137–151. doi: 10.1007/s13225-012-0216-3.

Kusari, P., Spiteller, M., Kayser, O., and Kusari, S. (2014). Recent advances in research on *Cannabis sativa* L. endophytes and their prospect for the pharmaceutical industry. In: Kharwar, R., Upadhyay, R., Dubey, N., Raghuwanshi, R. (eds) Microbial diversity and biotechnology in food security. Springer, New Delhi. doi: 10.1007/978-81-322-1801-2_1.

Kuzmanović, N., Puławska, J., Prokić, A., Ivanović, M., Zlatković, N., Jones, J.B., and Obradović, A. (2015). *Agrobacterium arsenijevicii* sp. nov., isolated from crown gall tumors on raspberry and cherry plum. Systematic Applied Microbiology 38(6):373-8. doi: 10.1016/j.syapm.2015.06.001.

Kuzmanović, N., Puławska, J., Smalla, K., and Nesme, X. (2018a). *Agrobacterium rosae* sp. nov., isolated from galls on different agricultural crops. Systematic Applied Microbiology 41:191-197.

Kuzmanović, N., Smalla, K., Gronow, S., and Puławska, J. (2018b). *Rhizobium tumorigenes* sp. nov., a novel plant tumorigenic bacterium isolated from cane gall tumors on thornless blackberry. Scientific Reports 8:9051.

Lassalle, F., Campillo, T., Vial, L., Baude, J., Costechareyre, D., Chapulliot, D., Sham, M., Abrouk, D., Lavire, C., Oger-Desfeux, C., Hommais, F., Gueguen, L., Daubin, V., Muller, D., and Nesme, X. (2011). Genomic species are ecological species as revealed by comparative genomics in *Agrobacterium tumefaciens*. Genome Biology Evolution 3:762-781. doi: 10.1093/gbe/evr070.

Lata, H., Chandra, S., Khan, I., and ElSohly, M.A. (2009a). Thidiazuron induced high frequency direct shoot organogenesis of *Cannabis sativa* L. In vitro Cellular and Developmental Biology - Plant 45:12–19.

Lata, H., Chandra, S., Khan, I., and ElSohly, M.A. (2009b). Propagation through alginate encapsulation of axillary buds of *Cannabis sativa* L.—an important medicinal plant. Physiology and Molecular Biology of Plants 15:79–86.

Lata, H., Chandra, S., Khan, I., and ElSohly, M.A. (2010). High frequency plant regeneration from leaf derived callus of high (Δ9- tetrahydrocannabinol yielding *Cannabis sativa* L. Planta Medica 76:1629–1633.

Lata, H., Chandra, S., Techen, N., Khan, I.A., and ElSohly, M.A. (2016). In vitro mass propagation of *Cannabis sativa* L.: a protocol refinement using novel aromatic cytokinin meta-topolin and the assessment of eco-physiological, biochemical and genetic fidelity of micropropagated plants. Journal of Applied Research on Medicinal and Aromatic Plants 3:18-26. doi: 10.1016/j.jarmap.2015.12.001.

Liao, H.L. (2021).The plant-growth-promoting fungus, *Mortierella elongata*: its biology, ecological distribution, and activities promoting plant growth. UF/IFAS University of Florida Extension, Publication #SL466. doi: 10.32473/edis-ss679-2021.

Lindström, K., and Young, J.P.W. (2011). International committee on systematics of prokaryotes subcommittee on the taxonomy of *Agrobacterium* and *Rhizobium*: minutes of the meeting, 7 September 2010, Geneva, Switzerland. International Journal of Systemic Evolutionary Microbiology 61:3089-3093.

Liu, Y., Zou, Z., Hu, Z., Wang, W., and Xiong, J. (2019). Morphology and molecular analysis of *Moesziomyces antarcticus* isolated from the blood samples of a chinese patient. Frontiers in Microbiology 10:254. doi: 10.3389/fmicb.2019.00254.

Liu, Z., and Li, Z. (2001). Micropropagation of *Camptotheca acuminata Decaisne* from axillary buds, shoot tips, and seed embryos in a tissue culture system. In Vitro Cellular and Developmental Biology - Plant 37:84–88. doi: 10.1007/s11627-001-0016-z.

Lubell-Brand, J., Kurtz, L.E., and Brand, M.H. (2021). An in vitro-ex vitro micropropagation system for hemp. HortTechnology 31:199–207. doi: 10.21273/HORTTECH04779-20.

Mafakheri, H., Taghavi, S.M., Puławska, J., De Lajudie, P., Lassalle, F., and Osdaghi, E. (2019). Two novel genomospecies in the *Agrobacterium tumefaciens* species complex associated with rose crown gall. Phytopathology 109(11):1859-1868. doi: 10.1094/PHYTO-05-19-0178-R.

Malamy, J., Carr, J.P., Klessig, D.F., and Raskin, I. (1990). Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. Science 250:1002–1004.

Malnoy, M., Jin, Q., Borejsza-Wysocka, E.E., He, S.Y., and Aldwinckle, H.S. (2007). Overexpression of the apple MpNPR1 gene confers increased disease resistance in *Malus* × *domestica*. Molecular Plant-Microbe Interactions 20(12):1568–1580. doi: 10.1094 /MPMI -20-12-1568.

Mandolino, G., and Ranalli, P. (1999). Advances in biotechnological approaches for hemp breeding and industry. Advances in Hemp Research 9:185–212.

Martínez, M.T., Corredoira, E., Vieitez, A.M., Cernadas, M.J., Montenegro, R., Ballester, A., et al. (2017). Micropropagation of mature *Quercus ilex* L. trees by axillary budding. Plant Cell Tissue Organ Culture 131:499–512. doi: 10.1007/s11240-017-1300-x.

Matthews, B.F., Beard, H., Brewer, E., Kabir, S., MacDonald, M.H., and Youssef, R.M. (2014). *Arabidopsis* genes, *AtNPR1*, *AtTGA2* and *AtPR-5*, confer partial resistance to soybean cyst nematode (*Heterodera glycines*) when overexpressed in transgenic soybean roots. BMC Plant Biology 14:96. doi: 10.1186/1471-2229-14-96.

Matthysse, A.G. (2006). The genus *Agrobacterium*. In: Dworkin M, Falkow S, Rosenberg E, Schleifer KH, Stackebrandt E, (eds). The prokaryotes. New York (NY): Springer; pg. 91-114. doi: 10.1007/0-387-30745-1_5.

McPartland, J.M. (1994). Microbiological contaminants of marijuana. Journal of the International Hemp Association 1:41–44.

McPartland, J.M. (1996). A review of *Cannabis* diseases. Journal of the International Hemp Association 3(1):19-23.

McPartland, J.M. (2003). Diseases of hemp (*Cannabis sativa* L.). American Phytopathological Society. [Accessed 2022 Sept 2]. www.apsnet.org/edcenter/resources/commonnames/Pages/Hemp.aspx.

Mihalyov, P.D., and Garfinkel, A.R. (2021). Discovery and genetic mapping of *PM1*, a powdery mildew resistance gene in *Cannabis sativa* L. Frontiers in Agronomy 3:2673-3218. doi: 10.3389/fagro.2021.720215.

Monthony, A.S., Page, S.R., Hesami, M., and Jones, A.M.P. (2021). The past, present, and future of *Cannabis sativa* tissue culture. Plants 10:185. doi: 10.3390/plants10010185.

Moore, L.W., Bouzar, H., and Burr, T. (2001). II. Gram-negative bacteria. In: Schaad NW, Jones JB, Chun W, editors. Laboratory guide for identification of plant pathogenic bacteria, 3rd ed. St. Paul (MN): APS Press; pg. 17–35.

Moore, L.W., and Putnam, M.L. (2020). Crown gall disease of nursery crops.In: Pacific northwest pest management handbooks. [Accessed 2022 Oct 21]. https://pnwhandbooks.org/plantdisease/pathogen-articles/pathogens-common-many-plants/bacteria-other-prokaryotes/crown-gall.

Mousavi, S.A., Osterman, J., Wahlberg, N., Nesme, X., Lavire, C., Vial, L., Paulin, L., de Lajudie, P., and Lindstrom, K. (2014). Phylogeny of the *Rhizobium–Allorhizobium–Agrobacterium* clade supports the delineation of *Neorhizobium* gen. nov. Systematic Applied Microbiology 37:208-215.

Mousavi, S.A., Willems, A., Nesme, X., de Lajudie, P., and Lindström, K. (2015). Revised phylogeny of *Rhizobiaceae*: proposal of the delineation of *Pararhizobium* gen. nov., and 13 new species combinations. Systematic Applied Microbiology 38:84-90. doi: 10.1016/j.syapm.2014.12.003.

Movahedi, M., Ghasemi-Omran, V.O., and Torabi, S. (2015). The effect of different concentrations of TDZ and BA on in vitro regeneration of Iranian *Cannabis* (*Cannabis sativa*) using cotyledon and epicotyl explants. Journal of Plant Molecular Breeding 3(2):20–27. doi: 10.22058/JPMB.2015.15371.

Morgulis, A., Coulouris, G., Raytselis, Y., Madden, T.L., Agarwala, R., and Schäffer, A.A. (2008). Database indexing for production MegaBLAST searches. Bioinformatics 24:1757-1764. doi: 10.1093/bioinformatics/btn322.

Ordentlich, A., Elad, Y., and Chet, I. (1987). Rhizosphere colonization by *Serratia marcescens* for the control of *Sclerotium rolfsii*. Soil Biology and Biochemistry 19(6):747-751. doi: 10.1016/0038-0717(87)90058-7.

Ormeno-Orrillo, E., Servin-Garciduenas, L.E., Rogel, M.A., Gonzalez, V., Peralta, H., Mora, J., Martinez-Romero, J., and Martinez-Romero, E. (2015). Taxonomy of *Rhizobia* and *Agrobacteria* from the *Rhizobiaceae* family in light of genomics. Systematic Applied Microbiology 38:287-291.

Page, S.R.G., Monthony, A.S., and Jones, A.M.P. (2021). DKW basal salts improve micropropagation and callogenesis compared to MS basal salts in multiple commercial cultivars of *Cannabis sativa*. Botany 99:269–279. doi: 10.1139/cjb-2020-0179.

Panday, D., Schumann, P., and Das, S.K. (2011). *Rhizobium pusense* sp. nov., isolated from the rhizosphere of chickpea (*Cicer arietinum* L.). International Journal of Systematic Evolutionary Microbiology 61:2632-2639.

Parte, A.C., Sardà Carbasse, J., Meier-Kolthoff, J.P., Reimer, L.C. and Göker, M. (2020). List of Prokaryotic names with standing in nomenclature (LPSN) moves to the DSMZ. International Journal of Systematic and Evolutionary Microbiology 70:5607-5612. doi: 10.1099/ijsem.0.004332.

Pest Management Regulatory Agency. (2022). [Accessed 2022 Oct 10]. www.canada.ca/en/health-canada/services/consumer-product-safety/pesticides-pest-management.html.

Pulawska, J. (2010). Crown gall of stone fruits and nuts, economic significance and diversity of its causal agents: tumorigenic *Agrobacterium* spp. Journal of Plant Pathology 92:S1.87-S1.98.

Puławska, J. (2016). Crown gall on stone fruit trees. In: ISHS Acta Horticulturae 1149: II International Workshop on Bacterial Diseases of Stone Fruits and Nuts, pg.37-42. doi: 10.17660/ActaHortic.2016.1149.7.

Puławska, J., Willems, A., De Meyer, S.E., and Sulec, S. (2012). *Rhizobium nepotum* sp. nov., isolated from tumors on different plant species. Systematic Applied Microbiology 35:215-220.

Punja, Z. K., Collyer, D., Scott, C., Lung, S., Holmes, J., and Sutton, D. (2019). Pathogens and molds affecting production and quality of *Cannabis sativa* L. Frontiers in Plant Science 10:1120. doi: 10.3389/fpls.2019.01120.

Punja, Z.K., and Holmes, J.E. (2020). Hermaphroditism in marijuana (*Cannabis sativa* L.) inflorescences – Impact on floral morphology, seed formation, progeny sex ratios, and genetic variation. Frontiers in Plant Science 11:718. doi: 10.3389/fpls.2020.00718.

Punja, Z.K. (2021). Emerging diseases of *Cannabis sativa* and sustainable management. Pest Management Science 77:3857–72. doi: 10.1002/ps.6307.

Punja, Z.K., Scott, C., and Lung, S. (2021). Several *Pythium* species cause crown and root rot on *Cannabis* (*Cannabis sativa* L., marijuana) plants grown under commercial greenhouse conditions. Canadian Journal of Plant Pathology 4(1):66-81. doi: 10.1080/07060661.2021.1954695.

Purkayastha, G.D., Mangar, P., Saha, A., and Saha, D. (2018). Evaluation of the biocontrol efficacy of a *Serratia marcescens* strain indigenous to tea rhizosphere for the management of root rot disease in tea. PLOS ONE 13(2):e0191761. doi: 10.1371/journal.pone.0191761.

Qu, L.J., and Qin, G. (2014). Generation and characterization of *Arabidopsis* T-DNA insertion mutants. Methods in Molecular Biology 1062:241-258. doi: 10.1007/978-1-62703-580-4_13. PMID: 24057370.

Ranaweera, K.K., Gunasekara, M.T.K., and Eeswara, J.P. (2013). Ex vitro rooting: a low cost micropropagation technique for tea [*Camellia sinensis* (L.) O. Kuntz] hybrids. Scientia Horticulturae 155:8–14. doi: 10.1016/j.scienta.2013.03.001.

Razumova, O.V., Alexandrov, O.S., Divashuk, M.G., Sukhorada, T.I., and Karlov, G.I. (2016). Molecular cytogenetic analysis of monoecious hemp (*Cannabis sativa* L.) cultivars reveals its karyotype variations and sex chromosomes constitution. Protoplasma 253(3):895-901. doi: 10.1007/s00709-015-0851-0.

Ream, W. (1989). *Agrobacterium tumefaciens* and interkingdom genetic exchange. Annual Review of Phytopathology 27:583-618.

Rech, E., and Pires, J. (1986). Tissue culture propagation of *Mentha* spp. by the use of axillary buds. Plant Cell Reports 5:17–18. doi: 10.1007/BF00269708.

Roy, A. T., Leggett, G., and Koutoulis, A. (2001). Development of a shoot multiplication system for hop (*Humulus lupulus* L.). In Vitro Cellular and Developmental Biology - Plant. 37:79–83. doi: 10.1007/s11627-001-0015-0.

Sabaratnam, Siva. (27 March 2019). “Pathogens of berry and vegetable crops in the Fraser Valley”. BISC 430 – Plant-microbe interactions. Simon Fraser University.

Sahoo, Y., and Chand, P. K. (1998).Micropropagation of *Vitex negundo* L., a woody aromatic medicinal shrub, through high-frequency axillary shoot proliferation. Plant Cell Reports 18:301–307. doi: 10.1007/s002990050576.

Schappe, T., Ritchie, D.F., and Thiessen, L.D. (2020). First Report of *Serratia marcescens* causing a leaf spot disease on industrial hemp (*Cannabis sativa*). Plant Disease 104(4):1248. doi: 10.1094/PDIS-04-19-0782-PDN.

Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012). NIH image to ImageJ: 25 years of image analysis. Nature Methods 9:671-675. doi: 10.1038/nmeth.2089.

Schroth, M., Weinhold, A., McCain, A., Hildebrand, D., and Ross, N. (1971). Biology and control of *Agrobacterium tumefaciens*. Hilgardia 40(15):537-552. doi:10.3733/hilg.v40n15p537.

Scott, C., and Punja, Z.K. (2020). Evaluation of disease management approaches for powdery mildew on *Cannabis sativa* L. (marijuana) plants. Canadian Journal of Plant Pathology 43:394–412. doi: 10.1080/07060661.2020.1836026.

Scott, M., Rani, M., Samsatly, J., Charron, J. B., and Jabaji, S. (2018). Endophytes of industrial hemp (*Cannabis sativa* L.) cultivars: identification of culturable bacteria and fungi in leaves, petioles, and seeds. Canadian Journal of Microbiology 64:664–680. doi: 10.1139/cjm-2018-0108.

Slusarkiewicz-Jarzina, A., Ponitka, A., and Kaczmarek, Z. (2005). Influence of cultivar, explant source and plant growth regulator on callus induction and plant regeneration of *Cannabis sativa* L. Acta Biologica Cracoviensia Series Botanica 47:145–151.

Sorokin, A., Yadav, N.S., Gaudet, D., Kovalchuk, I. (2020). Transient expression of the β-glucuronidase gene in *Cannabis sativa* varieties. Plant Signaling and Behavior 15(8):1780037. doi: 10.1080/15592324.2020.1780037.

Spencer, T.M., Gordon-Kamm, W.J., Daines, R.J., Start, W.G., and Lemaux, P.G. (1990). Bialaphos selection of stable transformants from maize cell culture. Theoretical and Applied Genetics 79:625-631.

Stachel, S.E., Nester, E.W., and Zambryski, P.C. (1986). A plant cell factor induces *Agrobacterium tumefaciens* vir gene expression. Proceedings of the National Academy of Sciences of the United States of America 83(2):379-383. doi: 10.1073/pnas.83.2.379.

Stack, G.M., Toth, J.A., Carlson, C.H., Cala, A.R., Marrero-González, M.I., Wilk, R.L., Gentner, D.R., Crawford, J.L., Philippe, G., Rose, J.K.C., Viands, D.R., Smart, C.D. and Smart, L.B. (2021). Season-long characterization of high-cannabinoid hemp (*Cannabis sativa* L.) reveals variation in cannabinoid accumulation, flowering time, and disease resistance. GCB Bioenergy 13:546-561. doi: 10.1111/gcbb.12793.

Suzaki, K., Yoshida, K., and Sawada, H. (2004). Detection of tumorigenic *Agrobacterium* strains from infected apple saplings by colony PCR with improved PCR primers. Journal of General Plant Pathology. 70:342–347. doi: 10.1007/s10327-004-0133-8.

The UniProt Consortium. (2021). UniProt: the universal protein knowledgebase in 2021. Nucleic Acids Research 49:D1.

Vlot, A.C., Dempsey, D.A., and Klessig, D.F. (2009). Salicylic acid, a multifaceted hormone to combat disease. Annual Review of Phytopathology 47:177–206. 10.1146/annurev.phyto.050908.135202

Wahby, I., Caba, J., and Ligero, F. (2013). *Agrobacterium* infection of hemp (*Cannabis sativa* L.): establishment of hairy root cultures. Journal of Plant Interactions 8(4):312–320. doi: 10.1080/17429145.2012.746399.

Wang, P.J., and Hu, C.Y. (1980). “Regeneration of virus-free plants through in vitro culture. In: Advances in biomedical engineering, Vol. 18, ed A. Fiechter A (Berlin, Springer Press), pg 61–99.

Wang, R., He, L.S., Xia, B., Tong, J.F., Li, N., and Peng, F. (2009). A micropropagation system for cloning of hemp (*Cannabis sativa* L.) by shoot tip culture. Pakistan Journal of Botany 41:603–608.

Wielgus, K., Luwanska, A., Lassocinski, W., and Kaczmarek, Z. (2008). Estimation of *Cannabis sativa* L. tissue culture conditions essential for callus induction and plant regeneration. Journal of Natural Fibers 5:199–207. doi: 10.1080/15440470801976045.

Yan, J., Li, Y., Han, X.Z., Che, W.F., Zou, W.X., Xie, Z., and Li, M. (2017a). *Agrobacterium deltaense* sp. nov., an endophytic bacteria isolated from nodule of *Sesbanis cannabina*. Archives of Microbiology 199(7):1003-1009. doi: 10.1007/s00203-017-1367-0.

Yan, J., Li, Y., Yan, H., Chen, W.F., Zhang, X., Wang, E.T., Han, X.Z., and Xie, Z.H. (2017b). *Agrobacterium salinitolerans* sp. nov., a saline-alkaline-tolerant bacterium isolated from root nodule of *Sesbania cannabina*. International Journal of Systematic Evolution and Microbiology 67:1906-1911.

Yancheva, S., and Kondakova, V. (2018). Plant tissue culture technology: present and future development. In: Pavlov, A., Bley, T. (eds) Bioprocessing of Plant In Vitro Systems. Reference Series in Phytochemistry. Springer, Cham. doi: 10.1007/978-3-319-54600-1_16.

Young, J.M., Kuykendall, L.D., Martinez-Romero, E., Kerr, A., Sawada, H. (2001). A revision of *Rhizobium* Frank 1889, with an emended description of the genus, and the inclusion of all species of *Agrobacterium* Conn 1942 and *Allorhizobium undicola* de Lajudie et al. 1998 as new combinations: *Rhizobium radiobacter*, *R. rhizogenes*, *R. rubi*, *R. undicola* and *R. vitis*. International Journal of Systematic Evolutionary Microbiology 51:89-103.

Young, J.M., Kuykendall, L.D., Martínez-Romero, E., Kerr, A., and Sawada, H. (2003). Classification and nomenclature of *Agrobacterium* and *Rhizobium* – a reply to Farrand et al. (2003). International Journal of Systematic and Evolutionary Microbiology 53(5):1689–1695. doi: 10.1099/ijs.0.02762-0.

Zahradnik, J., Nunvar, J., Parizkova, H., Kolarova, L., Palyzova, A., Maresova, H., Grulich, M., Kyslikova, E., and Kyslik, P. (2018) *Agrobacterium bohemicum* sp. Nov. isolated from poppy seed wastes in central Bohemia. Systemic Applied Microbiology 41:184-190.

Zhang, Y., Fan. W., Kinkema. M., Li, X., and Dong, X. (1999). Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the PR-1 gene. Proceedings of the National Academy of Science of the United States of America 96:6523-6528.

Zhang, X., Xu, G., Cheng, C., Lei, L., Sun, J., Xu, Y., et al. (2021). Establishment of an *Agrobacterium*-mediated genetic transformation and CRISPR/Cas9-mediated targeted mutagenesis in hemp. Plant Biotechnology Journal doi: 10.1111/pbi.13611.