# Optimisation and evaluation of masculinisation approaches to produce feminised seeds of industrial hemp (*Cannabis sativa* L.)



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**The University of Adelaide**

**Faculty of Sciences**

**School of Agriculture, Food and Wine**

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**Declaration**

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**Muhammad Nadeem**

31 October 2025

To my beloved daughters, Gulwareena and Hoorain:

I know I missed many birthdays, but I promise to make up for it.



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# Chapter 1

# General introduction

## Thesis summary/Abstract

Industrial hemp or hemp (*Cannabis sativa* L.) is a dioecious plant, with female plants being highly preferred due to their rich cannabinoid profiles. Commercially, feminised seeds (100% female) are highly sought-after, which are produced by crossing a female plant with a masculinized female plant. Numerous approaches are utilised to produce feminised seeds, but they are inconsistent, contradictory and lack detailed information. Therefore, the aim of this research was to tailor a robust protocol that is cost-effective, scalable and consistent.

Initially, different masculinising agents were screened to identify a robust masculinisation protocol. Among the tested treatments, daily application of 60 ppm colloidal silver (CS) demonstrated superlative masculinisation (100%), making it the most effective candidate. To enhance practicality, subsequent trials were performed to assess timing, dosage and number of applications. Three sprays of 200 ppm produced highest masculinisation (100%) and were further reduced to two sprays to reduce chemical input and overall operational costs. The two spray regime of 200 ppm achieved comparable efficacy (97%) and was identified as an optimal dosage.

The optimised protocol was further validated across two hemp cultivars MSD1 and Black Label (BL), revealing cultivar-specific variation: MSD1 exhibited 87% efficacy followed by BL (32%). Pollen health assessments in MSG1 and BL revealed no abnormalities and pollination efficiency was improved via manual intervention. Resulting feminised seeds exhibited excellent germination rates in both indoor and outdoor conditions, with no evidence of toxicity. Sex expression phenotyping was also performed in both conditions, BL under both conditions produced female plants. However, MSG1 produced few male flowers in indoor conditions. Metabolic profiling of feminised plants was performed in indoor conditions to evaluate THC levels and identify peak harvest time. Results revealed that all feminised seeds (BL) were under the compliance level, and highest cannabinoids were accumulated in the $8^{th}$ week of post flowering.

In the final part of the study, RNA-seq analysis was conducted to identify regulatory genes involved in CS-mediated sex modification. However, high levels of rRNA contamination underpowered the transcriptomic data for differential analysis. Overall, this study establishes a robust and scalable method for production of feminized hemp seed using CS. Further research should focus on validating the protocol in additional cultivars, assessing outdoor trials and elucidating the molecular pathways underlying sex reversal.

## Thesis Background

As a type of *Cannabis sativa*, industrial hemp, hereafter referred to as hemp, is one of the oldest cultivated crops, yet there is still limited scientific understanding of the species (Pollio 2016; Small 2015). Hemp was illegal for centuries due to an arbitrary classification and its close resemblance to marijuana or recreational cannabis (Small 2015). However, through updated classification and proven economic potential, it has now been legalized in many countries, including Australia (Burton et al. 2022; Small 2015). Hemp is a rich source of nutraceuticals, high quality fibre comprising bast and hurd and bioactive metabolites, as well as producing nutritious seeds for human consumption (Burton et al. 2022; Schultz et al. 2020). In fact, it has been shown to produce hundreds of metabolites including phytocannabinoids, phenolic compounds and terpenes, the majority of which are produced in female floral tissues (Mahlberg and Kim 2004; Small 2017; Potter 2014). These metabolites are highly sought after in the medicinal industry and possess multiple therapeutic applications (Rupasinghe et al. 2020).

Sex expression in hemp is defined as dioecious, with separate male and female plants determined by XY and XX sex chromosomes respectively (Shi et al. 2025). However, some hemp cultivars are monoecious, with separate male and female flowers on the same plant, and, in some cases, plants may become hermaphrodite (male and female organs in the same flower structure) during their life cycle (Punja 2020). This plasticity in sexual expression has been associated with a variety of stress mechanisms and exposure to phytohormones (Truta et al. 2007; Hall et al. 2012; Ainsworth 2000). Male plants are undesirable for medicinal purposes as seed production affects cannabinoid yield (Lubell et al. 2018; Lipson et al. 2021) Therefore, maximising female plants, with their associated floral structures, while minimising male plants within a population makes sound economic sense for commercial practices (Mejía et al. 2023; Flajšman et al. 2021).

Feminised seeds are utilised as they guarantee 99-100% female progeny (Timoteo et al. 2024) that can be particularly valuable for both medicinal and recreational producers. Such seeds can be produced when female plants are stressed to develop male flowers that produce female pollen - pollen which carries only X gametes (Lubell et al. 2018). Following pollination, the resultant seeds will be entirely feminised (XX) (Owen et al. 2023; Timoteo et al. 2024). This is a very unusual capability in the plant world, where only a few species including cannabis and cucurbit species, show this trait, and it can be manipulated (Hall et al. 2012; Martínez 2021), often called masculinisation, to produce feminised seeds, representing an economic advantage.

In cannabis production various stress-based methods have been developed to induce male flowers that carry only female pollen, thus enabling feminised seed production (Lubell et al. 2018; Chailakhyan et al. 1978; Mohan et al. 1979; Truta et al. 2007; Hall et al. 2012). However, most of these masculinisation techniques or protocols are unreliable, inconsistent, pose some safety risks (Owen et al. 2023), have been used only at small scales and reported on only anecdotally. Therefore, these protocols haven't been developed for commercial scale operations, nor have they been optimised to maximise the production of female seeds. Further, there is a lack of documented or scientifically tested details regarding downstream behaviour of plants growing from feminised seeds such as pollination capacity, seed yield, sex phenotyping and metabolic yield. Therefore, the work in this thesis concerns an investigation into forms of masculinisation that are robust/repeatable, safe, cost effective and scalable. Additionally, understanding the underlying mechanisms that drive this unusual capacity to produce female seeds would also progress our understanding of hemp sex regulation and provide possible alternatives for breeding strategies or targets.

**The objectives of this research were to;**

1) Evaluate various masculinising agents for their efficacy in converting female flowers into male floral structures that produce only female pollen. (Chapter 3)

2) Develop a robust masculinising protocol for hemp by optimising dosage and frequency of application. (Chapter 4)

3) Assess potency of the selected agent in different hemp cultivars. (Chapter 4)

4) Examine traits such as germination rates, seedling vigour, pollen viability, pollination capacity, seed yield and secondary metabolite production from plants grown from feminised seeds. (Chapter 4)

5) Better understand the genetic control of the changes that drive the production of male flowers producing female pollen on female plants. (Chapter 5)

**Thesis Structure**

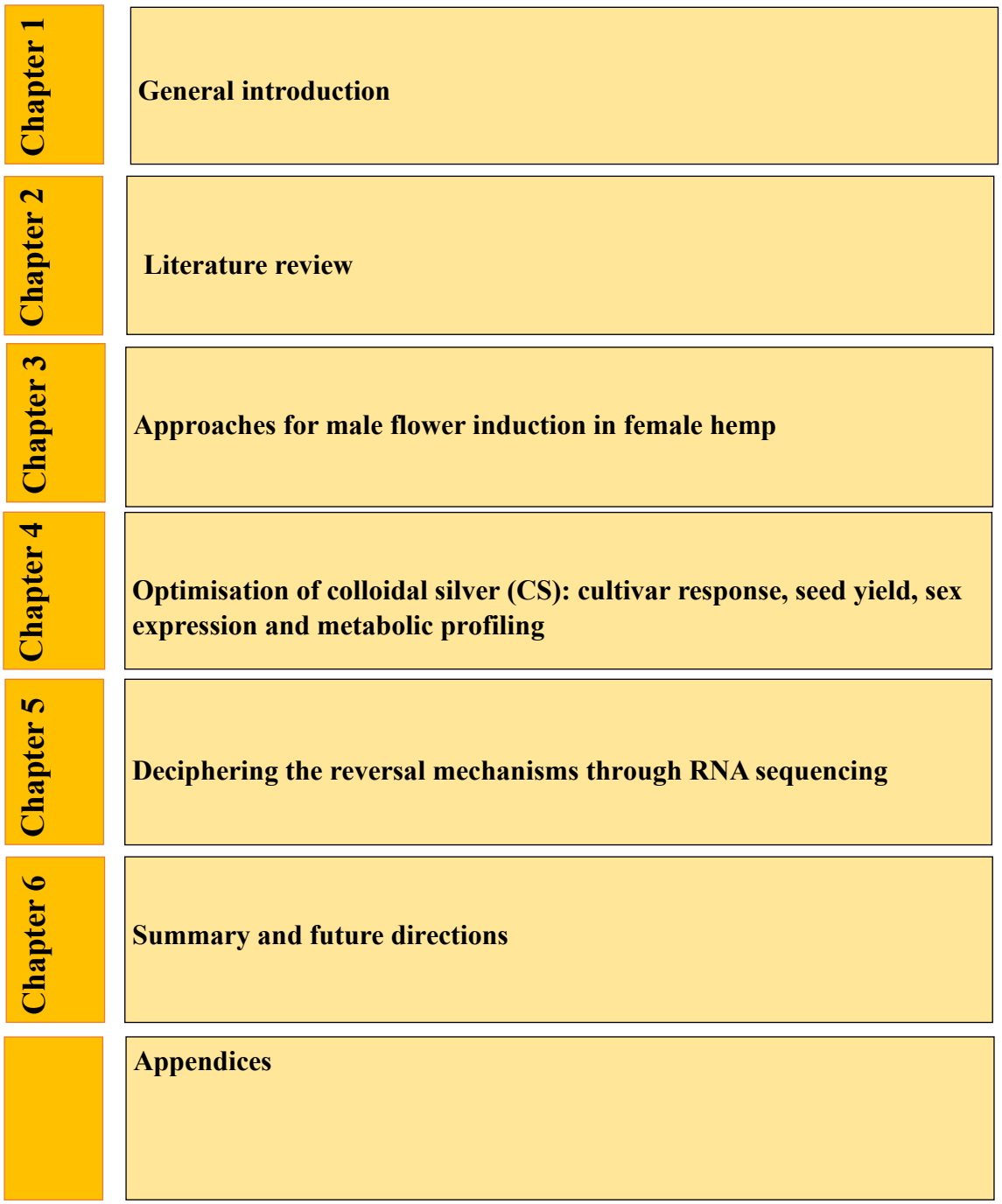


**Figure 1.** Graphic outline of thesis structure.

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# Chapter 2 Literature review

## 2.1 Cannabis taxonomy

*Cannabis sativa* L. is a flowering plant belonging to the family *Cannabaceae* and genus cannabis (Small 1973; Small et al. 1976). The taxonomic history of cannabis has been notoriously arbitrary and has significantly evolved over time (Pollio 2016; Koren et al. 2020). Initially, Carl Linnaeus classified cannabis as a single species. Later, Jean-Baptiste Lamarck suggested a second species that he called *Cannabis indica*, to signify a drug type plant (McPartland 2018). More recently, the genus *Cannabis* has been divided further into three sub-species, namely *C. indica*, *C. sativa* and *C. ruderalis* (McPartland 2018). But, due to the high degree of variability in the genus, monotypic classification has been adopted for *C.sativa* (Small et al. 1976). According to this classification, *C. sativa* is divided into two different chemotypes based on their differential Δ9-THC (Delta-9-tetrahydrocannabinol) profile (Small et al. 1973; Small et al. 1976; Koren et al. 2020). Industrial hemp or hemp refers to any species of the genus *Cannabis* having no more than 1% THC (dry weight), while varieties exceeding this level are recognized as medical, or recreational, cannabis (Strzelczyk et al. 2021).

## 2.2 Hemp applications

Hemp is an economical, sustainable and versatile crop (Karche 2019; Rehman et al. 2021). An imperative advantage is the fact that the entire plant is recoverable and holds myriads of applications in several fields (Figure 2.1). The stalk provides durable fibres that are used in fabrics, construction materials and bio composites (Jami, Karade, and Singh 2022; Ranalli and Venturi 2004; Shahzad 2012). Hemp seeds are a rich source of minerals, vitamins, edible proteins, unsaturated fatty acids and dietary fibres and are utilised in diverse food formulations and nutraceuticals (Burton et al. 2022; Pate 1999; Ross et al. 2000; Leizer et al. 2000; Crawford et al. 2012). Floral buds contain hundreds of bioactive compounds and are highly valued in the pharmaceutical industry (Crini et al. 2020; Naeem et al. 2023; Miyabe et al. 2022). Reflecting

this versatility, the industrial hemp market is likely to pass US$ 36 billion by 2030 (Kumar et al. 2024; Cerino et al. 2021), growing at a compound annual growth rate (CAGR) of 22%. The future of the hemp industry is indeed very promising (Jami et al. 2022); however, research is still in its infancy, and recognizing hemp's full potential will rely on continued integrative research and cross-industry alliances (Bernstein 2024).

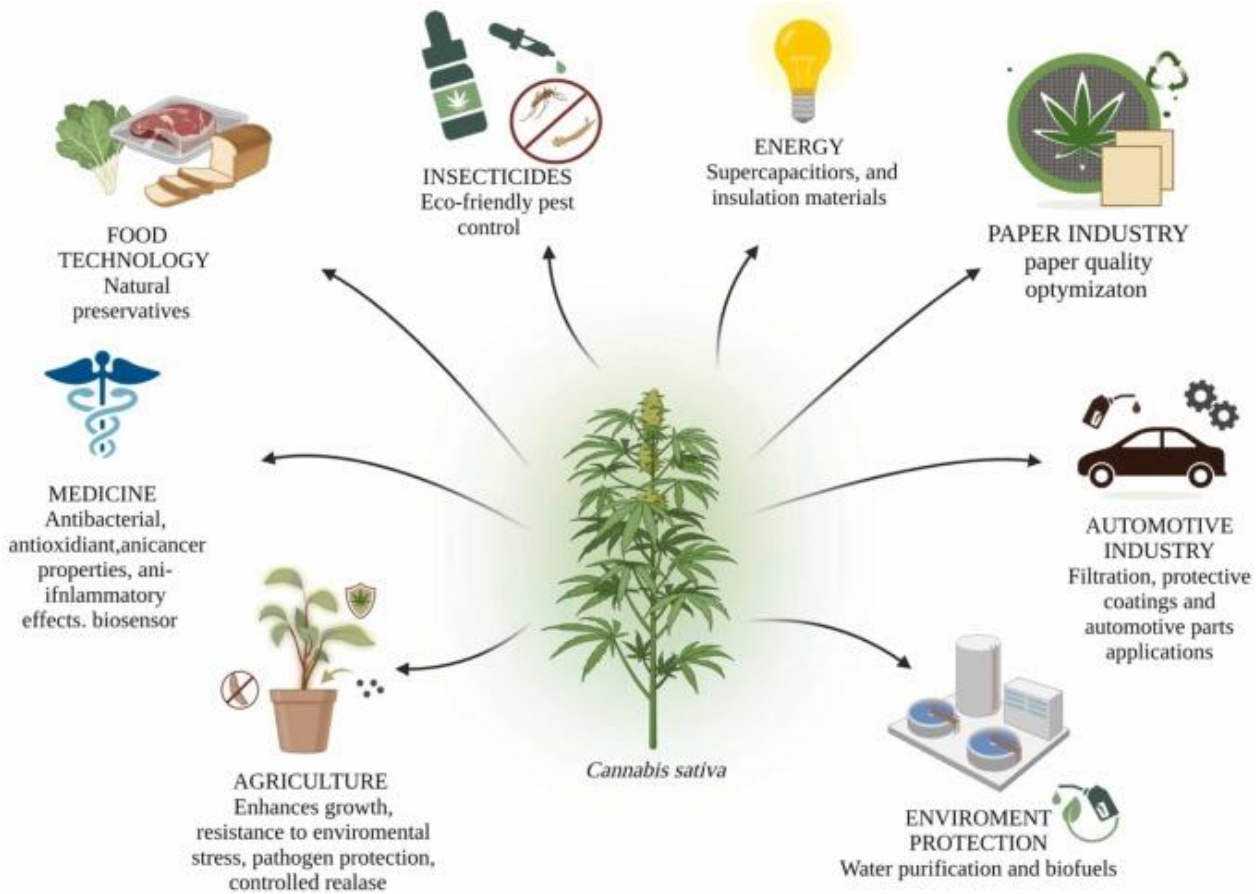


**Figure 2.1**. Uses of Hemp for different purposes. Reproduced from (Gorski et al. 2025)

## 2.3 Female plant anatomy

Cannabis naturally exhibits a dioecious nature, where male and female flowers are carried on separate plants (Clarke 1999). A key anatomical feature of female flowers is the presence of glandular trichomes on its surface (Alberti et al. 2025), which are abundantly distributed over the entire inflorescence, plus the sugar leaves (Figure 2.2) (Alberti et al. 2025). Glandular trichomes are of three types in cannabis: bulbous, sessile and stalked (Figure 2.3A-C) (Hammond and Mahlberg 1973). Stalked glandular trichomes are the largest and often considered the prime biochemical manufacturing hub of numerous cannabinoids, terpenes and flavonoids (Turner, Hemphill, and Mahlberg 1977; Mahlberg and Kim 2004; Livingston et al. 2020). These molecules are assembled at the base (source) of the trichome crown via specialised disk cells and then deposited (sink) in the overhead section of the structure (Figure 2.3D) (Livingston et al. 2020; Kim et al. 1991; Kim et al. 2003). The biosynthesis and accumulation of these metabolites depend mainly on trichome density, plant type, age, genotype, growing conditions and cultivation practices.

The most notable cannabinoids produced by hemp include cannabidiolic acid (CBDA), cannabigerolic acid (CBGA), cannabichromenic acid (CBCA), cannabinolic acid (CBNA), and cannabinodiolic acid (CBNDA) (Citti et al. 2018; Moreno et al. 2020). In fresh hemp tissues or raw products, most of these cannabinoids accumulate in the acidic form but may transform to their neutral form (CBD, CBG and CBC, etc.) via non-enzymatic decarboxylation reactions elicited by heat, aging or light (Filer 2022; Wang et al. 2016; Kim et al. 2022; Citti et al. 2018). Recently, interest in cannabinoids (excluding THC) has surged due to their strong anticancer (Pellati et al. 2018; Afrin et al. 2020), neuroprotective (Maroon et al. 2018), anti-inflammatory (Pellati et al. 2018) and analgesic properties (Capano et al. 2020). Since female plants possess a rich profile of these therapeutic metabolites, they are highly sought after for commercial applications and breeding practices.

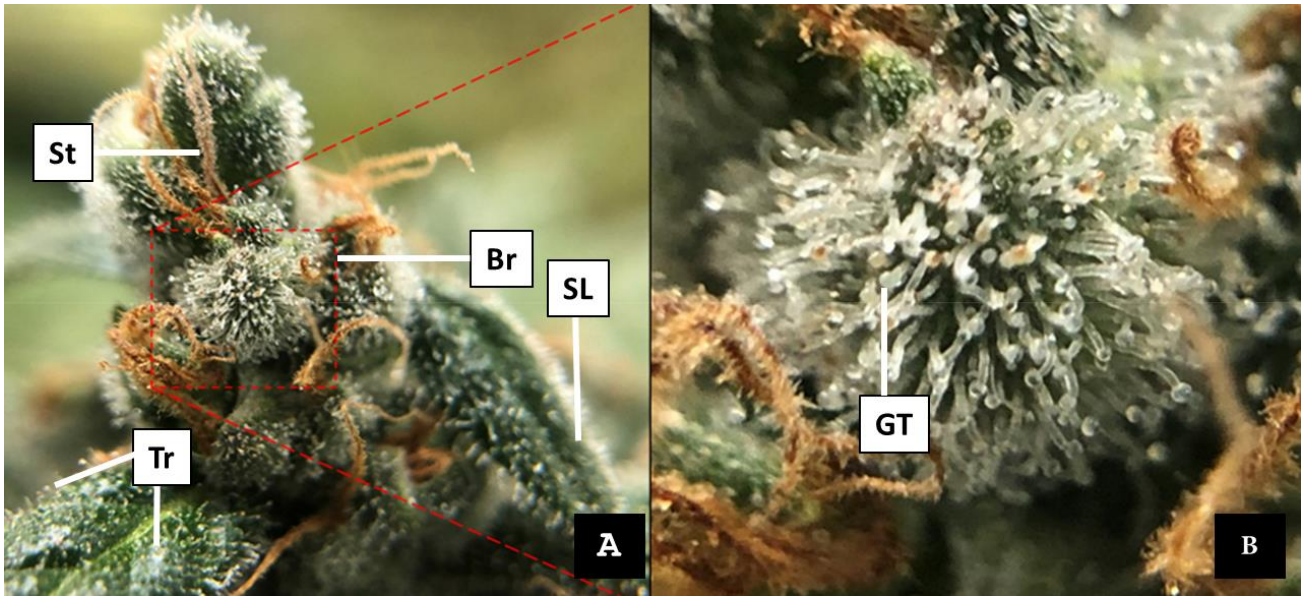


**Figure 2.2** Female inflorescence features. **A**) Female floral cluster showing: St;Stigma, Br: Bract, SL: Sugar leaves, and Tr: Trichomes (frosty appearance) **B)** Close up of a bract densely populated with glandular trichomes (GT). Adapted from (Oultram et al. 2021)

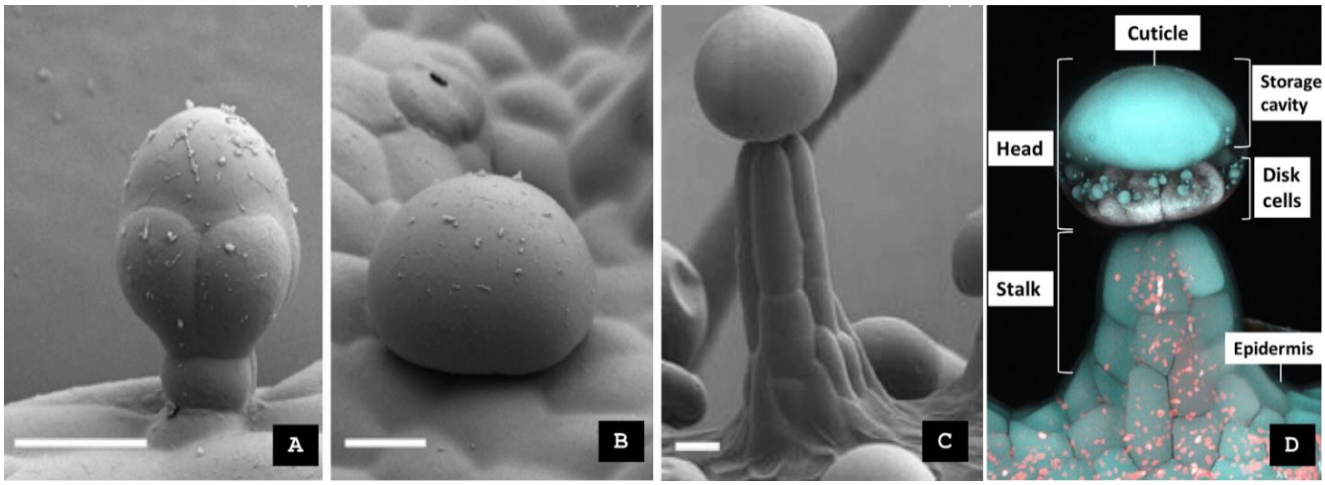


**Figure 2.3** Types of glandular trichomes found in cannabis. Cryo-SEM images of cannabis glandular trichomes, categorized as sessile **A**), bulbous **B**) and stalked **C**). Scale bars; 20 µm. **D**) Multi-photon microscopy closeup image of the stalked glandular trichome. Adapted from (Livingston et al. 2020).

### 2.3.1 Male plant anatomy

Male hemp plants are less bushy, taller and produce fewer glandular trichomes than females (Figure 2.4). Although male plants may be valuable in fibre crops, in cannabinoid-focused farming males are regarded as less useful and can be removed from the population in the early stages of growth (Lubell et al. 2018). Male plants can be distinguished from female plants via morphological, molecular and biochemical markers. These may be reliable but are impractical for large scale operations as well as being costly. Usually, plants can't be distinguished morphologically until flowering, although a recent method based on hyperspectral measurement has been developed which can distinguish males from females early in vegetative growth before flowering (Matros et al. 2023).

Generally, once gender is apparent, males can be culled from the population if desired (Lubell et al. 2018; Hall et al. 2012). A typical male plant can produce a huge amount of pollen – estimated at around 350,000 pollen grains per flower – and cannabis pollen can travel significant distances (Olejar et al. 2022). Given that hemp is wind pollinated, contamination from neighbouring crops can result in undesirable consequences in the following seed crop, including reduced oil yield and increased cannabinoid levels (Nimmala et al. 2024; Small et al. 2003). Successful pollination of females may therefore only require a limited number of strategically placed male plants, leading some growers to remove excess males (Nimmala et al. 2024). However, this is a very labour-intensive process and results in low yield per production cycle and wasted resources (DeDecker 2019; DiMatteo, et al. 2020). There is also the risk that failure to remove all males, or the appearance of hermaphrodite plants due to stress, can lead to pollination of medicinal cannabis crops. This can substantially reduce cannabinoid yield through the preparation for reproduction and the energy required for seed development (Lipson et al. 2021) as well as complicating downstream processing (DeDecker 2019; Olejar et al. 2022).

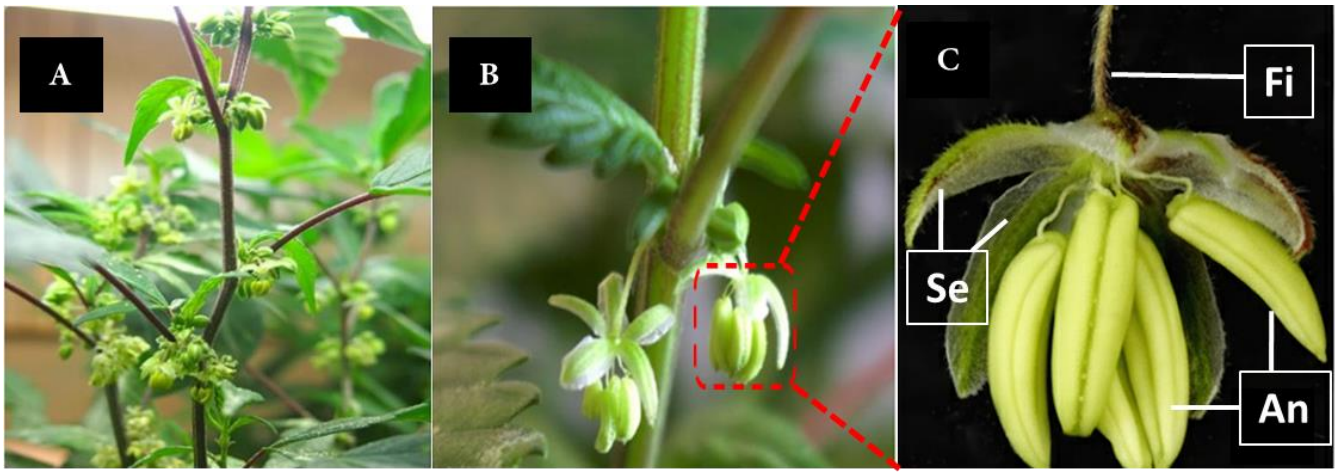


**Figure 2.4**. Morphological Features of male Inflorescences **A-B**). Male plant and flowers formed in constellations at leaf axils **C**). Closeup image of the cannabis male flower, Fi: Filament, SE: Sepals and An: Anther. Adapted from (Schilling et al. 2020).

## 2.4 Exclusive propagation of female lines

In breeding programs and across the cannabis industry female plants can be sourced via regular seeds, clones and feminised seeds (Owen et al. 2023; Timoteo et al. 2024; Chandra et al. 2017). Regular seeds are appropriate for large-scale farming but not ideal for medicinal purposes, as they produce both male and female plants at an approximate ratio of 1:1 (DiMatteo et al. 2020). Clonal propagation is an ultimate substitute to propagate guaranteed female lines (Clarke 1999), but is costly, time consuming and needs extra operational space (10-20%) for stocking cuttings and mother plants. Clonal propagules are also prone to pests and diseases if not managed properly (Page et al. 2021; Baiton 2024).

Tissue culture can mitigate this issue and can produce disease-free plants but it is even more cost restrictive and requires substantial skills (Monthony et al. 2021). It has been estimated that a clonal propagule costs US$5–10, while a tissue-culture propagated hemp plant can be up to 5-10 times more expensive (Kim et al. 2024; Baiton 2024; Monthony et al. 2021). Hemp farming is booming, with outdoor cultivation expanding and requiring hundreds of thousands of plants (Baiton 2024; Olejar et al. 2022), so clonal propagation will present an increased logistical and economic challenge as compared to seeds, which can be stored and transported much more easily (Baiton 2024). In addition, clones also require additional care at and following transplantation, to ensure survival, which further limits their potential applications.

The last choice is the use of hybrid feminised seeds, which offers a more robust platform for small or larger scale farming (Owen et al. 2023; Timoteo et al. 2024). However, quality and variability of such seeds have been a major challenge, combined with their greater up-front cost, so many growers have tended to grow clones or select regular seeds (Olejar and Park 2022; Alghanim et al. 2003; de Meijer et al. 1992). Potentially, feminised seeds could provide a cost-effective platform where a guaranteed population of female-only plants is required – for

example in medicinal cannabis production. However, there are few studies around the development of optimal feminisation protocols or the downstream effects on plant phenotype and performance in response to the stress profile that is necessary to achieve masculinisation followed by the production of female seeds.

## 2.5 Sex determination in Cannabis

*Cannabis sativa* is a diploid plant species with nine sets of autosomal and one pair of sex chromosomes (Petit et al. 2020). Male plants are heterogametic (XY) while females are homogametic (XX), with chromosome sizes from 80-100 Mbps (Moliterni et al. 2004a; Van Bakel et al. 2011; Divashuk et al. 2014). In hemp, sex expression is controlled by both genetics and environmental factors (Galoch 1978; Hall et al. 2012; Punja et al. 2020). Although each plant carries specific sex chromosomes, hemp sex expression is highly plastic (Truta et al. 2007) and can be altered via exogenous phytohormones and stressors (Truta et al. 2007; Lubell et al. 2018; Chailakhyan et al. 1978b; Mohan Ram and Sett 1979; Ainsworth 2000; Dellaporta et al. 1993; Heslop-Harrison 1957; Chailakhyan et al. 1978a; Ram and Sett 1979; Freeman et al. 2019). For instance, exposure to auxins and ethylene favour the development of female flowers (feminisation), while gibberellins and cytokinins stimulate the production of male flowers (masculinisation) (Truta et al. 2007; Hall, Bhattarai et al. 2012).

Plant hormones are thought to shape flower development by influencing the activity of key regulatory elements (Krizek et al. 2005; Yamasaki et al. 2003, 2005). The genes comprising the ABCDE model are of special interest as they have been shown to regulate the morphogenesis of both male and female reproductive organs in many plant species (Bowman et al. 2012; Stewart et al. 2016). In hemp, a recent study (Garcia-de Heer et al. 2025) shows that these genes also display clear sex-biased patterns of expression. For example, ethylene treatment suppresses

the activity of Class B genes, which are typically associated with stamen development, thereby directing flowers away from male traits and promoting female flower development.

Chemicals such as metallic ions, aminoethoxyvinylglycine and chloroethanephosphonic acid have been reported to alter sex expression in hemp (Mohan et al. 1979; Ram et al. 1982). Silver, cobalt chloride and lead ions prompt masculinisation, while copper and zinc induce feminisation (Chailakhyan et al. 1978a, 1978b; Ram 1982). The mode of action of these chemicals in plants is not yet fully deciphered; however, it is hypothesized that these agents might interfere with endogenous phytohormone levels or other regulatory elements involved in sexual organ differentiation (Truta et al. 2007). This biological flexibility results in the production of hermaphrodite plants or monoecious phenotypes (Moliterni et al. 2004b; Petit et al. 2020; Amaducci et al. 2015; Baldini et al. 2018). Monoecious phenotypes are often considered superior for hemp seed production, with a range of cultivars now available such as Futura 75, Fedora and Ferimon (Di Giacomo et al. 2023; Faux et al. 2016; Amaducci et al. 2008). They may also be utilised for production of feminised seeds and inbreeding (Faux et al. 2014).

## 2.6 Feminised Seed Production

Feminised seeds from treated parental plants provide 100% female progeny (Lubell et al. 2018; Sarath et al. 1979). Such seeds are produced by stimulating male flower structures, specifically anthers, on genetically female plants, which produce female pollen to allow pollination, or selfing, of the female flowers on the same plant (Baek et al. 2025). Masculinised flowers making female pollen contribute only “X” gametes and thus all developing seeds will carry “XX” gametes (Figure 2.5). This contrasts with conventional crosses between male and female plants contributing both “X” and “Y” gametes, resulting in varying sex expression (1:1) in the next generation (Lubell et al. 2018; Sarath et al. 1979; Owen et al. 2023). A diverse range of

chemicals have been reported to induce masculinisation followed by female pollen production and thus feminised seeds (Reviewed in Baek et al. 2025). These fall into two main categories in that they are either 1) plant growth regulators, including natural hormones like gibberellic acid (GA) or 2) chemicals which generate metal ions such as silver.

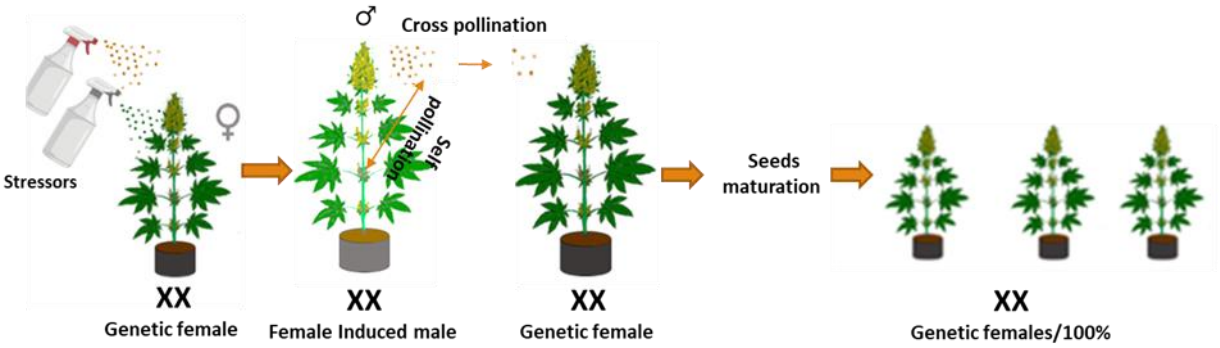


**Figure 2.5**: Schematic diagram of the production of feminised seeds.

## 2.7 Nanotechnology

Nanotechnology is an emerging discipline with many applications in various fields of science and technology (Fulekar 2010). This technology exploits nano-size structures with size ranges from 1–100 nm, known as nanoparticles (NPs) (Strambeanu et al. 2015). NPs exhibit distinctive physiochemical properties compared to their bulkier counterparts and are predicted to revolutionise many fields (Horikoshi et al. 2013), especially in agriculture where they hold a lot of promise and potential (Kale et al. 2021). In the last few years, metallic NPs have been the centre of attention in many fields of science (Schröfel et al. 2014).

Metallic NPs are mainly synthesized via chemical and biological approaches (Strambeanu et al. 2015). The former method is expensive, labour intensive and requires toxic chemicals for synthesis, which questions their safety and applicability in biological systems (Ijaz et al. 2020). The latter method of synthesis: the green method, is proven to be safer, eco-friendly and cost-effective (Iravani 2011). This approach uses biological resources such as plants, microbes or their derived products for fabrication of NPs (Marslin et al. 2018). The plant-based synthesis is considered more reliable, as microbes often present challenges such as pathogenicity, difficulties in culturing, and complex downstream processing (Nadeem et al. 2017; Nadeem et al. 2018).

In a simple process, bulk metallic salt is mixed with plant extracts, including foliage (Akhter et al. 2024), fruit (Singh et al. 2024; Yadav et al. 2022) and peel (Olabemiwo et al. 2021), where diverse metabolites (polyphenols, sugars and proteins) play dual roles in the fabrication process (Iravani 2011). They act both as a reducing agent, transforming bulk precursor salts into an elemental form, and as capping or stabilising agents and improve NP stability and stereochemistry (Cardoso et al. 2025). A schematic method of a possible green route is presented in figure 2.6. The green or biogenic route not only eliminates the need for toxic

chemicals but also produces nanoparticles with improved biocompatibility and lower toxicity (Srikar et al. 2016). The green route is therefore considered safer, making it highly desirable for agricultural applications (Marslin et al. 2018).

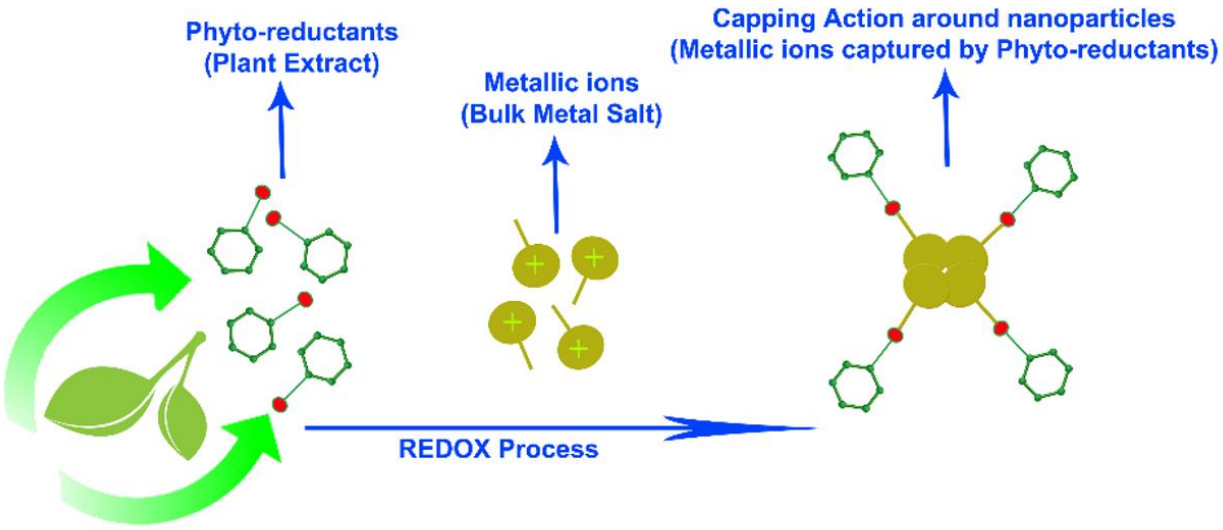


**Figure 2.6**. Schematic illustration of the green synthesis route for NP synthesis. Reproduced from (Marslin et al. 2018).

## 2.8 Silver nanoparticle interactions with plants

Silver nanoparticles (AgNPs) are valued for their broad-spectrum potential in a multitude of industries (Natsuki et al. 2015). Presently, they are utilised in medicine, textiles, cosmetic formulations and agriculture (Beyene et al. 2017). In agricultural practices, they have been used as a novel biocontrol agent, growth stimulant, floral preservative and gene delivery tool (Kale, Parishwad et al. 2021; Beyene et al. 2017). Although silver (Ag) is a non-essential element for plant growth, it is nonetheless readily taken up by plants (Tripathi et al. 2017). AgNPs mainly enter plants via cellular channels, stomata and plasmodesmata (Huang et al. 2022; Wang et al. 2023) and are expedited into cellular compartments via clathrin-mediated endocytosis or caveolin-mediated endocytosis, depending on their size (Tripathi et al. 2017; Huang et al. 2022; Sahay et al. 2010). Once internalised the NPs primarily accumulate in the cytoplasm, vacuole and middle lamella of the plant cell wall (Bao et al. 2016; de Paiva Pinheiro et al. 2021). A schematic diagram of NP entry and internalisation is shown in Figure 2.7.

AgNPs interact with the cell wall and elicit the production of diverse reactive oxygen species (ROS), including hydrogen peroxide ($H_2O_2$), hydroxyl radicals (•OH) and superoxide anions ($O^{2}\bullet^{-}$) (Jiang et al. 2014; Li et al. 2022; Bagherzadeh 2016). At the cellular interface elemental silver ($Ag^0$) is oxidised into silver ions ($Ag^0 \rightarrow Ag^+$) by oxidants such as $H_2O_2$ (Liu et al. 2010; Ma et al. 2012; Siddiqi and Husen 2022), and provoke ROS build up to create a **vicious cycle:** $Ag^0 + H_2O_2 \rightarrow Ag^+ + H_2O + e^-$ (de Paiva Pinheiro et al. 2020; de Paiva Pinheiro et al. 2021). Increased levels of ROS induce pores in the cell wall and optimises $Ag^0$ influx into the cellular matrixes, and soon after, the particles encounter the plasma membrane and induce even more supplemental ROS build up (de Paiva Pinheiro et al. 2021; Geisler-Lee et al. 2013; Anjum et al. 2016). All these interactions disrupt cellular functioning, block cell-cell communications and stimulate a cascade of stress signalling pathways (Srikar et al. 2016; Siddiqi et al. 2022; Baxter

et al. 2014). Overall, a summary of plant response to AgNPs and their effect on physiological, developmental and biochemical aspects is shown in Figure 2.8.

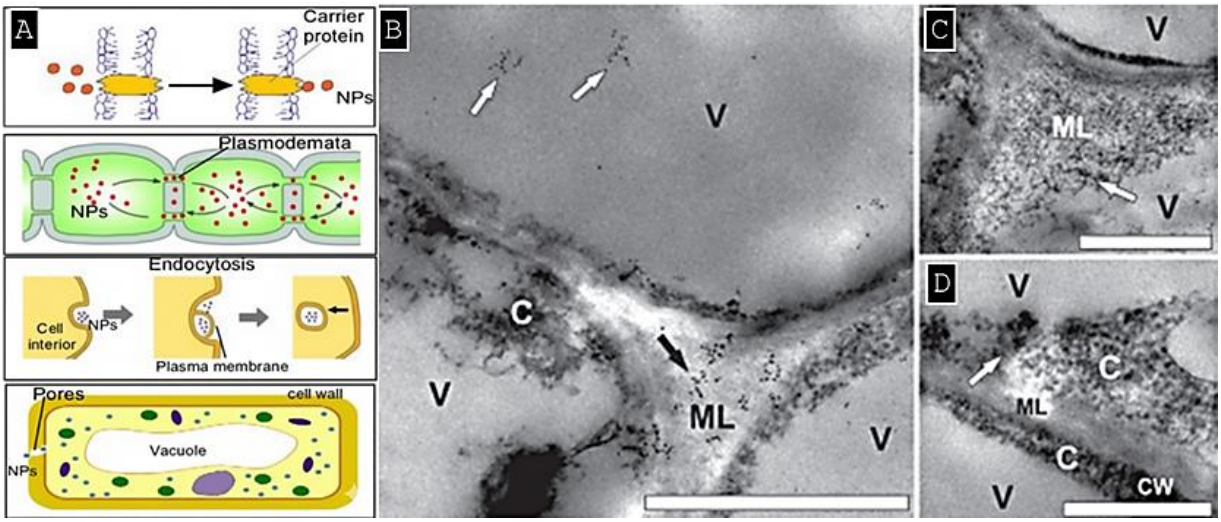


**Figure 2.7.** Nanoparticle entry and internalisation in plants. **A**) Probable routes of cellular uptake for NPs in the plant cell. **B-D**) AgNPs accumulation in vacuoles (V), middle lamella (ML), cytoplasm (C) and cell wall (CW). Scale bars: (b) 1µm; (c,d) 500 nm. Adapted from (Ghorbanpour et al. 2021; de Paiva Pinheiro et al. 2020).

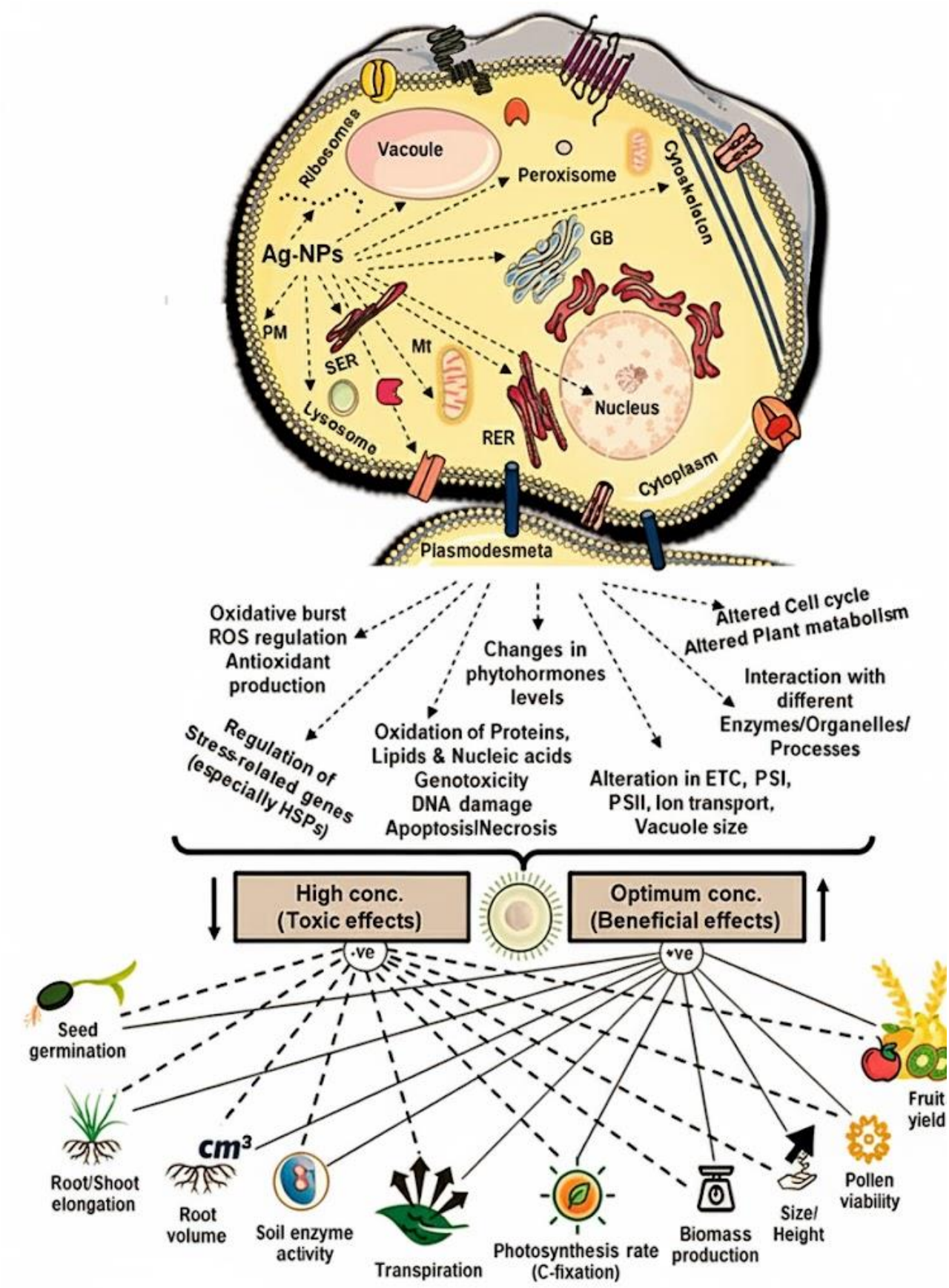


**Figure 2.8**. Overall plant response to Ag-NPs. Reproduced from (Siddiqi et al. 2022)

Comparative studies have shown that green synthesized silver NPs (GS-NPs) surpassed chemical counterparts in terms of stimulatory effects and exhibited reduced toxicity (Alabdallah et al. 2021; Galazzi et al. 2019). For instance, bio-mediated AgNPs have been described to improve soybean growth via stabilizing proteins and ATP levels, while chemical counterparts largely hampered those parameters (Mustafa et al. 2020). In a study by Zhang et al, (2008) it was documented that GS-NPs significantly improved growth in cucumbers while chemically synthesized AgNPs produced massive ROS increases and inhibited growth. Similar results have been noted for fenugreek, maize, onion and lettuce, where biogenic AgNPs improved overall growth, in contrast to the chemical equivalents which suppressed growth and provoked oxidative stress (Soliman et al. 2020). The improved performance of GS-NPs might be ascribed to their organic surface functionalization with organic groups, which allows stable release of Ag ions, reduces oxidative burden, and mediates more favourable interactions with plant tissues (Srikar et al. 2016; Hasan et al. 2022; Li, et al. 2012; Adamczyk et al. 2016). In contrast, chemically synthesized AgNPs often lack this biocompatible coating and tend to be more destructive (Marslin et al. 2018).

Overall, AgNPs use demonstrates a double-edged sword in agronomic practices: they have both synergistic and antagonistic effects on growth (Shukla et al. 2024). The border between beneficial and destructive effects is slender, which is further complicated by differential responses among plants, and effects of size and concentration (Gupta et al.2018; Salachna et al. 2019; Siddiqi et al. 2022). Nonetheless, the GS-NPs, owing to their ecofriendly synthesis, organic capping conjugates and minimal toxicity, provide a safer and more effective alternative to chemical approaches (Beyene et al. 2017) . However, their potential hasn't been fully harnessed, and studies are needed to further explore their biological potential and interactions with plants and toxicity, as they still contain silver.

## 2.9 Silver-induced (chemical) masculinisation in cannabis

Silver, either as a bulk salt ($AgNO_3$) or in the form of ions (silver thiosulphate/STS) has been reported to masculinise both medical and industrial hemp (Baek et al. 2025; Lubell et al. 2018; Sarath et al. 1979). Silver ions have long been known to impede ethylene perception in many plant species (Ram et al.1972; Yamasaki et al. 2011; Beyer Jr 1976; Saltveit et al. 1978; Veen 1979). Mechanistically, $Ag^+$ ions displace copper in the ethylene binding site of the receptor, permitting ethylene binding but restricting the subsequent conformational effects essential for ethylene signal transduction (McDaniel et al. 2012; Byers et al. 1972; Binder et al. 1999; Binder et al. 2007; Kendrick et al. 2008). Ethylene is a gaseous plant hormone and a key modulator involved in the development of female reproductive structures (Byers et al. 1972; Zhao et al. 2002; Papadopoulou et al. 2005; Kendrick et al. 2008; Wang et al. 2010). Hemp female flowers have been reported to exhibit higher transcription levels of ethylene related genes compared to male flowers, suggesting a role for ethylene in female sex expression (Garcia-de Heer et al. 2025). However, another transcriptomic study showed that silver induced conversion may be largely hormonal but not primarily dependent on ethylene (Adal et al. 2021). This indicates that sex reversal in hemp is a complex trait with multiple regulatory elements, plus their controlling factors, likely to be involved in hemp masculinisation (Faux et al. 2014).

Although the induction of masculinity by silver ions is largely believed to be hormonal (Faux et al. 2014; Adal et al. 2021), the role of ROS should not be overlooked. Silver ions trigger high levels of ROS production and have been shown to prompt oxidative stress in many plants (Bagherzadeh et al. 2016; de Paiva Pinheiro et al. 2021; Geisler-Lee et al. 2013). The oxidative burst damages membrane structure and triggers a cascade of stress responsive signalling pathways and hormones (Yuan et al. 2018). For instance, (Kaveh et al. 2013) reported that $Ag^+$ ions induced high levels of ROS accumulation, interfered with auxin, abscisic acid and ethylene expression and suggested that all are linked with hemp sex expression (Sun et al. 2017).

Recently, attention has shifted to silver nanoparticles (AgNPs), which appear to exert similar reproductive effects to STS (Flajšman et al. 2021). Their mechanism of action is still unclear, but it could be unrealistic to expect it to be the same as STS treatments. In fact, AgNPs not only release silver ions and induce oxidative stress (via ROS) but also exert mechanical stress via blocking cell-cell channels and causing aggregates in the cellular matrices (Kaveh et al. 2013; Angelini et al. 2022). Mechanistically, AgNPs seem to work through a dual mechanism: (1) the steady release of silver ions, permitting localized and persistent inhibition of ethylene receptors, and (2) the induction of oxidative stress via ROS coupled with mechanical stress (physical presence of NPs). For instance, (Angelini et al. 2022; Kaveh et al. 2013) reported that AgNPs provoked severe mechanical injury and elicited the release of salicylic acid, jasmonic acid and ABA. So currently in hemp it is unknown whether the observed hormonal changes are a direct effect of silver exposure or a secondary response to stress, particularly mechanical damage.

### 2.10 Gibberellins (hormonal induced masculinisation)

Gibberellins (GAs) are diterpenoid plant hormones associated with growth and reproductive organ development. Early studies reported that exogenous application of GA to genetically female plants triggered the appearance of male flowers among female inflorescences. High levels of endogenous GAs have also been found in genetic males supporting their key role in masculinisation (Adal et al. 2021; Faux et al. 2014), but studies of exogenous applications and their effects are only partly explored. In other plants such as spinach (*Spinacea oleracea*), the role of GA has been demonstrated and is mediated by DELLA proteins (West and Golenberg 2018). DELLA proteins act as negative regulators in gibberellin (GA) signal perception and transduction (Ito et al. 2018). When GA levels are low, DELLA proteins remain highly stable and repress the expression of male floral regulators (Class B genes) (Hou et al. 2008). In contrast, when the GA level rises (e.g., due to stress or exogenous supply), GA binds to GIBBERELLIN INSENSITIVE DWARF1 (GID1) and forms a GA–GID1 complex (Ueguchi-

Tanaka et al. 2005). The GA–GID1 complex then interacts with DELLAs and directs them for ubiquitination and subsequent proteasomal degradation. GA-GID-1 thereby revokes the repression of Class B genes, thus promoting male floral development (Sarwar et al. 2023). Their significance in male flower formation has been recently reported in hemp, but the relationship between GA and Class B gene regulation in this species remains unclear (Garcia-de Heer et al. 2025). In hemp, although GA's masculinising role mirrors that in other dioecious species like spinach, exogenous GA application studies are less abundant. Most current evidence comes from physiological observations, emphasizing a need for detailed experiments to define the developmental timing, tissue specificity, and interaction with other hormones such as ethylene.

### 2.11 Challenges with current feminisation approaches

Although STS is proven effective for hemp masculinisation there are several limitations that renders its application difficult for large scale operations (Owen et al 2023; Fitzgerald 2021). STS has a short shelf life and silver ions in the buffer precipitate over time, which might lead to inconsistent effects (Veen 1979, Owen 2023). In addition, most techniques also comprise numerous or repetitive applications, where the STS solution must be freshly made for each exposure to ensure stability, thereby increasing both exposure risk and operational costs (Owen 2023, Veen 1979, Baek et al. 2025). Although. silver at low exposure levels is generally considered safe (Ratte 1999; Steck et al. 2024). However, exposure to large doses or excessive intake can lead to cutaneous manifestations (argyria, bluish skin) and may also cause systemic effects, such as haemorrhage, pulmonary edema, bone marrow, renal, and hepatic necrosis (Fung et al. 1996; Ratte 1999; Wadhera et al. 2005). Silver has several oxidation states, such as Ag+, Ag2+, and Ag3+, with Ag+ being the most biologically active (Steck et al. 2024; Di Sotto et al. 2022). Since STS largely contains the most reactive form of silver ($Ag^{+)}$ it can pose a serious threat to humans and marine life (Quadros 2010).

The need to add wetting agents or adjuvants in some protocols (Owen et al. 2023), has been reported which further maximises the formulation steps and overall cost. STS application can also lead to inconsistent results. For instance, partial or even no masculinisation was reported when plants were sprayed with STS (Mejía-Londoño et al. 2023). STS has also been reported to induce pollen abnormalities (DiMatteo et al. 2020), developmental delays and deformation (Ram et al. 1982) in both hemp and medical cannabis. Consistent with literature studies, experiments in the Burton Laboratory also showed that STS had low masculinization efficacy and reduced growth in hemp (unpublished data; Alex James Clare, Honours thesis, 2020). Silver in ionic form is more destructive and acute ROS build up might not allow the plant to readjust, so leading to potential toxicity. Therefore, it is suggested that other robust, broad spectrum and safer alternatives should be tested for large scale operations.

AgNPs or colloidal silver (CS) based protocols have been shown to induce male flowers as efficiently as STS and can be used as an alternative (Flajšman et al. 2021). CS protocols are informally shared among growers and online, but their use lacks scientific detail and is mostly limited to cultivars of medical or recreational cannabis. CS is readily available over the counter and the purchase of pre-made solutions minimises operational handling, and the toxicity risk to human health. CS formulations provide a steady ion release in cellular matrixes which would allow plants to re-adjust and cope with the ROS burden and would produce healthy and fertile male flowers, which could be utilised for bulk scale seed production. However, their toxicity is still a concern to both humans and the ecosystem (Baek et al. 2025). The GS-NPs in this regard might mitigate these effects but their use has not been reported in hemp, therefore additional studies are required to assess their efficacy and potential. However, all AgNPs are still silver-based and raise concerns about exposure and toxicity. This makes it crucial to explore silver-free strategies for hemp masculinisation.

Novel ethylene inhibitors such as pyrazinamide (PZA) (Sun-Li, et al. 2017), plant hormones like gibberellic acid ($GA_3$) and mechanical stress can offer safer alternatives. However, these methods have not yet been thoroughly tested in hemp, and their effectiveness, stability, and reproducibility remain largely unknown.

### 2.12 Risks associated with feminised seeds

Feminised seeds provide a scalable platform for female plant production with reduced costs and labour requirements (Timoteo et al. 2024). However, they are not foolproof (Olejar et al. 2022). One of the leading risks is the potential for hermaphroditism via unexpected sex expression (Lubell et al 2018; Hall et al. 2012; Lipson et al. 2021). In normal female plants this phenomenon often results from external stressors, genetic instability and expression of latent hermaphroditic traits (Truta et al. 2007; Hall et al. 2012). Feminised seeds, like regular seeds, are not immune to such stressors and may carry predisposed traits, which can be expressed under optimal and suboptimal conditions (Olejar et al. 2022; Punja et al. 2020). Studies are limited on sex expression phenotyping in hemp but a recent study indicated that a farmer planted 14,000 acres of certified feminized high-CBD hemp and found that only 75% of the plants were true females, which revealed the hidden risk of using feminized seeds (Olejar et al 2022). It is essential to conduct a detailed assessment of seed quality. This assessment includes determining factors such as seed germination rate, seedling survival rate, and phenotypic sex expression (Timoteo et al. 2024).

Another challenging issue, for industrial hemp, is to maintain stable THC levels below the threshold limit (1%) (Olejar et al. 2022). Research studies have demonstrated that multiple environmental conditions, epigenetics and genetics can affect metabolite yield, including THC levels (Brousseau et al. 2021). THCA is synthesized from the precursor cannabigerolic acid (CBGA) and then catalysed by THCA synthase into its active form THC (Tahir et al. 2021).

Although, hemp doesn't produce high amounts of THCA it still carries the THCA loci and stress may trigger gene expression which then amplifies the THC level beyond legal limits, leading to "HOT" crops (high THC) and causing compliance issues with subsequent crop destruction and economic loss (Deventer 2018; Sunoj et al. 2023). This effect in the cultivar Yuma is most well-known in the hemp industry, where hemp plants spiked to 3.1% THC, compelling crop demolition (Deventer 2018).

The production of feminised seed relies on creating female homozygosity through selfing, which can increase the frequency of homozygous loci and expose recessive alleles, potentially causing inbreeding depression (Timoteo et al. 2024; Ainsworth 2000; Husband et al. 1996). Studies in dioecious plants closely related or similar in reproductive biology to hemp, including *Mercurialis annua* (annual mercury) and S*ilene latifolia* (white campion), showed varying but generally low to moderate inbreeding depression after selfing (Eppley et al. 2009; Teixeira et al. 2009). Inbreeding depression tends to be lower in typically outcrossing species like *Cannabis sativa* but still can result in instability and might affect phenotypic sex expression and cannabinoid profiles (Punja et al. 2020). While feminised seeds provide a practical solution for uniform female crops, their production poses an inherent genetic risk that requires auxiliary investigation and careful breeding strategies to preserve yield quality and cannabinoid potency.

## 2.13 Summary

Feminised seeds offer a scalable platform for female plant propagation. Hemp breeders have developed multiple protocols to produce feminised seeds. But such protocols lack scalability, may not be reliable, incur a cost and may carry an additional human health risk, leading to safety concerns. Therefore, a robust, safe, broad-spectrum and reliable protocol is needed to fulfil the growing needs of both the seed industry for production of both medicinal and recreational products In addition, the feminised seed should be tested to ensure seed quality, THC compliance and vigour, providing reassurance that any new protocols are robust and fit for purpose An additional benefit from these studies would be deciphering the mechanism of sex reversion which would progress our understanding of hemp sex determination and hormonal regulation.

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# Chapter 3

## Assessment of chemical and physiological stress methods for male flower induction in female hemp (*Cannabis sativa* L)

## 3.1 Introduction

Hemp is a dicoeous plant, meaning reproductive organs are carried on different unisexual, individual plants (Faux et al. 2014; Faux et al. 2013). Sex expression in hemp is a paramount trait as the female flower is arguably the single most valuable hemp component (Moliterni et al. 2004; Crini et al. 2020). Female flowers produce a wide array of specialised phytochemicals which contribute to the plant's distinctive pharmacological properties (Di Sotto et al. 2022; Asati et al. 2017). So far these include a wide array of identified compounds estimated at 42 polyphenols, 34 flavonoids, 120 terpenes and 125 cannabinoids, the majority of which are synthesized in the secretory glandular trichomes abundantly present on pistillate flowers (Radwan et al. 2021; Tanney et al. 2021; Livingston et al. 2020). In contrast, male flowers produce much lower levels of these metabolites and have a lower economic value in the medicinal industry but are valuable for fibre (Moliterni et al. 2004; Mansouri et al 2017). In addition, when male pollen fertilises female plants in a medicinal or recreational context, it can reduce their economic value (Meier et al 1998).

Currently, growers are likely to source cannabinoid-rich female chemotypes via vegetative propagation (i.e. cuttings). Clonal propagation is ideal for large scale production, but high cost, and stringent maintenance requirements remain the major challenges (Chandra et al. 2017; Timoteo et al 2024). In addition, somaclonal mutations in non-meristematic tissues build up over generations that may decrease plant vigour and deleteriously affect the metabolic profile (Malabadi et al. 2023; Trancoso et al. 2022). Tissue culture production of clonal plants can potentially mitigate these issues, but this approach is highly cost restrictive and impractical for large scale operations (Monthony et al. 2021). Recently, the industry has shifted towards developing or using feminised seeds, which can not only reduce costs and minimize disease risks but may also provide a reliable approach for large scale farming (Timoteo et al. 2024).

Therefore, developing feminised seeds could be very important to the floral hemp industry since only females are used in commercial production (Owen et al 2023).

Sex in hemp is primarily determined by genetics, with seeds carrying either XX (female) or XY (male) chromosomes (Malabadi et al. 2023; Moliterni et al. 2004). However, sex expression in hemp is highly plastic and can be altered with intervention of hormones or their antagonists (Fitzgerald 2021; Ram 1982; Hall et al. 2012). For hemp breeding practices and in the medicinal industry numerous protocols have been established to develop feminised seeds (Kim et al. 2024; Lubell et al. 2018; DiMatteo et al. 2020). However, conventional feminisation methods, relying on application of STS, have several limitations, including high cost, multistep formulations, the need for fresh stocks each time, safety concerns for human operators and they often produce inconsistent results (Kim et al. 2024; Ram 1982; Veen 1983). Thus, alternative approaches for sex manipulation and feminised seed production would be highly valuable in the hemp industry and more studies are needed to evaluate the efficacy of various exogenous agents and techniques for sex modification in industrial hemp.

More recently colloidal silver (CS), also alternatively called silver nanoparticles (AgNPs), has been reported to induce similar reproductive effects in hemp (Flajšman et al. 2021). However, the study lacks detailed information and was conducted on a medical cultivar. The CS approach offers advantages such as easy availability, strong stability and ease of operation, making it a more cost-effective and reliable alternative to STS (Baek et al. 2025). Albeit CS toxicity is still a concern for natural ecosystems and human operators and so alternative or less toxic forms of silver delivery should be investigated. Coating AgNPs with different organic functional groups has been reported to reduce toxicity in comparison to non-coated counterparts and could represent a suitable alternative (Sharma et al. 2014). Therefore, the aim of this present study was to screen various approaches including nanoparticles (commercially purchased CS and

green synthesized NPs made in the laboratory), hormones (Gibberellic acid), and pruning and novel ethylene inhibitors such as pyrazinamide (PZA) for the induction of male florets on female plants of industrial hemp, in order to assess their efficacy based on their masculinisation potential.

The decision to omit STS as a control, even though it is the current industry standard, was based on the fact that a large amount of documented evidence relating to its performance is already published and that this compound is toxic. Additional exposure risk to other users of the growth spaces was also deemed unacceptable. Under the South Australian licencing laws for cannabis cultivation, plant growth was restricted to nominated facilities at the Waite Campus which were shared by a number of researchers and students.

## 3.2 Materials and methods

### 3.2.1 Plant growth

All plants used in these experiments were propagated from healthy vegetative female plants of theMSG1 cultivar (Appendix 1 . Small (10-13 cm) branches were excised at a 45-degree angle below the 3rd node and immediately dipped in rooting hormone (indole butyric acid 3 g/L) (Gathera Hydroponics) and planted in a rockwool rooting cube (Grodan). Clones were maintained under a humidity dome with a 16/8 hour light dark cycle and sprayed daily with plain water. Once rooting was established, clones were transplanted into 4 L pots (substrate; coco coir) (Stealth Garden Supplies, Lonsdale, Australia) and placed in a controlled growth room maintained at 24-26 °C, 70% humidity and a 16/8 hour light/dark regime and supplemented with a mixture of COCOS A + B (3ml/L) (House, and Garden, Van De Zwaan, USA) and nitrogen (0.450 ml/L) (House, and Garden, Van De Zwaan, USA). The light regime was changed to 12/12 hours (dark/light) after 30 days of growth to induce flowering. At the flowering stage the humidity was reduced to 60%. Each pot was flushed with plain water after two doses of nutrients in both flowering and vegetative states to avoid nutrient burn.

### 3.2.2 Elicitor preparations

Colloidal silver was procured online from Discount Vitamin Express (https://tinyurl.com/2byt4ey7), while green synthesized nanoparticles were made as described in the results section. Tecnai G2 spirit transmission electron microscopy (TEM) at Adelaide microscopy was employed to characterise nanoparticles. Fresh stock solutions of Pyrazinamide (PZA) (Sigma-Aldrich) and $GA_3$ (Sigma-Aldrich) were made in distilled water at the required concentrations prior to each treatment. Amber-glass spray bottles were used for silver nanoparticles to avoid photodecomposition and separate spray bottles were used for all

treatments. Hemp female plants were thoroughly sprayed, targeting both sides of the leaves until the solution ran off completely.

### 3.2.3 Statistical analysis

All the experiments were conducted in six replicates. For statistical analysis, GraphPad Prism (Windows, v10.0) was used. Significant differences in mean values indicated by ANOVA were evaluated using Tukey's test ($\alpha = 0.05$). Data are presented as untransformed means and data are visualised with graph pad and Microsoft excel.

## 3.3 Results

### 3.3.1 Green synthesis and nanoparticle characterisation

Fresh orange peels were washed thoroughly and chopped into small pieces. About 100 grams of fresh washed slices were mixed with 300 ml of distilled water and boiled for two minutes. Then 100 ml of the filtered extract was mixed with 100 ml 0.1 mM $AgNO_3$. The solution was stirred for two hours at 60°C and subsequently centrifuged at 10,000 rpm for ten minutes. The supernatant was discarded, and the pellet residue was washed and centrifuged at 10,000 rpm three times with water to remove impurities. The pellet obtained was dried in an oven at 100°C, and a stock solution was prepared in distilled water for characterisation. Transmission electron microscopy (TEM) was used to compare a commercially produced CS solution with the green synthesised particles. The commercial CS particles had an average size of 5.96 nm, most of them were circular/oval shaped and they were uniformly distributed across the TEM field (Fig 3.1A). In contrast, the green synthesized nanoparticles were substantially bigger, exhibited a spherical shape but were less dispersed and a lot more clumped together, as shown in Figure 3.1B.

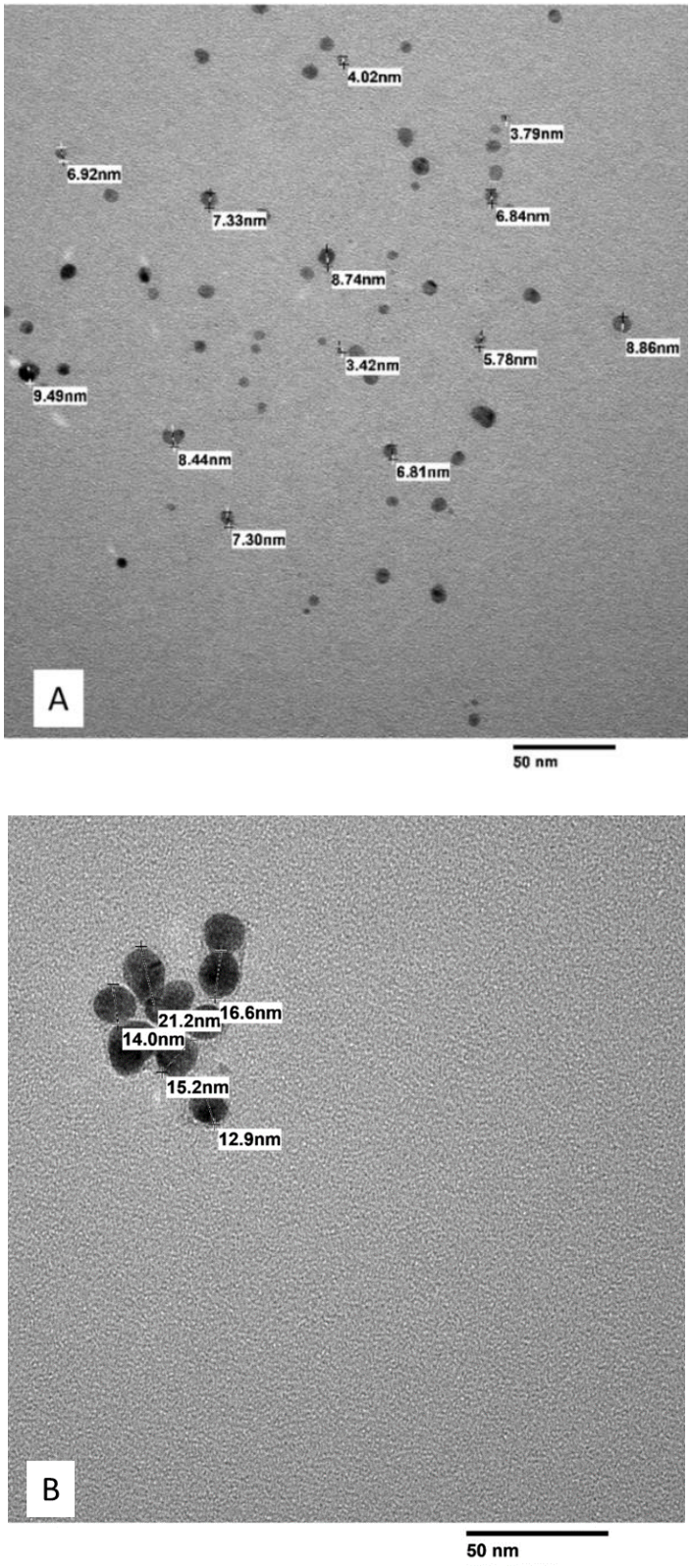


**Figure 3.1.** TEM micrographs of **A**). Commercial CS and **B**). GS AgNP's

### 3.3.2 Elicitor treatment

Seven different treatments were applied to evaluate the masculinisation potential of a range of chemical and physical measures (Table 3.1). For treatments T1-T4, 50 ml of stock solutions (30 and 60 ppm of CS and GS) were sprayed on whole plants (both sides of the leaves) once a day for fourteen days. Pyrazinamide was sprayed three times at three-day intervals over the whole plant on days 1, 3 and 6. $GA_3$ was applied to the shoot apex at a dose of 10 µg per day for five consecutive days, resulting in a total of 50 µg per plant, following the protocol of Ram et al. (1972). For T6, all fan leaves were removed by pruning. Group T0 represented the control where no treatment was made. All treatments were started on day 29 of vegetative growth and after 24hr the light regime was changed to 12/12hr to induce flowering.

**Table 3.1:** Treatments used to masculinise female MSG1 plants.

| Variant | Type and dosage | Number of applications |
|---|---|---|
| **T0** | Control (Distilled water) | 14 |
| **T1** | Colloidal silver (30 ppm) | 14 |
| **T2** | Colloidal silver (60 ppm) | 14 |
| **T3** | Green synthesised (30 ppm) | 14 |
| **T4** | Green synthesised (60 ppm) | 14 |
| **T5** | Pyrazinamide (1mM) | 03 |
| **T6** | Gibberellic acid (50ug total/plant) | 05 |
| **T7** | Pruning | |

For these optimisation experiments different variables were measured at day 35 post-flowering. This included counting the total number of inflorescences per plant and finding the masculinisation efficacy, calculated as the number of total inflorescences with male flowers divided by the total inflorescence number overall. A scale from 1 to 5 based on work published by (Lubell et al. 2018) was adopted to classify the percentage masculinisation where 1 = 0%

male flowers (all female inflorescences), 2 = 1% to 30% male flowers, 3 = 31% to 60% male flowers, 4 = 61% to 99% male flowers, 5 = 100% male flowers.

### 3.3.3 Masculinisation rate varied across different approaches

In comparison to the control plants (Figure 3.2A), colloidal silver (CS) applied at 60 ppm resulted in complete masculinisation with a score of 5 (Table 3.2), where all inflorescences developed into male flowers and no female flowers were apparent (Figure 3.2C). At CS 30 ppm, complete masculinisation was only noted on the terminal inflorescences, whereas partial conversion, with some remaining females, was observed on flowers on the middle and lower branches so these plants were scored at 4 (Figure 3.2B, Table 3.2). Green synthesized nanoparticles applied at both 30 and 60 ppm induced partial masculinisation where generally a mix of male and female flowers were observed on all inflorescences, also producing a score of 4 (Figure 3.3B and 3.3C, Table 3.2). All plants treated with silver nanoparticles (T1-T4) started to show signs of stamen development 14-16 days after the first treatments. All flowers in both treatments showed uniform development and began to open between 5-6 weeks after plant treatments started (Figure 3.4).

The $GA_3$ treated plants (T5) showed marginal conversion and only a few male flowers were induced, scoring 2 (Table 3.2). Unlike male flowers induced in T1-T4, those induced on $GA_3$ plants appeared only on the nodes and desiccated prior to opening (Figure 3.5). The remaining two treatments (T6-7) with applications of PZA or pruning didn't produce any male flowers and so were scored at 1. The untreated control plants only produced female buds which confirms that all plants used in these experiments were female and no male flowers were induced by other factors (Figure 3.2, 3.3 and 3.4A).

Overall, CS achieved 100% and 85% conversion at 60 and 30 ppm, respectively (Figure 3.6), whereas GS application achieved 70% and 76% conversion. The use of $GA_3$ only produced 10% male flowers where the rest remained female. Overall, the total number of inflorescences remained unchanged in all treatments, except for $GA_3$ where a significant reduction was noted (Table 3.2).

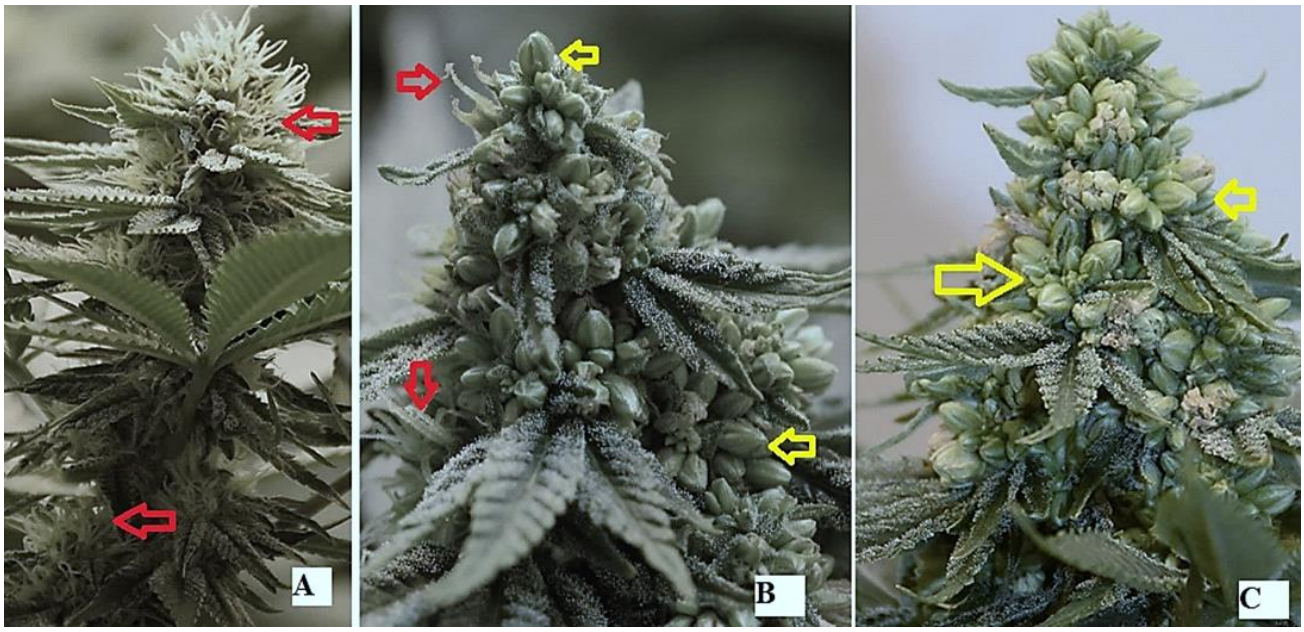


**Figure 3.2**: Images of treated female MSG1 plants **A**: Control, **B**: 30 ppm CS, **C**: 60 ppm CS (Yellow arrow indicates induced male, red arrow indicates the female stigma)

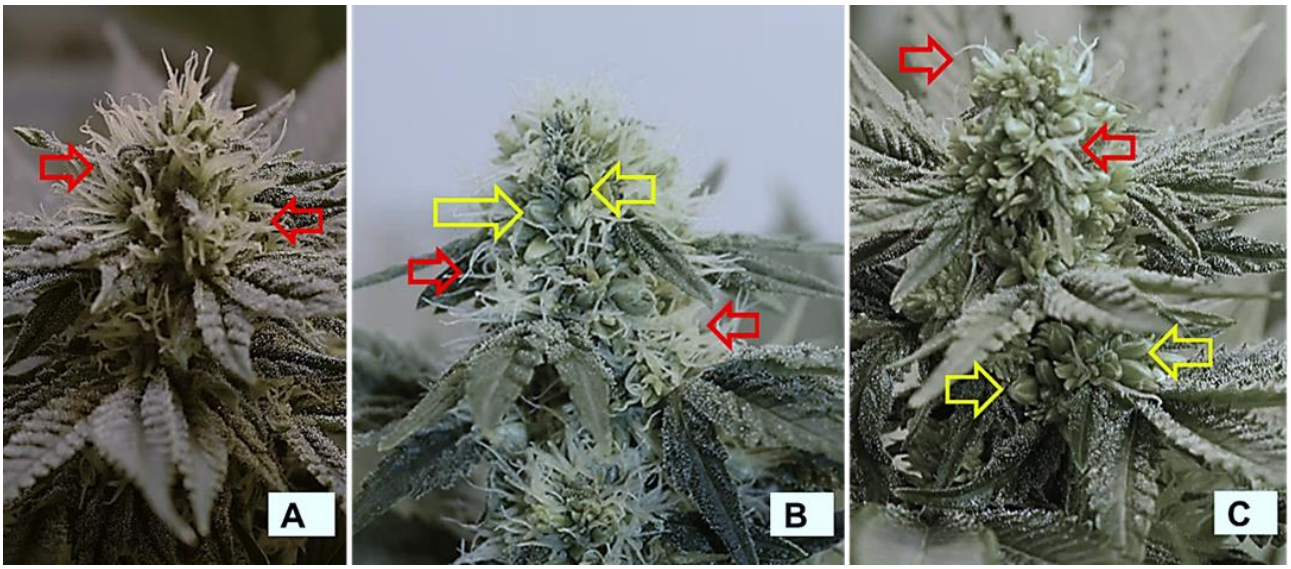


**Figure 3.3:** Images of treated female MSG1 plants. **A**) Control. **B**) 30 ppm GS. **C**) 60 ppm GS. (Yellow arrow indicates induced male, red arrow indicates female stigma)




**Figure 3.4:** Induced males at opening stage on plants treated with **A**) 60 ppm CS. **B**) 60 ppm GS.




**Figure 3.5**: Images of treated female MSG1 plants. **A**) Control. **B**) $GA_3$ treated plants. **C**) Male flower close-up. **D**) Dried male flowers. (Yellow arrow indicates induced male, red arrow indicates female stigma, blue solid arrow indicates dried male flowers)

**Table 3.2**: Influence of different treatments on number of inflorescences and degree of masculinisation of MSG1 plants (n=6). Means within a column with the same letter are not significantly different at the 5% level of probabolity (Tukey).

| Treatment | Number of all inflorescences | Masculinisation rating |
|---|---|---|
| Control | 94 [a] | 1 [a] |
| 30 ppm Colloidal silver (CS) | 93.5 [a] | 4 [b] |
| 60 ppm Colloidal silver (CS) | 94 [a] | 5 [c] |
| 30 ppm Green synthesised (GS) | 94.25 [a] | 4 [b] |
| 60 ppm Green synthesised (GS) | 93.75 [a] | 4 [b] |
| 50 µg Gibberellic acid (GA) | 51.7 [b] | 2 [d] |
| 1 mM Pyrazinamide (PZA) | 93.42 [a] | 1 [a] |
| Pruning | 94.67 [a] | 1 [a] |

Number of inflorescences and Masculinization rating. Masculinstion rating indicates: 1 = 0% male flowers (all female inflorescences), 2 = 1% to 30% male flowers, 3 = 31% to 60% male flowers, 4 = 61% to 99% male flowers, 5 = 100% male flowers.

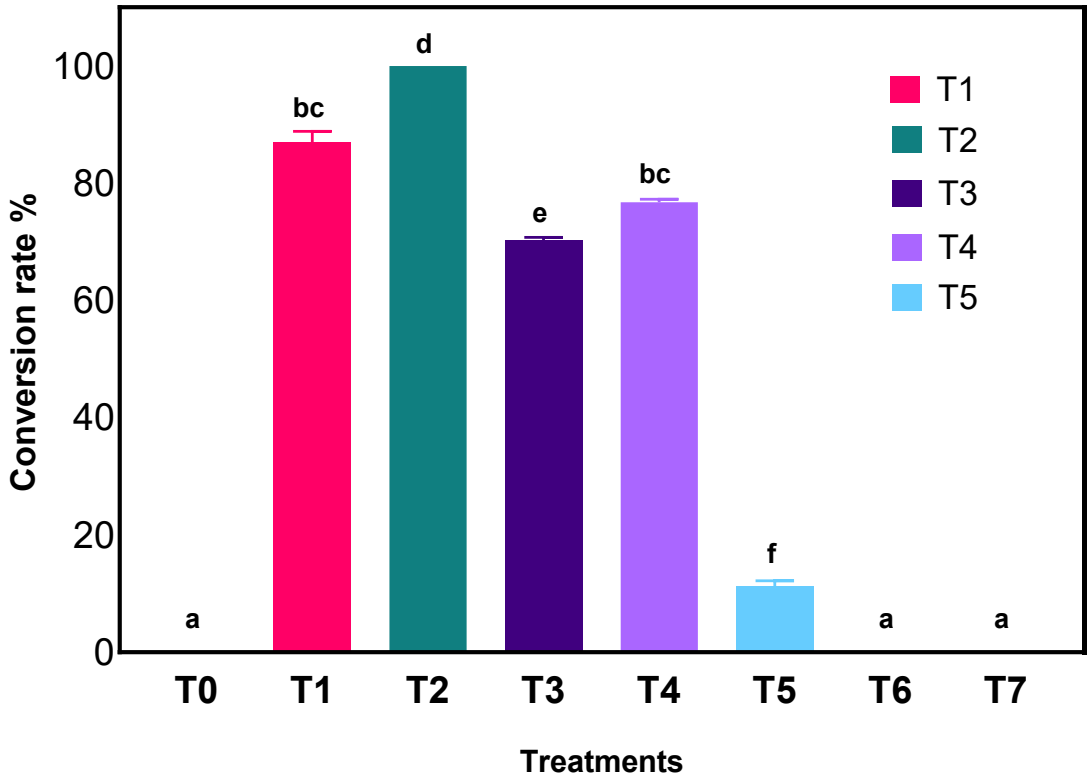


**Figure 3.6**: Percent of male flowers per total number of inflorescences from each different treatment (According to table 3.1). Mean values indicated by different letters are significantly different at the 5% level of probability (Tukey).

## 3.4 Discussion

A wide range of masculinisation approaches have been established in the hemp breeding and medicinal cannabis industries to produce feminised seeds (Owen et al. 2023). Consistently, chemical approaches have been effective and widely accepted commercially (Flajšman et al. 2021; Lubell et al. 2018). However, much of the information about chemical use is anecdotal, contradictory and lacks scientific details**.** The hemp seed industry is growing and could greatly benefit from robust masculinisation protocols to achieve bulk scale feminised seed production (Burton et al. 2022). It has been reported that CS is the most effective treatment (Flajšman et al. 2021) whilst GA is the most inexpensive method (Ram et al. 1972) but there is a lack of clear comparison and effectiveness for industrial hemp. In the quest to find a robust feminisation protocol, here we compared and evaluated the masculinisation efficacy of colloidal silver, green synthesized silver nanoparticles, $GA_3$, PZA and pruning for feminised seed production in industrial hemp.

Results indicated that CS induced superior masculinisation on MSG1 female plants, and a dosage dependent response was observed (Table 3.2 and Figure 3.6). These results align with the findings of (Flajšman et al. 2021), where CS induced male flowers on medical cannabis. However, these authors scored the masculinisation effects in a different way, choosing to count the number of male flowers produced per plant, thus reporting an average of ~379 male flowers per plant using a 30 ppm CS daily application for their chosen medical cannabis cultivar. Here, it was impractical to count the exact number of male flowers as so many were produced (Figure 3.2B and 3.2C). Instead, we chose to quantify the efficacy of each treatment in terms of estimated percentage conversion (Table 3.2), a metric that is adopted when precise counting is not possible. For this reason, the two studies are not directly comparable and there was also a

difference in application method. Flajsman et al. 2021 reported that CS was applied to whole plants, while we sprayed CS on both sides of the leaves. Hemp is a hypostomatous plant species (Haworth et al. 2024), with most stomata on the lower surface and spaying on both sides likely resulted in high absorption and efficacy.

Through the course of the experiments presented here, CS didn't prompt any phenotypic aberrations or developmental delays, effects that have been commonly reported in other published work (Lubell et al. 2018; DiMatteo et al. 2020; Ram and Sett 1979). For instance, STS was shown to induce effective masculinisation but significantly affected floral biomass, pollen viability and induced irreversible tissue necrosis (RAM 1982; Fitzgerald 2021). While in comparison with other milder masculinising agents such as aminoethoxyvinylglycine (AVG) (Fitzgerald 2021) and 1-methylcyclopropene (1-MCP) (Baiton 2024), CS achieved superlative masculinisation conversion. In this work, CS showed superior efficacy and minimal phytotoxicity suggesting that it can be used as a robust alternative for hemp seed feminization. Nevertheless, additional research is required to examine whether it is equally efficient for treatment across a range of cultivars.

The masculinisation efficacy achieved by treatment with GS nanoparticles was comparatively lower than that of CS and partial masculinisation was observed at both 30 and 60 ppm concentrations (Figure 3.3 and 3.6, Table 3.2). The superior efficiency of commercially manufactured CS might be attributed to its relatively smaller particle size (<10 nm) and homogeneous distribution (Figure 3.1A), since published studies suggest that small sized AgNPs are more efficiently internalised than bulker particles by plants (Wang et al. 2023). It is possible that clumps of GS NPs, as indicated by TEM, would be too large to enter via the stomata and so would remain on the leaf surface. This might be difficult to establish if the clumps were to be dislodged in the fixation

process necessary for TEM samples, although use of an environmental SEM may help. In addition, smaller NPs also exhibit higher surface area which allow more intimate contact with cellular receptors and membranes and provoke a broad range of responses (Yan et al. 2019; Yin et al. 2011). Although the mechanism of entry is unknown in hemp, we assume that a high influx of CS into the cellular compartments and better adherence to membranes provoked superior masculinisation. The GS particles were able to induce masculinisation, but further investigations are required to optimise synthesis protocols aimed at improving GS dispersion, to stop the clumping, and reducing particle size, which would be likely to enhance their consistency and effectiveness. There is a potential trade-off between the improved safety of the GS-NPs and the rate of masculinisation. Since each anther produces so many pollen grains, a 100% conversion frequency to male flowers may not be necessary, especially where either hand (indoor) or wind (outdoor) pollination can be easily achieved. If the synthesis could be improved and standardised, then the use of GS-NPs may be a viable future option.

The precise way in which AgNP's induce masculinisation is unknown in hemp, but the active ingredient ($Ag^+$) is known to impede ethylene signalling (Rodrıguez et al. 1999; Binder 2008). The role of ethylene in feminisation is well studied in other plants but its role in hemp remains unclear. For instance, Adal et al. (Adal et al. 2021), demonstrated that silver induced masculinity in cannabis is not primarily expedited by ethylene inhibition, but was majorly influenced by several hormones, indicating that the reversal involves complex hormonal cross talk rather than a single hormonal pathway. Silver nanoparticles have also been reported to provoke oxidative stress and mechanical damage in the cell wall (de Paiva Pinheiro et al. 2021). Such damage is often associated with reactive oxygen species formation and has been shown to trigger a cascade of stress hormones such as auxin, ABA and ethylene (Baxter, Mittler, and Suzuki 2014; Saed-

Moucheshi, Shekoofa, and Pessarakli 2014; Zandalinas and Mittler 2018; Huang et al. 2019). However, the ROS mediated responses and their role in hemp masculinisation remains unknown. Therefore, further studies are needed to fully decipher the masculinising effects and whether these changes are primarily due to hormonal antagonism or due to the secondary response from ROS or both.

MSG1 female plants treated with $GA_3$ showed very minimal masculinisation conversion. Treated plants developed non-viable staminate flowers, and a typical gibberellin effect (elongation) was observed (Figure 3.5). Although other authors have reported (Ram et al.1972; Chailakhyan and Khryanin 1978), that exogenous $GA_3$ has a strong masculinising potential in cannabis, we did not observe this here. The limited masculinisation response in our study might be attributed to cultivar difference or use of a different method of application. In one study (Chailakhyan et al 1978) they dipped roots in a GA solution, but this approach is impractical for breeding practices. Our findings are in accordance with the study of (Flajšmanet al. 2021), who reported that GA failed to induce significant numbers of male flowers in medical cannabis. This suggests that some cultivars might have a high level of insensitivity, or the exogenous GA has been degraded by endogenous GA-oxidases. Another possible explanation is the hormonal interplay, particularly between GA and ethylene (Li et al. 2017). Since GA-treated plants mostly retained a female phenotype, indicating that the masculinising effects of GA could have been overridden by female floral factors such as ethylene.

The antagonistic crosstalk between ethylene and GA is primarily mediated via DELLA proteins and is well demonstrated in plants (West et al. 2018). Ethylene stabilizes DELLA proteins and acts as a negative regulator of GA-responsive processes to maintain the female phenotype. We speculated that in the case of AgNPs, Ag+ might have repressed ethylene and DELLA accumulation, resulting in high expression of GA-

responsive genes and thereby promoting male floral characteristics, but this, or another pathway was obviously not triggered to any extent here as the masculinisation rate was very low.

Mechanical damage or physical conditions may also serve as stressors and can trigger hormonal fluctuations linked with sex determination (Braam 2005; Blake-Mahmud and Struwe 2020). Wounding or injury have been shown to result in high expression of jasmonic acid (JA) in plants (Blake et al. 2020). JA has been reported to support male sex expression and stamen development in multiple plant species (Acosta et al. 2009) and its crosstalk with other hormones such as ethylene and auxins is also well known (Song et al. 2013). To mimic these effects via mechanical stress, hemp plants were rigorously defoliated. However, contrary to our expectations pruning did not produce positive results with this cultivar, suggesting that mechanical stress or its induced signals may not be sufficient to stimulate male flowers.

Lastly, the novel ethylene inhibitor (PZA) treatment also failed to stimulate male flowers on hemp females. PZA is transformed to pyrazinoic acid (POA) and inhibits ethylene biosynthesis via blocking the precursor and final rate limiting enzymes (Sun et al. 2017; Zarei et al. 2024; Zarei and Ehsanpour 2023). The lack of effects could be associated with poor absorption, incorrect developmental timing, or suboptimal route of exposure, or this compound is just not effective.

Since the aim of the work in this chapter was to identify the most efficient treatment to induce masculinisation on female industrial hemp plants, the CS treatment at 60ppm was chosen to take forward to test on other cultivars, with supporting experiments to examine sex- expression stability.

## 3.5 Conclusion

In conclusion, among all the approaches assessed for inducing male flowers in MSG1 female hemp, commercial CS and green synthesized silver nanoparticles were the most effective, with CS demonstrating superlative efficacy. These findings highlight that CS can be used as a robust candidate for hemp seed feminisation. From an industrial perspective a CS induced male phenotype would be sufficient to produce bulk feminised pollen and seeds. Further research is now possible to optimise the protocol and reduce application frequencies to make the method as easy as possible to use. It would also be important to know if a streamlined protocol works with equal efficiency on a range of different cannabis cultivars. These investigations are presented in the following chapter.

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# Chapter 4

## Optimisation of colloidal silver (CS) protocol, cultivar response, seed yield, sex expression and metabolic profiling of feminised seeds

## 4.1 Introduction

In the hemp breeding and seed industry numerous protocols are utilised to produce feminised seeds (Baek et al. 2025). These approaches, including those summarized in Chapter 3, often require repeated applications (Flajšman et al. 2021) or large chemical dosages (Lubell et al. 2018), thereby increasing operational costs, chemical input, safety risks and complicating breeding operations (Fitzgerald 2021; Owen et al. 2023). Therefore, it is crucial to determine if fewer doses of sex-reversal chemicals can reliably induce male flowers in female cannabis for large-scale pollen production (Kim et al. 2024). The timing of application and the dose applied are factors (Timoteo et al. 2024) which are important to explore now that the most efficient agent has been identified through the research presented in the previous chapter.

In fact, much of the existing research and the protocols thereby developed, have only focused on the efficiency of the masculinisation process (Timoteo et al. 2024; Baek et al. 2025), while giving limited attention to the downstream outcomes (Baek et al. 2025). This gap is critical because the effectiveness of feminised seed production is not primarily determined by the successful induction of male flowers, but also by the viability, fertility and productivity of the pollen produced (Flajšman et al. 2021; Punja et al. 2020a). Despite its widespread application, systematic evaluations of how masculinisation treatments influence both male flower induction and seed productivity remain scarce (Baek et al. 2025; Hall et al. 2012). Until now only one study (Flajšman et al. 2021) has reported feminized hemp seed production through masculinisation, yielding a limited number of seeds (39 seeds per plant). However, with the hemp industry expanding rapidly (Huser 2020; Burton et al. 2022), such low output is inadequate for large-scale farming. Therefore, developing robust strategies to maximize yield and efficiently meet demand and supply is essential (Burton et al. 2022).

Cannabis is an outcrossing plant species and exhibits extensive genetic and phenotypic diversity, which often contributes to variable responses to diverse stimuli (Barcaccia et al. 2020; Small 2022; Kim 2024). For instance, certain cultivars have been shown to respond differently to drought (Mateva et al. 2024; Sheldon et al. 2021), salinity (Liu et al. 2016; Hu et al. 2019), and other biotic and abiotic stressors. Plasticity has particularly been observed in hemp sex expression, where some cultivars produce varying or inconsistent results (Baek et al. 2025; Lubell et al. 2018; Flajšman et al. 2021). This cultivar specific variation creates a complexity in the hemp seed feminisation process, as protocols that work efficiently in one cultivar may fail in another (Mejía-Londoño et al. 2023; Chailakhyan et al. 1979; Flajšman et al. 2021). Hence, it is imperative to develop a standardised approach across a range of cultivars to ensure reliable, reproducible and scalable production of feminised seed.

Feminised seeds only carry XX chromosomes, guaranteeing that they will produce only female progeny (Lubell et al. 2018; Green et al. 2001). However, feminised seeds may still inherit a genetic predisposition for hermaphroditism (appearance of males) which can be expressed under sub optimal (Baek et al. 2025) or even optimal growing conditions (Punja et al, 2020). Extrinsic stressors such as fluctuations in light cycles, temperature, nutrient or water imbalances, physical injury, pest and pathogen manifestations can trigger male flower formation in genetic females (Hall et al. 2012; Truta et al. 2007; Van der Werf et al. 1995; Malabadi et al. 2023). In the medicinal industry, hermaphroditic traits are decidedly undesirable, as they can negatively affect cannabinoid accumulation and complicate downstream processing (Malabadi et al. 2023; Lipson et al. 2021; Olejar et al. 2022). Stressors not only reduce floral quality and uniformity but can also elicit secondary metabolism changes and may lead to increased Δ9 THC (delta-9-tetrahydrocannabinol) levels (Hahm et al. 2025; Caplan et al. 2019). Higher levels of THC may increase commercial value for recreational cannabis; however, it can push industrial hemp plants above legal threshold levels (>0.3% dry

weight), creating regulatory compliance concerns (often called “Hot” plants). Consequently, due to these traits the quality and reliability of feminised hemp seeds are often questionable (Olejar et al. 2022), particularly if the parental plants or seed production process experienced stress. Such stress can also affect progeny growth and overall crop quality and the current understanding about the stability of sex expression and growth of feminised hemp seeds remains less explored. Therefore, this research aimed to optimize colloidal silver (CS) treatments to produce feminised hemp seeds by examining the timing and frequency of applications, while also evaluating their efficacy across different cultivars and assessing the effects on pollen traits, seed set and the sex expression of feminised seeds. In addition, the total cannabinoid levels, including THC accumulation, were analysed to determine peak harvest stage and to identify any potential spikes that could lead to non-compliance with legal THC limits.

## 4.2. Materials and methods

The experiments were carried out using female plants (clones) of three breeding cultivars of industrial hemp, namely MSG1, MSD1 and Black Label (all obtained originally from Mountain Strong Hemp, Colorado, USA). Certificates of analysis are provided in Appendix 1. Each cultivar is genetically unique and has a particular cannabinoid profile, rich in either CBD or CBG, and all are low in THC. All plants were propagated, maintained and grown as previously described in Chapter 3.

### 4.2.1 Pollen health assessment

Pollen quality was assessed using the fluorescein diacetate (FDA) stain as described by (Flajšman, Slapnik, and Murovec 2021). A stock solution of FDA was prepared in acetone at a concentration of 2 mg $mL^{-1}$ and stored at 4°C until use. Fresh pollen from MSG1 and Black Label generated using protocol T3.2 (CSB) was collected at anthesis (day 35) and stained with FDA at a final concentration of 1 ug/ml. After 15 minutes, stained pollen grains were examined using a fluorescence microscope (Nikon Ni-E), with filter sets (excitation: 455–485 nm; emission: 500–545 nm) for detection of green fluorescence. Pollen viability and fertility was also assessed in terms of pollination capacity and seed yield using both self and manual pollination of plants. In the later approach, fresh pollen from a single masculinised female was collected from dehisced anthers and brushed onto four female flowers at the $35^{th}$-$36^{th}$ days of flowering. Each experiment was conducted at a separate time to avoid pollen drift. Seed yield was counted after maturation and harvest (day 90).

### 4.2.2 Germination rate

Fresh feminised seeds of MSG1 (MSG1-F), Black label (BL-F) and non-feminised seeds were grown in a controlled environment room (CER) maintained at 24-26 °C, 70% humidity and a 16/8-hour light/dark cycle. Coca coir (House, and Garden, Van De Zwaan, USA ) was used as

the substrate and plantlets were sprayed from above with water every day until true leaves appeared. Germination rate was evaluated after one week and data was recorded using the following equation;

$$\textbf{Germination rate} = \frac{\text{germinated plants}}{\text{total seeds}}$$

### 4.2.3 Sex phenotyping and progenies characterisation

Feminised seeds of MSG1 (MSG1-F), Black Label (BL-F) and non-feminised MSG1 (control) (n=40) were grown and maintained in indoor growth cabinets under the following conditions: 24-26 °C, 70% humidity and a 16/8-hour light/dark. Two weeks after planting the nutrient supply was gradually increased by watering with COCA a/b and nitrogen (weeks 1-2 = plain water, week 2 = ¼ strength, week 3 = full strength). After six weeks of vegetative growth, the light cycle was changed to 12/12 light/dark hours to induce flowering. For outdoor phenotyping, germination was not efficient after direct sowing and so seeds were germinated indoors under the conditions described above. Two weeks after germination plantlets were gradually acclimatised to outside conditions over seven days. On day 1, hemp plantlets were exposed to sunlight for 2 hours and the exposure time was increased by one hour each day, reaching a total of 7 hours on day 7. After three weeks all plantlets were moved into an outdoor facility and planted in soil. Sexual phenotype was recorded over a three-month period (16 Dec 2024–16 Mar 2025). In addition, progeny characteristics including plant height, inflorescence number and branch number were also evaluated in feminized (MSG1-F) and non-feminized (control/MSG1) under indoor conditions.

### 4.2.4 Sample preparation and Cannabinoid analyses

Terminal floral buds (colas) were collected for cannabinoid analysis and placed in a paper container and subsequently dried in a Memmert UM400 oven at 37°C for 72 hours. The dried

floral tissues were enclosed in a moisture resistant (plastic) bag and stored in a cold room at 4°C until the extraction process. The dried floral tissues were then finely ground using a mortar and pestle. For extraction, briefly 50 mg of ground sample was mixed with 2ml of HPLC grade ethanol and each sample was sonicated for 15 minutes. Each sample was centrifuged at 15,000 rpm for five minutes at room temperature. Subsequently, the supernatant was collected and centrifuged again, and the final mixture was transferred into HPLC vials. A 2 µL clear extract was injected into the HPLC system for analysis . This protocol closely follows the methodology outlined in Phillips et al. (2025). Chromatographic analysis was performed on an Agilent 1260 Infinity LC system with a diode array detector at 240 nm, using a Kinetex® C18 column (50 × 2.1 mm, 2.6 µm, 100 Å). The mobile phase consisted of buffer A (0.1% formic acid in water) and buffer B (0.1% formic acid in methanol). The gradient began with 60% buffer B, increasing linearly to 85% over 10 minutes, then to 100% within one minute, held for two minutes, and re-equilibrated back to initial conditions for three minutes. Runs were carried out at a flow rate of 0.5 mL/min, injection volume of 2 µL, column temperature of 50 °C, with a total 16 minutes of runtime.

### 4.2.5 Statistical analysis

Significant differences in mean values indicated by ANOVA were evaluated using Tukey's test ($\alpha = 0.05$). All statistical analyses were performed using statistical software program R graph pad (10.4.1 Windows). Data are presented as untransformed means ± SE, and graphs were made via graph pad and Microsoft excel.

## 4.3. Results

### 4.3.1 Masculinisation of MSG1 plants with colloidal silver

MSG1 female plants were exposed to different doses of colloidal silver on the schedule shown in Table 4.1. For applications T1, T2 and T3 each plant was sprayed with 50 ml of CS solution of varying concentrations on days 1, 7 and 14. For T3.1 the 200-ppm dosage was also tested as a single application made on day 1 (CSA) or on days 1 and 7 (CSB) to assess the effects of timing and number of applications on masculinisation efficiency. For each treatment six replicates (clonal plants) were used and for the control group the same amount of distilled water was used instead of the CS solution.

**Table** 4.1. Schedule of CS applications on MSG1 plants.

| Treatment | Time | Number of applications | Concentration (ppm) |
|---|---|---|---|
| **T0** | | | Control |
| **T1** | Day 1_7_14 | 3 | 60 |
| **T2** | Day 1_7_14 | 3 | 100 |
| **T3** | Day 1_7_14 | 3 | 200 |
| **T3.1 (CSA)** | Day 1 | 1 | 200 |
| **T3.2 (CSB)** | Day 1_7 | 2 | 200 |

Across treatments T1 to T3, the 200-ppm application showed the highest masculinisation efficiency, where all inflorescences developed into male flowers and no female stigmas were observed on any inflorescences (Figure 4.1E). Partial masculinisation was observed when 100 and 60 ppm were used (T2 and T3). At 100 ppm male flowers developed on both terminal and lateral branches

(Figure 4.1D), whereas at 60 ppm, male florets were only found on lateral branches (Figure 4.1B-C). By counting the actual number of male flowers per total inflorescence, it was determined that 200 ppm produced 100% conversion, while 100 and 60 ppm showed 59 and 83% conversion respectively (Figure 4.3). Overall, the results indicated that higher CS concentrations increased masculinisation rates but did not affect total inflorescence number and overall growth (Table 4.2).

In the subsequent 200 ppm single (T3.1; CSA) and double dose applications (T3.2;CSB), results showed that a double dose also induced maximum masculinisation (97%) (Figure 4.3), where all inflorescences developed mostly male flowers with only a few stigmas (ranging from 1 to 4) observed on some buds (Figure 4.2C). The single dose variant (CSA) stimulated partial masculinisation (30%) (Figure 4.3), where male flowers only developed on lower branches (Figure 4.2A and B). For both treatments CSA and CSB, the total number of inflorescences remained unchanged (Table 4.2).

Plants transitioned from initial flowering to full flowering at the third week from the start of induction and a uniform transition was noted in all treatments. Similarly, no atypical toxicity or abnormality was observed in any of the treatment groups. Male flowers were first observed 14-16 days after the first spray application and were clustered together in the floral buds. Pollen shedding started after fifth week of post flowering in all groups. However, for T3 (200 ppm with three sprays) overcrowding was observed and some male flowers remain unopened.

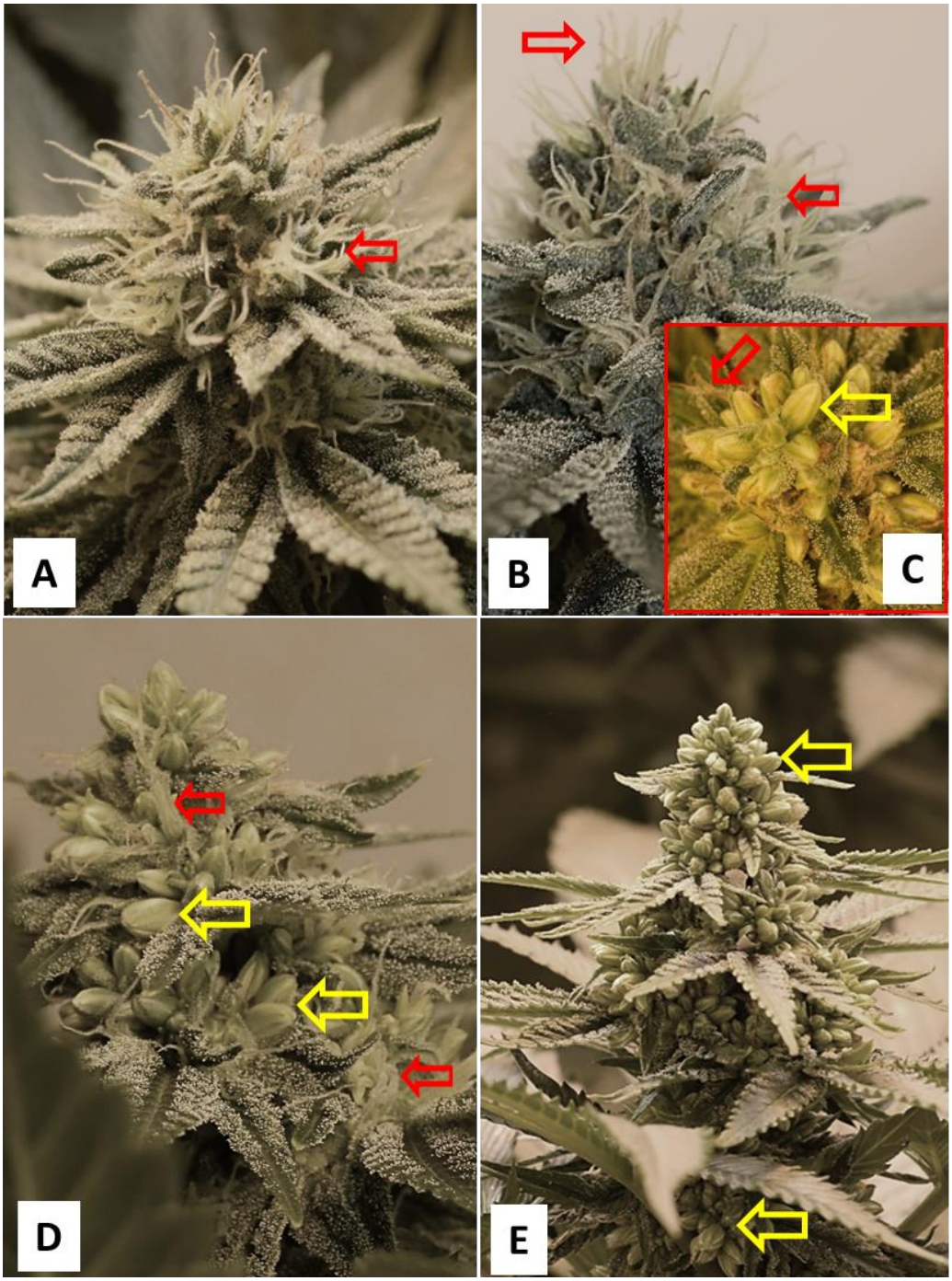


**Figure 4.1.** MSG1 female plants treated with different doses of CS (T1-T3):

**A**) Control MSG1 female plant terminal inflorescence (T0). **B**) Terminal inflorescences from plants treated with 60 ppm CS (T1). **C**) CS Lateral inflorescence from plants treated with 60 ppm CS. **D**) Terminal inflorescence from plants treated with 100 ppm (T2) CS. **E**) Terminal inflorescence from plants treated with 200 ppm CS (T3). (Yellow arrow indicates induced male, red arrow indicates female stigma).

**Table 4.2:** Total number of inflorescences on MSG1 plants treated with different dosages of CS (n=6). Mean values are followed by SE.

| Treatments | Number of all inflorescences |
|---|---|
| Control | 92.75±1.10 |
| 60 ppm | 92.25±1.78 |
| 100 ppm | 92±1.47 |
| 200 ppm | 92.25±1.22 |
| 200 ppm (Once) | 92.75±1.47 |
| 200 ppm (Twice) | 91.25±1.63 |

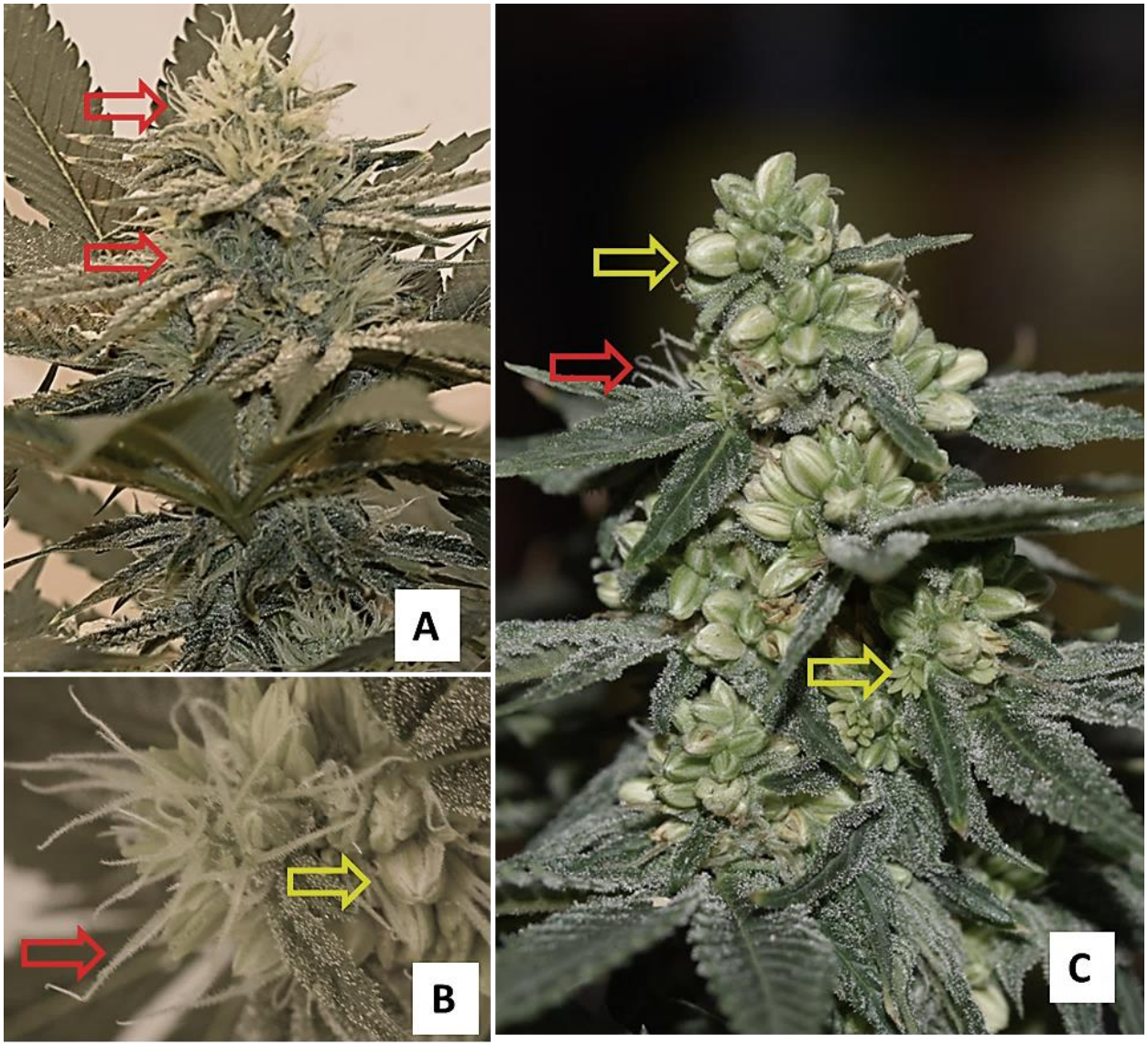


**Figure 4.2.** MSG1 female plants treated with CS dosages T3.1 and T3.2 (CSA and CSB):

**A**) Plants terminal inflorescence treated with T3.1 (Day 1/one spray). **B**) Plants lateral inflorescence treated with T3.1 (Day 1/one spray). **C**) Plants terminal bud treated with T3.2 (Day 1 -7/two sprays). (Yellow arrow indicates induced male, red arrow indicates female stigma)

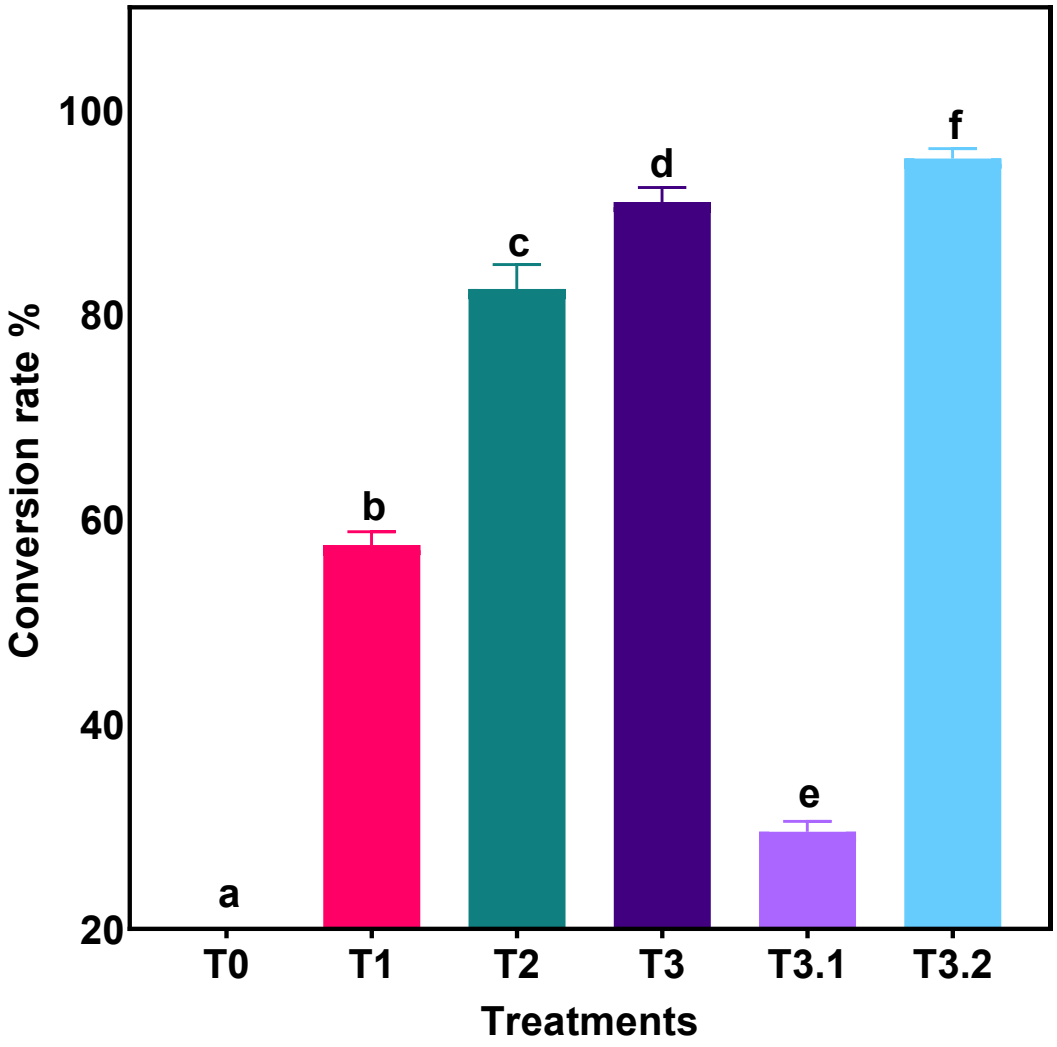


**Figure 4.3**. Percent of male flowers per total inflorescences treated with CS at 60, 100 and 200 according to Table 4.1. Mean values followed by different letters are significantly different at the 5% level of probability (Tukey). Horizontal bars represent SE (± SE).

### 4.3.2 Masculinisation of other cultivars with colloidal silver

Given that it has been demonstrated that cannabis is highly plastic and that different cultivars can exhibit markedly different responses to the same stimulus, the efficacy of the 200 ppm CS treatment (T3.2 CSB) was tested on the other cultivars MSD1 and Black Label (Table 4.3). Plants were sprayed with colloidal silver on days 1 and 7.

**Table 4.3.** MSD1 and Black Label sprayed with 200 ppm CS.

| Treatments | Time | Number of applications | Cultivar |
|---|---|---|---|
| **T3.2 (CSB)** | Days 1 and 7 | 2 | MSD1 |
| **T3.2 (CSB)** | Days 1 and 7 | 2 | Black Label |

Plants transitioned at 14-21 days from early flowering to full flowering and male flowers were observed at the third week after the first treatment. The highest conversion was recorded for MSD1, where 87% of the inflorescences developed male flowers (Figure 4.5). Complete masculinisation was observed on the apical inflorescences of MSD1 while some lower branches remain unchanged with only female flowers present (Figure 4.4B). The Black Label plants showed a 32% conversion rate (Figure 4.5), where male flowers only appeared on lateral branches (Figure 4.4D and 4E). For both cultivars control plants remained unchanged (Figure 4.4A and 4.4C). Pollen shedding occurred after 35 days from MSD1 plants and 37 days from Black Label plants, indicating a slight difference in floral development and pollen release. No negative effects were observed on either of the treated cultivars and inflorescence number also remain unchanged.

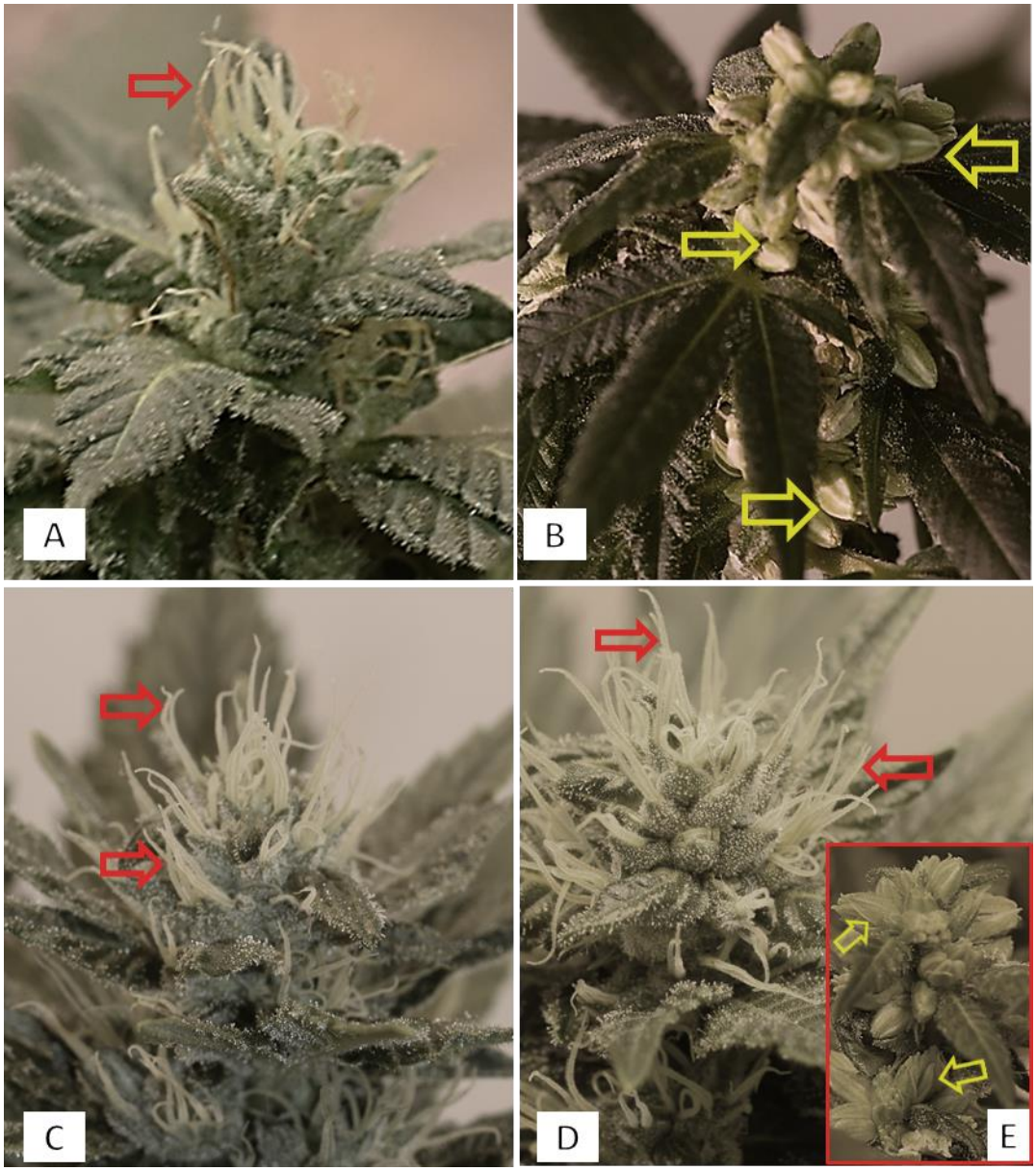


**Figure 4.4.** Differential cultivar response to two sprays (days 1 and 7) of 200 ppm CS:

**A)** MSD1 control terminal inflorescence. **B)** MSD1 terminal inflorescence from a plant treated with 200 ppm. **C)** Black Label control. **D)** Black Label terminal inflorescence from a plant treated with 200 ppm CS **E)** Black Label lateral inflorescence from a plant treated with 200 ppm CS. (Yellow arrow indicates induced male, red arrow indicates female stigma).

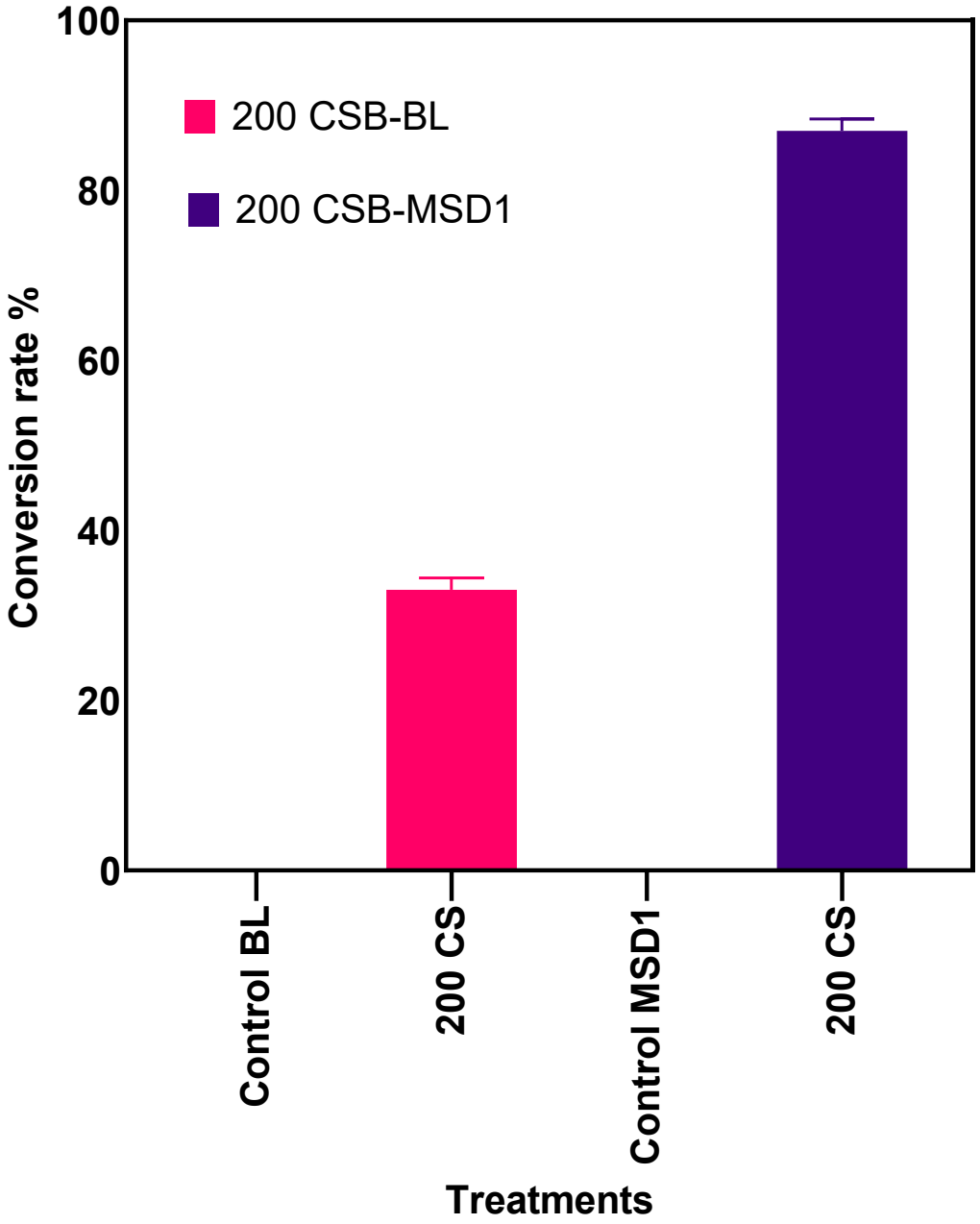


**Figure 4.5**. Percent of male flowers per total number of flowers on inflorescences treated with 200 ppm of CS (at day 1 and 7) for the MSD1 and Black Label cultivars (n=6). Horizontal bars represent SE (± SE).

### 4.3.3 Pollen attributes and seed yield

The vitality of the pollen produced by the CS-induced male flowers was characterised via staining and counted seed yield. Pollen quality (integrity) was assessed via staining with fluorescein diacetate (FDA), after which examination using a fluorescent microscope revealed that pollen from all cultivars had intact membranes and were mostly healthy (Figure 4.6A and 4.6B). Pollen viability was also assessed via seed yield as an indication that normal fertilisation of female flowers was unimpaired. Although it was noted that seed yield varied according to whether plants were allowed to self-pollinate or were manually pollinated. The highest number of feminised seeds resulted from the manual pollination method for both cultivars (Table 4.4). In comparison to self-pollination, a 24-fold increase was noted for MSG1, while an 18-fold increase was observed for Black Label with manual pollination.

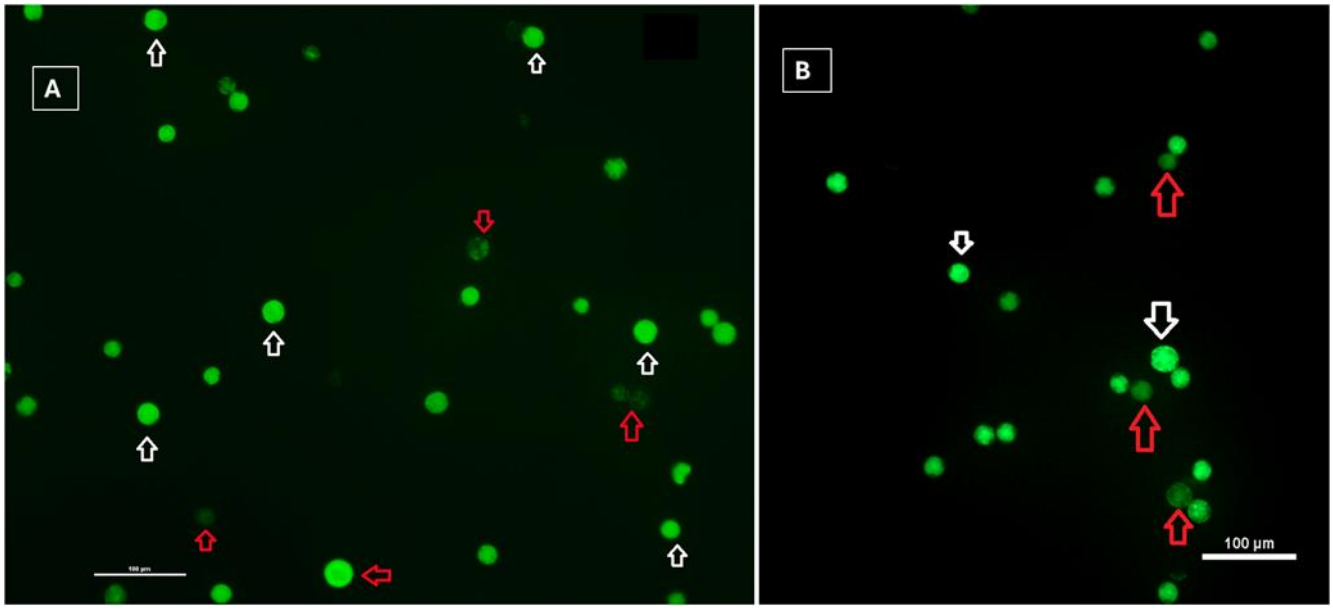


**Figure 4.6**: FDA staining of pollen grains (**A;** MSG1 **B**; Black Label) where white arrows indicate viable pollen and red arrows indicate non-viable pollen grains. Scale bars: (a) 100 µm; (b) 100 µm

**Table 4**.4. Seed yield from self and manual pollinations (n=6). Mean values are followed by SE.

| Treatments | Pollination method | Cultivar | Seed number |
|---|---|---|---|
| **Control** | | MSG1/ BL | 0 |
| **T3.2CSB** | Auto-self | MSG1 | 39± 1.6 |
| **T3.2 CSB** | Auto-self | BL | 45±2.8 |
| **T3.2 CSB** | Manual | MSG1 | 966±51.3 |
| **T3.2 CSB** | Manual | BL | 835±42 |

### 4.3.4 Germination rates of seeds from CS treated plants

The germination rates of the feminised seeds MSG1 (MSG1-F), Black Label (BL-F) and non-feminised (MSG1) seeds were tested under controlled environmental conditions in either soil or coca coir. Feminized seeds (BL-F and MSG1-F) exhibited germination rates of 91% and 85% in both substrates, respectively, while the non-feminized control (MSG1) showed a germination rate of 84% (Table 4.5). Germination started at day 4 after planting for all cultivars, and no signs of phenotypic abnormity were observed in the young seedlings. An outdoor sowing led to very low germination success due to waterlogged soil conditions, so plants were germinated under controlled conditions and transplanted when they were at the four true leaf stage.

**Table 4.5**. Germination rate of feminised and non- feminised seeds (control) planted indoors (n=40).

| Cultivar | Indoor soil | Indoor coca-coir |
|---|---|---|
| **Control (MSG1)** | 84% | 84% |
| **MSG1-F** | 85% | 85% |
| **BL-F** | 91% | 91% |

### 4.3.5 Sex expression and progeny characterisation

The sex expression of feminised seeds was assessed under both indoor and outdoor conditions. All Black Label seeds matured into a female adult plant in both settings (Figure 4.7C and 4.9, Table 4.6). Similarly, all MSG1-F plants grown outdoors were female (Figure 4.8). However, under indoor conditions, some MSG1-F plants developed male flowers, indicating 20% hermaphroditism (Figure 4.7A and 4.7B, Table 4.6), while non-feminized seeds grown under indoor conditions produced 36% male and 64% female plants (Table 4.6).

**Table 4.6:** Phenotyping of sex expression of plants grown from feminised and non-feminised seeds (Values in %) (n=40).

| Conditions | Cultivar | Female | Male | Hermaphrodite |
|---|---|---|---|---|
| **Indoor (n=40)** | **Control MSG1** | 64 | 36 | 0 |
| **Indoor (n=40)** | **MSG1-F** | 80 | 0 | 20 |
| | **Black Label** | 100 | 0 | 0 |
| **Outdoor (n=40)** | **MSG1-F** | 100 | 0 | 0 |
| | **Black Label** | 100 | 0 | 0 |

Progeny characteristics, including plant height, total inflorescence number and branch numbers were also assessed in indoor conditions in both feminised (MSG1-F) and non-feminised (MSG1) seeds However, no significant differences were observed between the two groups in the above-mentioned parameters (Table 4.7).

**Table 4.7.** Progeny characterisation of feminised and non-femisned seeds. Mean values are followed by SE (n=20).

| Cultivar | Height (cm) | Total Inflorescence | Total branches |
|---|---|---|---|
| **Control** | 47.50 ± 3.11 | 52.25 ± 1.71 | 17.50 ± 1.29 |
| **MSG1-F** | 48.50 ± 1.91 | 51.00 ± 0.82 | 16.75 ± 0.96 |

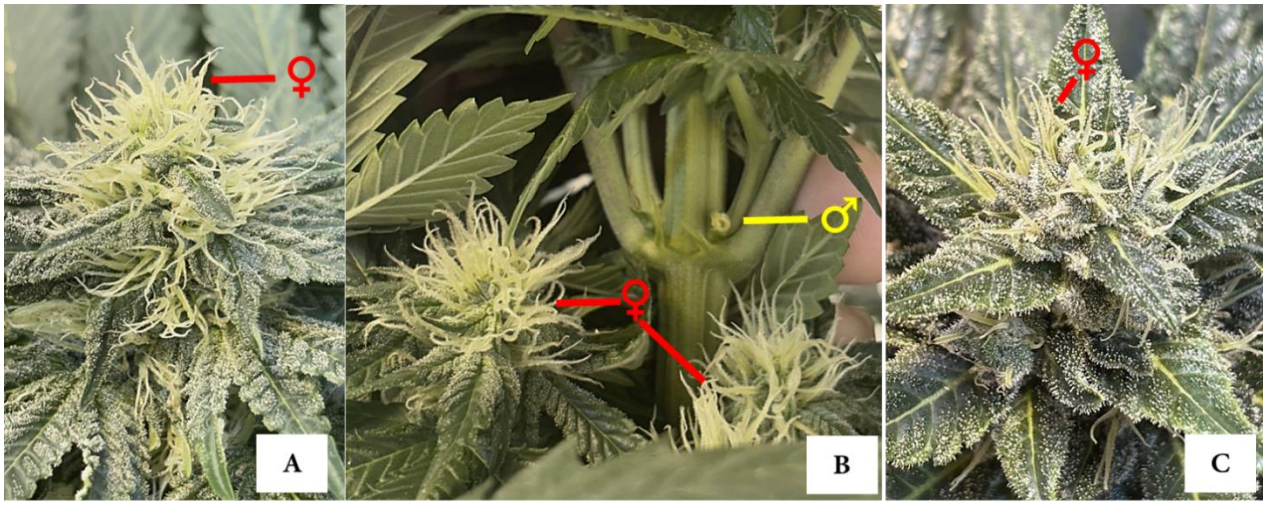


**Figure 4.7**: Indoor grown feminised plants: **A)** MSG1 true female. **B)** Hermaphrodite MSG1. **C)** Female Black Label plant. (Symbols indicating sex phenotype: ♀ Female, Male ♂).



**Figure 4.8.** MSG1-F plants grown outdoors.



**Figure 4.9**. Black Label plants grown outdoors

### 4.3.6 Temporal dynamics of cannabinoid accumulation in BL feminised seeds

Cannabinoid biosynthesis and accumulation was monitored over a time course during flowering of Black Label plants grown from feminised seed to identify peak harvest time and confirm THC levels. The highest levels of total cannabinoids were present at week 8 (post flowering) (Figure 4.10) and a decline was noted at the 9th week, suggesting a shift in metabolic activity. Plants at these times had both milky and amber trichomes (Figure 4.11). Results demonstrate that the feminised seed-derived plants did not show increased THC amounts, and levels remained well under the legal compliance limit of 0.3% (Figure 4.10).

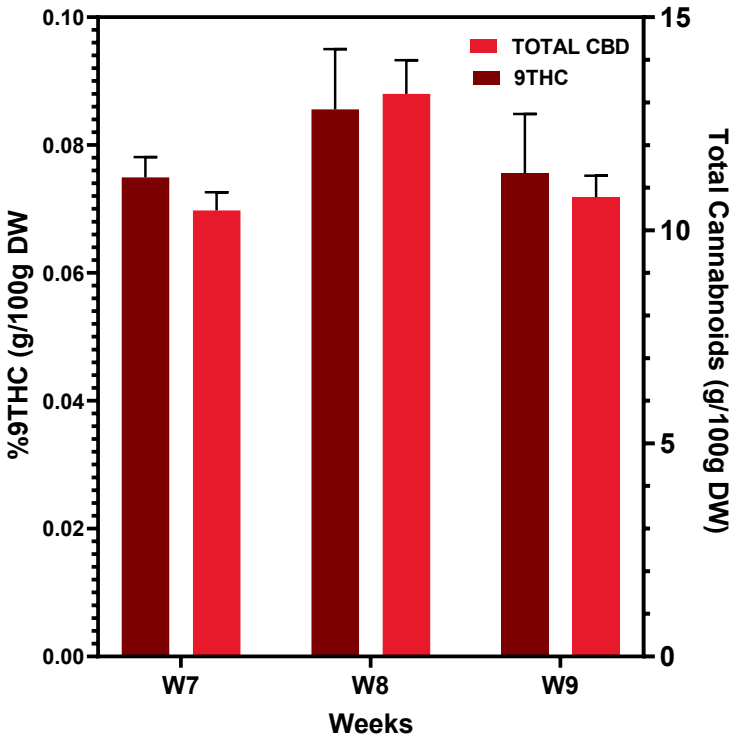


**Figure 4.10**. Total cannabinoids and 9-THC at weeks 7, 8 and 9 after the induction of flowering from Black Label plants grown from feminised seeds. Horizontal bars represent SE (± SE).




**Figure 4.11.** Terminal inflorescences on Black Label plants grown from feminised seeds at weeks 7, 8 and 9 after the induction of flowering.

## 4.4. Discussion

The optimisation trials presented in Chapter 3 suggested that CS exhibited strong masculinisation potential that could be effectively utilised for feminised seed production. However, the need for daily applications would be impractical and demanding for larger scale commercial operations. Operational feasibility is a critical factor in the development of a reliable protocol and minimal applications are preferred. The preliminary findings also suggested that increasing CS concentration improved masculinisation efficiency. Based on this assumption, the work in this current chapter assessed the effects of increasing CS dosage with a reduced number of applications allowing the development of a treatment schedule that is effective, feasible and scalable.

The results indicated that the degree of masculinisation was directly proportional to CS concentration, with a complete conversion to100% male flowers achieved with three sprays of 200 ppm (Figure 4.1E and Figure 4.3). These findings are in accordance with earlier studies in cannabis, which also observed a positive correlation between silver concentration and masculinisation efficiency (Ram 1982; Lubell et al. 2018). To reduce chemical input the ideal concentration was further narrowed down to single and double applications. With two applications of 200ppm of CS, a similar efficiency of 97% was achieved to the three sprays, whereas a single spray was markedly less effective, producing only 30% male flowers (Figure 4.3). When hemp plants transition from vegetative to reproductive growth under short-day conditions, female primordia remain plastic and highly responsive to stress-induced sex reversal. These results indicated that CS application during the early reproductive phase greatly enhanced the conversion of female floral meristems, converting them into male flowers, whereas late-stage applications, once male primordia were already visible, provided little to no added benefit. This stage-specific responsiveness is consistent with previous work by (Lubell

et al. 2018; Kim et al. 2024), who both reported that early or transitional floral tissues exhibit the highest masculinisation efficiency in response to STS, while late reproductive tissues are largely unresponsive.

From a commercial perspective, these findings carry important implications. The ability to completely masculinise plants so that almost all flowers become male and can supply XX pollen is commercially useful, as well as being scientifically interesting. Obviously, the total conversion of a plant to male is not helpful to produce feminised seed, since no female flowers remain to be pollinated. However, it provides an easy avenue to produce large amounts of female pollen from just a small number of plants, reducing spraying requirements which is beneficial from cost and safety perspectives and limiting the number of plants that need to be stressed. Efficient distribution of XX pollen from just a few strategically situated masculinised plants amongst an entire female population, particularly in an indoor growing operation would be an efficient platform for large scale production of feminised seed. Alternatively, the use of just a single application of 200 ppm CS could be used to convert around a third of the female flowers to males on each plant. This would then provide the option for self-pollination of each plant providing suitable conditions were provided i.e. sufficient air movement or manual pollen distribution. It would be an interesting exercise to calculate the economic costs of these two strategies in production settings but perhaps this might depend on multiple factors that are necessarily directly comparable.

Published data shows that across many stresses and under different growing conditions cannabis cultivars respond in a variety of ways (Sheldon et al. 2021; Liu et al. 2016), indicating that this plant species is phenotypically plastic (De Meijer et al. 1996). This characteristic means that responses can’t be predicted necessitating the trial of each cultivar individually for each set of conditions. Consistent with this observation, variable masculinisation responses were observed

among different cultivars exposed to the same dosage of CS. Results showed that there was a gradient of response with MSG1 being the most sensitive, outperforming MSD1 while Black Label displayed the lowest level of masculinisation (Figure 4.3, and Figure 4.5). In the same way, a variable response has been reported by other researchers (Kim et al. 2024; Lubell et al. 2018), where CBDA hemp and superwoman cultivars were found to be more responsive to a 0.3 mM STS application as compared with other hemp cultivars. A differential response was also observed in another study (Flajšman et al. 2021), where the MX-CBD-707 and MX-CBD-11 cultivars showed varying responses across all chemical (Silver/STS) and hormonal (GA3) treatments. Given that the treatment regime was the same for all cultivars in the present study and they were conducted simultaneously under the same conditions, data clearly shows that the masculinisation process in hemp does vary among cultivars and is likely to be genotype specific.

Differential responses to AgNPs haven't only been observed among cultivars of the same species but also between species (Ali et al. 2024; Fatemifard et al. 2024; Yang et al. 2025). In plants the effects of NPs are driven by multiple physiological, biochemical and molecular pathways (Mo et al. 2021; Al-Khayri et al. 2023; Khan et al. 2022; Wang et al. 2023). Stomatal aperture and anatomy, cuticle thickness, trichome density and cell structure all influence the internalisation rate and then response to the presence of foreign materials (Wang et al. 2023; Vaccarini et al. 2025; Koch, Bhushan, and Barthlott 2009; de Paiva Pinheiro et al. 2021). Plants also produce varied levels of compounds to combat heavy metal toxicity via their chelating properties(Maleki et al. 2017; Liu et al. 2023). In addition, differences in antioxidant systems (Nazir et al. 2024) and differential flowering behaviour may further shape how each plant balances growth, defence and reproduction under stress. Since cannabis is an outcrossing species and exhibits a wide range of these diversified traits (De Meijer et al. 1996; Petit et al. 2020; Sankari 2000) it is likely that each cultivar shows varied levels of chemical and physical

responses which overall affects the uptake, entry and physiological response to NPs at cellular levels. A limited amount is known about AgNPs uptake and interaction in hemp, so further studies are needed to explore these varied responses and decipher their origin.

To date various methods have been established to assess hemp pollen quality and viability (Owen 2023). CS-induced pollen was of excellent quality, and the treatment didn't induce any toxicity or deformity in the developing male germ cells (Figure 4.6). In contrast, abnormal or misshapen pollen grains have been reported with the use of other masculinisation agents (DiMatte et al. 2020; Moliterni et al. 2004; Fitzgerald 2021). However, staining and other viability methods are often inconclusive, and breeders tend to characterise pollen output via seed yield (Owen et al. 2023). When masculinised plants were left to self-pollinate seed development for all cultivars indicated pollen viability, but a very low seed yield was observed (Table 4.4). A low dispersal rate due to the absence of wind and pollinators in the indoor environment is likely to be the reason for this (Moliterni et al. 2004; Lubell et al. 2018) hence, a manual pollination method was adopted and notable 18- and 24-fold increases were seen in seed yield of Black Label and MSG1 respectively (Table 4.4). Therefore, we recommend using seed set to characterise hemp pollen vigour and suggest manual pollination for indoor breeding practices. However, standardizing pollen number and uniformly applying pollen on female flowers is still challenging (Owen et al. 2023), thus a standardized protocol is needed to precisely quantify pollen output and seed yield. Note: True male plants (control progeny) were removed at early flowering to prevent pollen drift and were therefore excluded from pollen viability assessments.

A published study has reported that Ag-NP exposure at the flowering stage induced reproductive toxicity, reducing seedling vigour and subsequent plant growth (Geisler-Lee et al. 2014). This concern is often raised around hemp masculinisation, where silver treatments may leave a stress memory or heritable changes in developing seeds that appear in the following generation (Owen

et al. 2023). However, in our study both feminised (BL-F and MSG1-F) cultivars showed excellent germination (Table 4.5), healthy seedling growth, and no signs of progeny toxicity, suggesting that Ag-NPs didn't induce changes in developing seeds.

Feminised seeds are particularly generated to produce guaranteed female plants (Fitzgerald 2021). Black Label feminised (BL-F) seeds exclusively developed a stable female phenotype when grown under both indoor and outdoor conditions (Table 4.6, figure 4.7C, figure 4.9). Similarly, MSG1-F seeds developed into females when grown outdoors (Table 4.6, Figure 4.8) but some female plants developed a low number of anthers (ranging from 1-3) under indoor conditions (Figure 4.7B), although no true male plants were observed for any cultivar in outdoor condition (Table 4.6). The presence of anthers on some female plants of MSG1-F in the indoor controlled environment marks these plants as hermaphrodites. This is not a rare occurrence limited to feminised seeds, since plants grown from regular seeds and clones may also undergo sex changes when conditions are unfavourable (Punja et al. 2020a; Flajšman et al. 2021; Hall et al. 2012; Small 2016) and in fact many important industrial hemp cultivars, such as Finola , Futura and Fibranova have been bred to be stably hermaphroditic, although they are more commonly called monoecious (Eržen et al. 2023; Pavlovic et al. 2019). Research studies suggest that allosomes are not only strictly involved in the determination of hemp sex expression, but some autosomal genes can also influence sex expression in many dicoeous plants and hemp (Baek et al. 2025; Shi et al. 2025; Charlesworth 2017). Considering the polygenic sex determinants of hemp (Baek et al. 2025; Faux 2014; Faux et al. 2013; Truta et al. 2007), it is speculated that male flower development might be triggered by environmental stimulus (humidity or temperature or both) or genetic instability which prompts the expression of male traits, not necessarily in response to stress events (Malabadi et al. 2024; Punja et al. 2020b). Further research is required across the entire cannabis field to investigate and understand the triggers and mechanisms of hermaphroditism in more detail.

Here no significant differences were observed in plant height, total number of inflorescences and branch numbers between plants grown from feminized (MSG1-F) and non-feminized (MSG1) seeds (Table 4.6), suggesting that CS treatment did not affect any vegetative or reproductive traits in the next generation. However, it should be noted that due to limited space availability and potential pollen drift, the experiment was conducted only on the MSG1 cultivar. Therefore, further studies with additional genotypes and larger experimental setups are warranted.

Determining the optimal harvest time is crucial for yield, quality and regulatory compliance (Adesina et al. 2020; Visković et al. 2023) across all types of cannabis; medicinal, recreational or industrial. In the current study, the highest levels of total cannabinoids found in plants grown from feminised seeds (BL) were recorded at the 8th week post flowering and then a decline was noted at nine weeks (Figure 4.10). For industrial hemp, used either for seed production for consumption, or less critically for fibre, the concern that environmental stresses such as high temperatures, humidity, drought and pest attack may trigger an increase in THC levels which pushes the crop above the legal compliance limits set by various countries, and even states in Australia, is of major concern to growers. Crops which exceed this limit are required to be destroyed causing significant economic losses (Olejar et al. 2022). As the application of CS is designed to purposely engineer stress, assaying the effects on THC levels in plants growing from the resultant feminised seed is important to monitor induced changes in these metabolites. Feminised seeds of Black Label fall into the type III chemotype, which specifies that THC doesn't exceed the threshold level (0.3%) (Figure 4.10) (Pacifico et al., 2008; Toth et al., 2020). and in fact, levels were consistently quite low at under 0.1% THC. Since the current study was only conducted under indoor conditions which rules out the presence of the soil microbiome and other environmental stressors, outdoor studies would be needed to assess the phytochemical profile and identify the optimal harvest stage for field-grown crops.

## 4.5. Conclusion

In the present study different concentrations of CS were systematically assessed to find an ideal dosage for peak hemp masculinisation. Two sprays of 200 ppm CS spaced seven days apart induced almost complete masculinisation and this was identified as the optimal dosage. The ideal dosage was further tested on other cultivars, and a differential response was noted. Excellent pollen quality and viability indicated that most of the masculinised flowers were viable, while seed set significantly improved through manual intervention. Under both indoor controlled and outdoor conditions feminised seeds produced female plants; however, some hermaphroditism was observed in MSG1 plants. Plants grown from feminised seeds were tested for cannabinoid levels, partly to identify an optimal stage for harvesting and to ensure that none of the female plants crossed the legal threshold THC levels. These findings offer significant implications for breeding and commercial cultivation, offering a robust and reliable approach for bulk masculinisation and feminised seed production. The finding that CS can also be used to convert 100% of the female flowers to male flowers carrying XX pollen also provides an ideal opportunity to study the production of male flowers in a controlled system. The use of this opportunity to understand more about flowering and sex determination in cannabis by examining changes in transcription patterns is explored in the following chapter.

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# Chapter 5

# Deciphering the mechanisms of colloidal silver-induced male flower formation in female *Cannabis sativa* through RNA sequencing

## 5.1. Introduction

Hemp (*Cannabis sativa*) naturally exhibits a dicoeous nature, although monecious or hermaphroditic plants also exist (Baek et al. 2025). Sexual dimorphism in cannabis is not visually apparent until flowering when plant sex becomes phenotypically evident (Campbell et al. 2021; Borthwick et al. 1954), although a non-invasive hyperspectral method can also be used on younger vegetative plants (Matros et al. 2023). Male flowers develop on cymose panicle inflorescences, wherein each floret includes five sepals and five short-stalked stamens (Schilling et al. 2020). In contrast, female plants develop compound raceme inflorescences with green bracts enclosing each flower, containing an unfertilised ovary and a short style with two feathery stigmas (Spitzer-Rimon et al. 2019; Shi et al. 2024).

The molecular mechanisms of flower development are well characterised in many plant species (Haughn et al. 1988; Kater et al. 1998; Causier et al. 2010; Litt et al. 2010). According to these studies, the flower formation process generally follows the ABC(DE) model (Searle et al. 2004; Causier et al. 2010; Bouché et al. 2016). This model describes the roles of different classes of genes (A, B, C, D, and E genes) involved in the development of different floral organs in four concentric whorls of sepals, petals, carpels and stamens (Bouché et al. 2016; Causier, et al. 2010). For example, petal development is regulated by class A+B+E genes, sepals by A+E, stamens by B+C+E , carpels by C+E and ovule identity are determined by C+D+E (Bouché et al. 2016; Angenent et al. 1995).

In the context of the ABC model, the male cannabis flower is quite minimal, comprising a first whorl containing five sepals, a missing second whorl and a third whorl encompassing five stamens, while the fourth whorl is again missing (Leme et al. 2020). The female flower is similar, with small sepals making the first whorl and the fourth whorl containing the carpel where both second and third whorls are missing (Shi et al. 2024). In hemp, the complete

absence of opposite-reproductive organs suggests that floral homeotic genes in male and female flowers are regulated differently from the earliest stages of development.

Hemp is a diploid (2n=20) and one of the few plant species in which sex chromosomes are well characterised (Divashuk et al. 2014). Hemp exhibits heteromorphic sex chromosomes, with females being homogametic XX and males heterogametic XY (Moliterni et al. 2004). But, even with the presence of this distinct XX/XY genetic framework, hemp shows remarkable plasticity (Monthony et al. 2025). This flexibility facilitates alteration of sexual phenotype where plants may display the opposing phenotype as characterised by chromosomal composition (Baek et al. 2025; Hall et al. 2012). Sex reversal in hemp may be due to environmental, physiological, hormonal or chemical stress (Khryanin 2002). The reversal mechanism/s is/are unclear but are largely assumed to be hormonal which modulate the floral differentiation and developmental processes (Baek et al. 2025; Adal et al. 2021).

Stimulation and inhibition of the hormonal pathways have both been shown to be effective in modulating sexual phenotypes (Chailakhyan et al. 1979; Ram et al. 1982; Lubell et al. 2018; Papadopoulou et al. 2005). The inter-play between floral sex identity and hormonal levels has been well examined in other species, particularly those of the *Cucurbitaceae* family (Pan et al. 2018; Martínez et al. 2021; Luo et al. 2023). In these species, female floral identity is predominantly controlled by ethylene (Manzano et al. 2011; Martínez et al. 2021; Luo et al. 2023; Pan et al. 2018), and silver, an effective suppressor of ethylene (Binder et al. 2007), has been shown to trigger masculinisation in *Cucumis melo* (Papadopoulou et al. 2005; Byers et al. 1972)*, Cucumis sativus* (Sunita et al. 2015; Beyer 1976) and *Cucurbita pepo* (Manzano et al. 2011)**.** Mechanistically, $Ag^{+}$ ions compete with copper ions at the ethylene binding domain of the receptor (Binder et al. 1999), allowing ethylene binding but restricting downstream conformational effects, essential for ethylene signal transduction (McDaniel et al. 2012; Byers et al. 1972; Binder et al. 1999; Binder et al. 2007; Kendrick et al. 2008). Decreased ethylene

levels in the primordia stimulate male floral factors, promoting stamen development while concurrently suppressing female traits (Martínez and Jamilena 2021; Wang et al. 2010).

The oxidation state of silver has also been reported to shape the overall response in plants (Siddiqi et al. 2022; Qian et al. 2013; Pociecha et al. 2021). For instance, (Kaveh et al. 2013) reported that both AgNPs and Ag+ ions induced oxidative stress and altered the expression of genes encoding enzymes that regulation the action of hormones including ethylene, auxins and ABA. However, AgNPs triggered a broader response as compared to their ionic counterparts (Kaveh et al. 2013). In addition to oxidation stress, silver also showed characteristics of mechanical stress and induced jasmonic acid, salicylic acid and high levels of other stress markers (Angelini et al. 2022). High levels of jasmonates have also been implicated in the development of stamens in *Zea mays* L (maize) and *Arabidopsis* (Acosta et al. 2019; Yuan and Zhang 2015; Acosta et al. 2009; Lunde et al. 2019).

The work in this thesis demonstrated that CS showed excellent masculinisation efficacy in hemp, but it remains unclear whether these changes were primarily induced by a direct interaction between silver and changes in gene expression, how this relates to hormone production and action, whether ROS acted as a secondary mediator, whether ROS played a supplemental role, or whether hormonal changes were merely incidental. The complete conversion of the female inflorescence to male flowers, albeit still carrying female XX pollen, provides a unique opportunity to use this system to try and understand the cues that trigger the genetic pathway determining the production of male flowers. The application of CS supplies a way to tightly coordinate the production of many male florets, providing synchronised tissues that can be investigated for gene expression. The work in this chapter was therefore aimed at investigating the regulation of male flower formation by treating female hemp plants with CS to induce male flowers, with a comparative control being provided by unsprayed female plants left to develop naturally. We employed RNA sequencing analysis to help elucidate the

regulatory genes relating to CS-mediated sex modification, in the hope that male and female pathways may contain key differentially expressed genes. These differences underlying male and female floral development could then be used to identify the putative relationships between floral homeotic genes, hormone synthesis genes and sex expression and could be usefully compared with the floral networks in other angiosperms and more particularly with the work in the *Cucurbitaceae* plus recent advances in hemp (Garcia-de Heer et al. 2025).

## 5.2. Material and methods

### 5.2.1 Plant materials and colloidal silver treatment

The experiment was conducted using female plants (clones) of MSG1 (Mountain Strong Hemp Colorado, USA). Plants were grown and maintained as previously described (Chapters 3 and 4). At day 27 of vegetative growth whole plants were treated with 50 ml of 200 ppm CS solution on both sides of the leaves, whereas control plants were treated only with distilled water. Plants were sprayed four hours before the dark cycle to avoid silver-light burns and after 24 hours ($28^{th}$ day) the light cycle was switched to 12/12 dark/light to induce flowering. The second application of CS was performed seven days after the first treatment.

### 5.2.2 Tissue collection for RNA analysis

Shoot apical meristem (SAM) tissues were manually harvested and instantly flash-frozen in liquid nitrogen and stored at -80 °C until further processing. SAMs were collected at two different stages: First-stage treatment samples (called T1) were harvested 48 hours after the initial treatment, while the second-stage samples (called T2) were harvested 72 hours following the second treatment. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol. A total of 16 samples were sent for RNA sequencing, comprising eight controls (four from each stage) and eight treatments (four from each stage).

### 5.2.3 RNA-Sequencing assessment

Initial quality control, library preparation and sequencing analysis were performed commercially at the DNA Sequencing Core Facility of the South Australian Genome Centre in Adelaide. RNA samples were QC'd using a Qubit for quantity, and tape station analysis was used to assess size distribution and RNA integrity. All samples returned an RNA Integrity Number (RIN) of above 9. Libraries were prepared according to the Nugen Universal Plus Total RNA-Seq protocol (Human rRNA and globin depletion). Equimolar pools were generated and

converted to MGI-compatible libraries. Quality assessment of reading libraries was performed using Fast QC v 0.12.1 and Fast P/Adapt Cut was used to remove low-quality reads and remaining adapter sequences from each dataset. The splice-aware STAR aligner 2.7.10b was used to align the remaining reads against the *Cannabis sativa* 'Pink Pepper' (Ryu et al. 2024) genome for reference sequence and annotation.

## 5.3. Results

### 5.3.1 Quality report

The initial quality checks (e.g., RNA integrity (RIN) and QC) indicated excellent and acceptable RNA quality (Table 5.1). The RNA sequencing of the libraries produced a total of 479 million reads.

**Table 5.1:** Quality report details of S1-S16.

| ULN | Sample Name | RIN | Total Clusters Passing Filter (Million) |
|---|---|---|---|
| 24-03693-S1 | Cs_T1 | 9.5 | 31.7 |
| 24-03694- S2 | Cs_T1 | 9.7 | 34.0 |
| 24-03695- S3 | Cs_T1 | 9.8 | 21.7 |
| 24-03696- S4 | Cs_T1 | 9.7 | 32.1 |
| 24-03697-S5 | Cs_T2 | 9.4 | 34.5 |
| 24-03698-S6 | Cs_T2 | 9.7 | 32.0 |
| 24-03699-S7 | Cs_T2 | 9.5 | 31.9 |
| 24-03700-S8 | Cs_T2 | 9.5 | 28.8 |
| 24-03701-S9 | Cs_C1 | 9.7 | 30.5 |
| 24-03702-S10 | Cs_C1 | 9.6 | 33.0 |
| 24-03703-S11 | Cs_C1 | 9.7 | 28.4 |
| 24-03704-S12 | Cs_C1 | 9.6 | 28.0 |
| 24-03705-S13 | Cs_C2 | 9.5 | 28.0 |
| 24-03706-S14 | Cs_C2 | 9.6 | 27.0 |
| 24-03707-S15 | Cs_C2 | 9.6 | 28.0 |
| 24-03708-S16 | Cs_C2 | 9.6 | 29.4 |
| | | | Total = 479M |

Raw reads were adapter-trimmed using Trimmomatic prior being screened. To assess the contamination rate in the RNA-seq data a stepwise approach was employed that integrated reference-based filtering, mapping, and comparative analyses based on (Herliana et al., 2023).

Initially, BBDuk was used with SILVA SSU and LSU reference sequences (both combined and separately) to remove potential ribosomal RNA contaminants.

### 5.3.2. Using minimap2 to map reads to SILVA LSU references

The quality of RNA-seq reads was evaluated by mapping to the SILVA large subunit (LSU) ribosomal RNA reference database using minimap2. The total number of paired end reads generated for each sample, the number of successfully mapped pairs, and the corresponding mapping percentages are summarized in Table 5.2. Across all samples, a high proportion of reads were aligned to the SILVA LSU reference, with mapping rates consistently above 85%. This outcome indicates that most reads represent ribosomal RNA sequences rather than messenger RNA, consistent with a high level of rRNA contamination. While the successful alignment to LSU references confirmed the presence of abundant ribosomal content in the dataset, it also demonstrated that a considerable fraction of sequencing effort was consumed by rRNA reads, thereby reducing the proportion of informative mRNA reads available for downstream transcriptomic analysis.

**Table 5.2.** Overview of RNA-seq percentage of reads that mapped to the SILVA LSU reference.

| Sample | Total Pairs | Mapped Pairs | Percent |
|---|---|---|---|
| 24-03693_S1 | 31587442 | 29723373 | 94.0987 |
| 24-03694_S2 | 33855332 | 31985803 | 94.4779 |
| 24-03695_S3 | 21581168 | 20067089 | 92.9843 |
| 24-03696_S4 | 31926641 | 29570112 | 92.6189 |
| 24-03697_S5 | 34347213 | 32493726 | 94.6037 |
| 24-03698_S6 | 31784446 | 29987576 | 94.3467 |
| 24-03699_S7 | 31751192 | 30078265 | 94.7311 |
| 24-03700_S8 | 28643023 | 26995933 | 94.2496 |
| 24-03701_S9 | 30298551 | 28036804 | 92.5351 |
| 24-03702_S10 | 32860470 | 30605797 | 93.1386 |
| 24-03704_S12 | 27803948 | 25882793 | 93.0904 |
| 24-03705_S13 | 27849367 | 26324389 | 94.5242 |
| 24-03706_S14 | 26860723 | 25302408 | 94.1985 |
| 24-03707_S15 | 27843307 | 26213140 | 94.1452 |
| 24-03708_S16 | 29295717 | 27665983 | 94.4370 |

### 5.3.3 rRNA Detection Across Samples Using SILVA and 5S Databases

The proportion of reads assigned to different ribosomal RNA (rRNA) databases was assessed across all RNA-seq samples (S1–S16) to evaluate compositional origin of the contamination. The results showed that more than 90% of reads in each sample were classified against reference databases, with only minor variation between categories (Figure 5.1). Reads mapped to the combined rRNA reference and the SILVA large and small subunit databases (LSU_SILVA128 and SSU_SILVA138) consistently reached above 93%, while slightly lower proportions (approximately 88–90%) were observed for the 5S_Eukaryota and SSU_SILVA138TAX categories (Figure 2). The overall high proportion of reads assigned to

rRNA references indicated a high level of contamination, although the classification performance remained consistent across samples.

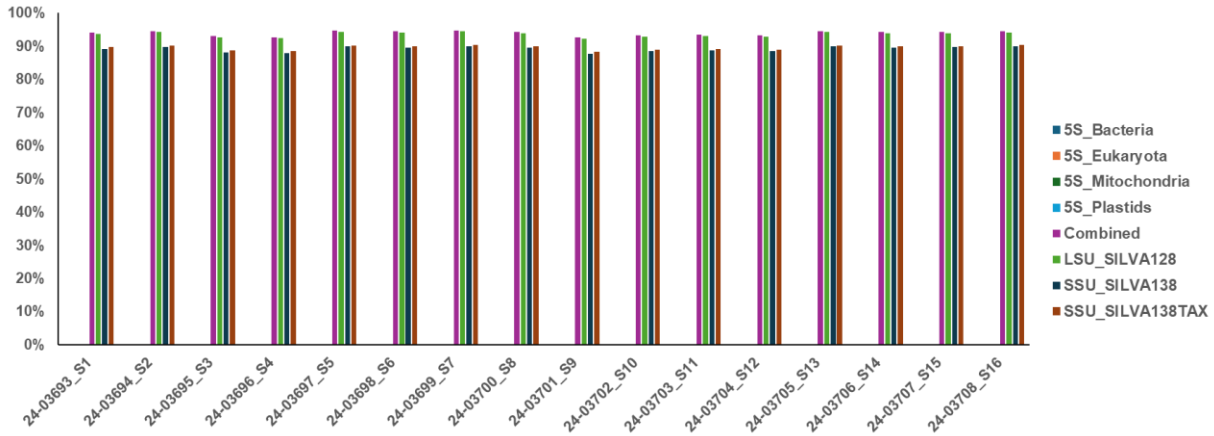


**Figure 5.1**. rRNA detection across samples using SILVA and 5S Databases.

### 5.3.4 Using minimap2 with the *Cannabis sativa* genome

In the next evaluation step RNA-seq samples were aligned to the *Cannabis sativa* reference genome using minimap2. Alignment statistics, including the total number of read pairs, mapped pairs, and high-confidence alignments (MAPQ ≥ 20), are presented in Table 5.3. The results showed that a large proportion of reads successfully mapped to the reference genome, with overall mapping rates exceeding 85% across samples. A high proportion of mapped reads also passed the MAPQ ≥ 20 threshold, suggesting reliable alignment. While these results confirm that sequencing reads are of sufficient quality for alignment, a substantial proportion of the mapped reads represent ribosomal RNA sequences and so their origin was investigated

**Table 5.3.** Alignment statistics, total number of read pairs, mapped pairs, and high-confidence alignments.

| Sample | Total Pairs | Mapped Pairs | Percent | Mappedpairs (Mapq>=20) | Percent (Mapq>=20) |
|---|---|---|---|---|---|
| 24-03693_S1 | 31,587,442 | 26,581,722 | 84.15 | 15392926 | 48.7312 |
| 24-03694_S2 | 33,855,332 | 28,095,876 | 82.99 | 16197928 | 47.8445 |
| 24-03695_S3 | 21,581,168 | 18,099,597 | 83.87 | 10478249 | 48.5527 |
| 24-03696_S4 | 31,926,641 | 26,513,874 | 83.05 | 16180879 | 50.6814 |
| 24-03697_S5 | 34,347,213 | 28,429,042 | 82.77 | 16063265 | 46.7673 |
| 24-03698_S6 | 31,784,446 | 26,490,026 | 83.34 | 14861638 | 46.7576 |
| 24-03699_S7 | 31,751,192 | 26,182,173 | 82.46 | 15071844 | 47.4686 |
| 24-03700_S8 | 28,643,023 | 23,670,247 | 82.64 | 14024663 | 48.9636 |
| 24-03701_S9 | 30,298,551 | 25,308,555 | 83.53 | 14920495 | 49.2449 |
| 24-03702_S10 | 32,860,470 | 27,307,625 | 83.10 | 16299888 | 49.6033 |
| 24-03703_S11 | 28,244,280 | 23,351,695 | 82.68 | 14217896 | 50.339 |
| 24-03704_S12 | 27,803,948 | 22,929,189 | 82.47 | 13807837 | 49.6614 |
| 24-03705_S13 | 27,849,367 | 23,011,090 | 82.63 | 12706814 | 45.6269 |
| 24-03706_S14 | 26,860,723 | 22,022,733 | 81.99 | 12763102 | 47.5159 |
| 24-03707_S15 | 27,843,307 | 22,617,348 | 81.23 | 12848649 | 46.1463 |
| 24-03708_S16 | 29,295,717 | 23,981,906 | 81.86 | 13870890 | 47.3478 |

### 5.3.5 Mitochondrial versus chloroplast rRNA reads

Ribosomal reads can originate from several cellular components including the nuclear, mitochondrial and chloroplast genomes. The *Cannabis sativa* reference genome contains the mitochondrial genome but lacks the chloroplast genome meaning that a subset of reads mapped to mitochondrial regions, while chloroplast-derived transcripts were not detected due to the absence of a chloroplast reference in the assembly. Annotation of the *Cannabis sativa* mitochondrial genome (accession NC_029855.1) revealed the presence of three ribosomal RNA (rRNA) genes: *rrnL* (large subunit rRNA; positions 369,248–370,983, minus strand), *rrn5* (5S rRNA; positions 381,429–381,544, plus strand), and *rrnS* (small subunit rRNA; positions 382,094–384,029, minus strand) (Figure 5.2) These genes form the mitochondrial ribosomal RNA components essential for mitochondrial translation.

**Figure 5.2.** rRNA mitochondrial loci in the *Cannabis sativa* genome assembly.

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 GNU nano 2.9.8                                                
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A BLASTN analysis of *rrnL*, *rrnS*, and *rrn5* sequences showed 100% identity with *Cannabis sativa*, *Arabidopsis thaliana* and other species (Table 5.4) confirming that these genes are either from the nuclear or mitochondrial genome. Further manual BLAST searches across unlimited databases revealed significant numbers of hits to chloroplast rRNA sequences, indicating that the remaining sequences are indeed from this organelle.

**Table 5.4.** BLASTN searches against different sequence databases.

| **BLASTN Result** | **Organism** | **Percent Identity** |
|---|---|---|
| ***rrnL*** | *Cannabis sativa* (Hemp) | 100.00% |
| ***rrnL*** | *Humulus lupulus* (Hops) | 99.80% |
| ***rrnL*** | *Arabidopsis thaliana* | 94.39% |
| ***rrnL*** | *Homo sapiens* (Human) | 88%–90% |
| ***rrn5*** | *Arabidopsis thaliana* | 100.00% |
| ***rrn5*** | *Logfia minima, Epilobium ciliatum, Retula pendula, Ribes stenocarpum, Jacobaea vulgaris* | 100.00% |
| ***rrns*** | *Cannabis sativa* (Hemp) | 100.00% |
| ***rrns*** | *Homo sapiens* (Human) chain 2, 18S ribosomal RNA | 75% |

## 5.4. Discussion

In summary, screening against the SILVA SSU and LSU databases using BBDuk and minimap2 revealed a very high ribosomal RNA (rRNA) signal, with more than 90% of reads classified as rRNA-like. Genome mapping with minimap2 to the available *Cannabis* reference, which included nuclear and mitochondrial sequences but excluded the chloroplast genome, produced approximately 45–50% high-confidence alignments (MAPQ ≥ 20) (Table 5.3). These alignments comprised both nuclear and mitochondrial reads, but not chloroplast-derived reads which can be surmised to make up the remaining subset to take the total of undesirable reads to 92-94% (Table 5.2). It should be noted that the reference genome used did not contain chloroplast sequences and that nuclear rRNA (rDNA) regions were not annotated. BLASTN comparison of rRNA sequences against plant and human references showed lower identity against the human sequences, suggesting limited cross-species similarity. As a result, the use of a human ribodepletion kit was not effective in removing nuclear or mitochondrial rRNA from the cannabis RNA samples, with presumably the same inefficiency against chloroplast rRNA sequences. Taken together, this unsuccessful ribodepletion step left the dataset unsuitable for further transcriptomic analysis, since differential expression analysis should be restricted to nuclear genes, with rRNA and organellar genes excluded to avoid confounding effects. For optimal RNA-seq analysis, a spliced aligner such as minimap2 with splice mode or STAR should be employed, and chloroplast and mitochondrial genomes should be included during quality control.

For future studies, a plant-specific or custom rRNA depletion kit should be used to remove unwanted sequences, or the mRNA fraction can be separated using an enrichment step based on polyA binding before the libraries are synthesised.

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# Chapter 6

# Summary and future directions

## Summary

Sex expression in hemp (*Cannabis sativa* L) is highly plastic and can be manipulated by environmental or hormonal factors (Monthony et al. 2025; Baek et al. 2025). This plasticity allows female plants to produce female pollen, enabling both self and cross pollination, which is a very unusual ability in the plant world. Through this pathway the resultant seeds will be entirely feminised (Lubell et al. 2018; Flajšman et al. 2021) providing a degree of downstream certainty for growers. Female plants produce high levels of cannabinoids and are valued in the medicinal industry (Soler et al. 2017). Currently, several protocols are utilised to produce feminised seeds (DiMatteo et al. 2020; Lubell et al. 2018; Baek et al. 2025; Kim et al. 2024) but these existing approaches are somewhat anecdotal where they have been developed in the recreational cannabis industry, and can be cost restrictive and risky for health, thus limiting their consistency and scalability for large-scale seed production. To meet the growing global demand for feminised hemp seeds, alternative, efficient and safer induction methods are needed (Burton et al. 2022). This research established a robust feminisation method that can reliably induce male flower formation in genetically female hemp plants, offering a viable alternative for large scale production of feminised pollen and seeds.

Based on previous studies indicating that stressors can alter sex expression in cannabis (Truta et al. 2007; Garcia-de Heer et al. 2025) the overarching hypothesis tested here was that the application of particular stresses can stimulate the production of male flowers carrying female pollen (XX) on female plants. In Chapter 3, various masculinising agents including commercially produced colloidal silver (CS), green synthesized silver nanoparticles made in the laboratory, gibberellic acid, pruning and pyrazinamide were screened to assess their masculinisation potency on the MSG1 cultivar. Among these tested stressors, commercial CS stimulated superlative masculinisation and was demonstrated to be an optimal masculinising

agent. However, the daily application regime was impractical for large scale operations and further optimisation was carried out to streamline the timing and dose of CS.

In Chapter 4, the selected masculinising agent (CS) was further optimized in terms of concentration, numbers of applications and timing during the flowering cycle based on published literature that suggested that these are the key factors that substantially impact masculinisation in cannabis (Timoteo et al. 2024; Kim et al. 2024). Results revealed that three applications of 200 ppm CS (spaced three weeks apart) resulted in complete masculinisation. The same concentration (200 ppm) was further narrowed down into single and double doses. Remarkably, two sprays of 200 ppm at early flowering (applied at seven-day intervals) induced near-complete masculinisation, and this regimen was identified as the optimal dosage and frequency. From a commercial perspective, these findings carry important implications. This approach requires minimal applications, can generate sufficient pollen from a single plant to pollinate many other females, and therefore supports large-scale, cost-effective feminised seed production.

The dual spray regime was also tested on additional cultivars MSD1 and Black Label as it is becoming clear that responses to a range of stresses can vary widely by cannabis genotype (Mateva et al. 2024).Results revealed that a differential masculinisation response was observed, indicating that masculinisation is indeed also genotype dependent. The pollen induced on MSG1 and Black Label plants was tested for viability and fertilisation capacity. Results indicated that the induced pollen of both cultivars was viable, while seed set substantially improved through manual intervention. Seed germination and plant vigour were also tested, and we found that both varieties had excellent germination rates and showed no phenotypic defects, unlike reports of other compounds used for masculinisation (DiMatteo, Kurtz, and Lubell et al. 2020).

Feminised seed quality is often questioned in terms of sex expression and THC levels. Sex expression of feminised seeds was tested in both indoor controlled and outdoor conditions, where Black Label produced a 100% female phenotype under both conditions; however, some hermaphroditism was observed in indoor grown MSG1 plants. Since the MSG1 population produced some male flowers on the feminised plants, further analyses on just the Black Label plants allowed the identification of peak cannabinoid production, and therefore harvest stage, and adherence to THC compliance levels. Analysis revealed that the highest levels of cannabinoids were accumulated in week eight after the start of flowering, and that THC levels were well under the compliance levels and therefore had not significantly changed from levels normally seen on untreated Black Label plants.

In the last chapter, the ideal dosage of CS identified in this work to cause complete masculinisation was used as a tool to generate material ideal for exploring the genetic basis of male flower production, which can also be viewed as a reversion from female to male. Control and treated floral tissues sprayed with CS at two timepoints, namely early and mid-stages of flowering, were collected and used for total RNA extraction. RNA sequencing was thus performed on a total of 16 samples producing excellent numbers of reads per sample. However, bioinformatic processing of the reads revealed an extremely high contamination rate with ribosomal sequences which has been attributed to the failure of the depletion step, designed to remove the typically high level of ribosomal RNAs, before the libraries were constructed. Disappointingly, this left only relatively low numbers of reads representing other transcribed genes which was not sufficient for a rigorous analysis of differentially expressed genes.

**Limitations and Future directions**

The green synthesized silver nanoparticle (GS-NPs) protocol presented a novel and sustainable alternative for hemp seed feminisation. However, large particle sizes and agglomeration likely reduced their masculinisation efficacy. Reaction conditions, substrate composition and precursor salt concentration have been shown to influence physiochemical properties (e.g., size and shape) of metallic nanoparticles (Yaqoob et al. 2020; Soni et al. 2011; Darroudi et al. 2011). All these properties play crucial roles in plant-NP interactions so optimising the above-mentioned parameters is therefore required to harness their true potential. For controlled synthesis and to prevent particle aggregation, the use of supplemental stabilising agents such as pectin, starch and cellulose (Patel et al. 2017), as well as alternative plant derived substrates like leaves, roots and fruits, should be further investigated.

However, the partial conversion rate achieved with GS-NPs, even with the larger particle sizes and the propensity to clump, could still be useful in hemp breeding practices. Since a single male flower can produce hundreds of thousands of pollen grains and if they are dispersed effectively, either via wind outdoors or airflow indoors, just a limited number of male flowers could still provide high seed yield. If the lower efficiency rate of using the GS-NPs is offset against sustainability and economic considerations, then even without the higher conversion rate this still may represent a very attractive option to growers and seed producers. Discussions with industry about these results should be a future action and if there is interest then the full suite of downstream tests, including assessment of pollen viability, fertilisation efficiency and absence of phenotypic defects and hermaphroditism should be pursued.

The present study was conducted in a controlled environment to minimise variability and for ease of application. Hence, it is also important to assess CS masculinising efficacy in outdoor conditions for future large-scale operations. The potency of CS applied outdoors may be

subjected to additional factors including high light intensity (Zhou et al. 2016), UV exposure (Gorham et al. 2012; Mittelman et al. 2015) and weather fluctuations which have all been shown to affect silver nanoparticles behaviour (Ellis et al. 2018). Potential runoff may also pose hazards to the soil microbiome and aquatic ecosystems (Ratte 1999). Therefore, outdoor studies are required to evaluate efficacy, stability, potential runoff and cost-effectiveness and ensure that laboratory findings translate reliably to field conditions.

Silver nanoparticles have been widely used as a biocontrol agent in agricultural practices (Lee et al. 2010). Apart from their antimicrobial potential, they have also been used as a novel genetic transformation tool for some plant species (Gunasena et al. 2024). However, their mode of uptake, internalisation route and interaction with cellular receptors is poorly described. Future studies using advanced microscopy should be conducted to better understand their entry, internalisation and accumulation.

Cannabis exhibits remarkable variability among cultivars and differential responses to the same stressors have been observed (Mateva et al. 2024; Sheldon et al. 2021). In the current study we also observed similar varied responses, where Black Label was less sensitive to CS than other cultivars. Since limited studies are available on such differential responses, further research is required to identify the underlying causes of this variation. In addition, considering the remarkable diversity in hemp, the CS protocol should be further tested in wider range of cannabis cultivars to assess its consistency, robustness and broad-spectrum potency. Since limited studies are available on this differential response, further research is needed to identify the underlying causes of this variation in hemp sex expression.

Although feminised seeds are designed to produce exclusively female plants, genetic disposition or stress may induce male flowers (Punja and Holmes 2020; Hall, Bhattarai, and Midmore 2012). This phenomenon is known as hermaphroditism and in some cases is decidedly

unwelcome in hemp farming (Olejar et al. 2022), although it also forms the basis of some very successful monoecious hemp cultivars. The appearance of male flowers on the MSG1 population under indoor conditions may be associated with either environmental factors (Borthwick et al. 1954), or genetic determinants (Punja et al. 2020). However, the absence of male flowers in the outdoor progeny suggests that a stress factor, potentially excessive humidity, was likely a contributing factor (Garcia-de Heer et al. 2025; Adal et al. 2021). However, some strains of cannabis also carry a predisposition for hermaphroditic traits (Punja et al. 2020). Further work would be needed to determine whether these changes in the MSG1 population were genetically preprogrammed, were triggered by environmental stress or CS.

Selfing similar genotypes may introduce deleterious alleles in progeny seeds. Although inbreeding in hemp may not appear straight away, multiple crosses may induce such traits (Kurtz et al. 2020). Therefore, comparative studies would be needed through several generations to assess steady state phytochemical levels in parental plants versus feminised progeny to avoid or detect early inbreeding depression.

While silver residues are less likely to be present in the seeds derived from cross pollination, selfing (same plant) may allow accumulation (Owen et al. 2023), warranting further studies and AgNPs have been reported to induce early seed abscission in *Ocimum* treated plants (Nejatzadeh et al. 2014). It would be beneficial to study whether the seeds contain silver residues, exhibit impaired development, or show alterations in their overall metabolic profile. In the present study we only analysed cannabinoid levels of Black Label plants grown from feminised seeds in indoor conditions. Since outdoor conditions differ greatly from indoor environments and the soil microbiome can also influence plant metabolism (Jamir et al. 2019), further studies should be conducted to assess the phytochemistry (e.g., cannabinoids and essential oils) of plants derived from feminised seeds grown in outdoor conditions.

The transcriptomic analysis attempted in Chapter 5 was severely undermined by the overwhelming presence of ribosomal RNA (rRNA) reads in the data set. This was likely to be due to using a non-customized human ribodepletion kit, which failed to remove rRNA from the cannabis RNA-seq libraries. If this work were to be repeated a plant-specific or custom rRNA depletion kit should be used to remove unwanted sequences or perhaps even better, mRNA enrichment to select the polyA fraction performed before library construction. The CS-induced reversal mechanism in hemp remains elusive. Current understanding suggests that hemp sex reversal is largely hormonal (Trancoso et al. 2022; Adal et al. 2021). However, the mechanisms controlling hormone biosynthesis, signalling and interactions and how they influence floral identity genes remains unclear. Experiments using single and combined hormone applications, along with the use of inhibitors can help determine whether hormones act independently or synergistically. Spectroscopic methods, including real-time ethylene quantification, could help determine ethylene contribution; however, due to its volatile nature, accurate measurement is challenging. Therefore, RNA-seq analysis serves as a more practical first step to help narrow down which hormones and other regulators may be involved. Silver nanoparticle exposure has also been shown to induce reactive oxygen species (ROS) and trigger oxidative stress (de Paiva Pinheiro et al. 2021). These signals can provoke a series of hormonal pathways and activate genes which are related to hemp sex expression (Baxter et al. 2014). However, their role in hemp masculinisation is unknown. Therefore, future studies can be conducted to confirm the role of ROS potential in hemp masculinisation. Chemical stress can be induced via application of hydrogen peroxide for example, and antioxidant enzyme levels can be measured over a time course that can be correlated to RNA-seq data.

The role of putative genes, including transcription factors and sequences that align with the ABC(DE) floral model defined for other plants should ideally be further verified and tested via overexpression and editing experiments, but this would also have to wait until an effective hemp

transformation system is available, There is no doubt that understanding the CS sex reversal mechanism carries significant value in the hemp breeding industry. This would aid in identifying marker genes associated with monoecious or bisexual plants, enabling breeders to manipulate female sex expression via targeted crosses without chemical applications and allowing growers to better regulate desirable traits such as fibre, seed, or flower bud production. The work described in this thesis therefore provides a springboard for future investigations that may eventually help researchers to understand more about these complex processes and, in collaboration with the hemp industry, use the findings to develop improved breeding practices.

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# Appendix I

## Certification of Cannabis Cultivars Used in This Thesis

botanacor

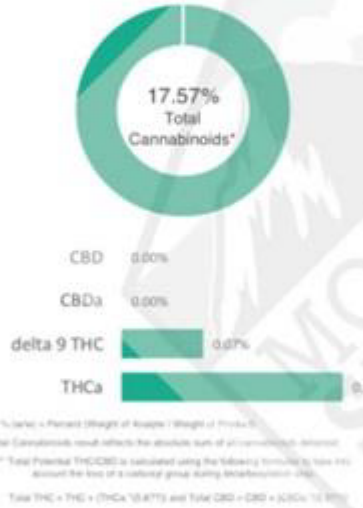
CERTIFICATE OF ANALYSIS

# MSG1

MBG 3

| | | | |
|---|---|---|---|
| **Batch ID:** | | **Test ID:** | 9298628.0056 |
| **Reported:** | 10-Jan-2020 | **Method:** | TM14 |
| **Type:** | Plant | | |
| **Test:** | Potency | | |

## CANNABINOID PROFILE




| Compound | LOQ (%) | Result (%) | Result (mg/g) |
|---|---|---|---|
| Delta 9-Tetrahydrocannabinolic acid (THCA-A) | 0.06 | 0.19 | 1.9 |
| Delta 9-Tetrahydrocannabinol (Delta 9THC) | 0.03 | 0.07 | 0.7 |
| Cannabidiolic acid (CBDA) | 0.06 | 0.00 | 0.0 |
| Cannabidiol (CBD) | 0.03 | 0.00 | 0.0 |
| Delta 8-Tetrahydrocannabinol (Delta 8THC) | 0.03 | 0.00 | 0.0 |
| Cannabinolic Acid (CBNA) | 0.08 | 0.00 | 0.0 |
| Cannabinol (CBN) | 0.04 | 0.00 | 0.0 |
| Cannabigerolic acid (CBGA) | 0.05 | 15.98 | 159.8 |
| Cannabigerol (CBG) | 0.03 | 0.77 | 7.7 |
| Tetrahydrocannabivarinic Acid (THCVA) | 0.05 | 0.00 | 0.0 |
| Tetrahydrocannabivarin (THCV) | 0.03 | 0.00 | 0.0 |
| Cannabidivarinic Acid (CBDVA) | 0.05 | 0.00 | 0.0 |
| Cannabidivarin (CBDV) | 0.03 | 0.00 | 0.0 |
| Cannabichromenic Acid (CBCA) | 0.04 | 0.50 | 5.0 |
| Cannabichromene (CBC) | 0.05 | 0.06 | 0.6 |
| **Total Cannabinoids** | | **17.57** | **175.70** |
| Total Potential THC** | | 0.34 | 2.37 |
| Total Potential CBD** | | 0.00 | 0.00 |

NOTES:
N/A

* Total Cannabinoids result reflects the absolute sum of all cannabinoids detected.

** Total Potential THC/CBD is calculated using the following formulas to take into account the loss of a carboxyl group during decarboxylation step.

Total THC = THC + (THCa *(0.877)) and Total CBD = CBD + (CBDa *(0.877))

## FINAL APPROVAL

| | |
|---|---|
| Michelle Gagnon<br>10-Jan-2020<br>1:04 PM | David Green<br>10-Jan-2020<br>2:32 PM |
| PREPARED BY / DATE | APPROVED BY / DATE |

*Testing results are based solely upon the sample submitted to Botanacor Laboratories, LLC, in the condition it was received. Botanacor Laboratories, LLC warrants that all analytical work is conducted professionally in accordance with all applicable standard laboratory practices using validated methods. Data was generated using an unbroken chain of comparison to NIST traceable Reference Standards and Certified Reference Materials. This report may not be reproduced, except in full, without the written approval of Botanacor Laboratories, LLC. ISO/IEC 17025:2005 Accredited A2LA Certificate Number 4329.02*

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MSD1

AURUM LABS

# Certificate of Analysis

## Cannabinoid Potency and Contaminant Analysis Report

Sample Name: 1301A
Sample Type: Plant, Flower - Cured, Indoor
Sample ID: 2003AU0050.03302
Batch ID:
METRC Tag: 1A4000712681398000000450

Windham Farms LLC

1403 Skyview Road

Bayfield, CO, CO 81122
(720) 584-3420
Lic. #403H-75195

## Cannabinoid Profile

| Analyte | LOQ | Amount % | Amount mg/g |
|---|---|---|---|
| THCa | 0.06 | 0.56 | 5.6 |
| Δ9-THC | 0.06 | 0.41 | 4.1 |
| Δ8-THC | 0.03 | ND | ND |
| CBDa | 0.06 | 16.95 | 169.5 |
| CBD | 0.06 | 3.71 | 37.1 |
| CBDVa | 0.03 | 0.13 | 1.3 |
| CBDV | 0.03 | 0.03 | 0.3 |
| CBN | 0.03 | ND | ND |
| CBGa | 0.03 | 0.91 | 9.1 |
| CBG | 0.03 | 0.33 | 3.3 |
| CBCa | 0.03 | 0.89 | 8.9 |
| CBC | 0.03 | 0.29 | 2.9 |
| CBL | 0.03 | ND | ND |

## Total Cannabinoids

| Analyte | Total* |
|---|---|
| THC | 0.90% |
| CBD | 18.58% |
| CBG | 1.13% |
| CBC | 1.07% |
| CBDV | 0.14% |

*Total is the sum of the neutral (active) cannabinoid and the completely converted acidic cannabinoid.

## Sample Photo



## Residual Solvent Analysis

| Analyte | LOQ | Limit | Amount | Status |
|---|---|---|---|---|

## Final Approval

Results Approved By:
Lucas Mason, M.S.
*Lab Director*

Results Analyzed By:
Joshua Reilly
*Analyst*

## Microbial Contaminants

| Analyte | Limit | Amount | Status |
|---|---|---|---|

**Received: 03/05/2020** **Tested: 03/06/2020** **Reported: 03/09/2020**

Definitions: LOQ= Limit of Quantitation, ND = Not Detected, CFU/g = Colony Forming Units per Gram

*This product has been tested by Aurum Labs using validated testing methodologies (unless specified in this report) and a Quality System as required by state law. Values reported related only to the product tested. Uncertainty information available upon request. Aurum Labs makes no claims as to the efficacy, safety or other risks associated with any detected or non detected levels of any compounds reported herein. This Certificate shall not be reproduced except in full, with the written approval of Aurum Labs.*

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# Black Label

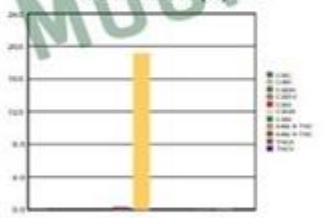
desert valley TESTING

| Sample Information | |
|---|---|
| Sample Identification | Black Label CBG |
| Laboratory Number | 1911282-01 |
| Batch Number | 1 |
| Matrix | Flower |

| Cannabinoid (HPLC) | % | mg/g |
|---|---|---|
| **Compound** | | |
| THCA | 0.14 | 1.4 |
| delta 9-THC | 0.05 | 0.5 |
| delta 8-THC | ND | ND |
| CBGA | 19.17 | 191.7 |
| THCV | ND | ND |
| CBDA | ND | ND |
| CBD | ND | ND |
| CBD-V | ND | ND |
| CBN | ND | ND |
| CBG | 0.34 | 3.4 |
| CBC | 0.11 | 1.1 |
| **Cannabinoids Total** | | |
| Max Active THC | 0.17 | 1.72 |
| Max Active CBD | 0.00 | 0.00 |
| T.Active Cannabinoids | 0.49 | 4.94 |
| Total Cannabinoids | 19.80 | 198.00 |

| Max Active Ratios |
|---|
| 0.00 : 1 CBD to THC |
| NA :1 THC to CBD |

Cannabinoid (%)



| RS (GCMS-MS) | ppm | RL |
|---|---|---|
| **Compound** | | |
| Acetone | NT | NT |
| Acetonitrile | NT | NT |
| Benzene | NT | NT |
| n-Butane | NT | NT |
| Carbon Tetrachloride | NT | NT |
| Chloroform | NT | NT |
| 1,2-Dichloroethane | NT | NT |
| Ethanol | NT | NT |
| Ethyl acetate | NT | NT |
| Ethylene oxide | NT | NT |
| n-Heptane | NT | NT |
| n-Hexane | NT | NT |
| iso-Butane | NT | NT |
| iso-Pentane | NT | NT |
| Methylene Chloride | NT | NT |
| n-Pentane | NT | NT |
| Propane | NT | NT |
| 2-Propanol (IPA) | NT | NT |
| Tetrahydrofuran | NT | NT |
| Toluene | NT | NT |
| Trichloroethene | NT | NT |
| o-xylene | NT | NT |
| Xylenes (p,m) | NT | NT |

Sample Image



| Terpenes (HPLC) | % | mg/g |
|---|---|---|
| **Compound** | | |
| alpha-Bisabolol | NT | NT |
| (-)-Borneol and (+)-Borneol | NT | NT |
| Camphene | NT | NT |
| Camphor | NT | NT |
| beta-Caryophyllene | NT | NT |
| trans-Caryophyllene | NT | NT |
| Caryophyllene Oxide | NT | NT |
| alpha-Cedrene | NT | NT |
| Cedrol | NT | NT |
| Endo-fenchyl Alcohol | NT | NT |
| Eucalyptol | NT | NT |
| Fenchone | NT | NT |
| Geraniol | NT | NT |
| Geranyl acetate | NT | NT |
| Guaiol | NT | NT |
| Hexahydrothymol | NT | NT |
| alpha-Humulene | NT | NT |
| Isoborneol | NT | NT |
| Isopulegol | NT | NT |
| Limonene | NT | NT |
| Linalool | NT | NT |
| p-Mentha-1,5-diene | NT | NT |
| beta-Myrcene | NT | NT |
| trans-Nerolidol | NT | NT |
| Ocimene Isomer 1 | NT | NT |
| alpha-Pinene | NT | NT |
| beta-Pinene | NT | NT |
| Pulegone | NT | NT |
| Sabinene | NT | NT |
| Sabinene Hydrate | NT | NT |
| gamma-Terpinene | NT | NT |
| alpha-Terpinene | NT | NT |
| alpha-Terpineol | NT | NT |
| 3-Carene | NT | NT |
| Ocimene Isomer 2 | NT | NT |
| gamma-Terpineol | NT | NT |
| Terpinolene | NT | NT |
| Valencene | NT | NT |
| Nerol | NT | NT |
| cis-Nerolidol | NT | NT |
| Total Terpenes | NT | NT |

| Metals | ppm | RL |
|---|---|---|
| **Compound** | | |
| Arsenic | NT | NT |
| Cadmium | NT | NT |
| Mercury | NT | NT |
| Lead | NT | NT |

| Percent Moisture |
|---|
| NT % |

| Water Activity |
|---|
| NT |

RL = Reporting Limit
NA = Not Applicable
NT = Not Tested
ND = Non Detected

MOUNTAIN STRONG HEMP

## Appendix II

## Career and Research Skills Training (CaRST) Certification

What is CaRST:

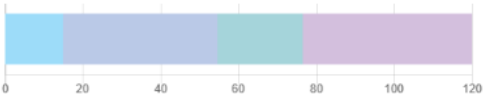
From the CaRST handbook:

"CaRST is a specialised training and development program for Higher Degree by Research (HDR) students at the University of Adelaide. CaRST was introduced to broaden the research training experience and prepare HDRs for a wide range of careers both within and outside academia. The program aims to facilitate a quality, in-depth research training experience for all HDRs and has been designed to meet the needs of our diverse researcher community."

Higher Degree Research (HDR) students enrolled in a PhD program must complete 120 hours of activities or courses distributed across four domains of the Researcher Development Framework before the submission of their thesis.

I completed the following activities to meet the requirements of the CaRST program.

# CARST CREDITS OVERVIEW




**120**
**Recognised Credits**

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Well done, you've reached your CaRST target! Download Completion Certificate

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| Date | Title / Summary | Total Credits | Domains |
|---|---|---|---|
| 04 Apr 2022 | IT Tools for Researchers: What, Why, How, and Where | 1 | Domain A |
| 12 Apr 2022 | Planning your Research Proposal | 3 | Domain A |
| 19 Apr 2022 | Writing the Introductory and Literature Review Sections of your Research Proposal | 2.5 | Domain A |
| 26 Apr 2022 | Writing the Theory, Methodology and Summary Sections of your Research Proposal | 2.5 | Domain A |
| 28 Mar 2022 | Researcher Profiles and Personal Branding | 1 | Domain B |
| 26 Sep 2023 | Thinking Resiliently as a Researcher | 1 | Domain B |
| 26 Oct 2023 | Understanding Skills | 1 | Domain B |
| 07 Nov 2023 | Creating an Outstanding LinkedIn Profile | 1 | Domain B |
| 09 Nov 2023 | Sourcing Jobs Outside Academia | 1 | Domain B |
| 15 Nov 2023 | Writing your Resume | 1 | Domain B |
| 22 Nov 2023 | Responding to Selection Criteria | 1 | Domain B |
| 27 Nov 2023 | Ace your interview | 1 | Domain B |
| 29 Nov 2023 | Building Connections | 0.5 | Domain B |
| 11 Dec 2023 | Career Control for Researchers | 13 | Domain B |
| 30 Mar 2022 | CaRST Information Session | 0 | Domain C |
| 07 Apr 2022 | How to Plan your PhD | 2.5 | Domain C |
| 16 Mar 2023 | CaRST Information Session | 1 | Domain C |
| 11 May 2023 | eProcurement Information Session | 1 | Domain C |

THE UNIVERSITY of ADELAIDE

This is to certify that

# Mr Muhammad Nadeem

has completed

## 120 hours

of Career and Research Skills Training

16 Jan 2025

*Katy Dolman*

*Manager, Researcher Education and Development*

*Date*

make history.

## Appendix III

## Conference attendance

I have presented my research at following events:

- **PhD Milestone**: Core Component of the Structured Program (CCSP)

“Cannabis sativa feminisation using silver nanoparticles”

- 28 November March 2022

- **PhD Milestone**: Major Review

“Cannabis sativa feminisation using silver nanoparticles”

- 28 April  2023

- **University of Adelaide Postgraduate Symposium**

“Cannabis sativa feminisation using silver nanoparticles”

25  September 2023

- **Presented at “Fourth biennial Australian Industrial Hemp Conference**”, Hunter Valley, NSW, April 2024

Cannabis sativa feminisation using silver nanoparticles.




# Certificate of Attendance

This is to certify that

# Mr Muhammad Nadeem

Attended: **Fourth Biennial Australian Industrial Hemp Conference 2024**, Oaks Cypress Lakes Resort, Pokolbin, New South Wales

**Publications**

Gold Bulletin (2023) 56:69–81
https://doi.org/10.1007/s13404-023-00335-1

REVIEW

# Microbial-mediated synthesis of gold nanoparticles—current insights and future vistas

Muhammad Nadeem[1] · Laiba Pervez[2] · Ameer Muhammad Khan[2] · Rachel A. Burton[1] · Sana Ullah[3] · Akhtar Nadhman[2] · Jonathan Celli[4]

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## Abstract

Nanotechnology is an emerging field with a multitude of applications in medicine, agriculture, and engineering. To date, several methods are used for the synthesis of metallic nanoparticles including chemical, physical, and green methods. The later method is preferred for the synthesis of NPs due to its safe nature, efficiency, and minimal toxicity. Recently, gold nanoparticles (AuNPs) have been attracting attention due to their excellent biocompatibility and diverse medical applications. A diverse group of microorganisms or their metabolites (bacteria, fungi, and yeast) have been utilized to synthesize AuNPs from their respective bulk salts. This review focuses on the resources exploited for biosynthesis of AuNPs, the current understanding about their synthesis, and the emerging trends, along with their mechanistic antimicrobial and anticancer applications. This review also highlights the current challenges and future potential applications of AuNPs.

**Keywords** Nanotechnology · Green synthesis · Gold · Nanoparticles · Antimicrobial · Anticancer

## Introduction

Nanotechnology is an emerging field with a multitude of applications in medicine, agriculture, electronics, imaging, and sensing [1]. Nanotechnology exploits material sizes ranging from 1 to 100 nm [2]. These nanoscale entities have properties that significantly exceed their bulkier counterparts, and this has raised substantial attention in both research and industry [3]. In the medical field, nanoparticles (NPs) have been implicated in several advances including formulation of novel drugs, the introduction of facile delivery systems, and targeted therapy [4, 5].

Presently, metallic NPs are synthesized via chemical, physical, and green methods [3]. To achieve a sustainable route, the green synthesis approach offers multiple benefits over the other two. This method is safe, reliable, cost-effective, and eco-friendly as compared to physical and chemical approaches which tend to use expensive and toxic chemicals as reducing agents [6, 7]. Additionally, the green method is less laborious and requires minimal downstream processing [8, 9]. This approach involves the use of microbes, plants, and other biological agents or their products for synthesis processes. The metabolites in these reactions act as a reducing and capping agent in the reduction and synthesis of NPs from their respective bulk salts [7].

With the advent of nanotechnology, a variety of NPs have been introduced; however, in recent years, focus has shifted to the use of gold nanoparticles (AuNPs) in research and industry [10]. Feasible synthesis, distinct optical properties, and minimal toxicity have contributed to the superiority of AuNPs over others [10, 11]. With such unique attributes, AuNPs have been exploited in electronics, medicine, and agriculture. These NPs are also synthesized via chemical, physical, and biological methods, each having their own pros

Laiba Pervez and Ameer Muhammad Khan contributed equally to this work.

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and cons. However, the green derived AuNPs are preferred over other approaches as this offers minimal cyto- and genotoxicity [12, 13].

Amongst various biological materials (plants and others), microbial-mediated NP synthesis exhibits several advantages, and AuNPs of various shapes and sizes have been synthesized in recent years using different microbial species [14]. Multiple reviews have been published on green synthesized AuNPs, but none has summarized their biological applications in detail. This review encapsulates the key features of AuNP green synthesis with special emphasis on microbial routes and the therapeutic potential of the resultant AuNPs.

## Green synthesis of AuNPs using bacteria

Bacteria have been reported as an excellent candidate to produce AuNPs (Table 1). Based on the location of synthesis, it is classified into two categories: intracellular and extracellular [15]. In the former case, gold ions are translocated inside of cells via ion channels, endocytosis, or carrier proteins, and later, it is reduced into AuNPs [16–21]. Such pattern of synthesis have been documented in the bacterium strains *Geobacillus*, *Streptomyces*, *Stenotrophomonas maltophilia*, *Pseudomonas denitrificans*, and *Halomonas salina* [22–25]. In contrast, when reduction occurs outside of cytoplasm, it is termed as extracellular synthesis method. The differential location of synthesis is supposed to depend on enzyme availability and other media parameters [17, 26]. However, in *Bacillus subtilis*, AuNPs were synthesized on the inside and outside of cell, which indicates that the reducing and stabilizing agents are found in both in and outside of cells [27]. In terms of ease and yield, extracellular bacteria are considered more efficient [24]. Literature study proposes that enzymatic reduction is one of the paramount method for production of AuNPs [15, 18]. Bacterial enzymes such as nitrate and NADPH-dependent reductases have been shown to play an important role in the biosynthesis of AuNPs in both modes [18, 28]. NADPH-dependent reductase was also found to act as a nucleating agents in the extracellular synthesis of AuNPs in *Pseudomonas aeruginosa* [29]. In this enzymatic-based reaction, gold ions $Au^{+3}$ are changed into $Au^{0}$ by electron transfer from NADH via NADPH-dependent reductase [30]. Owing to rich metabolic profile of bacteria, various bacterial metabolites have also been reported to produce AuNPs efficiently. For example, *Pseudomonas fluorescens* extract has been used to produce spherically shaped NPs with sizes ranging from 5 to 10 nm. The reduction and stabilization process reaction attributed to the presence of aromatic and organic compounds [23]. *Serratia marcescens* yielded 21–40 nm and 20–120 nm sized AuNPs from cell-free extracts and cell biomass solutions, respectively. In this case, prodigiosin (naphthoquinones with two fused rings, one of which is a benzene ring and the other is a six-membered ring with three carbon atoms and three oxygen atoms) acts as a reducing agent for AuNP synthesis. Bacterial proteins such as tyrosine, cysteine, tryptophan, and surfactin have also been reported to act as capping and reducing agents in the synthesis of AuNPs [27, 31]. A wide range of live bacterial cells or their extracts have been utilized for the synthesis of AuNPs and are discussed in Table 1. Multiple parameters such as temperature, pH, and other conditions affect the physical and morphological properties of AuNPs. Most of the literature studies suggest that temperatures ranging from 25 to 38 °C are commonly observed for nanoparticle synthesis, with a predominant occurrence of spherical shapes. Though bacteria offer a good platform for biosynthesis of AuNPs, but factors such as slow culture growth, poor metabolomic profile, and higher chances of contamination and pathogenicity are still important concerns.

## Green synthesis of AuNPs using fungi and yeast

Fungi are a rich source of enzymes, metabolites, and proteins which can act as both capping and reducing agents in the synthesis of AuNPs from precursor gold salts [30]. In contrast to bacteria, fungal strains grow faster, require less labor, and have minimal chances of pathogenicity [10]. In the past, multiple species of fungi have been exploited in the biosynthesis of AuNPs (Table 2). For instance, *Fusarium oxysporum* was used for the extracellular production of AuNPs by the reductive action of the nitrate-dependent enzyme and shuttle, quinone [53]. Intracellular synthesis of AuNPs was obtained using *Verticillium* fungal strains via the action of reductases in fungal cell walls and membranes [54]. *Penicillium brevicompactum* extract yielded spherical, rod, pentagonal, and hexagonal shaped nanoparticles of 20–80 nm. FTIR analysis showed the presence of amines, carboxylic, and phenolic [55]. In another study, *Aspergillus niger* extract yielded triangular shaped AuNPs. FTIR analysis showed the presence of amide I and amide II proteins and COO- groups as reducing agents [56]. In a similar fashion, intracellular production of AuNPs was observed when *Mariannaea* sp. extract was exposed to bulk gold salt. The groups analyzed by FTIR as reducing agents were OH groups in protein or carbohydrates, C-H vibrations, amide I and II, the aliphatic amine stretch (C-N), and carboxyl groups [57]. Both extracellular and intracellular synthesis was reported, and results showed that laccase was involved in extracellular synthesis of AuNPs, while ligninase was involved in intracellular synthesis. Similarly, the extracellular mode of

**Table 1** AuNPs synthesized from various bacterial species

| S. No | Specie name | Size (nm) | Morphology | Functional group | Characterization | Temperature (°C) and pH | Time | Color | Mode of synthesis | Ref |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *Enterococcus* sp. (2020) | 5–10 | Spherical | NR | UV/Vis, TEM | NR | 48 h | NR | Intracellular | [32] |
| 2 | *Paracoccus haeundaensis* (2019) | 20.93 | Spherical | Amines, nitro, alcohols, carboxylic acid | UV/Vis, TEM, DLS, FTIR | 70 °C | 15 min | Ruby red | Extracellular | [33] |
| 3 | *Shewanella loihica* (2018) | 2–15 | Spherical | NR | UV/Vis, SEM, TEM, XRD | 30 °C and pH 4, 7, 9 | 48 h | Pink | NR | [34] |
| 4 | *Micrococcus yunnanensis* (2018) | 53.8 | Spherical | -OH, -NH, -CH, amides | UV/Vis, TEM, SEM, XRD, TGA, FTIR | 30 °C and pH 7.8 | 60 h | Purple | NR | [35] |
| 5 | *Mycobacterium* sp. (2018) | 5–55 | Spherical | -COOH, -OH, -Cl, -NH2 | UV/Vis, FTIR, TEM | 35 °C | 24 h | Dark pink | Extracellular | [36] |
| 6 | *Halomonas salina* (2017) | 30–100 | Spherical | Amino, sulfhydryl, carboxylic acid | UV/Vis, TEM, XRD | 30 °C and 100 °C, varied pH | 24 h | Change in color | Extracellular | [29] |
| 7 | *Deinococcus radiodurans* (2016) | 43.75 | Spherical, triangular | Carboxyl, amines, phosphates, hydroxyl groups | UV/Vis, EM, XRD, DLS, FTIR, XPS | 32 °C and pH 7 | 8 h | Dark purple | Extracellular | [37] |
| 8 | *Sporosarcina koreensis* (2016) | 92.4 | Spherical | NR | UV/Vis, FE-TEM, XRD | 37 °C and pH 4–10 | 48 h | Deep purple | Extracellular | [37] |
| 9 | *Anabaena* sp. (2016) | 10–30 | Spherical | NR | UV/Vis, SEM | NR | 48 h | Purple | Intracellular | [22] |
| 10 | *Azotobacter vinelandii* (2016) | 10–30 | Irregular, spherical | NR | UV/Vis, SEM | NR | 4–5 days | Pink to red | Intracellular | |
| 11 | *Azotobacter chroococcum* (2016) | 10–30 | Spherical, irregular, triangular | NR | UV/Vis, SEM | NR | 4–5 days | Purple | Intracellular | |
| 12 | *Acinetobacter* sp. (2016) | 19 | Spherical, triangular, polyhedral | Amines, alcohols, C–Cl | UV/Vis, TEM, FTIR | 30 °C and pH 7 | 120 h | Purple | NR | [38] |
| 13 | *Pseudomonas veronii* AS41G (2015) | 5–25 | Crystalline | -NH2, -C0, amides, C–Cl | UV/Vis, FTIR, XRD, TEM | 25 °C | 10 min | Ruby red | Extracellular | [39] |
| 14 | *Streptomyces* sp. (2015) | 20–80 | Spherical | -OH, alkanes, amines | UV/Vis, XRD, FTIR, SEM, TEM | 30 °C | 96 h | Pink | Extracellular | [40] |
| 15 | *Pseudomonas aeruginosa* (2014) | 40 | NR | NR | UV/Vis, TEM | 37 °C | 24 h | Wine pink | Extracellular | [30] |
| 16 | *Streptomyces fulvissimus* (2014) | 20–50 | Variable morphology | NR | UV/Vis, TEM, EDX, XRD | 30 °C | 48 h | Red | Extracellular | [41] |
| 17 | *Stenotrophomonas* (2013) | 10–50 | Spherical | NR | UV/Vis, DLS, TEM | 30 °C | 16 h | Violet | Extracellular | [42] |
| 18 | *Bacillus subtilis* (2013) | 7 | Spherical | NR | UV/Vis, XRD, TEM | 30 °C and pH 2.4 | 24 h | Light pink | Extracellular and intracellular | [27] |

**Table 1** (continued)

| S. No | Specie name | Size (nm) | Morphology | Functional group | Characterization | Temperature (°C) and pH | Time | Color | Mode of synthesis | Ref |
|---|---|---|---|---|---|---|---|---|---|---|
| 19 | *Geobacillus* sp. (2013) | 5–50 | Hexagonal | Amines, aides, aliphatic C-H, C-N | UV/Vis, TEM, EDX, FTIR | 65 °C and pH 7.0 | 16 h | Intense purple | Intracellular | [23] |
| 20 | *Klebsiella pneumonia* (2013) | 35–65 | Spherical | Carbonyl, nitro compounds, amines | UV/Vis, XRD, TEM, FTIR | 35 °C | 6–24 h | Dark purple | Extracellular | [43] |
| 21 | *Marinobacter pelagius* (2012) | 10 | Spherical | Amines, carbonyls, N–H compounds | UV/Vis, DLS, TEM | 30–37 °C and pH 7 | 24 h | Pink | NR | [22] |
| 22 | *Bacillus licheniformis* (2012) | 50 | Spherical | Nitro, phenols, alkanes, esters, amides, alkynes | UV/Vis, FTIR, TEM | 37 °C and pH 7 | 24 h | Purple | Intracellular | [44] |
| 23 | *Streptomyces viridogens* (2011) | 18–20 | Rod, spherical | NR | UV/Vis, XRD, TEM | 28 °C | 24 h | Pink purple | Intracellular | [24] |
| 24 | *Shewanella oneidensis* (2011) | 12 ± 5 | Spherical | -NH, carbonyls, hydroxyls | UV/Vis, FTIR, XRD, EDX, TEM | 30 °C | 48 h | Deep purple | Extracellular | [45] |
| 25 | *Brevibacterium casei* (2010) | 10–50 | Spherical | Amides | UV/Vis, XRD, FTIR, TEM | 37 °C | 24 h | Dark purple | NR | [46] |
| 26 | | 40 | Spherical | Phosphate groups | UV/Vis, FTIR, zeta potential | 25 °C | 8 h | Cherry red | Intracellular | [25] |
| 27 | *Bacillus megaterium* (2008) | 1.9 ± 0.8 | Spherical | NR | UV/Vis, TEM | 26 °C and pH 3.2 | 9 h | Pink | Extracellular | [47] |
| 28 | *Rhodopseudomonas capsulata* (2007) | 10–20 at pH 7 | Varied morphology | NR | UV/Vis, XRD, TEM | 24 °C and varied pH (4, 7) | 48 h | Purple | Extracellular | [48] |
| 29 | *Escherichia coli* (2007) | 20–30 | Spherical | NR | UV/Vis, XRD, TEM | NR | 120 h | Purple | NR | [49] |
| 30 | *Plectonema boryanum* (2006) | < 10–25 | Cubic | NR | UV/Vis, SEM, XPS | 20–200 °C | 14 days | Brown | Intracellular | [50] |
| 31 | *Rhodococcus* sp. (2005) | 5–15 | Spherical | NR | UV/Vis, EM, XRD | 27 °C and pH 7 | 24 h | Purple | Intracellular | [51] |
| 32 | *Thermomonospora* sp. | 8 | Spherical | Amides | UV/Vis, FTIR, TEM | 50 °C and pH 9 | 120 h | Ruby red | Extracellular | [52] |

**Table 2** AuNPs synthesized from various fungal species

| S. No | Specie name | Size (nm) | Shape | Functional group | Characterization | Mode of synthesis | Reference |
|---|---|---|---|---|---|---|---|
| 1 | *Cladosporium* sp. | 5–10 | Spherical | CO–OH, -OH, amides | UV/Vis, FTIR, XRD, TEM | Extracellular | [63] |
| 2 | *Penicillium crustosum* | 100 | Spherical | OH, -CH, methylene, -NH, amines, carbonyl | UV/Vis, FTIR, AFM, DLS | Extracellular | [64] |
| 3 | *Pleurotus ostreatus* | 10–30 | Spherical | NR | UV/Vis, FTIR, TEM, XRD, Zetasizer | Extracellular | [65] |
| 4 | *Trichoderma hamatum* (2018) | 5–30 | Divers | Amides, C-H, -NH, C-N | UV/Vis, TEM, FTIR | Extracellular | [66] |
| 5 | *Aspergillus fischeri* (2016) | 50 | Spherical | NH, C-O, C–C, amides | UV/Vis, XRD, FTIR, TEM | Extracellular | [67] |
| 6 | *Cladosporium oxysporum* (2016) | 73.32 ± 21.80 | Spherical | Hydroxyl | UV/Vis, DLS, TEM, EDS analysis | Extracellular | [68] |
| 7 | *Penicillium aculeatum* (2016) | 60 | Spherical | OH, C–C, C-H, C-N, -COO- | UV/Vis, AFM, DLS, SEM, FTIR | Extracellular | [69] |
| 8 | *Aspergillum* sp. (2016) | 10–100 | Spherical | NR | UV/Vis, TEM | Intracellular/extracellular | [70] |
| 9 | *Bipolaris tetramera* (2015) | 261.73 | Spherical, hexagonal | NR | UV/Vis, DLS, TEM | Extracellular | [71] |
| 10 | *Nigrospora oryzae* (2014) | 6–18 | Spherical | OH, amides, methylene, carboxyl | UV/Vis, DLS, AFM, TEM, FTIR | Extracellular | [72] |
| 11 | *Aspergillus sydowii* (2014) | 8.7–15.6 | Spherical | NR | UV/Vis, TEM | Extracellular/intracellular | [73] |
| 12 | *Trichoderma* sp. (2014) | 59–61 | Spherical | NR | UV/Vis, TEM, EDAX, SEM | Extracellular | [74] |
| 13 | *Hypocrea lixii* (2014) | 60 | Spherical | NR | UV/Vis, TEM, EDAX, SEM | Extracellular | |
| 14 | *Trichoderma harzianum* (2014) | 26–34 | Spherical | N–H, OH, C = C, COO-, -C-O | UV/Vis, DLS, TEM, EDX, FTIR, XRD | Extracellular | [75] |
| 15 | *Alternaria alternata* (2014) | 12 ± 5 | Spherical, triangular | OH, CH, amides, CH, C-O, R-CH, C = 0 | UV/Vis, DLS, XRD, EDX, TEM, FTIR, AFM | Extracellular | [76] |
| 16 | *Aspergillus terreus* (2014) | 10–19 | NR | CH, NH, SH | UV/Vis, EDX, XRD, FTIR | Extracellular | [77] |
| 17 | *Penicillium aurantiogriseum* (2013) | 153.3 | Spherical | Amide, -COO-, OH, CN | UV/Vis, FTIR, SEM, DLS, Nano Zetasizer | NR | [78] |
| 18 | *Penicillium citrinum* (2013) | 172 | Spherical | Amide, -COO- | UV/Vis, FTIR, SEM, DLS, Nano Zeta Sizer | NR | |
| 19 | *Penicillium waksmanii* (2013) | 160.1 | Spherical | Amide, -COO- | UV/Vis, FTIR, Nano Zetasizer, DLS, SEM | NR | |
| 20 | *Fusarium acuminatum* (2013) | 8–28 | Spherical | -C-O, C-N, C = O, carboxylate groups | UV/Vis, FTIR, zeta potential, XRD, TEM | Extracellular | [79] |
| 22 | *Aspergillus fumigatus* (2013) | 85.1–210 | Spherical | C = O, C-N, N–H, OH | UV/Vis, FTIR, SEM, XRD | Intracellular | [80] |
| 23 | *Botrytis cinerea* (2013) | 1–100 | Triangular, hexagonal, pyramidal | NR | UV/Vis, TEM, EDX, SEM | Extracellular | [81] |
| 24 | *Penicillium rugulosum* (2012) | 10–50 | Spherical | OH, NH, carboxylic, phenolic, R-CH groups | UV/Vis, Bio-TEM,XRD, FTIR, XPS | NR | [82] |

**Table 2** (continued)

| S. No | Specie name | Size (nm) | Shape | Functional group | Characterization | Mode of synthesis | Reference |
|---|---|---|---|---|---|---|---|
| 25 | *Phoma macrostoma* (2012) | 100–200 | Spherical, triangular, rod shape | NR | UV/Vis, XRD, TEM | Intracellular | [83] |
| 26 | *Helminthosporium solani* (2012) | 2–70 | Rods, triangles, pentagons, stars | NR | UV/Vis, TEM, XRD, DLS, AFM | Extracellular | [84] |
| 27 | *Penicillium brevicompactum* (2011) | 20–80 | Spherical, triangular, hexagonal | OH, NH, carboxylic, phenolic, amides | UV/Vis, Bio-TEM, XRD, FTIR | Extracellular | [55] |
| 28 | *Penicillium chrysogenum* (2011) | 5–100 | Spherical, triangular, rods | NR | UV/Vis, XRD, TEM | Intracellular | [85] |
| 29 | *Neurospora crassa* (2011) | 32 | Spherical | Amines, carboxyl, sulfhydryl, hydroxyl | UV/Vis, SEM, TEM, EDS | Intracellular | [86] |
| 30 | *Cylindrocladium floridanum* (2011) | 5–35 | spherical | NR | UV/Vis, XRD, SEM, EDX, TEM | Intracellular | [87] |
| 31 | *Sclerotium rolfsii* | 25 | Triangular, hexagonal, decahedral, rod, spherical | Amides, carbonyl, NH, carboxyl, CN | UV/Vis, XRD, SEM, EDX, FTIR, TEM | Extracellular | [88] |
| 32 | *Aureobasidium pullulans* (2011) | 29 ± 5 | Spherical | Amide, aldehydes | UV/Vis, FTIR, TEM | Intracellular | [89] |
| 33 | *Fusarium* sp. (2011) | 125 ± 83 | Spherical | Amide, aldehyde, C = O, C = N | UV/Vis, FTIR, TEM | Intracellular | |
| 34 | *Penicillium* sp. (2010) | 30–50 | Spherical | NR | UV/Vis, XRD, TEM, SEM, EDX | Extracellular/intracellular | [90] |
| 35 | *Fusarium semitectum* (2008) | 18–50 | Spherical | Amide, N–H, carboxyl | UV/Vis, FTIR, XRD, TEM | Extracellular | [91] |
| 36 | *Colletotrichum* sp. (2003) | 8–40 | Spherical, irregular | Amides | UV/Vis, XRD, FTIR, TEM | Extracellular | [92] |
| 37 | *Rhizopus oryzae* | 10 | Nanocrystalline | Amide, carboxyl, phosphate groups | UV/Vis, FTIR, TEM, EDAX, AFM | Cell surface | [93] |
| 38 | *Verticillium* sp. (2001) | 20 ± 8 | Spherical | NR | UV/Vis, XRD, SEM, EDX | Intracellular | [54] |

synthesis by fungi is also considered efficient than intracellular mode [58]. In contrast to bacteria, fungus offers a sustainable route for fabrication of AuNPs, but further studies are needed to elucidate the synthesis mechanisms and tailor a robust protocol for commercial production. Additionally, to avoid pathogenicity avirulent strains should be screened only for their use in biomedical or agricultural practices.

Certain species of yeast have also been utilized for the synthesis of AuNPs. For instance, *Saccharomyces cerevisiae* produced AuNPs after 24 h of incubation, and a color change from yellow to purple indicated the synthesis of AuNPs extracellularly [59]. Using TEM analysis, spherically shaped NPs with a size range of 20–100 nm were observed. Spherical AuNPs ranging from 20 to 80 nm were synthesized using *Candida albicans*. The extracellular mediated AuNPs showed excellent antimicrobial properties against pathogenic bacteria [60]. *Pichia jadinii* has also been exploited in the fabrication of AuNPs. The intracellularly synthesized NPs were present in multiple shapes and 10 nm in size as revealed by TEM [61]. A non-conventional marine yeast strain, *Yarrowia lipolytica*, synthesized intracellular AuNPs at a lower pH [62]. Though a very little has been explored in yeast, but it offers a very safe and less pathogenic platform for synthesis of AuNPs and could be explored for scalable production.

## Biological applications of microbial-mediated gold nanoparticles

### Antimicrobial applications of AuNPs

Gold has been used for a long time against many microbial pathogens including bacteria, fungi, and viral pathogens [1]. Owing to their excellent biocompatibility, AuNPs exhibit lower toxicity compared to other metal nanoparticles [94]. In the past, microbial-mediated AuNPs have been deployed against many strains of pathogenic bacteria and have shown potential antimicrobial activity (Table 3). The localized surface plasmon resonance effect of AuNPs, higher interaction

**Table 3** Antimicrobial activity of biosynthesized AuNPs

| S. No | Bacterial sps | Microbes tested | Reference |
|---|---|---|---|
| 1 | *Streptomyces griseus* | *Enterococcus faecalis, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Vibrio fluvialis, Vibrio damsel, Candida albicans* | [110] |
| 2 | *Lignosus rhinocerotis* | *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Bacillus* sp. | [107] |
| 3 | *Phaffia rhodozyma* | *Cryptococcus neoformans* | [111] |
| 4 | *Brevibacillus formosus* | *Staphylococcus aureus, Escherichia coli* | [112] |
| 5 | *Deinococcus radiodurans* | *Staphylococcus aureus, Escherichia coli* | [37] |
| 6 | Fungus sps | Microbes tested | |
| 7 | *Trichoderma viride* | *Pseudomonas syringae, Escherichia coli, Shigella sonnei* | [74] |
| 8 | *Hypocrea lixii* | *Pseudomonas syringae, Escherichia coli, Shigella sonnei* | [74] |
| 9 | *Candida albicans* | *Staphylococcus aureus, Escherichia coli* | [108] |
| 10 | *Streptomyces viridogens* | *Staphylococcus aureus, Escherichia coli* | [24] |
| 11 | *Alternaria* spp | *Pseudomonas aeruginosa, Klebsiella* and *Escherichia coli, pneumonia*, and *Staphylococcus aureus* | [113] |
| 12 | *Bipolaris tetramera* | *Trichoderma* sp., *Enterobacter aeruginosa, Bacillus cereus*, and *Staphylococcus aureus* | [71] |
| 13 | *Aspergillus terreus* | *Escherichia coli* | [77] |
| 14 | *Pleurotus ostreatus* | *Staphylococcus auras* and *Escherichia coli* | [65] |

with thiol groups, and heat shock proteins also contribute to their higher antimicrobial potential than other metallic NPs [95, 96]. AuNPs also release gold ions and also contribute to additional antimicrobial activity [97]. Generally, AuNPs kill bacteria via elevating oxidative stress which impedes normal cellular functioning and reproduction [98–101]. When bacterial strains were exposed to bacterial-mediated AuNPs, a cascade of singlet oxygen species was generated. The high levels of these free radicles resulted in membrane disruption, nucleic acid fragmentation, and halted replication [102]. A scheme of potential antibacterial activity is shown in Fig. 1, while putative interactions of AuNPs with bacterial cell membranes are shown in Fig. 2. When compared to other synthetic approaches (chemical), microbial-mediated AuNPs showed better antimicrobial action [65, 103]. The antimicrobial potential of AuNPs was also found to be dependent on AuNPs size, shape, and bacterial strains. Though it showed excellent potential against both gram-negative and

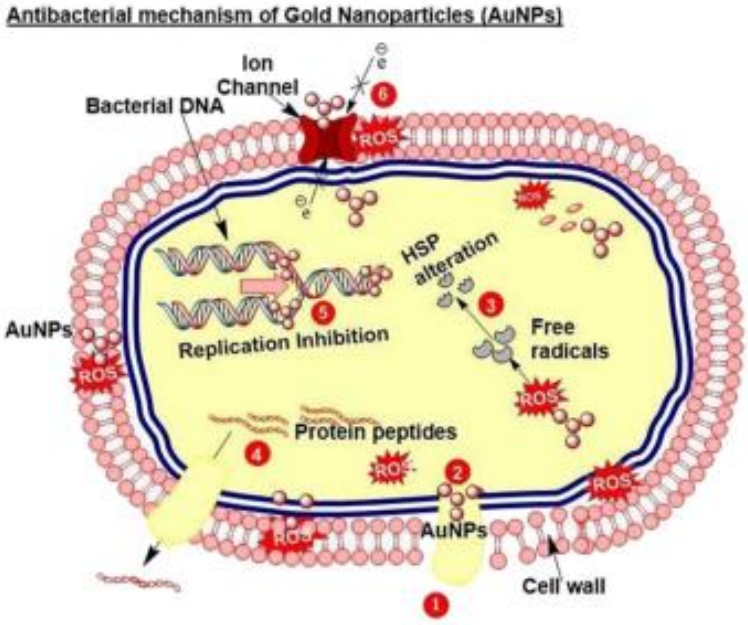


**Fig. 1** Antibacterial potential of bacterially derived green AuNPs. 1. AuNPs interaction with membrane of bacterial cell. 2. Translocation of AuNPs across membrane. 3. ROS generation damage proteins, especially heat shock proteins (HSP). 4. ROS denature proteins. 5. AuNPs inhibiting bacterial replication. 6. Membrane disruption

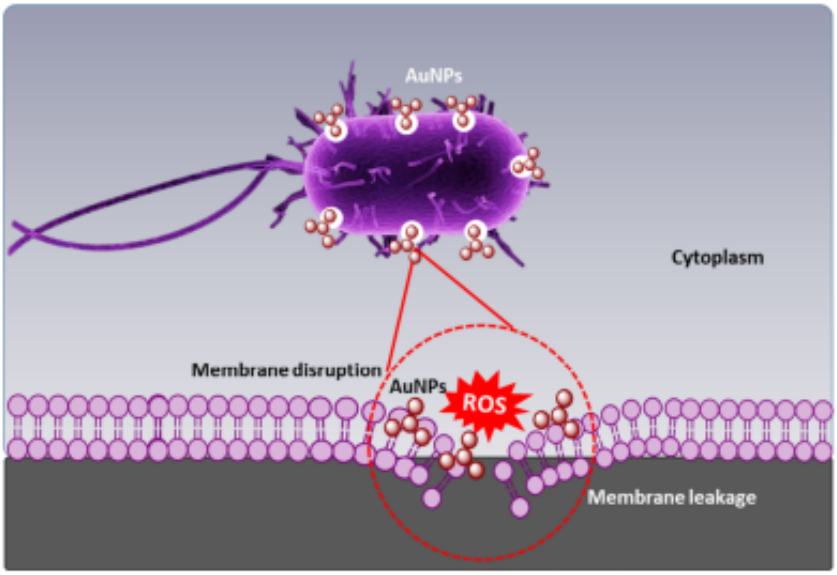


**Fig. 2** Putative mechanism of membrane destruction caused by microbial-derived AuNPs

gram-positive strains, but literature studies suggest that AuNPs were found most effective against gram-negative bacteria [104–107].

Bacterial-derived AuNPs have also been reported to show kills bacteria in the same way as chemical counterparts similar antimicrobial activities, i.e., impeding obstructing membrane permeability, distorting cellular structures inhibiting, inhibiting DNA uncoiling, and halting transcription processing [24]. Fungus species–mediated AuNPs have also been reported recently and have shown excellent antimicrobial potential against wide range of bacterial pathogens [34, 71, 74, 77, 108]. Their antimicrobial potential is associated with increase efflux of ions (K+) and ROS formation from membranes, followed by disintegration of membrane and organelles architecture, halting biofilm formation and replication machinery [108, 109]. Fungus mediated are both capable of inactivating gram-negative and gram-positive bacteria; however, they were found to be more potent against gram negative as compared to gram positive [77].

With such tremendous potential, AuNPs offer a versatile treatment against a wide range of microbes and multi-drug resistant (MDR) bacteria. Beside their antimicrobial potential, AuNPs also offer an excellent tool for targeted delivery of other antimicrobial agents into inaccessible parts of the body. Considering their safer profile, AuNPs could be also utilized as a biocontrol agent in food packings, agriculture, and health hygiene practices. However, their toxicity and safety should be investigated in both in vivo model organisms to translate these novel nanobiotics from lab to commercial practices.

## Anticancer potential of microbial-mediated gold nanoparticles

In the last few decades, AuNPs have attracted substantial attention in cancer theranostics [114]. In the past, they have been used in the treatment of various cancers [114–118]. In typical fashion, AuNPs target cancer cells via ROS-mediated apoptosis, DNA fragmentation, organelle malfunctioning, reducing cancerous protein expression, and hampering replication process (Fig. 3) [119–121]. Bacterial strain *Enterococci*-mediated AuNPs has been reported to exhibit excellent cytotoxicity (IC50 at 10 µg/mL) against human hepatocellular cancer cell lines. The anticancer potential was attributed cytochrome c dislocation, ROS-mediated premature apoptosis, and reduce expression of cancerous proteins in HepG2 cells [32]. In another study, *Paracoccus haeundaensis*–synthesized AuNPs were tested against lung and gastric cancer cell lines and showed excellent anticancer potential. Interestingly, the biogenic AuNPs did not harm normal cells [33].

Mycogenic AuNPs have also been reported to prompt inhibition of cancerous cells in same fashion as above. Fungal strain *Penicillium brevicompactum*–synthesized AuNPs showed excellent anti-cancerous potential against mouse myoblast C2C12 cancer cell lines [55]. Endophytic fungus–mediated AuNPs exhibited antitumor properties in breast cell line MCF-7 (IC50 38.23 µg/mL) via initiation of early apoptosis, DNA fragmentation, and reducing blood supply to cancer cells [63, 113]. Interestingly, the fungus-mediated AuNPs showed better anticancer activity than plant-made AuNPs [63]. Beside their anticancer potential, fungus-mediated AuNPs have

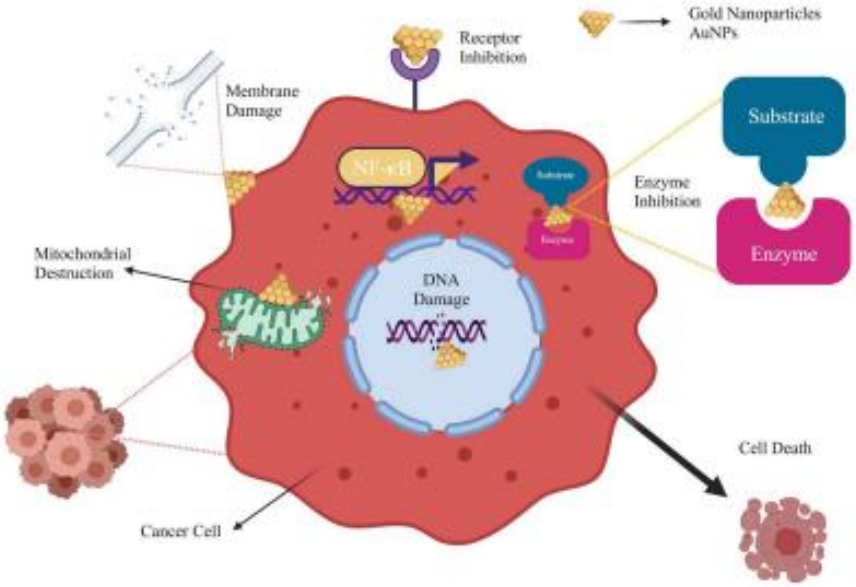


**Fig. 3** Anticancer potential of gold nanoparticles

also been reported in detection of liver cancer, which indicates their promising role in cancer diagnosis [122]. The anticancer potential of both bacterial- and fungal-mediated AuNPs appears to be both dose and cell line dependent [33, 55]. However, for many other studies, the exact detection mechanism is still elusive and has not been fully elucidated; thus, detailed studies are needed to decipher the exact mechanism at the molecular level and evaluate their efficacy in in vivo model organisms.

## Antiviral potential of microbial-mediated gold nanoparticles

Viruses have always been a threat to human existence and are very hard to treat as each have selective treatment options [123]. In the quest to find a reliable and versatile antiviral treatment, various metallic NPs have shown antiviral activities against a wide range of viruses [124]. For instance, *Oscillatoria* sp.–derived AuNPs showed excellent antiviral

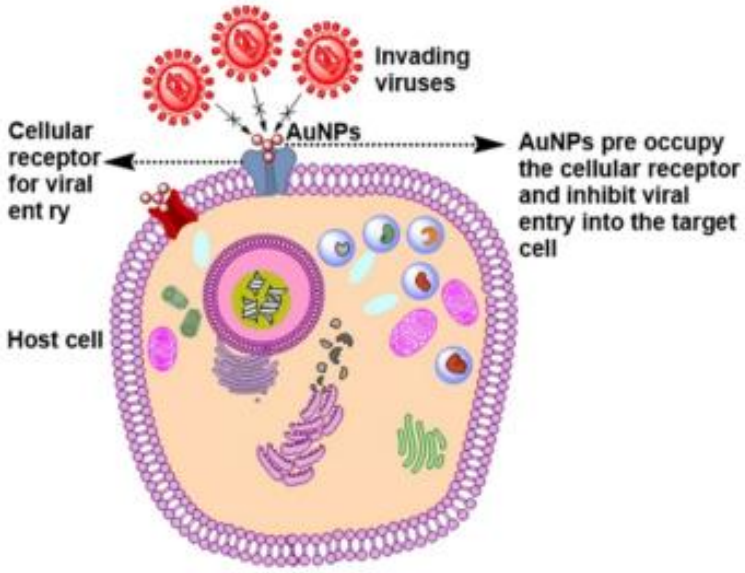


**Fig. 4** Antiviral potential of microbial-mediated gold nanoparticles

activity against herpes simplex virus (HSV-1). Upon exposure of AuNPs, a 90% reduction in cytopathic effect was noted in HSV-1 titer [125]. Less is known about the interaction of AuNPs with viruses than with other pathogens, but it is hypothesized that AuNPs block the cellular entry points (Fig. 4) and thus replication of viruses inside cell [126]. Beside their therapeutic potentials, it can be exploited either as novel antiviral drugs or as adjuvants. Thus, further studies should be conducted to investigate their antiviral potential against both RNA and DNA viruses and decipher their antiviral activities.

## Conclusion

Nanoscale materials have revolutionized medical, agricultural, and engineering fields and hold much promise for the future. AuNPs, have been the most exploited in this regard due to their safe nature and biocompatibility. In recent years, a green approach (using microbes) has been utilized in the fabrication of AuNPs and has showed significant advantages over other synthesis methods. This method is safe and eco-friendly and is energy efficient. Moreover, stable, biocompatible, and uniform structural nanoparticles are made via this approach. AuNPs have been exploited in industrial and medical sectors. In the medical field, they have shown potent antimicrobial, anticancer, and antiviral activities. Moreover, these NPs have also shown the potential to carry drugs and genes to various cellular compartments. However, these studies are nascent and detailed research needs to be conducted to fully elucidate their mechanisms, toxicity, and safety in in vivo model organisms. Finally, a detailed study should be conducted to investigate their potential against many incurable infections and diseases, in particular combating multidrug-resistant bacteria and several types of cancers.

**Abbreviations** *AuNPs*: Gold nanoparticles; *DLS*: Dynamic light scattering; *EDX*: Energy-dispersive X-ray spectroscopy; *EM*: Electron microscopy; *FTIR*: Fourier-transform infrared spectroscopy; *NR*: Not reported; *SEM*: Scanning electron microscopy; *TEM*: Transmission electron microscopy; *TGA*: Technique characterizes materials; *UV/Vis*: UV-visible spectroscopy; *XPS*: X-ray photoelectron microscopy; *XRD*: X-ray crystallography

**Author contribution** MN, MAK, LP, and SU: conceptualization, data collection, writing original draft and figure drafting; RB, A, and JC: writing—review and editing. All the authors read and approved the submitted draft.

**Data availability** NA.

**Code availability** NA.

## Declarations

**Competing interests** The authors declare no competing interests.

**Conflict of interest** The authors declare no competing interests.

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