Inducing Somatic Embryogenesis in Industrial Hemp (*Cannabis sativa)* Tissue Callus

by
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Abstract

Industrial hemp (*Cannabis sativa)* is a versatile plant known for its many uses. In recent years, there has been a renewed interest in the cultivation of hemp. The objective of this study was to use two specific strains of industrial hemp, *Cherry* and *Cherry Blossom,* for attempting somatic embryogenesis using plant tissue culture technology. Explants from both strains were placed on specific media to attempt callus generation. Both strains of hemp produced callus; however, the *Cherry Blossom* explants had a higher rate of callus production than the *Cherry* explants. This could have been due to the fact that the soil pH was not optimal for plant growth when the *Cherry* explants were transferred. The callus was transferred to five different liquid suspension cultures with varying amounts of plant hormones. Two out of the five liquid suspension cultures generated plant embryos in both strains of industrial hemp that were used. Some of the callus was unable to produce embryos, and this was more than likely due to the specific liquid suspension medium being used. The embryos failed to generate plantlets when placed on a nutrient medium. Further research should be done using the plant embryos to produce plantlets.

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Introduction

Industrial hemp (*Cannabis sativa)* has been one of the most important agricultural crops for centuries. *Cannabis* has long been used to make rope, lighting oil, and medicine, among other things (Allegret *et al.,* 2013). There has been renewed interest by many individuals and industries throughout the world, particularly for its wide array of uses (Johnson, 1999). There have been growing environmental concerns, such as the quality of soil and the increase in pollutants, resulting in a decrease in wood fibers, which has led to an ever-growing interest in the production of industrial hemp as a raw material (Ehrensing, 1998).

Hemp has very strong fibers, which is why it is known for its strength. Hemp has a faster than usual growth when compared to other natural fiber plants, and it is generally pest-resistant and drought-resistant. It is a plant that requires few pesticides and little fertilization. Furthermore, plant breeders have been able to develop different varieties of hemp with increased stem fiber content and very low levels of tetrahydrocannabinol (THC), the active ingredient of marijuana (Reddy and Sen, 2011). The cultivation of hemp has been fairly limited because it is often associated with the agent THC, which causes a hallucinogenic effect when consumed (Dingha *et al.,* 2019).

For the most part, hemp production was almost nonexistent in the United States for decades. However, the cultivation of hemp has resulted in interest with the growing demand for natural products and the rising production costs for raw material (Allegret *et al.,* 2013). Since hemp does have a wide variety of uses, there is an urgent need for creating varieties that can result in greater and faster growth in the hemp industry. One of the uses includes its application in the medicinal industry. The medicinal component of

hemp, cannabidiol (CBD), has many implementations. It is used for pain management and is effective in treating several childhood epilepsy syndromes. Based on specific studies, CBD was able to reduce the number of seizures or stop them all together. It is also used to aid in managing anxiety and insomnia (Grinspoon, 2018). If there were a protocol developed that could be used in inducing somatic embryogenesis in this versatile plant, the protocol could be used for mass production of hemp from a limited amount of plant tissue.

Plant tissue culture technology can be used in the development of a protocol for inducing somatic embryogenesis in industrial hemp callus (Feeney *et al.,* 2003). Somatic embryogenesis is the production of normal plant embryos. Somatic embryogenesis is a way in which plant tissue can be regenerated without genetically modifying the specimen. There are different factors that can contribute to either the success or the failure of somatic embryogenesis which include: the type of culture medium being used, the type and amount of growth regulators, the specimen, the origin of the explant, and the growth conditions (Nic-Can *et al.,* 2015). This technique of plant regeneration has been used successfully in several different plant species, some of which include woody species such as *Pinus taeda*, *Camellia sinensis,* and *Picea glauca* (Pais, 2019). Over the years, there have been strides in plant tissue and cell culture techniques. Understanding of a cell's growth and metabolism has been greatly enhanced through the improvement of these techniques. One way that plant embryos can be generated is through the initial production of callus. Callus is a mass of undifferentiated cells produced from explant tissues. The explant tissue can ultimately be derived from many different parts of the plant, such as the leaf or the stem. Callus is induced by transferring the explant tissue to

specialized callus-initiation media (Aitchison *et al.,* 1973). For successful callus initiation, the media have to have a specific amount of plant hormones (cytokinin and auxin). These two hormones play a significant role in plant growth and development. Auxin stimulates plant cells to elongate, whereas, cytokinins trigger cell division.

Once callus has been produced and it is viable, suspension culture can be used to generate new plant embryos. Suspension culture is a technique where cells grow in a moving liquid medium. Suspension cultures are established by placing well-developed callus in a liquid medium and continually agitating it by placing the medium on a rotating shaker. Once embryos are developed in this medium, plantlets can be generated from these plant embryos (Mineo, 1990). Studies have generated somatic embryogenesis in some industrial hemp, but at very low rates and only in specific varieties (Ślusarkiewicz-Jarzina *et al.,* 2005).

Thesis Statement

The objective of this study was to use Industrial hemp (*Cannabis sativa)* to produce a mass of undifferentiated cells, otherwise known as callus, which then was transferred to different types of media to attempt generation of plant embryos. The two varieties that were used to test for positive plant regeneration were *Cherry* and *Cherry Blossom.* These two varieties have been chosen because they contain high levels of CBD.

Materials and Methods

There are different types of media that can be used for callus induction of plant tissue. The medium that was used for this project was Murashige and Skoog basal medium, which is widely used in plant tissue culture (Thacker *et al.*, 2018). Before obtaining the explants (stems and leaves) from the hemp plants, hemp selection pressure medium was made. This specific medium initiates callus formation and contains anti-bacterial (PTC3) and anti-fungal (Daconil) compounds. The medium was made by using deionized water, MS salts, agar, Myo-inositol, Gamborg salts, sucrose, Daconil, PTC3 and two plant hormones, 2,4-dicholorophenoxyacetic acid (2, 4-D), which is an auxin, and kinetin, which is a cytokinin. This medium was prepared as described by Thacker *et al.* (2018). A beaker was filled with about 50 mL of deionized water; the rest of the elements, except the hormones and agar, were mixed into the beaker using a magnetic stirrer. Then, the beaker was filled up to 490 mL with deionized water and the hormones were added. The pH was adjusted to 5.6 by the addition of either an acid (hydrochloric acid) or base (potassium hydroxide) depending on the starting pH. The mixture was transferred to a flask, the agar was added, and it was autoclaved for one hour at 121 degrees Celsius at 15 pounds per square inch to sterilize it. After autoclaving, the liquid was cooled by placing the flask on the magnetic stirrer for about 45 minutes to an hour at 70 degrees Celsius and 200 rpm. The medium was poured into petri dishes in a biosafety hood, while flaming the neck of the flask every few petri dishes to minimize contamination. The petri dishes were left to sit overnight, after which they were sealed with Parafilm. The petri dishes were covered with aluminum foil and stored in a refrigerator. Fresh media were made throughout the project on an as-needed basis.

Both stems and leaves (explants) were collected from hemp plants by trimming the plants. The explants were placed in a sterilized 100 mL beaker. In a biosafety hood, the explants were sterilized by thoroughly rinsing in 100 mL of 70% alcohol solution for twenty seconds by holding the beaker and gently stirring it by hand. The explants were transferred to 100 mL solution of 2.5% bleach and 1% surfactant for twenty minutes, while being stirred continuously on a magnetic stirrer. After twenty minutes, the bleach solution was decanted and three rinses of 100 mL sterile deionized water were used to rinse the plants (Thacker *et al.,* 2018).

After being sterilized, the explants were dissected using a sterilized scalpel and forceps in a biosafety hood. The explant sections were transferred to the hemp selection pressure medium (MS basal medium) with the forceps. So as to not to over-stress the tissues, they were left on this particular medium for only one week. After this, the explant tissues were transferred to hemp non-selection pressure media, which lacked the anti-bacterial and anti-fungal compounds (Thacker *et al.,* 2018). All plates were incubated at 25 degrees Celsius.

Once callus had been produced (approximately four weeks), it was transferred to fresh growth media about every two weeks in order to produce as much callus as needed for embryogenic procedures. If the callus seemed to be lacking sufficient room for growth on the plate, the callus was split between two different petri dishes. The callus was then transferred to media that has been shown to generate embryogenesis in some industrial hemp, but at very low rates (Ślusarkiewicz-Jarzina *et al.,* 2005). This medium consisted of MS salts (0.433 g), thiamine (0.04 mg), myo-inositol (10 mg), sucrose (3 g), agar (0.8 g), naphthaleneacetic acid (NAA) (186.2 μL), and 2, 4-D (284.5 μL). All of the

measurements were based on per 100 mL of solution. The callus was continually monitored. Once the callus had initiated significant growth on the embryogenic medium, it was transferred to suspension cultures with different types and varying amounts of hormones to attempt generation of plant embryos. Five different liquid suspension culture media were made (Table 1). The media were made as previously described and stored in a refrigerator until needed.

To transfer callus into liquid suspension, two 50 mL flasks were autoclaved for 45 minutes at 121 degrees Celsius at 15 pounds per square inch. One was the empty receiver flask, while the other was the one that contained the liquid suspension medium. In a biosafety hood, necks of both flasks were flamed and the medium was transferred into the receiver flask. The callus was split using a sterilized scalpel and forceps, and transferred into the receiver flask as well. The neck was flamed again and aluminum foil was put over the opening of the flask and a rubber band was placed around the neck over the aluminum foil. The optimal amount of medium for a liquid suspension culture was about 10% of the flask volume that was being used.

The cells were allowed to grow (about four to six weeks) and were continually checked for the production of plant embryos. After procambial strands were generated in the liquid suspension, the callus was observed under a microscope to assess the generation of vascular tissue.

Table 1: Modifications of the N10D9 media for liquid suspension

	N10D9	M1	M2	M3	M4
MS salts	0.433 g	0.433 g	0.433 g	0.433 g	0.433 g
Thiamine	0.04 mg	0.04 mg	0.04 mg	0.04 mg	0.04 mg
Myo-Inositol	10 mg	10 mg	10 mg	10 mg	10 mg
Sugar 3%	3 g	3 g	3 g	3 g	3 g
NAA	46.55 μL	37.5 μL	75 μL		
2, 4-D	62.125 μL	37.5 μL	50 μL	100 μL	200 μL
Kinetin				100 μL	200 μL

The modifications listed (M1, M2, M3, M4) are the N10D9 media with different types and varying amounts of hormones. These measurements were per 100 mL of solution.

Once plant embryos were generated in the liquid suspension culture, they were transferred to a nutrient medium that mimicked artificial soil to attempt generation of plantlets. This nutrient medium was made by using 2.165 g of MS salts and 7.5 g of sucrose for 500 mL of solution. The pH was adjusted to about 6-6.5 with the addition of either an acid (hydrochloric acid) or base (potassium hydroxide). The solution was transferred to a 1,000 mL flask, 45 g of agar was added, and the medium was autoclaved. The medium was poured into petri dishes as previously described. The embryonic callus that was in the liquid suspension was poured into a sterile petri dish in a biosafety hood. The medium was extracted using a pipette and expelled into a beaker. The callus left in the petri dish, after the liquid suspension medium was removed, was transferred onto the nutrient medium. The plates were sealed using Parafilm and were placed on a north-

facing window sill for about 10 to 12 weeks to attempt to initiate the generation of plantlets. This was done to provide natural light. The embryos were transferred to fresh medium every other week for about three months.

Results

The explant sections for *Cherry* were placed on hemp non-selection pressure medium, MB2D2K, for one week. Twenty plates of this medium were made (Figure 1).

Figure 1: *Cherry* callus production on MB2D2K medium

After one week, the explants were transferred to the hemp-non selection pressure medium. The callus was transferred to embryogenic medium, N10D9, after one month. During this time, seven plates were contaminated and ten plates were successful in yielding viable callus. The callus was left to grow on this medium for another two months, during which, three more plates became contaminated. There was a 35% success rate for the *Cherry* explant sections generating callus (Figure 2). The remaining 65% of plates either failed to produce callus or were contaminated.

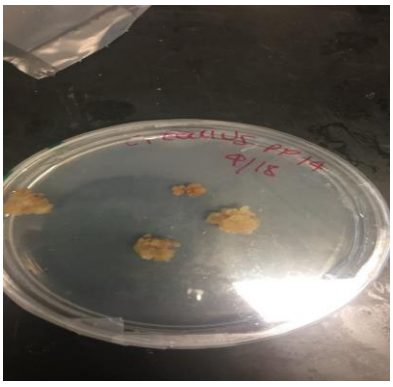

Figure 2: *Cherry* callus on N10D9 medium

Twenty plates of the MB2D2K were also made for the explant sections of *Cherry Blossom* (Figure 3).

Figure 3: *Cherry Blossom* callus production on MB2D2K medium

After one week, the explants were transferred to hemp non-selection pressure medium. The callus was allowed to grow on N10D9 medium for one month. This strain developed healthy callus within two months (Figure 4). *Cherry Blossom* had a 65% success rate. The remaining 35% of plates either failed to produce callus or were contaminated.

Figure 4: *Cherry Blossom* callus on N10D9 medium

For each liquid suspension medium that was made, two cultures were set up. One liquid suspension culture was for *Cherry* callus, while the other was for *Cherry Blossom* callus. Certain liquid suspension cultures generated plant embryos within six weeks of being in the liquid suspension medium (Figure 5-8). During this time, the medium was being changed to fresh medium every other week. The success rates for each culture are listed in Table 2. Once the plant embryos were transferred to the nutrient media, there was no production of plantlets at the end of three months.

Table 2: Success rate of plant embryos for each liquid suspension medium

	N10D9	M1	M2	M3	M4
Cherry	-	+	-	-	+
Cherry Blossom	-	+	-	-	+

+ = production of plant embryos
- = no production of plant embryos

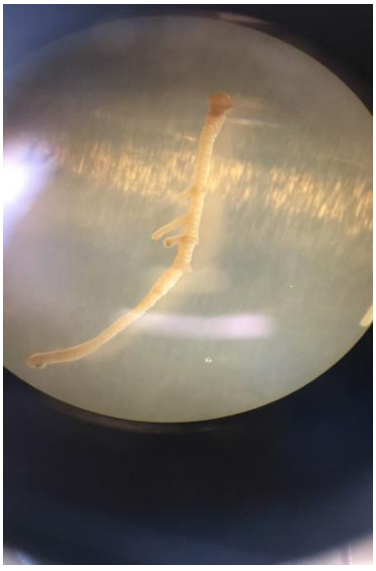

Figure 5: *Cherry* embryos in M1 liquid suspension

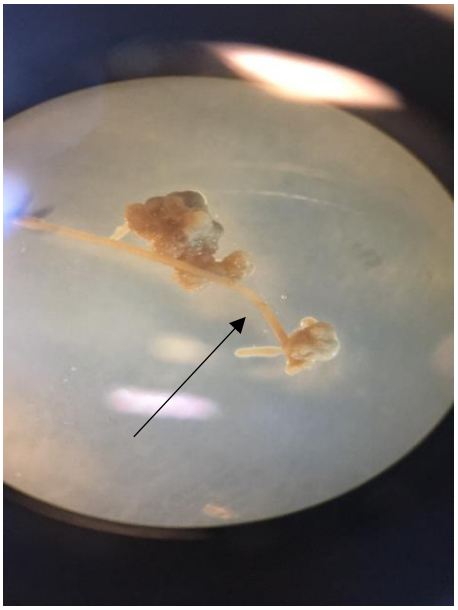

Figure 6: *Cherry Blossom* embryos in M1 liquid suspension

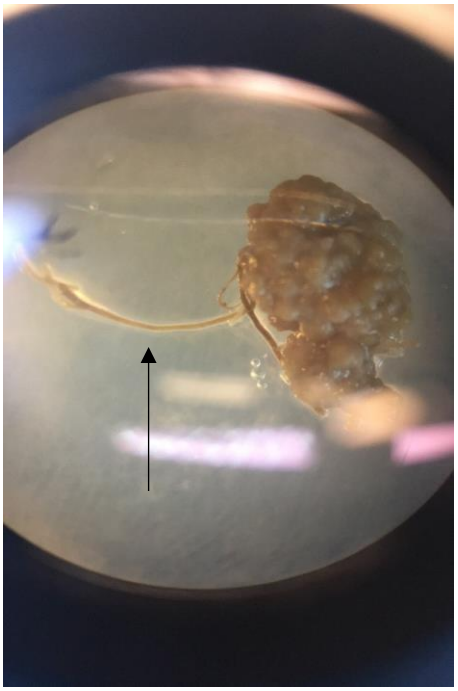

Figure 7: *Cherry* embryos in M4 liquid suspension

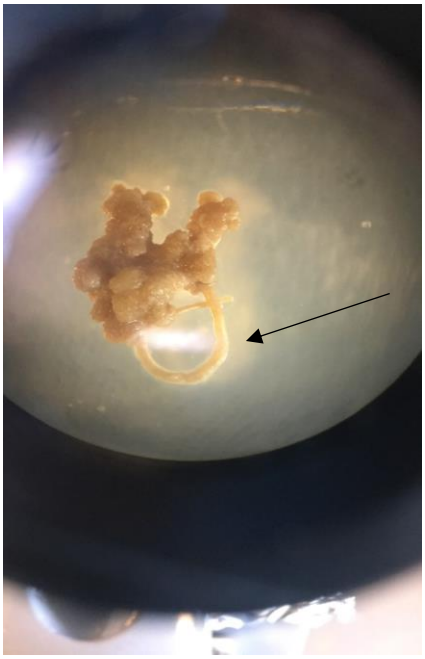

Figure 8: *Cherry Blossom* embryos in M4 liquid suspension

Discussion

Callus was successfully generated in both strains of hemp. There was a higher success rate in callus production with *Cherry Blossom* explants than with *Cherry* explants. There was a considerable difference in the success rates; however, this could very possibly be due to user error. The *Cherry Blossom* explants were transferred to the callus initiation medium after the *Cherry* strain had already initiated callus. Hence, the higher success rate in callus production for *Cherry Blossom* explants could have been simply due to a better understanding of the risks of contamination and proper sterilization techniques. Furthermore, when the explant sections were taken from *Cherry,* the soil pH wasn't optimal for plant growth. This could have been a factor in the failure of some explants in producing callus.

Both the M1 and M4 liquid suspension cultures were successful in producing plant embryos, regardless of the strain of hemp used. The M1 liquid medium contained two types of auxin, NAA and 2, 4-D, while the M4 liquid medium contained one auxin, 2, 4-D, and one cytokinin, kinetin. These specific plant regulators were chosen to induce somatic embryogenesis in this plant species because these hormones had been used to make the callus initiation media. In particular, 2, 4-D has been used to initiate embryogenesis in a few other plant species as well. This specific hormone was used in the initial experiments for inducing somatic embryogenesis in carrot (Stuart and McCall, 2011). It should be noted that both media types that were successful in producing embryos contained 2, 4-D. It is also interesting to note that somatic embryogenesis is most successful when using media that contain both an auxin and a cytokinin. Despite this, one of the media that was able to produce embryos had only two types of auxin. The

liquid suspension media that were unable to generate embryos differed only in the amount of the hormones used.

The inability of some of the callus to produce embryos was more than likely due to the liquid suspension medium used. Both strains were able to generate embryos in the same medium; hence, it is plausible to assume that the failure was not due to the specific strains being used. A few cultures did get contaminated so they were not able to be used for analysis of embryo production. Once the embryos were placed on nutrient media to attempt plant regeneration, there was no production of plantlets. Further research should be conducted on the generation of plantlets using the embryos. A different type of nutrient medium could be used for that study.

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