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Employing Aeroponic Systems for the Clonal Propagation of Cannabis

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TITLE:

Employing Aeroponic Systems for the Clonal Propagation of Cannabis

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SUMMARY:

This protocol is designed to provide instructional information for the clonal propagation of *Cannabis sativa* L. by implementing aeroponic systems. The method described here includes all necessary supplies and protocols to successfully reproduce desirable morphological and chemical properties in the genus *Cannabis*.

ABSTRACT:

This protocol describes the standardization of an efficient clonal propagation technique of hemp by utilizing aeroponic systems. Primary shoot cuttings were excised from two hemp varieties, named “Cherry Wine” and “Red Robin” (17–20% w/w CBD), that served as ‘mother plant’. An auxin precursor (indole-3-butyric acid) was applied to stimulate root development in the basal portion of the excised cuttings prior to placement in the system. Cuttings were lightly misted with the nutrient mist solution every three days to provide nutritional support as the solution contains the essential macronutrients, including nitrogen, phosphorus, and potassium. The aeroponic system water reservoir maintained a pH range between 5.0–6.0 and a water temperature between 68–72 °F. A submersible water pump was used to deliver water to the cuttings. The shoot tip cuttings were provided with 24 h of light per day for 10 days until root development occurred, upon which the rooted cuttings were transplanted for research purposes. These aeroponic systems have proven to generate desirable results for *Cannabis* propagation. The method described here alleviates potential time constraints that arise from traditional methods to allow for a more efficient means for the asexual propagation of *Cannabis*.

INTRODUCTION:

Cannabis sativa L. is an annual, dioecious, flowering plant classified in the family Cannabaceae. Cannabinoids, produced predominantly within glandular trichomes located on the outer

epidermal layer of bract tissues on female inflorescences[1], are becoming an increasingly popular research topic, primarily due to their progressively recognized medicinal properties. Cannabidiol (CBD) is the second most prominent cannabinoid found in *Cannabis* after Δ^9-tetrahydrocannabinol (THC) and is attributed to a host of medicinal benefits, including analgesic properties[2], anti-seizure properties[3], antidepressant properties[4], reducing the risk of diabetes[5], and treating various sleep disorders[6]. Due to the multitude of health benefits associated with the metabolites of the *Cannabis* plant, there is a growing demand for its commercial-scale production[7]. To meet this demand, cultivation methods are constantly being improved and reinvented to continuously supply consistent, high-quality plant material to the emerging *Cannabis* industry.

The propagation of *Cannabis* can be facilitated in two ways: sexual or asexual reproduction. An example of sexual reproduction is pollinating a female ovule with pollen from a male's stamen resulting in a seed that can be germinated. Seed germination is a reliable cultivation method that has been used for breeding and cultivation purposes where desirable phenotypical traits are selected in parental lines to improve the quality of the offspring *Cannabis* plants, including traits such as drought tolerance, insect resistance, increased yield, and increased potency[8]. However, unintended cross-pollination is an inherent risk when performing sexual reproduction, causing undesirable offspring, which leads to the potential loss of desirable traits or an introduction of unwanted traits. An example of this unintended pollination is highlighted by hemp growers receiving hemp seed pollinated with THC- producing pollen resulting in significant economic loss due to the non-compliant plants (>0.3% total THC w/w)[9]. Additionally, to generate a crop that consists of only females, a feminized seed must be sown instead of a non-feminized seed, which can lead to hermaphroditism and other undesirable traits leading to economic loss. To overcome the limitation of sexual reproduction of *Cannabis*, asexual reproduction has been widely practiced in commercial production models of the *Cannabis* industry[10].

Asexual reproduction of *Cannabis* requires only a single plant, which allows for the multiplication of a single genotype that allows for commercial production of plants carrying desirable agronomic and pharmaceutical traits. A common form of asexual *Cannabis* reproduction is to cut and insert small portions of a female plant into a soilless substrate[11] which is covered by a humidity dome to induce root formation. Although this method has proven successful, a common drawback is the accumulation of a high level of humidity (usually 80% or higher) inside the dome, providing an ideal growth environment for fungal pathogens, which can be detrimental to new, sensitive cuttings. Another form of asexual propagation is micropropagation using tissue culture, where sterile techniques allow for the propagation of insect, microbe, and virus-free *Cannabis* plant material in limited space[12]. This process, however, is expensive, time-consuming and requires trained laboratory technicians which are generally inaccessible for large-scale *Cannabis* facilities.

Very few published research reports exist on the clonal propagation of *Cannabis*. In order to provide a basis for the understanding of asexual reproduction of *Cannabis* for research purposes and industrial production, this study aimed to demonstrate the ease and accessibility of employing aeroponic systems for the clonal propagation of *Cannabis*. Aeroponic systems are

ideal for the asexual propagation of *Cannabis*, consistently supplying nutrient-rich water to the cuttings, inducing early root formation in a timely manner, and allowing for a plant to be maintained indefinitely if needed.

PROTOCOL:

1. Generation of a mother plant for clonal propagation

1.1 Select a healthy, female mother plant that exhibits desirable morphological and chemical characteristics specific to its intended use.

1.2 Allow the mother plant to reach the appropriate size (roughly 25 mature shoots) for clonal propagation (i.e., cuttings).

1.3 Allow the mother plants to remain in the vegetative growth stage (light: dark = 18 h:6 h) to promote shoot growth for future propagation.

2. Construction and preparation of aeroponic system

2.1 Begin by positioning the lid on top of the container (38.1 cm x 25.4 cm x 30.48 cm). Drill the desired number of holes into the lid while providing adequate space (preferably 3 cm) between each.

2.2 Position the water pump (**Table of Materials**) in the center of the container.

2.3 Pour 7–8 L of distilled water into the container so that the pump nozzle remains roughly 2.5 cm above the waterline.

NOTE: This ensures the submersible water pump (**Table of Materials**) is able to push water with enough force to spread across the container lid. Distilled water is recommended; however, regular tap water may also be used.

2.4 Situate the appropriate amount of Rockwool cubes (3.81 cm) (**Table of Materials**) or media cubes of choice into each slot. Turn on the pump and allow it to run for 24 h.

NOTE: Rockwool cubes are preferred due to their “anchoring” ability on the newly rooted cuttings that help keep plants upright following transplant.

3. Selecting and excising appropriate shoots

3.1 Collect shoots near the apical meristem using a sterilized scalpel or scissor. Cuttings are ~10 cm in length, ideally with several nodes.

NOTE: Cut the stem at a 45° angle. Cutting at a 45° angle increases the surface area of the basal portion of the cutting, allowing more space for root development. It's optional to make a small slit (1–2 cm) in the middle of the 45° cut to further increase surface area.

3.2 Remove all foliage except foliage present on the top three nodes.

3.3 Dip the newly excised cutting into the rooting solution containing indole-3-butyric acid (IBA) (**Table of Materials**) ~2–5 cm up from the base of the stem for ~5 s.

3.4 Insert the cutting into the center of a Rockwool cube positioned in the aeroponic system.

NOTE: The cutting insertion depth is to remain ~1-2 cm from the bottom of the Rockwool cube.

3.5 Spray the unrooted cuttings with the nutrient mist solution (**Table of Materials**) every 3 days.

3.6 Grow the cuttings with 18–24 h of light per day with a photosynthetic photon flux density (PPFD) of 100 µmol/m^2/s at 24–29 °C and 40%–60% relative humidity.

4. Aeroponic system maintenance and propagule health

4.1 Replenish the system with water at a pH between 5.0– 6.0 every 2–5 days.

4.2 Lightly mist the cuttings (one mist per cutting) with the nutrient mist solution (**Table of Materials**) every 3 days.

4.3 Add 5 mL of each nutrient solution (**Table of Materials**) to the reservoir every 3–5 days.

NOTE: The nutrient addition causes water to be brown and murky.

4.4 Add 15 mL of the algae and bacteria cleaning solution containing hypochlorous acid (0.028%) per 10 L of water every 5 days (**Table of Materials**).

5. Transplanting propagules

5.1 Select the cuttings with long, white, fibrous roots.

NOTE: Avoid cuttings with brown, slimy, and short root systems as this is an indicator for the presence of root rot and will usually take longer to acclimate to the new growing medium and can bring unwanted diseases.

5.2 Carefully dislodge the Rockwool cube from the system and untangle the roots.

5.3 Transplant the *Cannabis* propagules to 4 L nursery pot filled with a nutritious soil mix (**Table of Materials**).

NOTE: Watering immediately is recommended to prevent the roots from drying out.

6. Cleaning and storage of aeroponic system

6.1 When the system is no longer in use, wash with water and clean with 70% ethanol or another disinfectant.

6.2 Remove the filter from the water pump and rinse with water to remove debris.

6.3 Dry the system by wiping it down with paper towels or a washcloth.

6.4 Place the pump inside the tub with the lid on and store it until it is needed.

REPRESENTATIVE RESULTS:

To validate the efficiency of the described aeroponic system, a total of 10 and 12 healthy 14 cm long apical meristems were excised from the mother plants, 'Cherry Wine' and 'Red Robin', respectively (**Figure 1A**,**B**). After dipping into rooting induction media, the clones were placed into the system (**Figure 2A**). The construction and operation of an aeroponic system is shown as a schematic diagram in **Figure 2A**.

After 2 days of acclimation, all clones started to develop roots in 3–7 days and fully developed roots 37 cm in length after 10–14 days on the system, which was sufficient to be planted to a soil-filled pot (**Figure 2B**,**D**). **Figure 3** shows the average length of shoots and roots of each variety. The shoot length and root length were measured prior to transferring to the soil. The average length of shoots and roots were 24.8 cm ± 2.4 cm and 37.8 cm ± 2.5 cm for 'Cherry Wine' and 21.4 cm ± 2.1 cm and 39.7 cm ± 5.9 cm for 'Red Robin', respectively (**Figure 3A**,**B**). The differences between the two varieties were analyzed by two-way ANOVA, followed by Tukey's multiple compassions test, showing no significant differences in shoot and root lengths between the two varieties (n = 10–12, $p < 0.05$).

FIGURE AND TABLE LEGENDS:

Figure 1: A healthy mother plant that generates multiple shoots for clonal propagation. (A) Mature mother plants, "Cherry Wine" (front) and "Red Robin" (back) at ~4 months of vegetative growth, exhibiting numerous shoots ideal for propagation. (**B**) Approximate length (14 cm) for proper shoot excision for cloning *Cannabis*.

Figure 2: Establishment of the aeroponic system for Cannabis clonal propagation. (**A**) Schematic diagram illustrating the components of the aeroponic system (38.1 cm x 25.4 cm x 30.48 cm). (**B**) Aeroponic system fully occupied by "Cherry Wine" clones. (**C**) Inside the aeroponic system with clones exhibiting root growth. (**D**) Healthy root growth in Rockwool cube after 10 days in the aeroponic system.

Figure 3: Shoot and root length measurements for "Cherry Wine" and "Red Robin" after 10 days in the aeroponic cloning system. (**A**) A bar-graph and (**B**) table representing the length of

shoot and root length in the two hemp varieties. The differences in shoot/root lengths between two varieties were analyzed by two-way ANOVA, followed by Tukey's multiple comparisons test.

DISCUSSION:

With the increasing demand for *Cannabis* plants with consistent cannabinoid content, various clonal propagation methods have been exploited in *Cannabis* industry. The asexual propagation shows several advantages over sexual methods for large-scale, consistent production. An aeroponic propagation system is a modified version of a hydroponic system that utilizes an aerated nutrient-rich water mist to provide rapid root development. The described aeroponic system is composed of three critical steps, 1) generating a healthy female Cannabis 'mother' plant as a genetic source for a desired physical/chemical trait, 2) cutting shoots (apical meristems) and treating with root induction media containing the auxin precursor, IBA, and 3) acclimation of the propagule in the desired nursery pot.

The aeroponic propagation system permits the efficient production of hemp clones with clear advantages over different propagation methods. It includes 1) time and cost-saving through less labor-inducing healthy, mature root formation as soon as 5 days; 2) genetic homogeneity - allowing to produce genetically identical clones, excluding any genetic variations, to accommodate reproducible *Cannabis* research and industry applications; 3) uniformity in large-scale operations; and 4) less vulnerability to microbial pathogens – maintaining a relatively low level of humidity in the upper canopy of plants than the humidity level built inside of the container where root development occurs.

Although the aeroponic system offers a number of advantages, there are a few limitations presented that should be carefully addressed during practices. First, as all the nutrients are provided hydroponically, it can be easily contaminated with algal, fungal, and viral (e.g., hop latent viroid) pathogens leading to the malformation of the root system which is mitigated through regular treatment of anti-microbial/fungal/viral agent(s). Second, the widespread use of a single mother plant can be problematic for production facilities. Having a single genotype clone line as the only plant in a production model leads to a risk of entire crop loss if that genotype is susceptible to a given pest or pathogen that is present in that cultivation facility; thus, it is recommended to maintain multiple mother plants from diverse lineages that are used to propagate production clones with this aeroponic cloning system to limit economic loss from any given pest or pathogen[13]. It is advised to only vegetatively grow a mother plant for no more than 6 months before cloning a new mother plant to use as the future source for production clones. This prevents overgrown roots that can lead to unhealthy mother plants and lower cloning success rates, as well as overgrown canopies that can harbor pests and pathogens, which may be transmitted to the propagated clones.

The aeroponic system is industrially scalable in a cost-effective manner. **Figure 1** shows that at least 20 plant materials can be housed in a single container (38.1 cm x 25.4 cm x 30.48 cm). The system can be readily scaled up to hold more than 50 plants per unit without increasing costs on water, nutrients, and electricity. The advantages described here for the current method provide

a reason to implement the aeroponic cloning system into industry practices and research laboratories for the time-efficient and uniform propagation of *Cannabis*.

ACKNOWLEDGMENTS:

This work was supported by the Institute of Cannabis Research at Colorado State University-Pueblo. The authors wish to thank the Chuncheon Bioindustry Foundation for funding this publication, as well as Justin Henderson at Summit CBD for the generous donation for "Cherry Wine" seeds.

DISCLOSURES:

The authors have no conflicts of interest.

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Figure 1

A.

B.

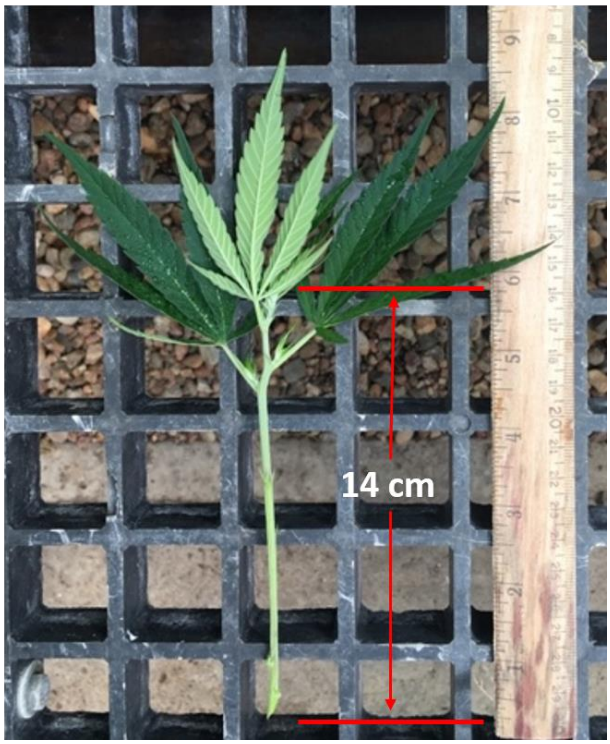


Figure 2

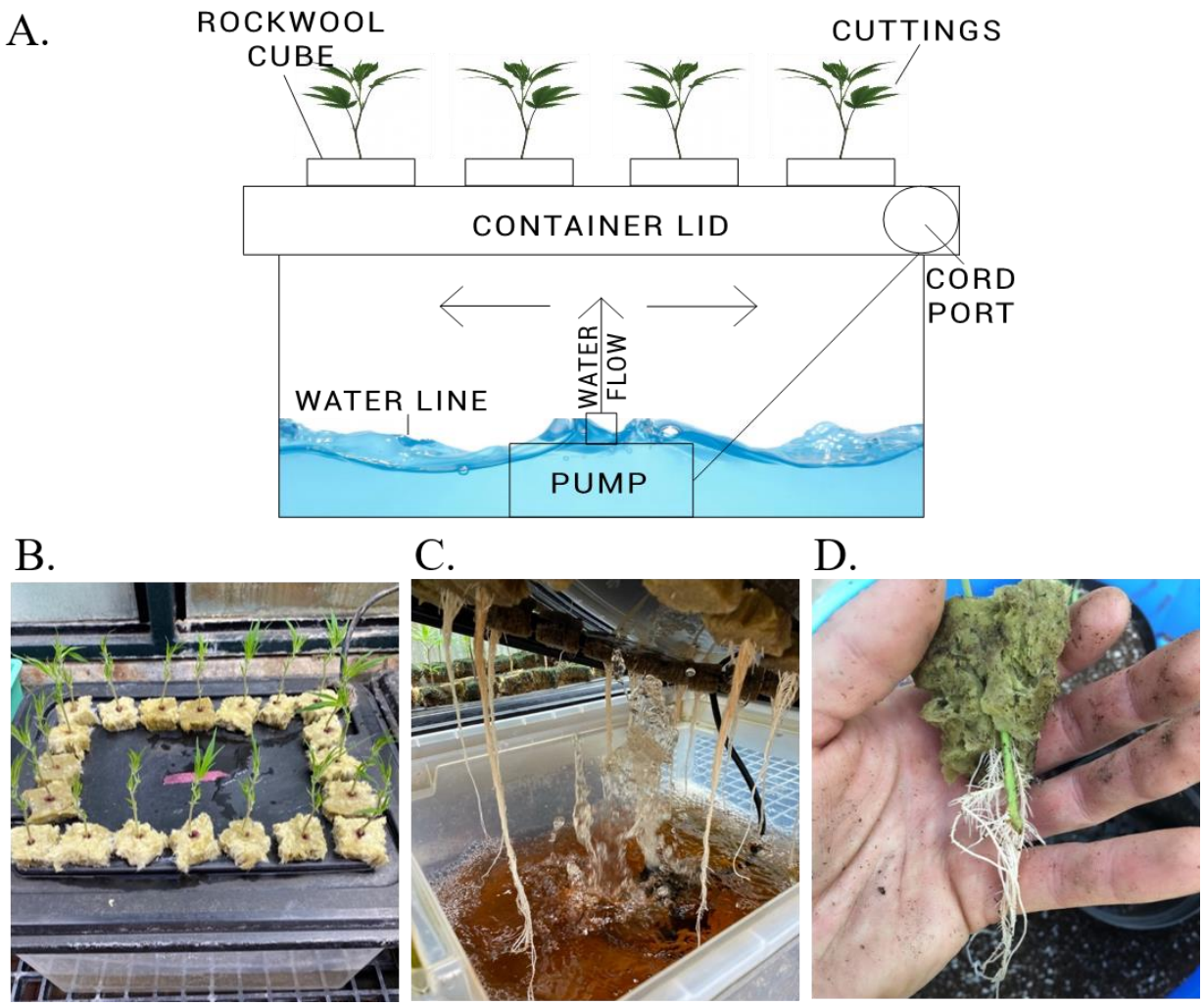
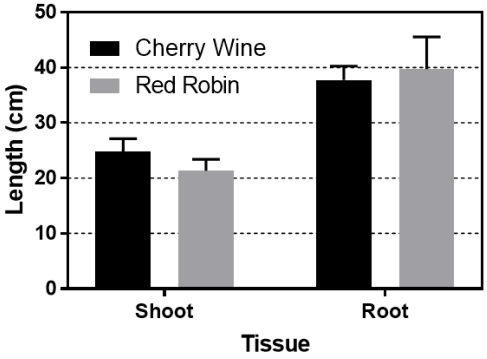


Figure 3

A.



B.

	Cherry Wine (n=10, mean±s.d.)	Red Robin (n=12, mean±s.d.)
Shoot length (cm)	24.8±2.4	21.4±2.1
Root length (cm)	37.8±2.5	39.7±5.9

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The authors carefully reviewed and proofread the manuscript. Thank you for your comment.

2. Please see if the Title can be concised. A suggested one is “Employing Aeroponic Systems for the Clonal Propagation of Cannabis”.

The title has been changed as suggested. Thank you.

3. Please define all abbreviations upon first use. For example, THC, etc.

In the first use, the acronym has been spelled out and the abbreviation has been used since then. Thank you.

4. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

The protocol was revised. Thank you.

5. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

The protocol was revised. Thank you.

6. Please avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly.

Phrases such as “could be”, “should be” and “would be” were deleted from the protocol. Thank you.

7. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?

The authors carefully reviewed the protocol steps. Thank you.

Step 3.1: Was 70% isopropyl alcohol used for disinfecting? What should be slit size? Please specify.

The use of 70% isopropyl alcohol in step 3.1 has been removed and stated as “using a sterilized scalpel or scissor’ (line 125). Using a sterilized apparatus will help to limit potential contamination that may occur. The slit size was specified to be 1-2 cm (line 130). Thank you.

Step 3.2: For how long were the cuttings dipped in IBA solution? Please mention.

The cuttings should be dipped in the IBA solution for approximately 5 seconds and is now described in step 3.3. Thank you.

Step 3.6: Please mention the temperature and humidity conditions.

The average temperature of 24-29°C and relative humidity of 40-60% was added to step 3.6. Thank you.

8. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Essential steps of the protocol were highlighted for the video. Thank you.

9. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

The entire discussion section has been re-written to elaborate the reviewer’s suggestions. Thank you.

10. Each Figure Legend should include a title and must include measurement definitions, scale bars, and error bars (if applicable). Titles and error bars have been added.
11. Please remove the titles and Figure Legends from the uploaded figures. The information provided in the Figure Legends after the Representative Results is sufficient. The titles and legends have been removed from the uploaded figures.
12. Figure 3 legend: Please remove company name (GraphPad Software, Inc., La Jolla, CA). This can be referenced in the table of materials. Removed and referenced in the table of materials.
13. Please do not abbreviate journal titles in References. All references have been reformatted according to the author’s guideline. Thank you.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
This is a very valid and useful protocol

Thank you!

Major Concerns:
I am not sure if this technology is truly scalable at an industrial level - this should be addressed in detail in a revision with some clear suggestions, especially for the "hemp sector".

The scalability at an industrial level has been discussed in the discussion section (lines 264-267). Thank you.

Minor Concerns:
The fact that you are using rockwool, instead of the foam holders that the airoclone uses, should be stressed further. I've always felt that the time that it takes for the naked roots to grab hold of substrate and be happy, negate the small time that it appears ready vs a rockwool clone. The continued flow of nutrients keeps it at the optimal level.

Rockwool cubes are used instead of neoprene collars due to the “anchoring” ability the rockwool cubes have on the newly rooted cuttings that help keep plants upright following transplant. This was added on lines 120-121 to clarify. Thank you for your comment.

Reviewer #2:
Manuscript Summary:
Reviewer comments on manuscript by T. Regas et al. on an aeroponic system for propagation of industrial hemp

Page Line Comments

1 22 Replace "asexual" with "clonal"

The word “asexual” was replaced with “clonal”. Thank you.

1 23 Delete "for the reproduction of"

The words “for the reproduction of” were deleted. Thank you.

1 23 Delete " or related species in the genus Cannabis" as these were not tested in this study. From observations of how cuttings are rooted in drug-type cannabis, this method will work but it should be tested first before claiming that all species will respond.

The words “or related species in the genus Cannabis” were deleted. Thank you.

1 32 Replace "meristematic" with "shoot tip". While the cuttings contained a meristem, they were more shoot tips than meristems

The word “meristematic” was replaced by “shoot tip”. Thank you.

1 33-35 Delete "Comparing to other traditional methods such as seed germination or clonal propagation using tray flats," as a direct comparison was not made in this study

The words “Comparing to other traditional methods such as seed germination or clonal propagation using tray flats” were deleted. Thank you.

1 36 Delete "fungal pathogen issues" as this was not tested. These cuttings can give rise to disease if they were originally infected. The method does not eliminate infection.

The words “fungal pathogen issues” were deleted. Thank you.

1 43 Insert "of bract tissues" after "epidermal layer"

The words “of bract tissues” were inserted after “epidermal layer”. Thank you.

1 45 Insert "after THC" after "found in Cannabis"

The words “after THC” were inserted after “found in Cannabis”. Thank you.

2 55 Delete "of female's stamen". Female flowers do not have stamens unless they are hermaphrodites, which is rare

The words “of female’s stamen” were deleted and replaced by “male’s stamen”. Thank you.

1 56 Seed germination is not used just for breeding purposes. Growers start a new strain from seed as well. Please correct.

The words “and cultivation purposes” were added after “breeding” to explain that seed germination is not just used for breeding purposes alone. Thank you for your comment.

1 63 Provide a reference for this observation

A reference has been added.

1 77 Insert "using" after micropropagation

The word “using” was inserted after micropropagation. Thank you.

1 78 Delete "extremely"

The word “extremely” was deleted. Thank you.

1 83 Insert "published" after "very few"

The word “published” was inserted after “very few”. Thank you.

1 87-88 Delete "and outcompete other methods by reducing the chance of fungal infection" as this was not determined or compared. This system can still give rise to problems with fungal infection if the mother plant was diseased. This can then spread regardless of the system being used.

The words “and outcompete other methods by reducing the chance of fungal infection” were deleted. Thank you.

3 113-114 The industry is now moving to replacing mother plants once every 6 months

Thank you for your comment. It has been changed to “It is advised to only vegetatively grow a mother plant for no more than six months”(lines 258-259).

5 207 Delete "Phytophthora" as root rot can be caused by other pathogens such as Pythium and Fusarium

The word “Phytophthora” was deleted. Thank you.

6 248 Replace "in the size of 37 cm" to "37 cm in length"

The words “in the size of 37 cm” were replaced with “37 cm in length”. Thank you.

7 255 Change $p>0.05$ to $p<0.05$

$p>0.05$ was changed to $p<0.05$. Thank you.

7 279 Replace "proceeding cultivation phases and requirements' with "producers"

The words “proceeding cultivation phases and requirements” was replaced with “producers”. Thank you.

7 290-291 Delete "acquired by progenies during cellular replication"

The words “acquired by progenies during cellular replication” were deleted. Thank you.

7 292-293 Delete "chemical and physiological uniformity - allows for the consistent production of cannabinoid and physiological traits" and replace with "3) uniformity in large-scale operations"

The words “chemical and physiological uniformity - allows for the consistent production of cannabinoid and physiological traits” was replaced with “3) uniformity in large-scale operations”. Thank you.

7 299 Insert "and uniform" before "propagation"

The words “and uniform” were inserted before “propagation”. Thank you for your comment.

General comment: Can the authors explain why they chose to use rockwool cubes rather than something like neoprene collars, which allow the cuttings to actually sit in the air and be misted by water. In the system the authors have described, it does not seem that the cuttings are being propagated in the air, they are sitting entirely in a rockwool block that is simply being watered by a pump. When they are describing how to place the cuttings in the rockwool blocks, they do not say if the cuttings should extend out of the bottom of the blocks but based on the figures they provided, it seems like the cuttings is kept entirely in the cube. Please clarify.

Rockwool cubes are used instead of neoprene collars due to the “anchoring” ability they have on the newly rooted cuttings that help keep plants upright following transplant. This was added in the lines 120-121 to clarify. The Words “The cutting insertion depth is to remain approximately 1-2 cm from the bottom of the rockwool cube” were added in the lines 141-142 to clarify. Once root initiation occurs, roots protrude from the rockwool cube and are suspended in air. Thank you for your comments.

The bottom of the container in Fig. 2 C looks very brown and murky. Is that normal?

Thank you for your comment. The bottom of the container appears brown due to the addition of bottled nutrients explained in the methods section (line 158). Thank you.